Diversity and Phylogenetic Analysis of Mosquito Species in Kole Wetlands of Thrissur, Kerala, and a Comparative Study on Susceptibility of *Aedes albopictus* (Skuse, 1895) and *Culex quinquefasciatus* Say, 1823, against Conventional Insecticides

Thesis submitted in partial fulfilment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY IN ZOOLOGY

Under the Faculty of Science

University of Calicut

by

ASHA A.V.

Under the supervision of

Dr. ANEESH E.M.
(Guide)
&
Dr. SUDHIKUMAR A.V.
(Co-Guide)

CHRIST COLLEGE (AUTONOMOUS)

IRINJALAKUDA - 680125, KERALA

MAY 2023

DECLARATION

I, ASHA A.V., hereby declare that the work embodied in the thesis **"Diversity and Phylogenetic Analysis of Mosquito Species in Kole Wetlands of Thrissur, Kerala, and a Comparative Study on Susceptibility of *Aedes albopictus* (Skuse, 1895) and *Culex quinquefasciatus* Say, 1823, against Conventional Insecticides"**, submitted to the University of Calicut in partial fulfilment of the requirements for the Degree of Doctor of Philosophy in Zoology is a bonafide record of the research work carried out by me under the supervision of Dr. Aneesh E.M., (Guide), Assistant Professor, Department of Zoology, University of Calicut, and Dr. Sudhikumar A.V., (Co-Guide), Assistant Professor, Head of the Department of Zoology, Christ College (Autonomous), Irinjalakuda. No part of the thesis has formed the basis for the award of any degree, diploma or other similar titles of any university.

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CERTIFICATE

This is to certify that the thesis entitled **"Diversity and Phylogenetic Analysis of Mosquito Species in Kole Wetlands of Thrissur, Kerala, and a Comparative Study on Susceptibility of *Aedes albopictus* (Skuse, 1895) and *Culex quinquefasciatus* Say, 1823, against Conventional Insecticides"** submitted to the University of Calicut for the award of the degree of Doctor of Philosophy in Zoology, is an authentic record of the work done by **Ms. ASHA A.V.,** under my supervision and guidance and no part of the thesis has formed the basis for the award of any degree/diploma or other similar titles of any University.

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This is to certify that **Ms. ASHA A.V.,** has completed the research work for the full period prescribed under the Ph.D. ordinance of the University of Calicut. This thesis **"Diversity and Phylogenetic Analysis of Mosquito Species in Kole Wetlands of Thrissur, Kerala, and a Comparative Study on Susceptibility of *Aedes albopictus* (Skuse, 1895) and *Culex quinquefasciatus* Say, 1823, against Conventional Insecticides"**, embodies the results of his investigations conducted during the period at which she worked as a research scholar. I recommend the thesis to be submitted for the evaluation for the award of the degree of Doctor of Philosophy in Zoology of the University of Calicut.

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ABBREVIATIONS

An.- Anopheles

Ae.- Aedes

Ar.- Armigeres

Cx.- Culex

Lt.- Lutzia

Ma.- Mansonia

WHO- World Health Organization

GIS- Geographical Information System

GPS- Global Positioning System

RS- Remote Sensing

QGIS- Quantum Geographic Information System

ASP- Active Server Pages

HTML- Hypertext Markup language

JAVA- Just Another Virtual Accelerator

CSS- Cascading Style Sheets

PHP- Personal Home Page

Arc IMC- Arc Internet Map Server

JE- Japanese encephalitis

DHF- Dengue hemorrhagic fever

DSS- Dengue Shock Syndrome

MoEF- Management of Environment and Forest

AFLP- Amplified Fragment Length Polymorphism

SNP- Single Nucleotide Polymorphism

DNA- Deoxyribonucleic acid

bp- Base pair

ITS- Internal Transcribed Spacer

CO1- Cytochrome Oxidase Subunit1

MEGA- Molecular Evolutionary Genetics Analysis

IGR- Insect Growth Regulator

dNTP- Deoxynucleoside triphosphate

PCR- Polymerase Chain Reaction

EtBr- Ethidium Bromide

BLAST- Basic Local Alignment Search Tool

NCBI- National Center for Biotechnology Information

BOLD- Barcode of Life Data System

NJ- Neighbor joining

ML- Maximum Likelihhood

APHA- American Public Health Association

pH- Potential of Hydrogen

$(NH_2)_2.H_2SO_4$- Hydrazine sulphate

$(CH_2)_6N_4$- Hexamethylenetetramine

KCl- Potassium Chloride

H_2SO_4- Sulphuric acid

EDTA- Ethylenediaminetetraacetic acid

TAE- Tris-acetate EDTA

NH_4Cl- Ammonium Chloride

NaCl- Sodium Chloride

$AgNO_3$- Silver Nitrate

Ag Cl – Silver Chloride

Na NO_3- Sodium Nitrate

K_2CrO_4- Potassium Chromate

Ag_2CrO_4- Silver Chromate

KNO_3- Potassium Nitrate

$MgCl_2$- Magnesium Chloride

TDS- Total Dissolved Solids

NTU- Nephelometric Turbidity Unit

DW- Distilled Water

DO- Dissolved Oxygen

BOD- Biological Oxygen Demand

LC- Lethal Concentration

ANOVA- Analysis of Variance

PAST- Paleontological Statistics Software Package for Education and Data Analysis

SPSS- Statistical Package for the Social Sciences

ABSTRACT

The Kole wetlands in Thrissur, Kerala, are a strip of shallow water that acts as an intermediate zone between terrestrial and marine ecosystems. They keep the water level between 0.5 to 1 meter below sea level and are situated in Kerala's central region, covering an area of 10187 hectares, spanning across the Mukundapuram, Chavakad, and Thrissur Taluks of the Thrissur District. These designated Ramsar sites are a unique part of the Vembanad-Kole wetland ecosystem. The area remains submerged under flood water for about six months each year, and during the Post and Pre-Monsson seasons, paddy and other agricultural practices are carried out. Additionally, several anthropological activities such as fishing, bird watching, clay mining, building construction, research, and recreation take place in the Kole lands. The Kole wetlands in Thrissur, Kerala, are a rich wetland ecosystem with a diverse range of species. Mosquitoes are an essential part of this ecosystem and are one of the most significant insect disease vectors globally.

Mosquitoes thrive and reproduce successfully in wetlands due to the availability of suitable breeding habitats and conducive environments. In Kerala, there has been a significant increase in the outbreak of mosquito-borne diseases in recent years. The presence of mosquitoes in wetlands poses a substantial risk of disease transmission to visitors and workers, thereby becoming a matter of public health concern. It is essential to accurately identify, map, and document the mosquito population and the factors that influence their proliferation to address this issue. This is the initial step in developing an effective vector control strategy.

The major study objectives were the diversity study of available mosquito species in the Kole wetlands of Thrissur, Kerala, and compare the susceptibility of *Aedes albopictus* and *Culex quinquefasciatus* against conventional insecticides. To understand the mosquito diversity, conventional and molecular identification of available mosquito species, their influencing factors, and GIS preparation of identified mosquito species were done in this study. The larval and adult mosquito populations

were collected by direct sampling method and identified using systematic keys. A stereo zoom microscope (Leica-M205C) was used for the identification in the laboratory. Dominance D, Simpson's 1-D, Shannon -H, Evenness H/S, Margalef, Biodiversity index, and Berger-Parker indices demonstrated the alpha diversity of the study area.

The study involved comparing two sites using the Jaccard and Sorenson Diversity indices and conducting Gamma diversity analysis. The 'PAST' software was utilised for the diversity analysis. The study area had three distinct seasons, namely Pre-monsoon, Monsoon, and Post-monsoon, each lasting four months. Monthly sampling was conducted over a period of two years from each sampling location, and the data collected were recorded. The number of mosquitoes collected in different seasons was analysed using a one-way ANOVA test to determine any variation. GPS readings were taken for each sampling location, and the mosquito species were recorded during the diversity study period. The available mosquito data were used to prepare a GIS map using the QGIS software. Additionally, water samples were collected from larval habitats during the monthly sampling.

During the study, water samples were collected and analysed to investigate the relationship between mosquito diversity and the physical and chemical characteristics of the larval habitats. The physico-chemical parameters, including temperature, pH, turbidity, conductivity, and chemical parameters, including total dissolved solids, dissolved oxygen, alkalinity, hardness, chloride, and salinity, were recorded simultaneously with the collection of water samples. The water samples were transported to the laboratory in sterile containers for chemical analysis, and the standard methods recommended by APHA were used to test the water parameters. To ensure the accuracy of identification, molecular identification through CO1 gene sequencing and phylogenetic analysis was conducted. A total of 20 mosquito species from five genera were collected and identified from various locations during the study period. GIS map of 3 sampling sites prepared with these identified mosquito species. The presence and abundance of mosquitoes vary in different seasons. Almost all mosquitoes except *Ph. cogilli* and *Ma. bonneae* were collected in either two or three seasons in a year. ANOVA test reveals that nearly all mosquitoes other than four species (*An. stephensi*, *Ph. cogilli*, *Cx. bitaeniorhynchus,* and *Ma. bonneae*) showed a

significant variation in different seasons. Species with no variation were either present in only one season (*Ph. cogilli* and *Ma. bonneae*) or had a uniform distribution in two (*Cx. bitaeniorhynchus*) or three (*An. stephensi*) seasons.

In this study, we tried to analyse the correlation between the physico-chemical parameters of water samples from different breeding habitats and mosquito diversity. Altogether 20 mosquito species were collected from different breeding habitats during the study period, and values of 10 water quality parameters were recorded. pH, Turbidity, Conductivity, TDS, Hardness, and Chloride exhibits significant correlation with the total number of mosquitoes collected. The rest of the parameters (Temperature, DO, Alkalinity, Salinity) do not correlate with the number of mosquitoes collected. Out of 20 species discovered, 9 mosquitoes express some correlation between the physico-chemical parameters of water samples from their breeding habitats.

DNA sequencing, molecular identification, and phylogenetic analysis were done with these collected species for species confirmation. Final nucleotide sequences of every collected species were deposited to NCBI GenBank and obtained an accession number. ORIGIN Genomic DNA isolation Kit used for DNA extraction. Agarose gel electrophoresis was conducted for the confirmation of the presence of DNA. The amplification reaction was performed using a DNA thermal cycler (Takara). The purified PCR product was sequenced using Sanger's sequencing method at Sci Genom Labs Private Ltd., Cochin, and IISc Bengaluru with ABI 3730XL automated sequencer. The trimmed COI sequences of forward and reverse obtained were aligned using ClustralW. The Final sequence was searched in NCBI BLAST for species confirmation. The partial COI gene sequence was deposited in GenBank (NCBI) for worldwide accession, and it can be used as a molecular barcode generation. Final nucleotide sequences were analysed using MEGAX to study phylogenetic relationships.

Insecticide susceptibility of *Ae. albopictus* and *Cx. quinquefasciatus* against conventional insecticides were checked by the standard WHO method. Comparison analysis of lethal concentration values of field and laboratory strain *Ae. albopictus* and *Cx. quinquefasciatus* revealed that laboratory strain was more susceptible than field strain mosquitoes in every insecticide. The remarkable difference in LC_{50} values

between strains of the same species may be the product of the evolution of insecticide resistance. This trait may be essential as a biological indicator of insecticide pollution. In the context of tested insecticides, synthetic pyrethroids were the most influential group of insecticides, and carbamate was the least effective. The insecticide efficacy varies, and the decreasing order of susceptibility follows Lambda-cyhalothrin > Deltamethrin > Temephos > Malathion > Propoxur. This study will provide information about available mosquito species and the prediction of mosquito-borne disease outbreaks in the Kole wetlands of Thrissur. The findings from the susceptibility test conducted on the identified mosquito species can aid in the efficient management of mosquito populations by minimizing the use of pesticides.

സംഗ്രഹം

കേരളത്തിലെ ഭൗമ-സമുദ്ര ആവാസവ്യവസ്ഥകൾക്കിടയിൽ ഒരു ഇടനില മേഖലയായി പ്രവർത്തിക്കുന്ന സമുദ്രനിരപ്പിനേക്കാൾ ആഴം കുറഞ്ഞ തണ്ണീർത്തടങ്ങളാണ് തൃശ്ശൂർ ജില്ലയിലെ കോൾപാടങ്ങൾ. തൃശൂർ ജില്ലയിലെ മുകുന്ദപുരം, തൃശൂർ, ചാവക്കാട് താലൂക്കുകളിലായി 10187 ഹെക്ടർ വിസ്തൃതിയിൽ കേരളത്തിന്റെ മധ്യഭാഗത്തായി വ്യാപിച്ചു കിടക്കുന്ന ഈ തണ്ണീർത്തടങ്ങൾ വേമ്പനാട് കോൾ തണ്ണീർത്തട ആവാസവ്യവസ്ഥയുടെ സവിശേഷ ഭാഗമാണ്. വർഷത്തിൽ ഏകദേശം ആറ് മാസത്തോളം വെള്ളത്താൽ മൂടപ്പെട്ടുകിടക്കുന്ന ഈ പ്രദേശം അന്തർദേശീയ തലത്തിൽ ശ്രദ്ധിക്കപ്പെട്ടിട്ടുള്ള റാംസാർ സൈറ്റുകളാണ്. കാലവർഷം എത്തുന്നതിനു മുമ്പും ശേഷവുമുള്ള മാസങ്ങളിൽ ഇവിടെ നെല്ലും മറ്റു വിളകളും കൃഷി ചെയ്തുവരാറുണ്ട്. ഒരു പ്രധാന പ്രാദേശിക വിനോദ സഞ്ചാരകേന്ദ്രമായ ഇവിടെ പക്ഷിനിരീക്ഷണം, ഗവേഷണ സംബന്ധമായ പ്രവർത്തനങ്ങൾ, മത്സ്യബന്ധനം, കളിമൺ ഖനനം, കെട്ടിട നിർമ്മാണം മുതലായവയും വ്യാപകമായി നടത്തി വരുന്നുണ്ട്.

തൃശൂരിലെ ഈ കോൾപാടങ്ങൾ വൈവിധ്യമാർന്ന ഒരു ആവാസവ്യസ്ഥയാണ്. ജന്തുജന്യരോഗവാഹകരിൽ പ്രധാനികളായി കണക്കാക്കപ്പെടുന്ന കൊതുകുകൾ ഈ ആവാസവ്യവസ്ഥയുടെ പ്രധാന ഘടകമാണ്. കൊതുകുകൾക്ക് വളരെ വേഗത്തിൽ പ്രജനനം നടത്തുവാനും പൂർണ്ണവളർച്ച പ്രാപിക്കുവാനും അനുകൂലമായ സാഹചര്യങ്ങൾ ഈ തണ്ണീർത്തടങ്ങൾ പ്രദാനം ചെയ്യുന്നു. കേരളത്തിൽ അടുത്ത കാലങ്ങളിലായി കൊതുകുജന്യ രോഗങ്ങളുടെ വ്യാപനത്തിൽ ഗണ്യമായ വർദ്ധനയുണ്ടായിട്ടുണ്ട്. കർഷക തൊഴിലാളികൾക്കും കൃഷിയിടം സന്ദർശിക്കുന്നവർക്കും രോഗങ്ങൾ പകരാനുള്ള സാദ്ധ്യത കൂടുതലായതിനാൽ കോൾപാടങ്ങളിൽ പെറ്റുപെരുകുന്ന കൊതുകുകൾ വലിയ ഒരു പൊതുജനാരോഗ്യപ്രശ്നമായി മാറുന്നുണ്ട്. ഈ പ്രശ്നം പരിഹരിക്കുന്നതിന് കൊതുകുകളിലെ ഇനങ്ങൾ, അവയുടെ വ്യാപനത്തെ സ്വാധീനിക്കുന്ന ഘടകങ്ങൾ എന്നിവ കൃത്യമായി തിരിച്ചറിയുകയും വിശദവിവരങ്ങൾ രേഖപ്പെടുത്തുകയും ചെയ്യേണ്ടത് അനിവാര്യമാണ്. ഫലപ്രദമായ കൊതുകുനിയന്ത്രണ രീതികൾ വികസിപ്പിക്കുന്നതിനുള്ള പ്രാരംഭഘട്ടമാണിത്. തൃശൂരിലെ കോൾപാടങ്ങളിൽ കാണുന്ന കൊതുകുകളുടെ വൈവിധ്യം മനസ്സിലാക്കുക, പരമ്പരാഗത കീടനാശിനികൾക്കെതിരെയുള്ള ഈഡീസ് ആൽബോപിക്ടസ്,

കൂലക്സ് ക്യുൻകിഫാഷിയേറ്റസ് എന്നീ കൊതുകുകളുടെ സംവേദനക്ഷ മത താരതമ്യം ചെയ്യുക എന്നിവയുമായിരുന്നു പ്രധാന പഠനലക്ഷ്യങ്ങൾ. കോൾപാടങ്ങളിൽ കാണപ്പെടുന്ന കൊതുകുകളുടെ വൈവിധ്യം വിശകലനം ചെയ്യുക, അവയുടെ സാന്നിധ്യം രേഖപ്പെടുത്തുന്നതിനായുള്ള ജി.ഐ.എസ്. മാപ്പ് തയ്യാറാക്കുക, കൊതുകുകളുടെ സാന്ദ്രതയെ സ്വാധീനിക്കുന്ന ഘടക ങ്ങളെ കുറിച്ചുള്ള വിവരങ്ങൾ ശേഖരിക്കുക എന്നിവയും പഠനത്തിൽ ഉൾപ്പെ ടുത്തിയിട്ടുണ്ട്. കൊതുകുകളെ കൃത്യമായി തിരിച്ചറിയുന്നതിനുവേണ്ടി പരമ്പ രാഗത വർഗ്ഗീകരണ രീതികളെ പിന്തുണച്ചുകൊണ്ട് ജനിതക ഘടകങ്ങളായ മൈറ്റോകോൺട്രിയൽ ഡി.എൻ.എ. സീക്വൻസുകൾ താരതമ്യം ചെയ്തു കൊണ്ടുള്ള നൂതനവിദ്യകളും പഠനത്തിനുവേണ്ടി ഉപയോഗിച്ചിട്ടുണ്ട്.

പഠനത്തിന്റെ ഫലമായി കോൾപാടത്തിലെ വിവിധ സ്ഥലങ്ങളിൽ നിന്നായി അഞ്ച് ജനുസുകളിൽ പെട്ട 20 ഇനം കൊതുകുകളുടെ സ്പീഷീസുകൾ കണ്ടെത്തിയിട്ടുണ്ട്. ഇവയുടെ സാന്നിധ്യം ചിത്രീകരിച്ചുകൊണ്ട് മൂന്ന് സാംപ്ലിങ്ങ് സൈറ്റുകളുടെയും ജി.ഐ.എസ്. മാപ്പ് തയ്യാറാക്കി. മൺസൂൺ കാലത്തും അതിനു മുമ്പും ശേഷവും കോൾപാടങ്ങളിൽ കാണപ്പെടുന്ന കൊതുകുകളിൽ നാല് സ്പീഷീസുകൾ ഒഴികെ മറ്റെല്ലാം അവയുടെ സാന്നി ധ്യത്തിലും എണ്ണത്തിലും വ്യത്യസ്തത പ്രകടിപ്പിച്ചിരുന്നു. പഠനത്തിന്റെ ഭാഗ മായി വിവിധ പ്രജനന കേന്ദ്രങ്ങളിൽ നിന്നും ശേഖരിച്ച ജലസാമ്പിളുകളുടെ ഭൗതിക രാസഘടകങ്ങളും അവയിൽ നിന്നും ലഭിച്ച കൊതുകുകളുടെ എണ്ണവും തമ്മിലുള്ള പരസ്പര ബന്ധം വിശകലനം ചെയ്യാൻ ശ്രമിച്ചിരുന്നു. പത്തോളം ഭൗതികരാസഘടകങ്ങൾ പരിശോധിച്ചതിൽ ആറ് ഘടകങ്ങൾ കൊതുകുകളുടെ സാന്ദ്രതയുമായി അനുകൂലമായോ പ്രതികൂലമായോ ബന്ധ പ്പെട്ടു കിടക്കുന്നതിനായി കണ്ടെത്തിയിട്ടുണ്ട്. 20 ഇനങ്ങളിൽ 9 തരം കൊതു കുകൾ ഈ ജലഘടകങ്ങളോട് ഇത്തരത്തിലുള്ള ബന്ധം കാണിക്കുന്നുണ്ട്.

ശേഖരിച്ച കൊതുകുകളുടെ സ്പീഷീസ് സ്ഥിരീകരണത്തിനായി ഡി.എൻ.എ. സീക്വൻസിങ്ങ്, മോളിക്യൂലാർ ഐഡന്റിഫിക്കേഷൻ, ഫൈലോജെനറ്റിക് അനാലിസിസ് എന്നീ നൂതന മാർഗങ്ങൾ സ്വീകരിച്ചു. കണ്ടെത്തിയ എല്ലാ സ്പീഷിസൂകളുടെയും ഫൈനൽ ന്യൂക്ലിയോടൈഡ് സീക്വൻസുകൾ എൻ. സി.ബി.ഐ. ജെൻബാങ്കിൽ നിക്ഷേപിക്കുകയും ആക്സഷൻ നമ്പർ നേടു കയും ചെയ്തു.

ഡബ്ല്യു.എച്ച്.ഒ. നിർദ്ദേശിച്ച പ്രോട്ടോക്കോൾ പ്രകാരം ഈഡീസ് ആൽബോ പിക്ടസ്, കൂലക്സ് ക്യുൻകിഫാഷിയേറ്റസ് എന്നീ സ്പീഷീസുകൾക്ക് പര

മ്പരാഗത കീടനാശിനികളോടുള്ള സംവേദനക്ഷമത പരിശോധിച്ചു. ഈ രണ്ടു സ്പീഷീസുകൾക്ക് ഇത്തരം കീടനാശിനികളോടുള്ള സംവേദനക്ഷമത വ്യത്യസ്തമാണ് എന്നാണ് ഈ പരിശോധനാഫലം വെളിവാക്കുന്നത്. കൂടാതെ ഈ രണ്ടു ഇനങ്ങളിലും കോൾപാടങ്ങളിൽ നിന്നും ശേഖരിച്ച കൊതുകുകളേക്കാൾ സംവേദനക്ഷമത കാണിച്ചത് ലബോട്ടറിയിൽ വളർത്തിയ കൊതുകുകൾ ആയിരുന്നു. ഒരേ ജീവിവർഗ്ഗങ്ങളിൽ കാണുന്ന ഈ വ്യതിയാനം കീടനാശിനി പ്രതിരോധത്തിന്റെ ജൈവിക സൂചകമായി ഈ കൊതുകുകളെ പരിഗണിക്കുന്നതിലേക്ക് നയിക്കുന്നു. കൂടാതെ ഈ രണ്ട് സ്പീഷീസുകളിലും പരിശോധിച്ച കീടനാശിനികളോടുള്ള സംവേദനക്ഷമത വ്യത്യസ്തമായിരുന്നു. സംവേദനക്ഷമത ഏറ്റവും കുറവ് കാർബണേറ്റ് വിഭാഗത്തിൽപ്പെട്ട പ്രോപ്പക്സർ എന്ന കീടനാശിനിയോടും കൂടുതൽ സിന്തറ്റിക് പൈറത്രോയ്ഡ് വിഭാഗത്തിലെ ലാംബ്ഡ സൈഹലോത്രിൻ എന്ന രാസപദാർത്ഥത്തിനോടും ആയിരുന്നു.

തൃശൂർ കോൾപാടങ്ങളിലെ കൊതുകുകളുടെ ഇനങ്ങളെക്കുറിച്ചുള്ള വിവരങ്ങളും ആ കൊതുകുകൾ പരത്തുന്ന രോഗങ്ങളുടെ മുന്നറിയിപ്പും ഈ പഠനം നൽകുന്നുണ്ട്. സംവേദനക്ഷമത പരീക്ഷണങ്ങളുടെ കണ്ടെത്തലുകൾ പരിസ്ഥിതി മലിനീകരണം കുറച്ചുകൊണ്ടുള്ള കൊതുകുനിയന്ത്രണ കീടനാശിനി പ്രയോഗത്തിന് സഹായമാകുന്നതാണ്.

GENERAL INTRODUCTION

Mosquitoes

Insects come under the invertebrate Phylum Arthropoda as they lack backbone along with crustaceans, arachnids, and other groups. The common characteristics shared by arthropods are their hard exoskeleton with jointed legs. Many creatures of this group are identified as pests of different kinds, as some are agricultural pests, and some are pests to other animals and humans. Mosquitoes belong to the family Culicidae of the order Diptera (Eldridge, 2008). There are about 3,719 described mosquito species in the world (Harbach, 2023). Mosquito-transmitted illnesses are a constant concern to public health because they may spread various infections from animal to animal, mostly harming humans. Numerous mosquito genera are extensively scattered around the world, although some are thought to be endemic (Rueda, 2007). Mosquitoes thrive in conditions similar to lentic aquatic environments, such as stagnant or sewage water, tanks, natural and artificial containers, coconut shells, tree holes, leaf axils, gutters, etc. (Mafiana,1989; Aigbodion, 2005; Aditya et al., 2006).

Mosquito classification includes three subfamilies that differ in their characteristics and stages of the lifecycle. The mosquito subfamilies are named Anophelinae, Culicinae, and Toxorhynchitinae. The members of Anophelinae are referred to as Anophelines and Culicinae as Culicines. As their females require blood to complete their life cycle, these subfamilies are crucial in public health. In contrast to these two subfamilies, Toxorhynchitinae females lack bloodsucking mouthparts and do not consume a blood meal. This species is significant because it may feed on aquatic creatures and other mosquito larvae's natural predators during their larval

stage. *Culex*, *Aedes,* and *Mansonia* are the three common genera coming under Culicinae, and the genus *Anopheles* contains almost all the species coming under Anophelinae. *Toxorhynchites* is the only genus which comprises all the species that belong to the subfamily Toxorhynchitinae (Resh and Carde, 2009).

Biology

The biology of mosquitoes explains them as bilaterally symmetrical. The adult form bears an exoskeleton with three principal regions: the head, thorax, and abdomen. Large compound eyes are present on the ovoid-shaped head. The head has five appendages: a proboscis, two antennae, and two maxillary palpi. The thoracic divisions between the head and abdomen are the prothorax, mesothorax, and metathorax. Jointed legs are seen attached to each thoracic subdivision, and functional wings appear on the mesothorax region. Halters, a pair of wings characterised as knobbed structures, are connected to the metathorax. The abdomen comprises ten segments, of which the last three are specialised for reproduction and excretion. The mosquito adults resemble Chironomidae, Chaoboridae, Dixidae, and other Nematocera, having immature aquatic stages. The appearance of scales on the wing veins and wing edges and the presence of a long, forward-projecting proboscis designed for piercing and sucking distinguishes mosquitoes from similarly shaped dipterous flies. In contrast to an adult, the larva is composed of soft, membranous tissues in the thorax and abdomen, and hardened, sclerotized plates in the head that allows for the swimming movements and doubling of the body when cleaning the mouth or palatal brushes (Rueda, 2007).

Mosquito adults resemble miniature flying midges. A lengthy, narrow proboscis specialised for piercing skin and sucking blood, as well as long, delicate wings

covered with microscopic scales, distinguishes1 most female mosquitoes from similar insects. Scales like females also cover the wings of the male mosquitoes, but their proboscis is designed to feed on plant juices and other sugar sources. Male mosquitoes are smaller than the females of the same species and bear noticeably longer and hairier maxillary palps. The mosquito larvae and pupae colour range from yellowish tan to black. A structure called a siphon, an air tube, is seen at the terminal abdominal segments of most of the larvae, although some species lack them (Resh and Carde, 2009).

Culicine adult females have a proboscis suited to pierce vertebrates' skin and drain their blood. Their bodies are typically positioned parallel to the skin surface of their hosts during feeding. Adult female Anophelines have proboscis modified for piercing vertebrate skin as well, but while blood feeding, they position themselves at around a 45° angle. Mosquito eggs are also different. Culicine females lay solitary eggs (*Aedes, Psorophora*), boat-shaped rafts of around 100 eggs (*Culex, Culiseta*), or clusters of eggs linked to floating plants (*Mansonia, Coquilletidia*). *Anopheles* eggs are placed single; however, they feature ornate floats that reach to the sides. *Anopheles* eggs are frequently observed in groups on water surfaces, generating geometric patterns. *Toxorhynchites* eggs are deposited individually on water surfaces (Resh and Carde, 2009).

Life Cycle

The life cycle of a mosquito contains four stages: egg, larva, pupa, and adult. The adult stage is free-flying, while the previous phases are aquatic. The amount of time needed for a mosquito to complete its life cycle varies depending on food availability, environmental conditions, and mosquito species. Some mosquitoes may

complete their life cycle in as little as 8 to 10 days if the conditions are right. Finding mosquito eggs is one approach to locating mosquito breeding places. Mosquito eggs are sometimes dispersed in rafts on the waters' surface, and they can also be placed individually on the waters, surface or in arid environments that are occasionally inundated. Mosquito eggs are white during the initial stage but become dark brown to black within a few hours. Mosquito eggs can be seen in various shapes and sizes, most being football or boat-shaped and about 0.02 to 0.04 inches in length. The eggs can hatch in 2 to 3 days in warm water, and in dry circumstances, some mosquito eggs can remain latent for months (Jackman and Olson, 2002).

Mosquito eggs develop into wigglers, long larvae rarely more than 1/2 inch long. The body of a wiggler is divided into three sections: a tiny head, an expanded thorax, and an elongated, cylindrical-shaped abdomen. Wigglers are exclusively found in water and eat minute plants, animals, and organic waste floating in the water for the most part. With their brush-like mouthpieces, they sift food particles from the water. Some mosquito larvae are predators that rely on the larvae of other mosquito species. In 4 to 10 days, most mosquito larvae mature, progressing through four instars (growth stages) before transitioning into the pupal stage. Food, temperature, and species influence the length of a mosquito's larval growth phase (Jackman and Olson, 2002).

Mosquito larvae take on unique postures in the water when eating or breathing. The larvae of most species breathe through an air tube towards the end of the abdomen, protruding the air tube through the waters' surface and hanging head down at an angle to the waters' surface, with only the tip of the breathing tube touching the waters' surface. The larvae of *Anopheles* mosquitoes are an exception. They do not

have air tubes and prefer to float flat on the waters' surface. Applying a surface coating of some petroleum compounds in standing water is one approach to suppress mosquitoes in the larval stage. This film stops the larvae from breathing and kills them (Jackman and Olson, 2002).

The pupal stage is a stage in the life cycle of mosquito that dwell in water and adults that live on land. Mosquito pupae need not consume food, and they spend most of their time on the waters' edge and only relocate when disturbed. Mosquito pupae are called tumblers since they twirl in the water when disturbed. Mosquito pupae are comma-shaped and breathe through air tubes at the surface of standing water, just like the larvae. The pupal head and thorax are joined, and the front part of the body is substantially expanded. A pair of trumpet-shaped respiratory tubes protrude from the rear of the thorax and are utilised to breathe air at the waters' surface (Jackman and Olson, 2002).

Adult female mosquitoes generally get nutrition from carbohydrates like plant sap, nectar, etc. Nevertheless, most species' females consume blood meal as a protein supplement before egg laying. Generally, the mating happens swiftly in the air near the emergence site. Female mosquitoes can fertilize all of their egg after a single mating by carrying sperm in their body. Male mosquitoes generally die soon after the breeding season is completed. Still, female mosquitoes live for around a week to a month, although this might vary based on various environmental variables. Some species overwinter as engorged mated females who can survive up to 6 months or more (Jackman and Olson, 2002).

Breeding Habitats

Mosquitoes are a large group of dipterans found worldwide, with approximately more than 3000 varieties and subspecies, and seen in a wide range of environments, from deserts at or near sea level to high alpine meadows at elevations of 3000 meters or more. Mosquitoes are flying insects that live on land, but their juvenile stages live in water. Larvae and pupae of diverse species can be found in ponds, ditches, puddles, swamps, marshes, water-filled tree rot holes, rock pools, plant axils, melting snow pools, abandoned tyres, tin cans, and other sorts of standing water. Some species are active during the year's hottest months, while others are more suited to the cooler months. Many mosquito species are infrequently observed and rarely represent a hazard to human or domestic animal health or well-being. Other species, on the other hand, are plentiful, regularly encountered, and quickly attack humans, pets, and cattle. A few of these genera can transfer microbial organisms that cause malaria, encephalitis, and other severe human and animal illnesses (Resh and Carde, 2009).

Feeding Behaviour

Plant juice is the primary energy resource both in male and female mosquitoes. Besides this, females consume vertebrate blood for their egg development. This female behaviour is an essential trait of mosquitoes, which was thought to have evolved several times from ancient species specialised for sucking plant fluids or preying on other insects. Even though the methods by which female mosquitoes find acceptable blood-feeding hosts have been researched, the exact explanation of this behaviour still needs to be determined. The soundest reason is that they can detect carbon dioxide-rich, warm, wet environments, although additional elements exist.

Some research has shown that chemicals like lactic acid, a component of human perspiration, might operate as an attractant (Resh and Carde, 2009).

A female mosquito would not usually generate a batch of eggs unless she has consumed a blood meal to provide sustenance for her ovarian growth. Autogeny is the ability of some strains or individuals of various species to produce eggs without needing a blood meal. Autogeny is the ability of some strains or individuals of various species to produce eggs without a blood meal. Only the initial batch of eggs can utilize these proteins, which are carried over from the larval stages for their egg development. Anautogeny is when a single female needs a blood meal to develop every batch of eggs. Some mosquitoes have specific invertebrate hosts; for example, one of the western tree hole mosquitoes, *Ochlerotatus sierrensis*, feeds exclusively on animals, and the northern house mosquito, *Culex pipiens*, feeds entirely on birds. But *Culex tarsalis*, the encephalitis mosquito, feeds on birds and animals, and this dual host preference was one characteristic of an effective vector of disease pathogens. The selection of a specific host was sometimes used to describe this relative host specificity. However, more than host preference is required since it overlooks host availability, host defensive behaviour, and other aspects unrelated to mosquitoes. Neurohormones control this blood-feeding urge, which can be artificially induced using juvenile hormones or their analogues. Usually, multiple blood meals are rare but do occasionally occur in the gonotrophic cycle of some species (Resh and Carde, 2009).

Mosquito blood intake is a complicated process. The saliva injection into the vertebrate host's feeding wound makes it more accessible. Saliva is produced by salivary glands located in mosquitoes' thorax. Saliva contains various compounds, including molecules that prevent vertebrate blood from clotting. A blood meal takes

2–3 days to digest, depending on the ambient temperature. The movement of muscular pumps in the heads of female mosquitoes allows them to sucking blood. Blood passes through the mosquito's digestive tract and into a tissue known as the midgut. When blood reaches the midgut, it is quickly encased in a thin sheath called the peritrophic membrane, produced by cells at the midgut's front (Resh and Carde, 2009).

Role in Ecology

The distribution and quantity of immature and adult mosquito populations and how they are impacted by geographic dispersion, elevation, weather, climate, vegetation, and seasons are all part of mosquito ecology. Ecology is a problematic and imperfect science. For example, some elements that affect vegetation patterns may likewise control mosquito population dispersal. However, vegetation may have direct mosquito-controlling effects, such as protecting mosquito larvae from predators when they are in aquatic vegetation. Finding specific cause-and-effect links is almost impossible due to the often immensely complicated nature of the cause-and-effect web (Eldridge, 2008).

Many people believe mosquitoes are one of the last species to be considered for conservation. Mosquitoes can be terrible during the rainy season, biting furiously under the shadow of trees or near salt-water mangroves. Even while mosquitoes bother most people, they serve a crucial role in the food chain, especially in watery ecosystems like the Everglades. Mosquito larvae are generally at the bottom of the food chain. They are a vital food source for small fish like the mosquito fish (Gambusia) in freshwater regions, feeding medium-sized fish like the blue gill and brim. These species serve food for bigger fish, such as garfish and largemouth bass.

The alligator, birds, and people all eat bass and gar. Egrets, spoonbills, and wood storks are the wading species that profit from the mosquito food chain. The mosquito fish and killifish become caught by the hundreds as the mosquito ponds dry up between rainstorms. At the receding ponds, wading birds converge to eat concentrated, easily accessible fish. Furthermore, without mosquito larvae, local fishers and anglers would have fewer fish to capture in many coastal places (U.S. Department of the Interior 2005). Mosquitoes are essential to wetland ecology because they provide food for birds, bats, amphibians, fish, and macroinvertebrates (Webb and Russell, 2007).

One of the most harmful insect pests to humans and animals is the mosquito. Female mosquitoes that bite people and animals can not only annoy them but also transfer various disease-causing germs. To effectively manage mosquitoes, it is necessary to understand their life cycle, recognize different types of mosquitoes and know what methods work best for certain species and places (Jackman and Olson, 2002). Mosquitoes can act as bioindicators of their living environment. For this reason, the diversity of insects has long piqued the curiosity of entomologists and those involved in other environmental programs (Devi and Jauhari, 2005). The most important group of insects in terms of public health is mosquitoes. Each year, they spread diseases that cause millions of fatalities, including filariasis, dengue fever, Japanese encephalitis, and malaria. *Anopheles, Culex, Aedes*, and *Mansonia* are the most common mosquito genera in India that transmit these illnesses (Das et al., 2007).

Wetlands

Wetland is an ecotone between terrestrial and aquatic environments, generally with shallow water-holding lands (Cowardin et al., 1979). Three significant aspects of wetlands are (I) the land must be seasonally or perpetually submerged under flood water; otherwise, water present for at least seven consecutive days during the growing season, (II) the land must support aquatic flora (III) the substratum of the water bed holds the water for a long time, due to this the anaerobic upper layer is formed on the hydric soil (Watzin, 1992). The wetland ecosystem is capable of controlling and maintaining its biotic and abiotic components. Wetlands control flood and sedimentation and offer shelter for many organisms. Compared to other biomes, wetlands are highly productive environments comprising a wide variety of species (Mitsch, 1994). Mosquitoes are one of the principal inhabitants of wetlands with a significant spatial and temporal variation. Through their blood-feeding behaviour, these mosquitoes annoy the population that resides in and surrounds this habitat and spread several illnesses among them (Schafer, 2004).

Flood water management, water quality improvement, carbon sequestration, and pollution removal are all essential social, economic, and ecological benefits provided by wetlands (Rey et al., 2012). Wetlands also have a high aesthetic and recreational value, making them very desired for increasing human well-being and, as a result, promoting local rural economies through increased tourism and the establishment of new communities (Medlock and Vaux, 2015).

Importance of wetlands

Ponds and wetlands provide vital ecosystem services such as stormwater management in aquatic life habitat, and ecosystem health and stability, in addition to

beautifying the environment. Ponds and wetlands reduce stormwater runoff, which traps and delay stormwater flow, and they aid in filtering and cleaning rainfall and runoff water, as well as replenishing groundwater aquifers. In the landscape, ponds and wetlands support a variety of flora and wildlife, including birds, bats, aquatic insects, fish, and frogs, all of which feed on mosquitoes. In addition to these benefits, ponds and wetlands provide many recreational possibilities, including fishing, swimming, boating, and hunting. Although ponds and wetlands can boost mosquito numbers in some cases, mosquito predators such as fish and other aquatic species will typically keep mosquito populations in check if the pond or wetland maintains a well-balanced ecosystem (Ladd and Frankenberger, 2003).

India has a diverse range of interior and coastal wetlands due to its large geographical area and varying topography and climate. The wetlands of Kerala are also well-known. Agricultural products, fish, fuel, fibre, fodder, and a variety of other daily essentials were given by these wetlands to the area's population. As long as human involvement was kept to a minimum, the ecosystem was self-cleaning due to its all-encompassing balancing nature. However, the need for growth, which dictates which courses to choose, disturbs the natural balance. Urbanization and its developmental activities in the form of roads, railways, and other lines of communication disrupted the wetlands' connectivity. They destroyed large tracts of coastal vegetation, upsetting the entire complex ecology. Rapid urbanization encroached on the rich and luxuriant mangrove forests, while industrial development did cause not only pollution but also prevented any regeneration opportunities; modern shrimp farms brought in the final onslaught - the invasive species. With its dense population, Coastal Kerala can no longer withstand such assaults. The destruction of Kerala's wetlands is not a unique incident. Wetlands are in danger

worldwide, contaminated, drained, or filled to make space for development. In recent years, the rate of wetland destruction has accelerated. As a result, wetlands have become one of our planet's most endangered ecosystems (Sabu and Ambat, 2007).

Wetland Mosquitoes

Mosquitoes, members of the Family Culicidae of the order Diptera, are among the most visible residents of wetlands (Schafer, 2004). Agricultural growth can potentially change the ecosystem in a way that favours mosquitoes, and agriculture-induced sedimentation can delay or obstruct streams and reduce water depth (Dian and Changxing, 2001). These activities increase the number of mosquito homes available while lowering the water temperature for vector growth (Norris, 2004). Wetlands have high productivity relative to other ecosystems and provide a home for a diverse range of plants and animals (Mitsch, 1994; Keddy, 2000). Mitsch describes the problematic link between mosquitoes and wetlands, claiming that mosquitoes are one of the sources of incompatibility between human civilization and wetlands in many regions of the world.

Mosquito populations will fluctuate depending on the degree of moisture and air temperature in all wetlands. Mosquitoes can travel and concentrate in these smaller regions of wetness during drought seasons, when the amount of water in some wetland areas may be reduced to small or shallow pools, while overall numbers of floodwater mosquitoes tend to drop during dry years (Shaman et al., 2002a; Shaman et al., 2002b). When former wetland regions get inundated following rain storms, mosquito populations thrive in locations where wetlands have been drained. Following rain, mosquito populations can grow due to intermittent wet, muddy or

shallow stagnant water and a lack of mosquito predators. Mosquitoes that spread disease and reproduce solely in stagnant water are among them (IDNR Fact Sheet; Jensen, 1999; Shaman et al., 2002a; Shaman et al., 2002b).

Mosquitoes can live in a variety of environments and tolerate harsh weather. The geographical location, water level fluctuation, size, permanency, water turbulence, and water's organic content all influence mosquito species composition in a water body (Tennessen, 1993). A wetland's nature and location will impact the species that live there. Every mosquito species has its preferred procreation grounds with favourable factors. The general impression is that wetlands serve as mosquito breeding grounds and generate some mosquito nuisances. Although mosquitoes can reproduce in almost any water source, these wetlands can be used to limit mosquito habitat (Russell, 1999). Birds, amphibians, dragonflies, and diving beetles have all been used to measure the beneficial biological diversity of wetlands (Chovanec and Raab, 1997). Many insects, including mosquitoes and biting midges, are crucial elements of biological diversity in wetlands. Among these, mosquitoes are the most important inhabitants of wetland insects because of their capacity for nuisance, enormous presence, and role as carriers for human and animal illnesses. Conversely, mosquitoes are essential for measuring biological variety since they interact with aquatic and terrestrial fauna in wetlands. Most species include information on environmental needs, and each mosquito species' presence necessitates particular aquatic and terrestrial environments for larvae and adults (Schafer, 2004).

Kole Wetlands

Vembanad-Kole wetland ecosystem in Kerala, comprising an area of 151250 ha, has been a Ramsar site since 2002. Thrissur Kole wetland is a unique part of this Ramsar

site. Within this ecosystem, the Kole land covers an area of about 13,632 ha spread over the Thrissur and Malappuram districts. They are low-lying tracts located 0.5 to 1 m below mean sea level between 10º20' and 10º40'N latitude and between 75º58' to 76º11'E longitude. A unique feature of the Kole land is that it remains submerged under floodwater for half of the year during the southwest monsoon when the water level rises to 5.5 meters. A network of primary and cross canals provides external drainage and connects the different areas of the Kole to the rivers. This wetland comes under the administration of Mukundapuram, Chavakkad, Thrissur, and Thalappilly taluks of Thrissur district and Ponnani taluk of Malappuram district (Johnkutty and Venugopal,1993).

Since 2002, Kole wetlands have been considered a Ramsar Site (Islam and Rahmani, 2008), a dominant Bird Area since 2004 (Islam and Rahmani, 2004), and a High-Value Biodiversity Area since 2009 (MoEF, 2009). The southern boundary of the Kole wetland is the northern bank of the Chalakudy River, and the northern boundary is the southern bank of the Bharathapuzha River (Johnkutty and Venugopal, 1993). Kole refers to the particular cultivation practice carried out from October to April. 'Kole,' a Malayalam word, indicated bumper yield or high returns if floods did not damage the crop. The area from Velukkara in the South on the Chalakudy River bank in Mukundapuram taluk to Mullasserry of Chavakkad taluk and Tholur-Kaiparamba areas of Thrissur taluk is designated as 'Thrissur Kole' (Francis and George, 2013).

The location is particularly unusual in terms of physiography. The whole track is the result of human-modified fluvial estuarine agencies. The area has no notable relief characteristics, consisting of a large flat ground surface mixed with uplands. The region is saucer-shaped, with plains in the interior and gradually rising elevation

towards the edges. The terrain around the rice fields has steep slopes that are terraced and planted with perennials such as areca nut and coconut, as well as annuals such as bananas, yams, and other root crops. The reasonably flat plateau areas mingle with the hills. The arid plains off the coast of Kole have flat terrain and are covered in coconut plantations (Johnkuty and Venugopal, 1993).

GIS preparation with available mosquito species

Mosquitoes, like other species, exhibit regional variation due to many variables, including environmental variability, habitat, and host preferences (Zhong et al., 2003). Understanding the relationships between habitats, environmental conditions, and the presence of young mosquitoes (Diptera: Culicidae) is critical for effective mosquito management (Devi and Jauhari, 2007). Researchers, government, and business professionals increasingly use Geographic Information Systems (GIS) to deal with spatially connected data (Kistemann et al., 2002). Recently GIS and GPS technology were applied to gather pertinent information on the location and size of mosquito larval sites. These data help estimate insect populations' density, select an insecticidal product, and employ the best techniques to create a logical, environmentally friendly strategy to control nuisance mosquitoes and vector-borne diseases (Zou et al., 2006).

GIS is a system that combines computer hardware, software, and geographic data to gather, store, update, analyse, and display all types of data related to spatial distribution (Kistemann et al., 2002). Thanks to modern information technology, GIS systems can now be integrated with database technology and digital mobile field data-gathering devices with GPS assistance. The ability to link information about where things are (location and geometry data) with properties about what they

are like (attribute data) allows users better to understand the ecology and spatial phenomena and their relationships. Such data might only be evident with sophisticated query, selection, analysis, and presentation procedures (Rydzanicz et al., 2011).

Seasonal variation

Insect pest abundance is largely determined by essential components of any biological system, such as prey-predator relationships and resource competition, especially in juvenile phases. Seasonal variations may affect the availability of resources such as food, breeding sites, and the development of immature insect life forms. Franklin and Whelan, 2009; Tsurim et al., 2013; Hoshi et al., 2014, suggest that this pattern might lead to a shift in the number of people when the seasons change.

Mosquitoes have distinct seasonal rhythms in almost every species. These patterns differ slightly based on the geographic location where individual populations reside. In certain animals, each year only produces a single population. These are known as univoltine species. Even under ideal meteorological circumstances, certain species, such as *Aedes tahoensis*, whose larvae grow on melting snow, have life cycles that do not allow for subsequent generations. Obligatory univoltine is the name given to this phenological pattern. Other species, particularly those with broad geographic ranges, may have generations in warmer portions of their range (multivoltine) but just a single generation in colder areas (monovoltine). However, if the temperature is hot, a second generation may emerge. This pattern is facultative univoltine (Eldridge, 2008). Floods and droughts can trigger mosquito epidemics because they provide breeding grounds for mosquitos whose dry eggs stay viable and develop in

still water. These drastic climate shifts may also diminish the number of natural mosquito predators, increasing adult mosquito populations (Epstein, 2000; Santos et al., 2002).

Physico-chemical parameters and mosquito diversity

Mosquitoes breed in virtually all types of lentic aquatic settings. The aquatic bodies provide a home for mosquito larvae, and the size and nature of these water bodies influence the density and dispersal of the larval mosquito population. The quantity and quality of larval mosquito populations are influenced by food, predators, and environmental competition (Sunahara et al., 2002; Carlson et al., 2004). The breeding environment is critical for mosquito population dynamics since several vital life cycle stages occur, such as larval development, adult emergence, resting, swarming, and adult mating (Dass and Mariappan, 1998).

The type of plant cover, water quality, food availability, and predator abundance influence the larval population density in a water body. Mosquitoes frequently use tiny, shallow water bodies (for example, tree holes and containers), which are abundant in nutrients, have no or deficient dissolved oxygen levels, and have high salinity. Mosquito predator levels (such as fish and aquatic macroinvertebrates) are often inadequate or non-existent in these settings, giving mosquito larvae a haven to survive and grow. As a result, artificial wetlands with high-nutrient standing water and plant cover might provide ideal circumstances for mosquito larval development, causing issues (Tennessen, 1993). Stormwater chemical deposits can be deadly to mosquitoes and predator animals. Mosquito larvae are more tolerant to contaminants than many potential predators and are more likely to re-establish them faster than most other wetland creatures (Russell, 1999).

Assessing larval mosquito habitats in terms of species composition and resources can aid in a better understanding mosquito bio-ecology and related control actions (Aditya et al., 2006). In addition, accurate identification of distinct mosquito species and population monitoring are critical for mosquito management. An intelligent information system would assist us in anticipating sickness and advise government and public health specialists on how to respond (Tsai et al., 2012). The most excellent way to combat these unhealthy circumstances is to better understand the vectors and their habitats. Because good health is a prerequisite for economic progress, placing these vectors in the proper context is necessary.

Barcoding and Phylogenetic analysis of mosquitoes

Mosquito identification in surveillance systems now relies on dichotomous keys for the morphological identification of specimens. This method is time-consuming, needs specialised expertise, and can be challenging when trying to discriminate between visually identical species or identify damaged specimens. DNA barcoding is an additional form of identification that has the potential to overcome these existing constraints (Batovska et al., 2016). Short standardized genomic segments are used as markers for species identification in DNA barcoding. Species differ in their DNA sequences, shape, ecology, and behaviour. As a result, a specific gene or gene fragment may be used to identify a species in the same way retail barcodes uniquely identify each consumer product, at least in theory (Mallet and Willmott, 2003).

In reality, we would anticipate DNA barcoding to function more slowly since genuine DNA sequences are susceptible to all the inherent intricacies of molecular evolution and might exhibit significant variation among species. (Mallet and

Willmott, 2003). Unlike retail barcodes, they are not routinely given to entities one by one. Nonetheless, if successful, DNA barcoding promises to automate specimen identification by determining the sequence of the barcode region, avoiding the complexities of morphological identifications, and prompting proponents to argue for the establishment of a system that could eventually be applied to all life (Tautz et al., 2003; Blaxter, 2004; Savolainen et al., 2005).

Because of its effortless organization, maternal inheritance, and potential to offer more phylogenetic information than individual genes, the mitochondrial genome has been utilized to deduce the phylogeny of numerous taxa (Talavera and Vila, 2011; Cameron, 2014; Hao et al., 2017). Deep-level phylogenetic links have been reconstructed using both mitochondrial and nuclear gene sequences. The ability of different types of sequences to recover clades at different taxonomic levels is a significant problem in molecular systematics (Springer et al., 2001). Caravas and Friedrich (2013) analysed nuclear and mitochondrial sequence partitions from various dipteran species and concluded that mitochondrial sequences should be used even in deep phylogeny reconstruction. The mitogenome phylogenetic analyses were successfully applied to various insect taxa. Species identification was made with several insect orders like Orthoptera (Fenn et al., 2008), Hymenoptera (Cameron et al., 2007), Heteroptera (Hua et al., 2009), and most crucially, the Neotropical Anophelines (Foster et al., 2017). Given the scarcity of molecular-based evolutionary studies of Culicidae species, they used the mitochondrial genomes of 102 representative species to study phylogenetic connections and estimate divergence periods among genera.

The following are unquestionably some of the direct advantages of DNA barcoding:

(i) DNA barcoding offers uniform and sophisticated identification techniques for systematic outputs to the broadest range of end-users possible, including in biomedicine (parasites and vectors), agriculture (pests), environmental testing, and customs (trade in endangered species). (ii) It relieves taxonomists of the additional burden of identifications, allowing them to focus on more critical tasks like delimiting taxa, resolving relationships, and discovering and describing new species. (iii) It pairs up different life phases of the same species (e.g., seedlings, larvae). (iv) It provides a bio-literacy tool for the general public. (Savolainen et al., 2005).

Phylogenetic tree and barcode

Genome taxonomic techniques rely on various DNA sequences for species identification (Kurtzman, 1994; Wilson, 1995). These sequences can be thought of as genetic "barcodes" buried in every cell. Discrimination against life's diversity is a minor issue from a combinatorial standpoint. To produce 100 billion unique identifiers, the Universal Product Codes used to identify retail items use ten different numbers in 11 locations. Although genomic barcodes only comprise four alternative nucleotides at each place, the number of sites that may be inspected is enormous. The assessment of just 15 of these nucleotide sites yields 415 (1 billion) codes, 100 times the number necessary to distinguish life if each taxon were individually branded (Hebert et al., 2003).

Insecticide susceptibility

Culex, Anopheles, Aedes, and *Mansonia* are the most common disease-transmitting and nuisance-causing mosquitoes. The principal mosquito-borne illnesses in India include Malaria, Filariasis, Japanese Encephalitis (JE), Dengue fever, and Dengue hemorrhagic fever (DHF) (William, 2000). Mosquito vectors are the leading causes

of sickness, and death globally, with over 350 mosquito species capable of spreading the infection (Taubes, 1997). Vector management has long been essential to the continuing global campaign to combat mosquito-borne illnesses (World Health Organization, 2000 a,b,c).

Mosquito control with chemical pesticides has been practiced since the 1940s, from the introduction of organic insecticides (Yap et al., 2003). Chemical pesticides are divided into four categories by the World Health Organization: I. organochlorines, II. organophosphates, III. carbamates, and IV. pyrethroids (World Health Organization, 1997). Long-term usage of pesticides, on the other hand, can lead to resistance. World Health Organization (WHO) defined the term resistance as "the capacity of a variant of an organism to resist toxicant dosages that would be deadly to the majority of individuals in a normal (susceptible) population of the species" (World Health Organization, 1957). According to Vythilingam et al., (1992), as insect populations are exposed to pesticides, the frequency of one or more resistance genes in the population rises, leading to the development of insecticide resistance. This resistance poses a significant challenge to mosquito control and disease management.

Genetic variety in the population is formed through mutation, genetic recombination, gene flow, natural selection, and genetic drift that operate on it. Because many disease vectors live in agricultural sectors, they are likely to be exposed to pesticides to control agricultural pests. Agricultural pesticides account for over 90% of all insecticides used worldwide. The widespread use of pesticides in agriculture has raised fears of increasing selection pressure for the evolution of insecticide resistance in disease vectors. This resistance might have a detrimental impact on controlling vector-borne diseases (Overgaard, 2006). Metabolic resistance

occurs when a pesticide undergoes metabolic transformations such as increased detoxification or sequestration, limiting the pesticide's ability to bind with a target insect protein and induce death. Esterases, cytochrome P450 monooxygenases, and glutathione-S-transferases have all been implicated in imparting resistance in several investigations (Vulule et al., 1999; Vontas et al., 2001).

Non-target creatures such as aquatic and terrestrial invertebrates and aquatic vertebrates are incredibly poisonous to insecticides typically employed for mosquito control, and there have been concerns regarding the impact of ULV treatments on these animals (Paul et al., 2005; Amweg et al., 2006; Paul and Simonin, 2006; Weston et al., 2006; Davis et al., 2007; Schleier et al., 2008). This study aims to gather species-specific data on the mosquitoes residing in the Thrissur Kole wetlands, including their seasonal variation. It also involves examining the waterbody's physico-chemical parameters and their relationship with the mosquito population. Additionally, the study will assess the effectiveness of commonly used insecticides on two key vector mosquito species.

Objectives

1. To study the diversity of mosquito species in selected areas of Kole wetlands of Thrissur district and to construct phylogenetic tree.
2. To study the correlation, if any, between physico-chemical parameters of breeding water sample and mosquito diversity in the study area.
3. To study the susceptibility status of *Aedes albopictus* and *Culex quinquefasciatus* mosquito species in Kole wetlands of Thrissur, against conventional insecticides.
4. To study the influence of seasonal variation on mosquito species of Kole wetlands.
5. To initiate Geographical Information System with different mosquito species.

Organization of thesis

Chapter 1: Mosquito diversity in Kole wetlands of Thrissur with GIS, and its Seasonal variation and Influence on physico-chemical parameters.

Chapter 2: Phylogenetic analysis of different mosquito species in Kole wetlands of Thrissur, Kerala.

Chapter 3: A Comparative study on susceptibility of *Aedes albopictus* and *Culex quinquefasciatus* against conventional insecticides

REVIEW OF LITERATURE

Wetland mosquitoes

Wetlands, which are intermittently or permanently covered by water, have a wide range of origins, geographical locations, water regimes, and chemistry. A wetland has characteristics of a distinct ecosystem because it is regularly or sometimes soaked with water. The particular flora suited to its exceptional soil conditions separates wetlands from other land types or water bodies. "Regions of marsh, fen, bog, or water, whether natural or artificial, permanent or temporary, with water that is lentic or lotic, fresh, brackish, or salt, including areas of seawater with a depth of fewer than six meters at low tide," is how the Ramsar Convention defines wetlands. EPA (the United States Environmental Protection Agency) or the Wetlands Reserve Program also defined wetlands similarly to the Ramsar convention. They termed wetlands "those areas that often flood or get saturated with surface or groundwater for long enough to maintain a preponderance of flora typically suited to survive in saturated soil conditions under normal conditions" (Abraham, 2015).

There are three types of wetlands (Chase and Knight, 2003): a) permanent wetlands that never dry, where the predators limit mosquito abundance. b) seasonal wetlands that dry yearly, where specialist predators and competitors are well adapted to predictable drying and thus limit mosquito abundance by lowering emergence rates (as a result of competitor density), increasing larval mortality, or avoiding oviposition. c) semi-permanent wetlands are wetlands that only dry out during droughts (i.e., when precipitation and the water table are at an all-time low). Mosquito predators and competitors are eliminated in such settings; they are

generally linked with permanent waterways and cannot tolerate drying, so they must recolonize after a drought. As a result, the number of wetland mosquitoes might explode, as mosquitoes have short generation rates compared to their predators and can spread swiftly between environments. Mosquitoes demonstrate fast population growth in semi-permanent wetlands in the years following a dry episode before invertebrate predator populations build up.

Wetlands have a unique interaction with mosquitoes, and the larval stages require a constant water supply. On mosquito ecology, there are several reference books. Lounibos et al., (1985) published an early reference book that included community and population dynamics, ecology, epidemiology, and the impact of genetics on the insect's life choices. Service (1993) examined the literature on ecology, sampling, and modelling mosquito populations, and remained the essential reference work. There is also national or regional literature utilized by mosquito control agencies, such as Russell (1993)'s work in southeast Australia, which offers a concise review of the ecology of common mosquitoes and their usual habitat features.

Mosquito Diversity Studies

The discovery that mosquitoes may transmit human filariasis by Patrick Manson in 1877. This work marked the beginning of medical entomology, and the first concrete indication that Arthropods were involved in transmitting human illnesses. Mosquitoes were shown to be involved in the spread of Malaria (1898), Yellow fever (1900), and Dengue (1903) not long after their discovery in 1877 (Philip and Rozenboom, 1973). Mosquitoes have garnered significant attention from entomologists and health professionals worldwide since important findings that they have a role as carriers of human illnesses. As a result, fundamental research, notably

in mosquito taxonomy, received a considerable boost, and entomologists started characterizing and naming many species that had previously been unknown. These investigations have resulted in a large body of literature on mosquito taxonomy, biology, ecology, disease relationships, and other topics, all of which are essential components in the epidemiology and management of mosquito-borne illnesses.

Compared to the rest of the globe, there is less literature about the Indian mosquito. Several publications about Indian mosquitoes were published in the early twentieth century. Grassi (1899) published the first work on Indian *Anopheles* taxonomy, which was followed by publications by Giles (1902), Theobald (1901, 1902, 1910), Liston (1901), James (1902), Cogill (1903), and many more. James and Liston made a significant contribution to the study of Indian *Anopheles*, and they published a book in 1911 that was extensively used as a reference for identifying species. Puri (1931) wrote a comprehensive book on the subject of substantial investigation of Indian *Anopheles* larvae. In the following year, 1932, Edwards wrote "Genera Insectorum," a significant publication on the systematics and classification of the Culicidae. Christophers published 'Fauna of British India' in 1933, describing Tribe Anophelini, and that was a critical revolutionary work in the early 90s. The names and systematics of the species, adult bionomics, breeding habitat, distribution, and vectorial capacity were all covered in depth in this volume. Christopher portrayed and named 43 mosquito species from four subgenera under the genus *Anopheles*.

While *Anopheles* mosquitoes received more attention than Culicines, they were entirely ignored until Barraud (1934) did a great job on British Indian Culicines. From 1923 until his book "Fauna of British India" published in 1934, Barraud published a series of studies in the Indian Journal of Medical Research under the heading "Revision of Culicine Mosquitoes of India". In the Culicinae subfamily,

Barraud recognized 16 genera and 245 mosquito species. There are 110 species and 12 subgenera in the genus *Aedes*. Barraud's 'Fauna of British India' is the best resource for information on Indian Culicinae. The release of these two monumental publications on British India's fauna represented a watershed moment in mosquito research in the Indian subcontinent. India is a tropical nation with a diverse range of flora and fauna. Mosquitoes are insects essential to public health because they spread illnesses, including malaria, filariasis, dengue fever, Japanese encephalitis, and many other viral diseases worldwide. A proper understanding of mosquito population variety, preference habitat selection of vector species, and their distribution would aid in developing an adequate mosquito population management plan and, as a result, the prevention of mosquito-borne illness outbreaks (Amala and Anuradha, 2011a). Mosquitoes (Diptera: Culicidae) are major disease vectors that are emerging and re-emerging. The ability to determine areas of potential pathogen transmission and conduct environmental health assessments in protected and intervened areas, such as areas with human interventions like oil and mining exploration, road and highway construction in forest areas, tourism developments, new urbanizations etc. requires knowledge of mosquito biodiversity (alpha, beta, and gamma diversity) and species distribution (Navarro et al., 2015).

The ability to effectively suppress mosquito populations and, consequently, mosquito threat in public health depends on knowledge of mosquitoes' biodiversity in a given area. This knowledge provides sufficient information on population diversity, distribution patterns, and preferred habitat selection. (Reuben, 1978). It is believed that diversity analysis' alpha, beta, and gamma components are used as an effective way to compare mosquito diversity with landscape structure (Whittaker, 1972). A community's alpha diversity (α) is considered the unique richness of the

homogenous group existing in a landscape. The degree of replacement in the particular composition between distinct landscape communities is referred to as beta diversity (β). Finally, gamma diversity (γ) is defined as the unique richness of the grouped communities that make up a landscape as a result of both alpha and beta diversities. This approach of biodiversity analysis may be used to investigate not just the impacts of climatic, physical, and biological factors on biodiversity but also the consequences of human pressure (Halffter, 1998).

Mosquito management necessitates a thorough investigation of species diversity, composition, habitats, breeding grounds, and biting preferences throughout time and space. It is critical to accurately identify and know the bionomics of the species involved in transmission since understanding faunal diversity and vector management is typically a significant component of disease control. Species documentation is integral to biodiversity study because it helps safeguard genetic resources and control pests and vectors. (Saha and Saha*.,* 2021).

International Diversity Studies

Mosquito populations may increase locally in aquatic settings such as artificial wetlands. While artificial wetlands may be planned and maintained to clean wastewater, mosquito control is crucial, according to Martin and Eldridge (1989). They also mentioned mosquitoes were a big issue in the early days of using oxidation ponds until the application of proper mosquito-prevention measures. In their study on wetland mosquitoes from 2004, Karpiscak et al., outlined several unanticipated corollaries of created wetlands. They spoke about how different types of mosquitoes have a place to live and shelter from the foliage in the shallow marshes. They also reported 12 mosquito species from the Arizona wetlands used for the study. Sarneckis (2002) reported on the physical distinctions between each

wetland and the elements that lead to the formation of prospective mosquito habitats in urban wetlands after researching "Mosquitoes in Constructed Wetlands in South Australia." According to the analysis, wetlands with open water bodies, steep sides, and minimal vegetation had no or very few mosquitoes. In contrast, shallow, protected wetlands having poor water quality, and few predators, can rises a high population of mosquitoes. Schafer researched "Mosquitoes as a Part of Wetland Biodiversity in Sweden" in 2004. The observer concluded that environmental factors such as wetland type, size, and surrounding topography influence mosquito diversity and assemblages and commended mosquitoes as they are a difficult-to-ignore wetlands dweller. Hribar collected mosquitoes through carbon dioxide-baited light traps throughout 2005 to study "Relative abundance of mosquito species on Big Pine Key, Florida, the United States of America". The author reported that 20 different species of mosquitoes had been gathered, and the most often collected species throughout the investigation were *Anopheles atropus, Culex bahamensis, Deinocerites cancer,* and *Ochelrotatus tritaeniorhynchus.*

Dale and Knight (2008) studied a topic on "Wetlands and Mosquitoes, and they concluded that communication and cooperation between mosquito control and wetland management agencies are required to limit mosquito habitats. They also discussed the crucial mosquito control measures practised in wetlands, the climatic environment, land use changes and their adverse impact on public health. "Species composition and dynamics of adult mosquitoes in southern Portugal" was researched by Osorio et al., in 2008. *Anopheles algeriencis, Anopheles, maculipennis*, *Coquillettidia richiardii*, *Culex pipiens*, *Culex theileri*, *Culex univittatus*, *Cliseta annulata*, *Cliseta longiaerolata*, *Ochelrotatus caspius*, and *Uranotaenia unguiculata* were among the 1,14,928 female mosquitoes he discovered. The most prevalent

species were *Ochlerotatus caspius* and *Culex pipiens*. After performing a study on "Mosquito management and wetlands," according to Berg et al., (2010), complete or permanent mosquito eradication is not a feasible or realistic goal since mosquitoes are a natural component of the wetland ecosystem. To the greatest extent practicable, we should avoid creating or maintaining site features that favour mosquito production to decline mosquito populations.

Medlock and Vaux (2011) researched "Assessing the possible implication of wetland expansion and management of mosquitoes in Britain, and summarised the number of mosquito species likely to be influenced by wetland development. Mitigation measures that utilise water and vegetation management affect the life cycle of these species in a range of wetland settings lower mosquito levels without impacting biodiversity accidentally. After completing a study on "Differences in mosquito (Diptera; Culicidae) biodiversity across diverse temperatures and land-use categories in Eastern Spain," Mari and Jimenez (2011) collected and identified a total of 11,279 mosquitoes belonging to 29 species. During their study, they reported the highest mosquito diversity in wetter and non-anthropized areas. Confalonieri and Costa (2012) researched mosquito vector diversity (Diptera: Culicidae) in Caxiuana, Para, Brazil, and found that *Culex portesi* dominated the region, accounting for around 84% of the specimens collected. This study also explained the impact of different diversity indices in the selected mosquito community.

In 2013, Mazzacano and Black researched in the United States on "Ecologically sound mosquito control in wetlands." According to the observer, ecologically sound mosquito management would necessitate good communication between land managers and mosquito control agencies and adaptive management to maximize mosquito control when site characteristics, weather, and biotic community change.

In 2013, 15355 mosquito specimens from 25 species among six genera were collected and identified in protected natural parks in the Valencian Autonomous Region (Eastern Spain). Inland Mountainous Areas (IMAs) had the most variety, indicating a low degree of interspecific dominance in these communities, whereas Coastal Wetlands and Marshes had the lowest diversity (Bernues and Jimenez, 2013). In their study, diversity of mosquitoes (Diptera Culicidae) in protected natural parks from the Valencian autonomous region (Eastern Spain), They collected 25 different mosquito species coming under six genera: *Aedes, Anopheles, Culex, Culiseta, Ochlerotatus*, and *Uranotaenia.*

Ilahi and Suleman (2013), after conducting a study on "Species composition and relative abundance of mosquitoes in Swat, Pakistan," recovered 21 species in 5 genera. *Cx. quinquefasciatus* showed the maximum frequency of occurrence, followed by *An. maculatus*, *Cx. pseudovishnui, An. annularis, An. stephensi*, *Cx. bitaeniorhynchus*, *An. splendidus*, and *Cx theileri* and rest of the species occurred infrequently. They concluded that the most favourable mosquito breeding site is rice fields, river margins, temporary pools, and springs. Lutomiah et al., (2013) conducted a study on "diversity, abundance, and distribution of mosquito vectors in selected ecological region of Kenya: public health implications," and a total of 5, 24,269 mosquitoes belonging to 11 genera and 101 species were collected. Thirty of these species are known vectors of arboviruses endemic to Kenya. The most abundant mosquito collection by site was recorded in Grassia at 37%, followed by Baringo at 31%, with Mt Elgon giving the most petite collection at 0.002%. The most remarkable diverse group was the genus *Aedes*, followed by *Aedomyi Anopheles, Coquelettidia, Eretmapodite, Uranotaenia, Theobaldia*, *Mansonia, Harpagomyia*, *Culex* and *Ficalbia.* Bagheri et al., investigated the West Azerbaijan

Province's marsh mosquito population and their WNV infection. In this investigation, 2143 specimens total 1541 adults and 602 larvae were gathered. *Anopheles maculipennis, Cx. hortensis, Cx. pipiens, Cx. theileri, Culiseta longiareolata*, and *Ochlerotatus caspius* were among the six species collected and identified (Bagheri et al., 2015). Mohlmann et al., identified 40 different mosquito species from southern Sweden, the central part of the Netherlands, and central Italy. Sampling locations often focused on (i) wetlands, (ii) farms, and (iii) peri-urban habitats in three countries at different latitudes across Europe. This study unveiled that the highest species diversity area was Wetlands in Sweden. However, the other two countries recorded a maximum number of species collected from Farm sites (Mohlmann et al., 2017).

Duque et al., investigated the ecology and diversity of mosquitoes in an Amazonian village similar to Kichwa Amazonian communities in Ecuador. They also focused on studying pathogens spreading from forest regions to urban. They used an intense and quick technique to assess the richness, ecology, and probability of mosquito viruses translocating from the forest to the city and the sensitivity of prospective mosquito vectors of illnesses. They concluded the study area resides a remarkable set of mosquitoes (33 species, H' 2.76), including four new species records for Ecuador (Duque et al., 2019).

National Diversity Studies

After Brazil, Indonesia, Malaysia, and Thailand, India is ranked fifth in mosquito biodiversity (Foley et al., 2007). Devi and Jauhari (2005) conducted a study on the habitat biodiversity of mosquito richness in some areas of Garhwal (Uttaranchal), India. They discovered that the area harbours 45 species from 3 genera, with 17

species of *Anopheles*, 15 species of *Aedes*, and 13 species of *Culex* following closely behind. They also noted that areas along rivers or in thick forests had greater mosquito diversity than those closer to non-forested or thin-forested areas. Aditya et al*.,* (2006) studied the Larval habitat and composition of different mosquito species in the Darjeeling Himalayas, India. They identified six immature mosquito species belonging to 4 genera, *Aedes, Armigeres, Culex*, and *Toxorhynchus*, with a significant difference in temporal variation in their relative and absolute numbers.

In three different phytogeographic zones in the Garhwal area of India, Devi and Jauhari, 2007, researched the variety of mosquitoes and their habitation. Thirty-four mosquito species from five genera were gathered over these three locations at different elevations. They concluded that the geographic location and kind of breeding environments could influence mosquito diversity in their study. Dutta et al., (2010a) did a similar survey of Devi and Jauhari in another hilly state of northeast India. They discovered a total of 58 mosquito species under 11 genera; of these, 21 species were recorded for the first time from this Nagaland state, e.g., *Aedes aegypti* and *Culex fuscocephala*. 11 mosquito species recorded earlier were not detected in their study. Dutta et al. (2010b) also surveyed the "Mosquito biodiversity of Dibru – Saikhowa biosphere reserve in Assam, India," and a total of 52 species of mosquitoes under 11 genera have been detected. The genus *Anopheles* was the predominant, followed by *Culex, Aedes, Mansonia, Armigeres, Minomyia, Ochlerostatus, Malaya, Toxorhynchytes, Ficalbia,* and *Aedomyia*.

In Rajathanikottai village of Dindigul District, Tamil Nadu, India, Amala et al., (2011) performed research on mosquito diversity, collecting a total of 1440 mosquitoes from four genera and seven species. Twenty-two mosquito species from

six genera were found. In their study on the Mosquito diversity in the forest ecosystem of Sirumalai hills, Dindigul district of Tamilnadu, Amala and Anuradha (2011a) collected 320 mosquitoes from 6 different species in total. The frequently collected and most common mosquito species during the study were *Aedes, Culex, Anopheles, Armigeres, Uranotaenia* and *Toxorhynchites*. Chilika Lake is a brackish water lagoon located near the mouth of the river Daya, which ends in the Bay of Bengal. This backwater wetland is one of the important Ramsar sites on the east coast of India in the Puri, Khurda and Ganjam districts of Odisha. Dash and Hazra, in 2011, Researched "Mosquito Diversity in Chilka Lake Area, Orrisa, India" and reported 22 mosquito species from six different genera, namely *Anopheles, Aedeomyia, Aedes, Armigeres, Culex*, and *Mansonia*. The results of the biostatistical analysis revealed that the Culicines were more diverse than the Anophelines in the research region.

Amala and Anuradha (2011b) conducted a mosquito diversity study titled "Diversity of mosquitoes in three foothill villages of Sirumalai hills Dindigul, India," They concluded their finding with a collection of 505 mosquitoes from 12 species and 4 genera in total. There was no comparable variation in the alpha diversity index calculated for the three villages. The dominant species in the collection was *Armigeres subalbatus*, followed by two primary vectors, *Aedes aegypti* and *Culex quinquefasciatus*. Sundaravadivelan et al., (2011) studied inter-generic bio-variability and relative abundance of adult female biting mosquitoes in wet and dry land areas of selected villages in the semiarid zone. In this study, *Culex* species was highly noticed, followed by *Aedes*, *Anopheles*, and *Armigers*. Balakrishnan et al., (2011), after conducting a study on "A survey on mosquito diversity in Parangipettai coast, Southeast coast of Tamilnadu, India," concluded

that a total of 337 individuals of mosquito species belonging to 3 genera, five families, and seven orders were collected from all the three habitats. 107 individuals were counted during the summer, 148 individuals during the wet season, and 82 individuals during the winter.

Karthaikairaj et al., (2013) studied on "Biodiversity of mosquitoes in a sub-urban area, Thiruthangal, Viruthunagar district, Tamil Nadu, India," and there were ten species of mosquitoes collected belonging to 4 genera (*Aedes, Anopheles, Armigeres* and *Culex)*. Among these, *Aedes* and *Culex* were more diverse around the study site. Varshini and Kanagappan conducted a mosquito diversity survey (2015) in three places in Tamil Nadu's Kanyakumari region. They identified three mosquito genera: *Anopheles, Aedes*, and *Culex. Anopheles* and *Aedes* mosquitoes are more widespread during the rainy season, while *Culex* mosquitoes were more common during the dry season.

Senthamarai and Jebanesan (2016) proposed a study to reduce mosquito breeding places in the Vellore district of Tamil Nadu to minimise vector-borne illnesses. All probable permanent and temporary water sources were examined to determine the mosquitoes' location, quantity, and species. They collected a total of 696 mosquitoes, including *Ae. aegypti, Ae. albopictus, An. stephensi, Cx. quinquefasciatus,* and *Cx. tritaeniorhynchus*. The principal breeding grounds for mosquitoes were old tyres, still ponds, and various containers. The presence of *Aedes, Anopheles*, and *Culex* mosquitoes suggests the presence of the disease. Knowledge of breeding environments would help develop a mosquito vector control plan. In the northern section of West Bengal, 50 distinct mosquito species belonging to eight different genera have been identified, providing information on their larval habitat. The genus *Anopheles* is responsible for the bulk of the species found and

Toxorhynchites and *Orthopodomyia*, on the other hand, have the smallest number of species. Various lentic aquatic settings have been documented to have immature mosquitoes. Most species were found in the concrete tanks, followed by the temporary pools, ground pools, and tree holes (Saha and Saha, 2021).

Several mosquito species from different genera have been identified in several sites in Kerala. Due to Kerala's notoriety for filariasis and malaria, researchers were enticed to investigate mosquito variety in the early 1900s. The first to note the presence and spread of mosquitoes in Kerala were done by James (1902), Giles (1902), Theobald (1901, 1902, 1905, 1910), James and Liston (1911), Horne (1914), Kamath (1917), Cruickshank and Wright (1914), Brunetti (1920), and Covell (1927, 1931). Iyengar contributed significantly (1938) to this scenario. Iyengar's investigation on the epidemiology of filariasis in Travancore identified several mosquito species from various genera. Covell and Harbhagwan studied Wayanad's mountainous and heavily forested regions in 1938. They discovered 19 species of *Anopheles*. Mathew (1939) also reported many malaria vector varieties in the old Travancore state. Numerous studies have identified the three *Mansonioids* predominating in Kerala (*Ma. annulifera, Ma. uniformis,* and *Ma. indiana*). Iyengar,1938; Singh et al., 1956; Nair and Roy, 1958; Nair, 1962; Chandrasekhar et al., 1976; Kumar et al.,1989 mentioned the vectorial significance of *Mansonia* mosquitoes and their importance in the filariasis spread. 13 mosquito species were also identified in Trivandrum by Daniel et al., in 1986.

In research on "*Aedes* mosquitoes in the arboviral epidemic prone region of Kottayam district, Kerala, India," Jomon et al., (2009) discovered six species of *Aedes* mosquitoes belonging to four subgenera. In terms of dispersion, *Aedes albopictus* out performed all other *Aedes* species. *Aedes* species showed a great

degree of adaptability to different eating behaviours. Jomon and Thomas (2010) published "A study on *Culex* mosquitoes with specific reference to Japanese encephalitis vector in Kottayam district, Kerala, India." They discovered 3662 mosquitoes belonging to 16 species, including 9 Japanese encephalitis vectors. The most common and widely distributed species was *Culex tritaeniorhynchus*. After performing a study on "Mosquito vectors in low-lying regions of Kottayam District, Kerala, India," Jomon and Valamparampil (2013) discovered 3,079 mosquitoes belonging to seven genera and 28 species. Twenty of the 28 species collected were potential disease vectors.

Balasubramanian and Nikhil (2013) studied mosquito fauna in several locations in Kerala's Alappuzha and Kottayam districts to learn more about mosquito variety. They discovered 44 mosquito species belonging to 21 subgenera and 11 genera in Alappuzha, whereas 21 species belonging to 14 subgenera and nine genera in Kottayam. Balasubramanian et al., 2015 investigated the seasonal change in container production and *Aedes albopictus* (Skuse) and *Aedes aegypti* Linnaeus infestation levels in the Alappuzha district. They analysed surveys, 1010 homes, and 3770 water-holding containers during the dry and wet seasons and discovered 1606 pupae and all immatures of *Aedes albopictus*. During July, the highest Breteau and Pupal indices were discovered to be 89.22 % and 39.5%, respectively. All of these indices were highly significant during the rainy and dry seasons.

For six months, from February to July 2016, Thankachan and Gopinath surveyed the variety of mosquito species in plantation regions of the Mananthavady municipal area, Wayanad district. The mosquito larvae and adults were collected and identified using the proper keys. They found 17 different types of mosquitoes, divided into six

genera: *Anopheles, Culex, Aedes, Armigeres, Uranotaenia*, and *Tripteroides*. The most prevalent genus was *Aedes* (10), and *Culex* (3), *Anopheles* (1), *Uranotaenius* (1), *Tripteroides* (1), and *Armigeres* (1) were the following genera (Thankachan and Gopinath, 2017). Understanding the diversity of mosquito species in Kerala's Ernakulam district, Radhakrishnan conducted a mosquito diversity analysis from October 2017 to August 2018. He acquired information about the current species diversity of larvae and adults. The result revealed 26 species of mosquitoes from the six different genera *Anopheles, Culex, Aedes, Mansonia Armigeres*, and *Toxorhynchites*. *Anopheles* (11) was the most prevalent genus, followed by genera *Toxorhynchites* (1), *Culex* (6), *Aedes* (5), *Mansonia* (2), and *Armigeres* (1). *Ae. albopictus Cx. tritaeniorhynchus, Cx. gelidus, Cx. quinquefasciatus,* and *Ma. uniformis* were found throughout the research period. This investigation discovered vectors for filariasis, dengue fever, chikungunya, Japanese encephalitis, and malaria (Radhakrishnan, 2019).

GIS

A geographic information system is a type of information service that enables the input, storage, retrieval, modification, analysis, and output of spatial or geographically linked data. "Remote sensing" refers to any technique for learning about the ground without touching it. The many methods of obtaining sensed data include satellites, radars, and aerial pictures. GPS is a constellation of twenty-four satellites that permits the precise measurement of coordinates at any place on or near the earth's surface (Kamel et al., 2011). GIS is a broad word encompassing a wide range of datasets from several sources, including RS and GPS. As a result, GIS is frequently referred to as the basis of spatial technology, with the ability to analyse

combined datasets and display the results as meaningful information to aid decision-making (Saxena et al., 2009).

GIS has been used with great precision for mapping, monitoring, visualizing, retrieving, analysing, and modelling geo-referenced data. GIS has been shown to map vector diversity and ecology, illness prevalence, disease transmission, and regional dispersion, among other things (Zhou, 2007). Furthermore, the effectiveness of GIS for disease surveillance and health data administration is unrivalled. Using an Application Programming Interface (API) to extract epidemiological disease data from a web mapping GIS is critical. This is possible because to the integrated customized online mapping GIS (ASP, Net, HTML, Java, Python, CSS, PHP, Arc IMS, Geo ext, C, C++, Visual Basic, Arc objects) with user interface capabilities for browsing, querying, and table sorting (Palaniyandi, 2013).

The current global warming phenomenon is well-known, and we are already seeing changes in the ranges of native nuisance mosquito species due to global climate change. Additionally, there are now confirmed cases of tropical Asian mosquitoes, the introduction of new diseases, and the reappearance of formerly endemic vector-borne viruses at higher latitudes, including in the Netherlands and Germany. (Scholte et al., 2007; Becker, 2008; Pluskota et al., 2008). We are seeing changes in ecological niches that are becoming more appropriate for mosquitoes with medicinal and veterinary significance (Gratz, 2004). Databases may help to anticipate future vector-borne illness distributions due to global temperature change and the likelihood that the vectors are native nuisance mosquitoes or immigrant species found only at lower latitudes (Schafer and Lundstrom, 2009).

Numerous studies have demonstrated the value of GIS and remote sensing in locating the aquatic habitats of vector mosquitoes (Zou et al., 2006; Caphina et

al., 2009). Realistically, mosquito control strategies most compatible with the area's cultural and economic conditions are more likely to be adopted. However, the approach should be tailored to the requirements and resources available, with the primary goal of preventing mosquito annoyance and the potential or actual transmission of vector-borne disease. It should also use cutting-edge technologies. They compared the relevant biological parameters (larval emergence, abundance, and development) of the various nuisance mosquitoes (Diptera: Culicidae) involved in the GIS database of mosquito breeding sites in irrigation fields in Wroclaw, Poland, and improved the efficacy and sustainability of the control program. GPS allows for better alignment with terrain characteristics and boosts accuracy. This map depicts the specific locations of mosquito breeding sites and their estimated sizes based on geometry. Using microbial larvicidal chemicals enables more accurate, better-timed aerial treatments and cost reductions. This approach should make it more difficult for prospective invading pests or carrier mosquitoes to develop, aiding the management of local species (Rydzanicz et al., 2011).

To record infestation by the two species and their ecological preferences, Kamgang et al., 2013 conducted comparative research in three Cameroonian towns (Sahelian domain: Garoua; equatorial field: Douala and Yaounde). There were high and varying levels of pre-imaginal *Aedes aegypti* and *Aedes albopictus* infection. They incorporate GIS with their collected data to describe the pattern of variation in various regions and future vector control strategies (Kamgang et al., 2013). The Indian subcontinent has a diverse geographical environment to sustain a large population and faces significant challenges in vector-borne disease outbreaks. India has become an endemic host for filariasis parasites and a hotspot for malaria, dengue, chikungunya, and JE viruses. The geographical variation of vector

biodiversity, vector abundance, and the active infection state of vector-borne disease transmission, as well as monitoring for epidemic control and management, are mapped using integrated hybrid approaches of remote sensing, GPS, and GIS (Palaniyandi et al., 2016).

The presence of favourable mating, survival, and longevity conditions is the main factor influencing how much vectors propagate. Many facets of environmental change may now be remotely observed because of advances in remote sensing technology. When combined with geographic information systems (GIS), these technologies can define local and landscape-level variables affecting disease and vector dispersion. Aruna et al., (1998) described a coastal malaria vector species utilising the GIS distribution of *Anopheles sundaicus*, and the results were quite encouraging. Thematic maps of ecological characteristics, such as forest cover, height, rainfall, and temperature toposheets, were digitised by the Survey of India. The optimum range of each environmental parameter for *An. dirus* was found and retrieved from digitised maps using GIS (Srivastava et al., 2001).

In 1967, 1970, 1982, 1988, and 1996, Delhi saw dengue epidemics caused by distinct dengue virus strains. Dengue virus types 1, 2, and 3 have all been found during previous dengue outbreaks in Delhi, but one form has usually predominated. In 1996, one of the most significant outbreaks of DHF/DSS in North India occurred in Delhi and surrounding areas, with the Den-2 virus being the primary circulating serotype. For researching surface climatic conditions for vector population modelling, a combination of high spatial resolution data for land use and land cover categorization and regular coarse resolution environmental satellite data for monitoring environmental fluctuation would be optimal. Using geotagged dengue cases reported from homes in North East (NE) Delhi, an analysis was conducted to

determine the parameters that significantly influence modelling high dengue-prone regions. Furthermore, this information was utilized to identify hotspots by utilizing the GPS locations of dengue-prone and confirmed cases in 2013. Based on the investigation findings, they sought to simulate the locations that are sensitive to dengue cases. They also tried two distinct techniques to model the eventual effects of dengue cases in the form of sensitive zones. The results of their study will help prioritize effective preventative and control measures in locations across India that are similar (Prakash and Kumar, 2014).

In order to create maps for identifying high-risk areas and implementing disease management at the appropriate time and in the appropriate manner, RS (Remote sensing) and GIS (Geographical Information System) offer excellent opportunities to collect data on a various environmental factor, such as vegetation and water bodies, vector abundance, and changes in land use and land cover. Kumari and Kant devised a project to create thematic maps of six chosen villages in Rohtak and Mewat districts utilizing RS and GIS technologies between 2008 and 2013. The high Annual Parasite Incidence (API) of the villages Kalanaur (3.48) in Rohtak district and Ujina (5.1) in Mewat district was strongly associated with the number of water bodies in the respective villages, indicating appropriate circumstances for Anopheline mosquito development. As a result, they highlighted the risk variables linked with high malaria transmission in six PHCs in Haryana's Rohtak and Mewat districts, allowing for the development of targeted malaria control measures (Kumari and Kant, 2016).

However, there still needs to be a more comprehensive understanding regarding the distribution and variety of mosquito-associated viruses and their associated vectors, notably in China. Atoni et al., 2020 gave the first thorough dataset of the variety and

distribution of these viruses and their associated vectors in China to improve their understanding (including Taiwan, Hong Kong and Macau). Quality-control procedures were used, and geographic data on the prevalence of mosquito-associated viruses and associated mosquito vector species were retrieved. This collection includes 2,428 records of geo-referenced mosquito species and mosquito-associated virus occurrences at various administrative levels in China. Japanese encephalitis, Dengue, Banna, and Culex flavivirus are the most common mosquito-associated viruses. *Culex tritaeniorhynchus*, *Aedes albopictus*, and *Culex pipiens pallens* are the most common mosquito vectors. This geographic dataset provides an overview of the distribution and variety of viruses linked to mosquitoes in China, and it is also helpful for other spatial and risk-assessment analyses.

Barcoding

Accurate species classification and identification are crucial for biological study. The species were recognised and classified using a morphological key-based method. However, there are several problems with conventional morphology-based taxonomy methods. A novel and straightforward approach to taxon identification is needed due to the apparent limitations of conventional taxonomic identification techniques. Mosquitoes continue to be a significant source of annoyance and the transmission of fatal illnesses to humans. The study of vector-borne illness surveillance and management requires accurate identification and categorisation of mosquito vectors. Traditional morphology-based identification methods take time and need more accurate identification to identify species. As a result, a multidisciplinary strategy incorporating morphological and molecular approaches is required (Paramasivan et al., 2013).

Recognising, categorising, and labelling organisms primarily depends on physical characteristics. Carl Linnaeus created it in the 18th century, and his taxonomic method is still widely practised. Taxonomists now consider physiology, behaviour, and population biology in categorizing new species. Genetic variation has played a vital role in distinguishing the diversity of life since the discovery of DNA and acknowledging its significance in heredity. Morphological identification of species is restricted since it ignores phenotypic plasticity, individual genetic diversity, or morphological complexity (for example, cryptic taxa or keys designed solely for a specific gender or life stage) (Hebert et al., 2003). DNA-based identification can fill in these gaps while adding additional biological miscellany to what is currently known. Furthermore, in taxonomic investigations, molecular-based approaches are commonly employed for species identification of viruses, bacteria, and protozoa (Pace, 1997; Edwards and Rohwer, 2005; Adl et al., 2007). Whole genome sequencing and other sequencing-based technologies are used to discover diversity in a broader sense. Another method is to look for biological markers like microsatellites, AFLP, or SNPs (Arif et al., 2010). However, this strategy is inappropriate and would be costly and time-consuming because of the biological variety of taxa like Arthropoda. Analysis of tiny gene segments, often known as DNA barcoding, provides a better technique to distinguish species (Hebert et al., 2003; Stoeckle, 2003).

It has been questioned whether gene segment would be the most fantastic match for this sort of inquiry because the rates of molecular evolution vary depending on which piece of a genome is being examined. However, since it lacks introns and seldom undergoes recombination, the mitochondrial genome appears to be more suited for barcoding than the nuclear genome in mammals. The maternal inheritance

of mitochondrial genes is also advantageous for barcoding (Saccone et al., 1999). Barcoding research has previously concentrated on mitochondrial ribosomal (12S, 16S) DNA genes. However, insertions and deletions (indels) are prevalent in these genes, confounding sequence matching (Doyle and Gaut, 2000). The ITS2 region of nuclear ribosomal DNA appears to be a superior candidate for barcoding in plants and fungi (Chen et al., 2010).

It is challenging to choose which of the 13 protein-coding mitochondrial genes to use in mammals. Prior barcoding studies have focused on the cytochrome b oxidase gene. However, another gene that needs attention is COI (Hebert et al., 2003). Because it contains sections with very conserved sequences, universal primers created for this gene are reliable. (Folmer et al., 1994). COI's phylogenetic signal is more vital than that of other mitochondrial genes. It appears to have a higher output, and base substitution at the third position of a nucleotide is more common. As a result, the gene's pace of evolution is relatively rapid (Knowlton and Weigt, 1998; Stoeckle, 2003).

The first evolutionary tree of Culicidae was constructed by Ross (1951) based on an intuitive interpretation of comparative bionomics and morphological data. The tree reflected the traditional division of the family Culicidae into three subfamilies: Anophelinae, the basal lineage; Toxorhynchitinae, the intermediate lineage; and Culicinae, the prominent derived lineage. The relationships illustrated by Ross (1951) are those more or less traditionally accepted and unchallenged by later workers until Harbach and Kitching (1998). The ability to precisely identify the target species has immediate medical and practical ramifications, particularly when establishing vector control measures.

Mosquito taxonomy has traditionally relied on morphological traits, cytogenetics, and isoenzyme markers. The molecular technique has recently considerably increased species identification accuracy (Kumar et al., 2007). DNA can be obtained and analysed from specimens in all developmental stages of both sexes, fresh, preserved in alcohol, dried, or frozen. These also make genomic DNA-based molecular species identification advantageous because they can be applied to specimens and situations unsuitable for morphological taxonomy (Marrelli et al., 2006). Cytochrome c oxidase subunit 1 (COI) is one of the mitochondrial genes thought to have the most reliable amino acid sequence, making it beneficial for taxonomic research (Knowlton and Weigt, 1998).

Traditional identification methods have drawbacks in distinguishing sibling and closely related mosquito species and depend on life stage and specimen quality. However, DNA-based identification methods using molecular markers such as the nuclear ribosomal internal transcribed spacer (ITS), which does not require intact or unharmed specimens, could overcome these limitations. Genomic DNA is often extracted from complete mosquitoes, legs, wings, and other body parts. By amplifying the ITS markers, the study looked at other sources for genomic DNA isolation, such as eggshells, larval and pupal exuviae. In a study, the ITS2 marker was also shown to distinguish *Aedes aegypti* and *Aedes albopictus* by creating amplicons of 330 and 520 bp. Genomic DNA from these other sources was also used to support the species-specific PCR to distinguish the *Culex vishnui* subgroup of mosquitoes (Dhananjeyan et al., 2010).

2012, Wang et al., using DNA barcoding, identified the most common mosquito species in China. Based on morphological criteria, 404 mosquito specimens were gathered and designated 15 genera, 122 species, and subspecies. Despite the

sampling site, members of the same species clustered together in a Neighbourhood-Joining tree based on COI sequence similarity. Daravath et al., amplified and sequenced the COI gene molecular sequence in Hyderabad in 2013. The data was reported to the NCBI gene repository and revealed that the COI gene is identical to that of several geographically distributed *Aedes albopictus* species. Within the Indian population of *Aedes albopictus*, the Hyderabad area species displays substantial diversity in amino acid proportion. In order to create a barcode library for mosquitoes, Ashfaq et al., conducted the first DNA-based examination of mosquitoes in Pakistan in 2014 and discovered that genetic diversity levels vary between species.

The cytochrome oxidase subunit I (COI) gene of *Armigeres subalbatus* was sequenced by Bindu and Sebastian in 2014 to determine its connection to other mosquito species and to provide a database for genetic barcoding of *Ar. subalbatus.* COI gene fragment from *Armigeres subalbatus* gave rise to a 522-bp product uploaded to GenBank. The *Ar. subalbatus* from Kerala was identical to the *Ar. subalbatus* from Pakistan, demonstrating no regional variation. Full-length DNA barcodes (658 bp) were constructed from 172 wild-caught *Culex* specimens taken from 11 Turkish regions by Gunay et al., 2015.

Ismail et al., (2016) used molecular methods to investigate the genetic diversity of *Aedes albopictus* in two dengue-infested locations in Subang Jaya, Selangor: Taman Bukit Kinrara (TBK) and PJS7. Cytochrome oxidase 1 (CO1) genes obtained from field-collected mosquitoes were examined and compared to the USM laboratory strain (F135), as well as sequences from the GenBank. The field-collected mosquitoes from TBK and PJS7 are genetically identical, according to the findings of this study. Singh and Vashist, 2017 investigated both nucleotide

sequence and the proportion of a specific nucleotide since both metrics are critical for understanding variance across four Anopheline mosquito species. They discussed how mitochondrial DNA-based identification has consistently been a reliable technique. The COII gene has been discovered to be highly beneficial in identifying and differentiating *Anopheles* species.

Diez-Fernandez took mosquito larvae and adults from southern Spain in 2018 and used a molecular technique to amplify and sequence a segment of the mosquito's cytochrome c oxidase subunit one gene (barcoding region). The mosquito sequences isolated in Spain had a 99% similarity with sequences from two *Aedes* mosquito species, *Aedes vittatus* and *Aedes cogilli*, and a 94 % similarity with sequences from other *Aedes* species, according to a blast comparison of the mosquito sequences isolated in Spain with those deposited in public databases. *Aedes cogilli* is only known to exist in India; there are no records of this species in Europe.

After being molecularly typed in Portugal, *Aedes albopictus* sequences from other parts of Europe showed significant similarity in early phylogenetic analyses. Arboviral RNA was examined in adult mosquitoes, and a quick monitoring response was started locally to assess its dispersion and abundance. A total of 103 specimens were collected, with 52 immatures and 51 adults. There were no harmful viruses found. Even though the acquired data indicate a low abundance of the population introduced locally, the possibility of *Ae. albopictus* dispersion and probable establishment in Portugal have aroused concerns about autochthonous mosquito-borne illness epidemics (Osorio, 2018). Being a tropical island in southern China, Hainan is vulnerable to outbreaks of sickness caused by mosquito-borne viruses. Most mosquito species on Hainan Island lack population genetic diversity. Li et al., 2020 describe the genetic diversity of the leading species of mosquito in Hainan as

well as the variety of other mosquito species. In 2018 and 2019, field populations of adults or larvae were gathered from 12 areas on Hainan Island. 1,228 identified mosquito samples were utilised to sequence gene fragments of the mitochondrial Cytochrome C oxidase subunit I (COXI). Nine unconfirmed mosquito species and 23 recognised one species from *Aedes, Armigeres, Culex, Mansonia,* and *Anopheles* were found. The mosquito species with the highest population densities in Hainan were *Aedes albopictus, Armigeres subalbatus,* and *Culex pipiens quinquefasciatus.*

Insecticide Susceptibility

DDT (Dichloro-diphenyl trichloroethane) was initially used to control mosquitoes in 1946, and the first example of DDT resistance was discovered in insects in 1947. More than 100 mosquito species have been resistant to one or more insecticides, with Anophelines accounting for more than half of them (Sajith et al., 2015). Many vital improvements in insect, vector and disease management have resulted from the creation and evolution of the chemical industry. However, the benefits of these advancements have yet to be felt equitably across the world. Pests and vectors continue to cause issues in many underdeveloped nations and some industrialised countries where scientific remedies exist. Dinitro-o-cresol, the first synthetic organic insecticide, was initially used in 1892, and by the 1930s, a variety of similar chemicals had been created and found limited usage (Cremlyn, 1978). Insecticides' increased efficacy as instruments for pest control has led to their rising domination as the favoured strategy for insect management since the 1920s. Towards the end of the 1930s, a succession of remarkable discoveries introduced new synthetic insecticides with massive potential for broad usage, reinforcing the emphasis on a chemical approach to pest control. Dichlorodiphenyltrichloroethane, better known as

DDT, was launched in 1939 as the "wonder" pesticide of the chlorinated hydrocarbon group (Becker et al., 2010).

The development of organophosphates (OP) in Germany followed DDT which had been in the works since Lange and von Krueger produced organofluorophosphate esters in 1932. In the 1950s, carbamates were created in Switzerland. The discovery and establishment of photostable pyrethroids, primarily in Japan and the United Kingdom, in the 1960s and 1970s resulted in significant modifications in pesticide use. These highly effective, biodegradable chemicals may be applied at rates as low as 20 g/ha in the field, which is 10–100 times lower than traditional insecticides and results in a smaller residual load in the environment (Becker et al., 2010).

Spending more than a hundred billion dollars per year on vector-borne disease impact on humans and the global loss caused by insects, controlling these organisms became critical for the long-term improvement of human health, agricultural sectors, and related industries. Pesticides have become an essential component of globally integrated vector control programs due to addressing these critical human needs. Insecticides used to control mosquitoes are classified into four chemical groups: chlorinated hydrocarbons, organophosphates, carbamates, and pyrethroids, and an additional class known as insect development regulators (IGRs) (Becker et al., 2010).

The earliest generation of insecticides comprised arsenicals like stomach poisons. Second-generation insecticides were generally contact type, including chlorinated hydrocarbons, organophosphates, carbamates, and pyrethroids. Williams proposed in 1976 that analogues (juvenoids) of juvenile hormones may be utilised as insect-specific control agents against which pest species cannot acquire resistance after his significant research into the diverse physiological effects of this juvenile hormone.

This array of chemicals was dubbed "third-generation insecticides" by him. The entomopathogenic properties of several microorganisms have given rise to the fourth generation of insecticides. Since the outset, commercial bacteria-based preparations have been offered. Standardized bacteria-based solutions have been available since the early 1980s and are essential to mosquito control management (Becker et al., 2010).

Mosquitoes, fleas, lice, sandflies, tsetse flies, and triatomine bugs are all key disease vectors controlled with insecticides. In 1955, the World Health Organization (WHO) advised that residual house-spraying of DDT be used to eradicate malaria, the most common vector-borne human illness. The pesticide euphoria, however, faded quickly, and the WHO formally switched from malaria eradication to malaria control in 1976. This significant transition from malaria eradication to primary health care was a contentious subject, prompting a dramatic shift in WHO language. Various factors influenced this choice, including the emergence of DDT resistance in a wide range of mosquito vectors. In 1975, the WHO estimated 256 million people lived in areas where malaria control efforts were hampered by DDT and BHC resistance. (This did not include the African area, which accounts for 90% of malaria cases and has already seen DDT resistance in *Anopheles gambiae*, the primary malaria vector). Resistance concerns continued with the advent of more recent pesticides like organophosphates, carbamates, and pyrethroids. Many control programmes have switched from spraying pesticides liberally inside homes to concentrating their activities on bed nets. Pyrethroids are the insecticides of choice for targeted spraying because they have the rapidity of death needed to protect the person within the bed net and the safety margin needed for insecticides used in close proximity to people. The current focus of resistance research is on rational resistance

management and molecular resistance mechanisms to avoid the development and spread of resistant vector populations. In an effort to "roll back" malaria, WHO and the World Bank have initiated large initiatives in Africa with the assistance of other crucial institutions and the scientific community. One of the main challenges addressed by these initiatives is the existence of two developing foci of pyrethroid resistance in the most prominent African malaria vector, *An. gambiae* (Hemingway and Ranson. 2000).

Organophosphates (OPs), carbamates (Cs), organochlorines (OCs), and pyrethroids (PYs) are the most common pesticide classes. In general, they work by poisoning insects' neural systems, which are pretty similar to mammals' neurological systems. Because of its small size and quick rate of metabolism, a small dose of pesticide can be lethal to an insect. Although such a concentration is not deadly to humans, it can cause harm. Due to similarities in the components of the nervous system, non-pest insects, humans, animals, and pets may be harmed by insecticides, making it very impossible to develop insecticides that only affect insect pests (Prato et al., 2012).

OCs have variable chemical structures but always have chlorine and chlorinated hydrocarbons. These organochlorine pesticides especially DDT had variety of drawbacks and benefits. OCs have significant unexpected end results despite the advantages of being affordable and efficient against target species. OCs are incredibly stable, slow to decay in the environment, soluble in fats, and appear nontoxic to animals, and they were formerly considered perfect. However, the movement of ions, including calcium, potassium, chloride, and sodium, across neurons is altered and disrupted by these chemicals. Regrettably, permanence and fat solubility are also considered unfavourable characteristics: OCs may bioaccumulate in the fat of humans and animals as they go up the food chain. These

pesticides' widespread usage and conveyance pollute animals worldwide, even in the Arctic and Antarctic, where OCs are not utilised. One of the pioneer symptoms of the unexpected repercussions is a decrease in the number of birds that hunt on animals exposed to DDT. DDT causes an unexpected weakening of bird eggshells, resulting in the mortality of babies. OCs like DDT is now mostly outlawed in developed nations, but they are still made and used in underdeveloped countries, where they are exposed to the formers (Prato et al., 2012).

In general, phosphoric acid esters are referred to as OPs. These compounds were created as potentially dangerous biowarfare weapons in the 1940s (nerve gases). Many countries kept contemporary derivatives like sarin and VX that provide complex management problems today. A wide range of OPs has been developed to search for pesticides that target certain insect species while being less harmful to mammals. OP parathion was first used as an alternative to DDT because it was believed to be more effective and sensitive. As a result, parathion has a higher short-term (acute) lethality than DDT, which has led to several human fatalities. The carbamate ester is the functional group is present in carbamates. OPs and Cs influence, a neurotransmitter, called acetylcholine that is crucial for nerve cell transmission that is common components in both insects and animals. One nerve cell produces acetylcholine, which activates another, through synaptic transmission. Although this stimulation needs to stop quickly for proper transmission of nerve impulses. Acetylcholinesterase, a particular enzyme, that can break down acetylcholine. OPs and Cs, block this enzyme which causes nerve cells to malfunction, and hence the name acetylcholinesterase inhibitors given to these pesticides. structural changes between the different OPs and Cs determines the effectiveness and degree of acetylcholinesterase blocking. Nerves are incredibly

efficient in inhibiting acetylcholinesterase permanently, whereas pesticides only do so momentarily. These pesticides' toxicity poses serious health risks, and researchers are working to produce new insecticides with lesser unforeseen effects (Prato et al., 2012).

PYs are the most recent class of insecticides, which were developed in the 1980s. Their ancient forms were commercially used in the 1800s, named pyrethrum, the natural extract of Chrysanthemum flowers. Over the last two decades, their use has risen steeply. Like OCs, OPs, and Cs, the primary target of PYs is the neurological system by influencing the transport of sodium ions (Na+) into and out of nerve cells that become hypersensitive to neurotransmitters. However, the chemical structure of PYs is different from OCs, OPs, and Cs, and they can have various harmful effects on insects and even mammals due to structural variations. PYs last longer in the environment than natural pyrethrum, which is unstable in sunlight and breaks down (Prato et al., 2012).

When utilising currently available pesticides to control malaria, insecticide resistance is a severe issue (Enayati and Hemingway, 2010). For instance, DDT was employed initially to manage mosquito populations in 1946; however, the first instances of DDT resistance were noted in 1947, and DDT resistance at various degrees has since been observed for more than 50 species of *Anopheles* mosquitoes, including several malaria vectors (Hemingway and Ranson, 2000). Since resistance tends to develop after pesticide switchovers, adopting new insecticides for malaria control, such as OPs, Cs, and PYs, only slightly improved the malaria management approach (Hemingway and Ranson, 2000). DDT usage in agriculture was formerly believed to be the leading cause of resistance to malaria vectors since agricultural regions breed many malaria vectors (Mouchet, 1988). It is currently believed that

using synthetic PYs worsens DDT resistance. (Diabate et al., 2002). DDT and PYs both have the same target, making the construction of a cross-resistance mechanism easier (Martinez-Torres et al., 1998). Furthermore, evidence of increasing resistance gene frequency due to IRS or ITN programs is concerning (Karunaratne and Hemingway, 2001). PYs, the only class of insecticide authorised to use on ITNs (Zaim et al., 2000), are progressively used in African IRS programs. Moreover, there has been a dramatic increase in reports of PY resistance in malaria vectors over the past decade (Santolamazza et al., 2008); additionally, PYs are popularly used in regulating agricultural pests around the world (Ranson et al., 2011).

In most cases, pesticide resistance is caused by two primary mechanisms: a) It keeps changing the insecticide target site and reduces its binding; b) it increases rates of insecticide metabolism (changes in the levels or impacts of detoxification proteins) and reduces the insecticide's ability to reach the target site (Hemingway et al., 2004; Ranson et al., 2011) These processes, alone or in the mixture, resulting in an extraordinarily high level of resistance to all existing pesticide classes (Hemingway et al., 2004).

International Susceptibility studies

Following a study on the "Malathion susceptibility test of *Anopheles stephensi mysorensis* in southern Iran" in 1974, Manouchehri, concluded that *An. stephensi mysorensis* is still susceptible to malathion after ten years of application in homes at a rate of 2g/m2 2-3 rounds per year, and adult susceptibility remains at the same level as it did in 2003. In their study "Pesticide susceptibility/resistance status in *Aedes* (stegomyia) *aegypti* and *Aedes* (stegomyia) *albopictus* (Diptera: Culicidae) in Thailand," Jirakanjanakit et al. 2017 discovered that the chosen mosquito species varied in their susceptibility to insecticides. The pesticide tested was effective

against *Aedes albopictus*. After completing an "Insecticide susceptibility test of A*nopheles minimus s.l, Aedes aegypti, Aedes albopictus*, and *Culex quinquefasciatus* in northern Thailand," Somboon et al. (2003) found that *Anopheles minimus s.l* was still susceptible to DDT and permethrin.

In a study titled "Susceptibility and irritability levels of main malarial vectors to synthetic pyrethroids in endemic areas of Iran," Borhani (2004) found that *An. stephensi* were susceptible to all tested insecticides except DDT, while *An. culicifasciatus* was susceptible to all tested insecticides. In same year, Vatandoost et al., also calculated the *Cx. quinquefasciatus* mosquito's sensitivity to several pesticides in both lab and field settings. They took mosquitoes out of drainage water to treat them as field strains and performed susceptibility tests under WHO guidelines. They concluded that the *Cx. quinquefasciatus* lab strain exclusively exhibited resistance to DDT. The insecticides DDT, bendiocarb, malathion, permethrin, deltamethrin, lambda-cyhalothrin, and etofenprox were ineffective against the strain from the field. The field-collected mosquitoes exclusively displayed cyfluthrin sensitivity. These results suggest that frequent pesticide usage for agricultural and domestic pest management may result in resistance in the wastewater mosquito, *Cx. quinquefasciatus*.

Pridgeon et al., (2008) conducted an experiment in which 19 pesticides with different modes of action were assessed against *Aedes aegypti, Culex quinquefasciatus* Say, and *Anopheles quadrimaculatus* Say in order to calculate the relative efficacy of pesticides in controlling adult mosquitoes in Florida. They tested the effectiveness of 19 insecticides with various modes of action on adult *Cx. quinquefasciatus* Say, *An. quadrimaculatus* Say, and *Ae. aegypti*. Results showed that different mosquito species had varying susceptibilities to pesticides,

demonstrating the necessity of selecting the most effective substance for the mosquito species that were least vulnerable to control mosquitoes successfully.

In 2010, Husham et al. set out to investigate the dengue vector *Ae. aegypti*'s pesticide susceptibility in Port Sudan City. Nine entomological stations were chosen to collect *Aedes aegypti*, which were in their aquatic stage, and collected testing samples. *Ae. aegypti* mosquitoes were gathered and treated following WHO standards. Technical malathion (5%) and lambda-cyhalothrin (0.05%), deltamethrin (0.05%), DDT (4%) and bendiocarb (0.1%) were the pesticides studied. Results discovered that *Aedes aegypti* was sensitive to the insecticides deltamethrin 0.05%, bendiocarb 0.1%, tolerable to the lambda-cyhalothrin 0.05%, and resistant to the insecticides DDT 4% and malathion 5%, respectively. They concluded that the control of pesticide resistance requires the use of synergy. In addition, new non-chemical alternatives must be employed, considering the increase in mosquito population resistance.

In Khartoum, Sudan, Abdalmagid et al., (2011) attempted to determine the sensitivity of adult and larval *Cx.quinquefasciatus* mosquitoes to the insecticides malathion, temephos, lambda-cyhalothrin, and permethrin. Malathion and the two pyrethroids showed resistance when used against *Cx. quinquefasciatus.* The average KDT 50 genes are thought to be high and show resistance. However, it was shown that larval stages were sensitive to temephos. They recommended that as a management technique to extend the effectiveness of the present pesticides, the vector control unit should think about using insecticide rotation. In addition, it was advised to use pesticides rationally and regularly check for insecticide resistance to slow the development of this problem. Ekloh et al., (2013) planned to evaluate the pyrethroid resistance of *Cx. quinquefasciatus* mosquitoes in some western regions of

Ghana. Long-lasting pyrethroid-treated nets are used in Ghana to suppress malaria vectors. Operations to control malaria may be impacted by the high degree of pyrethroid resistance seen in some West African *Culex quinquefasciatus* populations. The study aimed to determine how vulnerable *Culex* mosquito populations towards deltamethrin (pyrethroid pesticide) in various areas of Ghana's Western region. Female mosquitoes were aged 1-3 days who had not been fed were subjected to bioassays adopting WHO diagnostic test kits and methods. The findings show a considerable variance in the KDR gene of tested mosquitoes gathered from various regions of Ghana.

Greece and other European nations saw a high rate of mosquito-borne illnesses such as the West Nile Virus (WNV) in the 2010s. Fotakis et al., 2017 declared to explore the mosquito species composition and population dynamics in locations more susceptible to the spread of vector-borne diseases. They also looked into the pesticide resistance of the main pests and disease vectors. They observed a high density of mosquito species in the study area, most of which were vectors for the spread of illness. Interestingly, they found some pesticide resistance in these mosquitoes. They said in their final remarks that not just vector control programmes; Conversely, mosquitoes may develop this type of resistance via pesticide usage in agriculture.

Oyewole et al. (2018) used World Health Organization suggested Pesticides to evaluate the sensitivity status and knock-down data of local *Anopheles* mosquito species in Southwest Nigeria. *Anopheles* species larvae were gathered from four villages in Ila-Orangun from naturally contaminated water sources. Adult females subjected to tests with diagnostic kits regarding susceptibility to six insecticides (0.05% lambda-cyhalothrin, 0.75% permethrin, 0.05% deltamethrin, 4% Dichloro-

Diphenyl-Trichloroethane (DDT), 1% fenitrothion, and 0.1% bendiocarb). It was discovered that *Anopheles gambiae* was vulnerable to fenitrothion and bendiocarb but resistant to lambda-cyhalothrin, permethrin and deltamethrin, and DDT. The sorts of pesticides/insecticides used for agricultural operations and public health initiatives in the research region may be responsible for the susceptibility pattern seen. To prevent the spread of insecticide resistance and ensure the effectiveness of the intervention programmes, it is necessary to routinely evaluate the susceptibility pattern of the insecticides employed in malaria vector control techniques.

In Bangladesh, arboviral illnesses like dengue and chikungunya create serious public health risks. Al-Amin et al., 2020 intended to assess the prevalence of pesticide resistance in *Ae. aegypti,* explore the potential roles of detoxification enzymes, and change target site sensitivity as resistance mechanisms. Permethrin, deltamethrin, malathion, and bendiocarb bottle bioassays were carried out with female field-collected mosquitoes. They talked about their findings with *Ae. aegypti* populations had high levels of permethrin resistance, with diagnostic dosage mortality ranging from 0% to 14.8%. Higher permethrin dosages caused significant resistance (5.1-44.4% mortality). The need for adopting alternative *Ae. aegypti* management methods were highlighted by identifying widespread pyrethroid resistance, several resistance mechanisms, and regular monitoring of *Ae. aegypti* susceptibility patterns in Bangladesh with a better understanding of susceptibility changes across time and location, enabling the development of effective management tactics.

In Kenya, insecticide resistance is becoming a bigger problem for controlling malaria vectors. The microbiome of *Anopheles gambiae* from Tulukuyi village, Bungoma, Kenya, with various permethrin resistance profiles, was comparatively described in the context of information relating the mosquito microbiota with

pesticide resistance. 2–3-day-old, virgin, non-blood-fed female F1 offspring of field-caught *An. gambiae*. were treated five times (107.5 g/ml) with the discriminating dosage of permethrin using the CDC bottle bioassay. Following the bioassay, 50 resistant and 50 susceptible mosquitoes were examined for kdr East and West mutations before being processed one at a time for microbiological study utilising high throughput sequencing that focused on the universal bacterial and archaeal 16S rRNA gene. This study is the first account of unique *An. gambiae* microbiota linked to strong pyrethroid resistance. The results show that resistant and susceptible mosquitoes have differing abundances of specific bacterial taxa and point to a microbe-mediated mechanism of pesticide resistance in mosquitoes. These findings also point to the fixation of the kdr gene mutation in this population of mosquitoes, preventing further investigation into its relationships with the microbiota of mosquitoes but supporting the idea that any microbe-mediated mechanism of insecticide resistance would probably be metabolic (Omoke et al., 2021).

Dengue fever and malaria are the two most common mosquito-borne illnesses in Thailand. 3,051 cases of malaria and 9,494 cases of dengue fever were reported in total in 2021. Populations of *Ae. aegypti* were resistant to permethrin and deltamethrin across Thailand. Previous investigations have discovered and confirmed that the primary kdr mutations, often base pair changes V1016G, F1534C, and S989P, are linked to pyrethroid resistance. Along the Thai-Myanmar border, research was done to evaluate the pesticide sensitivity status in *Anopheles* spp. and *Ae. aegypti* mosquitoes. In order to better understand the mechanism of resistance in *Ae. aegypti*, particularly in regions where dengue and malaria are co-endemic, the prevalence of kdr alleles and their relationship with observed phenotypes were also reported. *Aedes aegypti* was shown to be genetically resistant

to the Pyrethroid pesticide group, and *Anopheles* malaria vector resistance was also discovered. Dengue and malaria vector control programmes, such as fogging in hotspot communities to increase the amount of mosquito vector resistance present in peri-domestic settings, are debatable. This occurrence provides valuable information for routine vector control monitoring to prevent the development of pesticide resistance in mosquitoes (Pusawang et al., 2022).

National Susceptibility Studies

Bansal et al., (2007) studied "Relative susceptibility of some common mosquito vector larvae to synthetic insecticidal compounds in North-western Rajasthan." Their conclusion said *Anopheles* was the most susceptible to the organophosphates tested. Within the tested organophosphates, temephos was the most effective, followed by fenitrothion, fenthion, and malathion. Selvi et al. (2010) did research on "Insecticide susceptibility and resistance development in malathion selected *Aedes albopictus* (Skuse)." They found that larvae of *Aedes albopictus* were less sensitive to malathion than adults. According to Singh, *Aedes aegypti* and *Aedes albopictus* were sensitive to malathion, permethrin, deltamethrin, lambda-cyhalothrin, and cyfluthrin. Singh et al. (2011) did a study on the "Susceptibility status of dengue vectors against several insecticides in Koderma (Jharkhand), India. "Harish et al. (2011) studied "Present susceptibility status of *Culex quinquefasciatus*, say to four insecticides at Mysore, India." They recommend that spinosad is a highly effective bioinsecticide against mosquito larvae and may be helpful in the management of *Culex quinquefasciatus*, particularly in situations where local strains are highly resistant to other insecticides.

Nearly half of the world's population is under the threat of mosquito-borne infections, resulting in approximately a million fatalities annually. India reported 1.5 million confirmed cases of malaria in 2009, with over 1,000 deaths. Many *Anopheles* species are involved in the spread of malaria. *An. subpictus* Grassi and *Anopheles stephensi* Liston (Diptera: Culicidae) were frequently discovered during the survey activity in the arid and semi-arid zone of Rajasthan and Gujarat. These malarial vectors were collected between 2004 and 2007 from various locations in the Arid and Semi-Arid Zone of India. The susceptibility studies were carried out using diagnostic doses of larvicides fenthion, temephos, chlorpyriphos, and malathion. Both species showed varying levels of tolerance to DDT and malathion in most locales. Both species' adults were sensitive to deltamethrin. Both Anopheline species' larvae showed some signs of resistance to temephos and malathion but were vulnerable to chlorpyriphos and fenthion (Tikar et al., 2011).

One of the vectors for dengue and chikungunya is *Aedes albopictus*, and the establishment of pyrethroid resistance in this species might be a significant problem for managing the vector. In 2015, Kushwah et al. assessed *Ae albopictus'* knockdown resistance (kdr) mutation status in several Indian populations and its pesticide susceptibility to DDT and pyrethroids. They gathered mosquitoes from several states in India, and then using a WHO test kit, they conducted an adulticide test. The voltage-gated sodium channel (VGSC) gene mutation F1534C kdr was detected in mosquitoes using PCR genotyping. With varying mortalities ranging between 61 and 92%, adult bioassays on five populations of *Ae. albopictus* showed different degrees of resistance against DDT. The *Aedes* population in Kerala showed early resistance to permethrin, and in Delhi, resistance against deltamethrin was noticed. All other mosquitoes were devoid of

resistance against both of these synthetic pyrethroids. None of the individuals were observed with kdr mutations in the DDT, deltamethrin, or permethrin resistance.

Synthetic pesticides are typically used in vector control techniques to control lymphatic filariasis spreading *Culex quinquefasciatus* mosquitoes. The extensive and unrestrained usage of these synthetic pesticides would develop insecticide resistance in mosquito vectors. Field populations of *Cx. quinquefasciatus* were gathered from three districts in the northern portion of West Bengal and tested for resistance to a variety of insecticides, including 5% malathion, 0.05% deltamethrin, 0.05% lambda-cyhalothrin, 0.5% permethrin, 0.1% propoxur, 4% DDT, and Temephos. In order to determine the involvement of detoxifying enzymes in the emergence of pesticide resistance, qualitative and quantitative enzyme assays were also carried out. This investigation showed that *Cx. quinquefasciatus* field populations across the investigated locations exhibited extensive resistance (Kushwah, 2015).

Because they consume blood for the entirety of their lives, mosquitoes are perfect vectors for transmitting a broad range of pathogens, including the fatal malaria parasite Plasmodium falciparum, filarial nematodes, and the viruses that cause yellow fever, dengue fever, and West Nile. Insecticides are the most crucial element in the current worldwide strategy to manage mosquito-associated illnesses, which prioritises vector control of mosquitoes. In the past, widespread insecticide spraying significantly reduced the spread of mosquito-borne diseases and, in some places, even eliminated malaria. However, mosquito-borne diseases are now once again on the rise, mainly due to mosquitoes developing resistance to the commonly applied insecticides. Therefore, we must develop new methods for stopping resistance development, managing resistant mosquitoes, and lowering the prevalence of

mosquito-borne diseases. To do this, we must better understand how insecticide resistance develops in mosquitoes and the mechanisms governing insecticide resistance (Liu, 2008).

GENERAL METHODOLOGY

Mosquito Diversity

Study Site

Thrissur Kole wetland is a 10187-ha area located between 10^0 20' and 10^0 40' N latitude and between 75^0 58' and 76^0 11' E longitude in the central region of Kerala. Thrissur Kole lands extend in Mukundapuram, Chavakad, and Thrissur Taluks of Thrissur District.

Sampling Design

Muriyad Kole, Palakkal Kole, and Adat Kole were finalised as the sampling sites. Adat Kole divided into Ambakkad, Puranattukara, Kururpara, and Ambathonnu. Locations of Palakkal Kole were Chiyyaram, Palakkal, Kanimangalam, and Nedupuzha. Muriyad Kole included Ananthapuram, Nambyamkavu, Konthipulam, and Nandipulam. Regular monthly sampling was done from 12 different locations of these three sampling sites. Random sampling methods were used in our study, and both juvenile and adult mosquitoes were collected. During sampling GPS reading and meteorological data were recorded from the sampling locations.

Mosquito Sampling and Diversity Analysis

Random sampling methods were used in this study. Eggs collected from Natural oviposition sites, Artificial ovitraps also used for egg collection. Larval collection done with Pipettes, Dippers, Aquatic nets. Adult specimen collected using Attractant and Non-attractant traps, sweeping insect nets, aspirator. Duration of diversity study from June 2016 to May 2018. Identification of collected specimen done standard

taxonomical keys. Dominance D, Simpson's 1-D, Shannon -H, Evenness H/S, Margalef, Biodiversity index, and Berger-Parker indices demonstrated the alpha diversity of the study area. Jaccard and Sorenson Diversity indices were used for comparison of two individual sites. Gamma diversity analysis were also done. Diversity analysis done with 'PAST' software.

Seasonal Variation

Pre-monsoon, Monsoon, Post-monsoon are the three distinct seasons in our study area, each of them having four months. Pre-monsoon starts in February and ends in May. Duration of Monsoon season spreads between June and September; later, the Post-monsoon season began (October- January). Monthly sampling for two years was done from every sampling location and recorded the collection data. The variation in the number of mosquitoes collected in different seasons was analysed with a one-way ANOVA Test.

GIS Preparation

GPS readings of each sampling location were taken. Mosquito species from all locations were recorded during the diversity study period. These available mosquito data are used in the GIS preparation. QGIS software was used for GIS preparation.

Correlation between physico-chemical parameters of the water sample and number of mosquitoes collected

Water samples from larval habitats were also collected during monthly sampling. Analysis was done with these water sample to check the correlation between mosquito diversity and physicochemical parameters of larval habitats. Physical parameters recorded at the same time of collection. Sampled water transported to laboratory in sterile bottles for chemical analysis. Ten standard water quality

parameters (Temperature, pH, Turbidity, Conductivity, TDS, DO, Alkalinity, Hardness, Chloride, Salinity) were selected for correlation analysis. All water parameters tests conducted as per APHA procedure.

DNA Barcoding

Collection and preservation

Mosquitoes were collected and morphologically identified and stored in 70% ethanol at -20º C. Appropriate code number was assigned and used as voucher specimen

Genomic DNA Extraction and PCR amplification

One of the thoracic legs used for the Genomic DNA extraction (ORIGIN Genomic DNA isolation Kit). Agarose gel electrophoresis conducted for the conformation of the presence of DNA. The amplification reaction was performed by using a DNA thermal cycler (Takara). Primer - forward and reverse primers (forward primer with DNA sequence 5'-GGTCAACAAATCATAAAGATATTGG-3' and the DNA sequence of reverse primer 5'TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994). Initial denaturation at 95°C for 5 minutes, amplification was made through 30 cycles, each consisting of a denaturation at 95°C for 10 seconds, annealing at 50°C for 1minutes, extension step at 72°C for 45 seconds final extension at 72°C for 3 minutes.

Agarose Gel Electrophoresis

The PCR products were resolved on 2 % TAE agarose gel stained with EtBr. A Gene Ruler (Thermo Scientific; GeneRuler 100bp DNA Ladder. #SM0242) was used to determine the size of the product. EtBr act as an intercalating agent on the bases of DNA and imparts an orange colour to DNA under ultraviolet light.

PCR product purification

After confirming the PCR amplification of the corresponding COI fragment, the remaining portion of the PCR product was column purified by using Fermentas, GeneJET PCR purification kit. The GenElute TM PCR Clean up Kit is designed for rapid purification of single stranded or double stranded PCR amplification products from other components in the reactions such as excess primers, nucleotides, DNA polymerase, oils and salts from the PCR products. The purified product was again resolved on 2% agarose gel to check the presence of DNA.

DNA sequencing and phylogenetic analysis

The purified PCR product was sequenced using the Sanger's sequencing method at Sci Genom Labs Private Ltd., Cochin, and IISc Bengaluru with ABI 3730XL automated sequencer. The trimmed COI sequences of forward and reverse obtained were aligned using ClustralW. The Final sequence was searched in NCBI BLAST for species confirmation. The partial COI gene sequence was deposited in GenBank (NCBI) for worldwide accession. It can be used as a molecular barcode generation. Final nucleotide sequences were analysed using MEGAX to study phylogenetic relationship

Susceptibility

Insecticide

Lambda cyhalothrin, Deltamethrin, Malathion, Temephos, Propoxur (Technical grade) purchased from "New India Surgicals, Calicut, Kerala, India

Mosquito sampling and colony maintenance

Aedes albopictus, Culex quinquefasciatus (field strain and laboratory strain) selected for susceptibility analysis. *Aedes albopictus* and *Culex quinquefasciatus* mosquitoes

from different localities of Thrissur kole wetlands and identified by classical taxonomic methods. F1 progeny larvae of field-collected mosquitoes were subjected to larval bioassay. Laboratory colonised insecticide-free *Aedes albopictus*, and *Culex quinquefasciatus* were maintained in the Communicable disease research laboratory, St. Joseph's College. Collected larvae were reared into adults in laboratory conditions (Temperature 26±2, larval food prepared by mixing yeast and dog biscuit). Adult mosquitoes fed with 5% sucrose and blood meal provided on the third day of emergence.

Larval Bioassay

The standard WHO procedure was followed for determining larval susceptibility (Brown, 1986). Accordingly, the larvae subjected to different concentrations of insecticides whose stock solutions were prepared using distilled water as the solvent. 1mg/ml deltamethrin, lambda-cyhalothrin, malathion, propoxur, and temephos stock solution was prepared in water. Test concentrations were prepared by adding 1ml insecticide containing solution to 249 ml of water in 500 ml capacity beaker and stirred vigorously for 30 seconds with a glass rod. For the control, 1ml of distilled water or absolute alcohol as required were added to 249 ml of dechlorinated water instead of insecticide. To each of the beakers containing different test and control, 25 late third or early fourth instar larvae were released with the help of a strainer. Six serial Test concentrations of insecticides were prepared for larval bioassay. Mortality was recorded after 24 hours. Unmoved and moribund larvae treated as dead. The LC_{50} and LC_{90} values for insecticides were calculated by the dosage mortality regression line using probit analysis (Finney,1971).

Statistical Analysis

SPSS version 22 was used for the calculation of lethal values of tested insecticides. LC_{25}, LC_{50}, LC_{90} values were calculated for discussing the result of larval bioassay with experimented insecticides. One way ANOVA analysis were done with the observed values of larval bioassay.

CHAPTER 1

Mosquito diversity in Kole wetlands of Thrissur with GIS, and its seasonal variation and influence on physico-chemical parameters

1.1 Introduction

Wetlands are a unique waterlogged habitat with distinctive abiotic and biotic environmental features. Regarding their origin, topographical position, aquatic structure, and interaction among them, these aquatic systems can be able to support a large amount of floral and faunal variety (Abraham, 2015). According to public health organizations, Wetlands are favourable breeding ground for vector mosquitoes that transmit arboviruses and parasites (Dale and Knight, 2008; Schafer et al., 2008). The Thrissur Kole lands are a shallow-water low-lying strip of the aquatic system that serves as an intermediate area between terrestrial and marine ecosystems, keeping the water level between 0.5 m and 1m below sea level. These Ramsar sites are topographically located in Kerala's central region, covering an area of 10187 hectares, and extending across the Thrissur District's Mukundapuram, Chavakad, and Thrissur Taluks. The monsoon water provided by the two major rivers in Thrissur inundates the Kole lands for about half of the year. Paddy and vegetable agriculture performed for the remaining half. (Srinivasan, 2010). Agriculture's by-product deposition might alter the depth and characteristics of the water system, allowing mosquitoes to reproduce (Norris, 2004; Dian and Changxing, 2011).

Mosquitoes from the Culicidae family of the order Diptera are among the most visible residents of wetlands (Schafer, 2004). Agricultural growth can potentially change the ecosystem in a way that benefits mosquitoes, and agriculture-induced sedimentation can delay or obstruct streams and reduce water depth (Dian and

Changxing, 2011). These environmental changes increase the number of mosquito homes available while lowering the water temperature for vector growth (Norris, 2004). Wetlands have high productivity relative to other ecosystems and provide a home for a diverse range of animals (Mitsch, 1994; Keddy, 2000). Mitsch describes the problematic link between mosquitoes and wetlands, claiming that mosquitoes are one of the sources of incompatibility between human civilization and wetlands in many parts of the world.

Mosquito-borne infections are the most common type of epidemic disease in tropical areas, wreaking havoc on the economies and public health systems of these developing nations. In recent years, these outbreaks have resurfaced in the temperate area (Rezza et al., 2007; Danis et al., 2011; Ndoen et al., 2012). In tropical nations where the illness has spread, mosquito-borne diseases contribute significantly to infection burden, mortality, poverty, and devitalization (Yang et al., 2002). Infected areas have seen an increase in unique illness occurrences and significant discomfort in recent years, prompting experts to conclude that mosquito control is the best answer to this riddle (Invest and Lucas, 2008). Due to the massive outbreak of mosquito-borne diseases, monitoring the mosquito species in the Kole wetlands of Thrissur is essential.

Insect pest abundance is determined mainly by essential components of any biological system, such as prey-predator relationships and resource competition, especially in juvenile phases. Seasonal variations may affect the availability of resources such as food, breeding sites, and the development of immature insect life forms (Franklin and Whelan, 2009; Tsurim et al., 2013; Hoshi et al., 2014). This pattern of ecological change might lead to a shift in the number of mosquitoes when the seasons change. All mosquitoes require stagnant water bodies for their early

juvenile development, and the aquatic system's fundamental physicochemical properties might impact these juveniles' growth and maturity (Garba and Olayemi, 2015). Gravid mosquitoes select oviposition locations depending on the availability of water supplies, their quality parameters, and the presence of potent predators (Shililu et al., 2003; Fillinger et al., 2004; Piyaratne et al., 2005).

Factors such as the quantity and types of salts, dissolved solids, eutrophication level, turbidity, mud deposition, vegetation in the environment, temperature, light, shadow, and pH influences mosquito larvae's oviposition, survival, and geographic dispersal (Tren, 2002). The physicochemical characteristics of rice fields and nitrogen-based fertilizers employed in agricultural growth create ideal conditions for mosquito larval population proliferation and spread. The richness of the larval mosquito population is barely affected by paddy height, aquatic temperature, dissolved oxygen, ammonia, or nitrogenous nitrogen. Nitrogen and ammonia levels can be raised by sprinkling nitrogen-based fertilizers over the area, eventually sustaining the robust larval population (Sunish and Reuben, 2001; Muturi, 2008).

It is well known that accurate breeding site mapping is essential for achieving successful results in contemporary mosquito control programmes (Becker et al., 2010). The site's characteristics and the use of ecologically friendly mosquito control methods would lead to the evaluation of various larvicidal formulations. GIS has become a vital tool for processing, analysing, and displaying geographical data in ecological research, disease ecology, and environmental and public health problems, as evidenced by recent advancements (Kistemann et al., 2002; Graham et al., 2004). The GIS program allows users to create interactive searches, analyse spatial data, change data and maps, and effectively define nuisance and vector mosquito habitats that are impacted by environmental variables. It allows for the generation of

forecasting maps for planning control methods on local and large sizes (Tourre et al., 2008; Dongus et al., 2009; Stensgaard et al., 2009), as well as the creation of maps based on field monitoring (Tourre et al., 2008). GIS programs that improve data analysis techniques maximize the value of additional information and enable better selection of single or combinations of available mosquito control options and larvicidal formulations.

A Geographic Information System is computer hardware, software, and geographic data set that collects updates and displays all types of geographically oriented data (Kistemann et al., 2002). The nature of the survey, logistics, and documentation of mosquito management models can be influenced and expanded by GIS and information technology (Becker et al., 2010). Thanks to modern information technology, GIS systems can now be combined with database technology and GPS-enabled digital mobile field data-gathering devices. GIS has become an indispensable tool for geographic data recording in ecology, the environment, and public health (Kistemann et al., 2002; Graham et al., 2004). Identification and mapping of mosquito breeding habitats are crucial in mosquito diversity research since the data can pinpoint specific areas of concern and reduce the use of pesticides. It allows it to generate field monitoring maps and forecasting maps for mosquito control plans (Tourre et al., 2008; Dongus et al., 2009; Stensgaard et al., 2009).

1.2 Methodology

1.2.1 Study site

The Thrissur Kole wetland is a 10187-ha area located between 10° 20’ and 10° 40’ N latitude and between 75° 58’ and 76° 11’ E longitude in the central region of Kerala

(Figure 1.1). Thrissur Kole lands extend in Mukundapuram, Chavakad, and Thrissur Taluks of Thrissur District. This area spreads from Velukara in the south, the northern bank of the Chalakudy river, and the Tholur and Kaiparambu areas of Thrissur thaluk in the north. The following area of Thrissur Taluk is named Ponnani Kole. Wetlands, paddy fields, rocky pools, tree holes, coconut trees, ditches, etc., are the different breeding habitats of mosquitoes in Kole wetlands (Johnkuttty and Venugopalan, 1993).

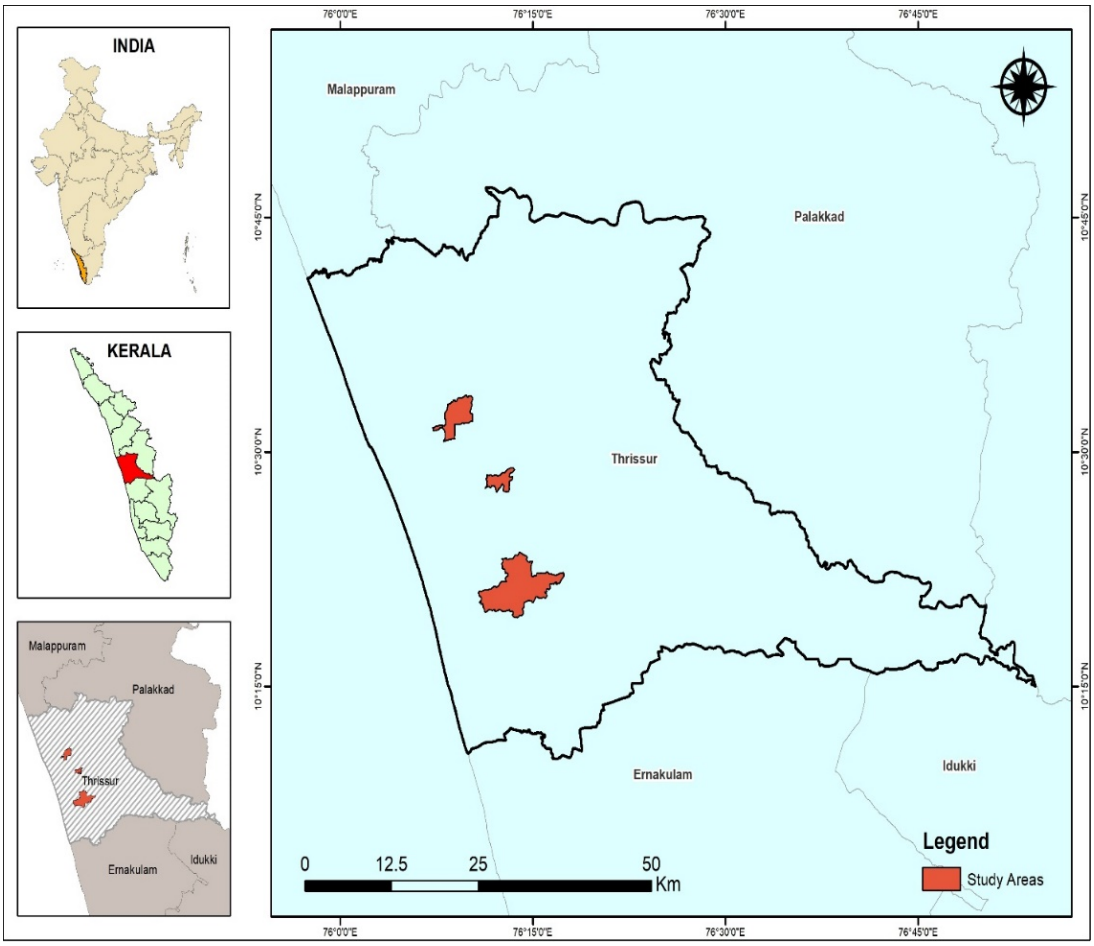

Figure 1 Location map of Thrissur Kole wetlands, Kerala

1.2.2 Sampling Design

Muriyad Kole, Palakkal Kole, and Adat Kole were finalised as the sampling sites. These sampling sites of Thrissur Kole wetlands are geographically distributed from

the southern to the northern region. Three sampling sites were again divided into four locations each. Site 1 Adat Kole divided into four locations Ambakkad, Ambathonnu, Kururpara, and Puranattukara. Site 2 Palakkal Kole had four sampling locations, namely Chiyyaram, Kanimangalam, Nedupuzha, and Palakkal. Site 3 Muriyad Kole included Ananthapuram, Konthipulam, Nambyamkavu, and Nandipulam regions. Regular monthly sampling was done from 12 locations of these three sampling sites. Random sampling methods were used in this study, and both juvenile and adult mosquitoes were collected. During sampling, GPS reading and meteorological data were recorded from the sampling locations. Table 1.1 shows the site, area, and geographical details of 12 sampling locations.

Table 1.1 Sampling Location Characteristics

Location	Site	Area	Lattitude	Longitude
Location 1	Adat	Ambakkad	N 10º 33′ 01.2″	E 76º 09′ 10.2″
Location 2	Adat	Ambathonnu	N 10º 31′ 29.7″	E 76º 07′ 18.0″
Location 3	Adat	Kururpara	N 10º 32′ 27.5″	E 76º 08′ 35.0″
Location 4	Adat	Puranattukara	N 10º 30′ 08.5″	E 76º 09′ 48.2″
Location 5	Palakkal	Chiyyaram	N 10º 28′ 51.3″	E 76º 13′ 14.1″
Location 6	Palakkal	Kanimangalam	N 10º 28′ 13.1″	E 76º 12′ 20.6″
Location 7	Palakkal	Nedupuzha	N 10º 28′ 16.7″	E 76º 12′ 16.7″
Location 8	Palakkal	Palakkal	N 10º 28′ 34.2″	E 76º 12′ 49.3″
Location 9	Muriyad	Ananthapuram	N 10º 21′ 56.9″	E 76º 15′ 15.3″
Location 10	Muriyad	Konthipulam	N 10º 22′ 37.4″	E 76º 13′ 16.7″
Location 11	Muriyad	Nambyamkavu	N 10º 20′ 42.6″	E 76º 13′ 14.1″
Location 12	Muriyad	Nandipulam	N 10º 23′ 05.6″	E 76º 14′ 08.8″

1.2.3 Mosquito Diversity study

1.2.3.1 Egg Sampling

Eggs are the first life stages of the mosquito; they were either collected from their breeding habitats or artificial ovitraps. Floating eggs collection done with aquatic nets, pipettes, and dippers. Gathered eggs were appropriately labelled and transported to our laboratory for larval and adult mosquito rearing and identification.

1.2.3.2 Larval Sampling and Identification

Larvae were collected from various breeding habitats of mosquitoes using pipettes, aquatic nets, and dippers. Properly labelled plastic containers were used to store the collected specimen and shifted to the laboratory. Larvae were treated with hot water (40° C) to kill them and identified with the help of taxonomical keys (Christopher, 1933; Barraud, 1934; Black, 1968; WHO 1975; Gilles, 1993; Nagpal and Sharma 1995; Das and Kaul1998; Nagpal et al., 2005; Tyagi et al., 2015, etc) under Stereo zoom microscope (Leica-M205C). Accurate species-level identification had some difficulty in the juvenile stages of mosquitoes; such larvae were reared into the adult. Unidentified larvae were reared into adults in laboratory conditions (Temperature 26±2, larval food prepared by mixing yeast and dog biscuit). Adult mosquitoes were fed with 5% sucrose.

1.2.3.3 Adult Sampling and Identification

Resting and host-seeking adult mosquitoes were collected using sweeping nets and an aspirator. Adult mosquito capturing was also done with various attractant and non-attractant traps, like light traps, CO_2 traps, etc. Collected mosquitoes were transferred to the laboratory to conduct the identification procedures. Both captured and reared adult specimens were killed properly with chloroform application and

performed detailed examination and dissection under Stereo zoom microscope (Leica-M205C). Species identification could be complete with the help of conventional morphological keys of Christopher, 1933; Barraud, 1934; Black, 1968; WHO 1975; Gilles, 1993; Nagpal and Sharma 1995; Das and Kaul1998; Nagpal et al., 2005; Tyagi et al., 2015, etc. Identified species were pinned and labelled carefully and stored in Communicable Disease Research Laboratory, St. Joseph's College, Irinjalakuda.

1.2.3.4 Statistical Analysis

Various fundamental diversity indices analyses were carried out to explain the mosquito collection data from three sampling sites (Site 1- Adat Kole, Site 2- Palakkal Kole, and Site 3- Muriyad Kole). Dominance D, Simpson's 1-D, Shannon - H, Evenness H/S, Margalef, Biodiversity index, and Berger-Parker indices demonstrated the alpha diversity of the study area. Jaccard and Sorenson Diversity indices were used to compare two individual sites. Analysis of gamma diversity were also performed. 'PAST' software was used for all the diversity analysis.

1.2.3.4.1 Alpha Diversity Analysis

The Dominance index demonstrates the abundance of the commonest taxa or species in a community ($D = \sum n (n-1) / N (N-1)$. The value of the Dominance index lies between 0 to 1. Dominance index '0' indicates that all taxa in a community are equally present, and '1' shows one taxon has complete dominance over the entire population. When the D value increases, the diversity becomes lower.

Simpson's index (1-D) is calculated from the Dominance index (The addition of D and 1-D is always 1). It defines the chance of randomly getting two individuals from

different species in a community. Values ranging from 0-1 values near 1 show higher diversity.

Shannon diversity index (H' = -[Σ(pi×lnpi)] where pi represents the proportion of individuals found in the i^{th} species and ln denotes natural logarithm) is a widely accepted one illustrating the species diversity of a community that explains abundance and evenness.

The Evenness index (E) explains the number of individuals from different species in the same community. The formula E = H/S is used to calculate the Evenness index where H = Diversity index and S = Number of species found. The greater value of the evenness index indicates the stability of the community.

Berger-Parker dominance is simply the number of individuals in the most dominant taxon relative to *n*. It explains the proportional importance of the most abundant species. This index uses only some information available from the sample. It is based on sample size and richness ($D = \frac{n_{max}}{N}$, where n_{max} represents the number of individuals in the species with the highest density, and N represents the total number of individuals in the sample).

Margalef's index $\frac{(S-1)}{\ln N}$, S stands for all species together as a straightforward indicator of species richness. N is the overall sample size, while ln is the natural logarithm. It counts the number of different species in each area or community.

1.2.3.4.2 Beta Diversity Analysis

The Jaccard similarity coefficient is another name for the Jaccard index. The similarity of the two sites is compared using it. $Sj = \frac{a}{a+b+c}$, a = species found in all sites, b = species found in site 1 c = species found in site 2. Sorenson index

calculated using the formula $\frac{2a}{2a+b+c\,a}$ = species found in all sites, b = species found in site 1 c = species found in site 2.

1.2.4 Seasonal Variation

Pre-monsoon, Monsoon, and Post-monsoon are the three distinct seasons in our study area, each having four months. Pre-monsoon starts in February and ends in May, and the Monsoon season spreads between June and September; later, the Post-monsoon season begins (October- January). Monthly sampling for two years was done from every sampling location and recorded the collection data. The variation in the number of mosquitoes collected in different seasons was analysed with a one-way ANOVA test.

1.2.5 GIS preparation

GPS readings of each sampling location were taken. Mosquito species from all locations were recorded during the diversity study period. These available mosquito data are used in the GIS preparation. QGIS software was used for GIS preparation.

1.2.6 Correlation between physico-chemical parameters of the water sample and the number of mosquitoes collected

Water samples from larval habitats were also collected during monthly sampling. Analysis was done with these water samples to check the correlation between mosquito diversity and the physico-chemical parameters of larval habitats. Physical parameters were recorded at the same time of collection, and transported the sampled water to the laboratory in sterile bottles for chemical analysis. Ten standard water quality parameters (Temperature, pH, Turbidity, Conductivity, TDS, DO,

Alkalinity, Hardness, Chloride, and Salinity) selected for correlation analysis. All water parameters tests were conducted as per APHA procedure (APHA, 1998; Welsh and Smith,1960; Winkler,1888).

a) Temperature

Temperature is one of the principal parameters of the self-purification of water. Temperature is measured using a mercury thermometer having a scale marked for every 0° C.

Procedure

A mercury thermometer is immersed in the water sample up to the mark specified by the manufacturer and reads the temperature after equilibration.

b) pH (APHA, 4500 H^+ B)

pH meter consists of pH 4 and pH 7 calibrator knob. Another knob is used for the temperature settings. The pH meter calculates the pH of different types of water. The pH meter is standardized using a buffer solution of pH 4 or pH 7. pH 4 buffer solution is made by adding pH 4 tablets to 100ml water. The pH 7 buffer solution is made by adding pH 7 tablets to 100ml water. The pH meter electrode is always dipped in either distilled or normal water to avoid dryness. Here the average room temperature is 32°C and is set in a temperature setter. The electrode is washed first, dipped in either pH 4 or 7, and adjusted on the calibrator. Once it is calibrated, the electrodes are dipped in different water samples, and the pH value is noted. A higher pH value indicated the purity of the water. pH was positively correlated with electrical conductance and total alkalinity.

Apparatus Required

pH meter, Beaker

Procedure

All the samples are taken in the beaker one by one. The pH value is recorded for the entire sample using the pH meter.

c) Turbidity (APHA, 2130 B)

Dissolved particles in the water cause Turbidity, the opacity of water or fluid. Finding the turbidity value is one of the most critical water quality tests. Increased sedimentation and siltation can harm the habitat areas for fish and other aquatic life. Particles also provide attachment places for other pollutants, especially bacteria and metals. That is why turbidity readings are potential pollution indicators in a water body.

Apparatus Required

Turbidity meter

Determination of turbidity

Turbidity is measured using the instrument called the digital Nephelo turbidity meter.

Reagents:

The solution I: Dissolved 1.00 gm hydrazine sulphate, $(NH_2)_2$. H_2SO_4, in distilled water and made up to 100 ml

Solution II: Dissolved 10.00 gm hexamethylenetetramine $(CH_2)_6N_4$ in distilled water and made up to 100 ml.

Solution III (Stock turbidity suspension): Mixed 5ml of solution I and 5 ml of solution II; allow standing for 24 hrs and diluting to 100ml. This solution would have a turbidity of 400 NTU.

Solution IV (Standard turbidity Suspension): Diluted10 ml above solution III to 100 ml to have a solution with a turbidity of 40 NTU.

Prepared a calibration graph in the range of 0 to 40 units by carrying out appropriate dilutions of solutions III and IV above and took readings on the turbidity meter. Once this instrument was calibrated using the standard solutions, the turbidity of the sample could either be read directly from the instrument or the calibration curve.

Procedure

Distilled water was poured into a caped tube after adequate wash. The turbidity of distilled water is always measured as zero, and it is used as a reference. Then, the testing water sample was transferred into the beaker, and the turbidity of the beaker was measured using the turbidity meter. The settings of the turbidity meter were managed to keep zero while reading the turbidity value of distilled water. The same procedure is repeated for all the samples.

d) Conductivity (APHA, 2510 B)

Conductivity is the measurement of the capacity of an electrolyte solution to conduct electricity. Conductivity and T.D.S (total dissolved solids) correlate directly in various cases. Dissolved inorganic solids that carry negatively charged anions like sulphate, chloride, nitrate, and phosphate or positively charged cations such as sodium, calcium, magnesium, iron, and aluminium determine the conductivity value in water. Various organic compounds like phenol, oil, sugar, and alcohol decrease the electrical conductivity of water.

Standardization of the instrument was done with KCl solution, and known conductance values were observed. Standardization with KCl was the prior step to determine the conductivity of different water samples. A buffer that gives a value of 1413 is then used to find the values of testing water samples.

Apparatus required

Conductivity meter**,** Potassium Chloride (KCl)

Procedure

KCl was dissolved in distilled water and prepared KCl solution. The conductivity of KCl is checked using a conductivity meter. If there was some error in the conductivity of KCl, then the settings of the conductivity meter were adjusted accordingly. Then, the sample was taken in the beaker, and the conductivity of the water is measured. The same procedure is repeated for all the samples. Hence, record the conductivity of all the samples.

e) Total dissolved solids (APHA, 2540 C)

The phrase used to describe the inorganic salts and trace quantities of organic stuff present in a solution in water is total dissolved solids (TDS). The principal constituents are calcium, sodium, magnesium, and potassium cations and carbonate, hydrogen carbonate, chloride, sulfate, and nitrate anions.

$$\text{TDS} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Volume of sample}} \times 1000 \times 1000$$

Apparatus required

Beaker. Hot plate, Weighing machine

Procedure

Took a 100 ml capacity beaker and its initial weight (Dry weight of the beaker). Took 60 ml of water sample into the beaker, then placed for complete evaporation on a hot plate. After evaporation, the final weight was recorded, and TDS was determined using the abovementioned formula.

f) Dissolved Oxygen

Oxygen consumption was determined by Winkler's method (1888), modified by Welsh and Smith (1961). Oxygen in the sample readily oxidises divalent manganese hydroxide to its higher valance state-this oxidation precipitates a brown hydrated oxide after adding NaOH and KI. Manganese reverts to a divalent state and liberates iodine from KI equivalent to the original DO content upon modification. Titrate the liberated iodine against sodium thiosulphate solution, and starch is used as an indicator.

Water samples were collected in a 300 mL BOD (Biological Oxygen Demand) bottle with special care to avoid air bubbles and tightly closed with a stopper. After removing the bottle stopper, added 1 mL of the manganous sulphate solution and 1 mL of the alkaline-potassium iodide-sodium azide solution at the surface of the liquid. Then the plug was replaced to avoid further bubble formation and shaken well several times by flip-flopping the bottle. Added 1 ml of concentrated sulfuric acid to run down the bottle's neck and dissolved the residue with vigorous shaking. The corresponding volume of the 200 mL original sample corrects some sample loss during the addition of reagents used for titration. The volume was calculated using the formula:

mL of sample to titrate = 200 x $[\frac{300}{(300-2)}]$ = 201 mL

Procedure

Took 201 ml of sample from BOD bottle to conical flask and titrate against 0.0250 N sodium thio-sulfate until the blue colour of the solution disappeared. Recorded the volume of sodium thiosulfate used and estimated dissolved oxygen by the formula

mg/L oxygen consumption = $\frac{\text{mL titrant x normality of titrant x 8000}}{\text{equivalent volume of sample titrated}}$

g) Alkalinity (APHA 2320 B)

The ions of the weak acid are what give natural water its Alkalinity. Water's Alkalinity has the power to neutralize acids. Bicarbonates and hydroxy carbonates are significant sources of natural Alkalinity. Carbon dioxide reacts with calcium and magnesium carbonate in the soil to form a significant quantity of bicarbonates. Humic acid-like organic solvents also provided Alkalinity to the soil.

Principle:

The Alkalinity of water was estimated by titrating it with a standard acid solution in the presence of a pH indicator.

Reagents:

1. 0.02M H_2SO_4: Took 200 ml of 0.1N sulfuric acid and dilute it to 1000 ml of distilled water. Standardized this solution against a standard sodium carbonate solution of 0.02M.

Procedure

About 25 ml of the sample was taken in a conical flask, and add 1 or 2 drops of methyl orange indicator and titrated against a standard sulphuric acid solution. The endpoint colour changed from golden yellow to orange-red.

$$\text{Total alkalinity, mg/L as } CaCO_3 = \frac{[(A-B) \times N \times 1000 \times 50]}{25} = \quad \text{mg/L}$$

Where,

A = mL titration for sample

B = mL titration for blank

N= Normality of Sulphuric acid Solution

h) Hardness (APHA, 2340 C)

Calcium and Magnesium are principal cations causing hardness. Calcium and Magnesium ions react with EDTA to form soluble complexes. The colour change of a suitable indicator such as Eriochrome black T. Calcium and Magnesium form a wine-red colour complex indicates the reaction's completion. Therefore, adding EDTA solution breaks the complex, and a new complex is formed and gives blue colour.

Reagents:

1. Standard EDTA solution (0.01M): Weigh 3.723 g analytical reagent grade disodium ethylenediamine tetraacetate dihydrate, dissolved in distilled water and dilute to1000ml.
2. Buffer solution (ammonium Hydroxide + Ammonium Chloride): Dissolved 16.9 g NH_4Cl in 143 ml concentrated ammonium hydroxide.
3. Erichrome black T indicator: Mixed 0.5 g dye and 100 g NaCl to prepare a dry powder mixture.

Procedure

Took about 25 ml of the collected water sample in a conical flask. Added about 1 ml of ammonium hydroxide buffer solution and titrated against 0.01M EDTA using Erichrome Black T as an indicator. At the endpoint of the reaction colour changed from wine red to blue

$$\text{Total hardness} = \frac{\text{M x V x 1000 x 100}}{25} = \quad \text{mg/L}$$

M – Molarity of EDTA

V- Volume of EDTA used

i) Chloride (APHA, 4500 Cl^- B)

Chloride in the form of chloride ions is one of the major inorganic ions in water and wastewater. Chloride is widely distributed in nature, generally in NaCl, KCl, and $CaCl_2$ salts, and it constitutes approximately 0.05% of the lithosphere. A tremendous amount of chloride in the environment is present in the oceans. The dissolving of salt deposits is the cause of the occurrence of chloride in natural waterways, the contamination resulting from the salting of roads to control ice and snow, discharges of effluents from chemical industries, sewage discharges, etc. The presence of chloride ions in potable water may create a salty taste. Besides chloride, the dominating cations, like Ca and Mg, cause a similar salty nature to water. Some waters with a chloride concentration of 250 mg/l could generate salt in the palate. On the other hand, if the dominating cations are Ca and Mg, the usual salty taste may be present in waters with as much as 1000 mg/l of sodium. Sodium chloride is a common food component and goes through the digestive system. However, the chloride content in wastewater is greater than that in raw water. Chloride may be in high concentrations Along the seacoast region because of saltwater leakage into the sewage system, and the industrial process may also increase it. Chloride concentrations in samples have been determined by using AgNO3 included argentometric titration method. AgNO3 reacts with chloride ions to form silver chloride. End point of reaction indicated by the actual colour generated by the reaction of silver nitrate with potassium chromate solution, which is added as an indicator.

Principle:

$$NaCl + AgNO_3 \rightarrow Ag\,Cl + Na\,NO_3$$

$$K_2CrO_4 + 2Ag\,NO_3 \rightarrow Ag_2CrO_4 + 2\,KNO_3 \quad \text{(Red brown)}$$

Reagents:

1. 0.0282N $AgNO_3$: Dissolve 2.395 g $AgNO_3$ and diluted to 500 ml. Standardized against NaCl of 0.0282N
2. Potassium chromate Indicator: Dissolved 50 g K_2CrO_4 in 1000 ml DW
3. Standard Sodium Chloride (0.0282N): Dissolved 824.1 mg NaCl (dried at 140^o C) in 500 ml DW.

Procedure

About 50 ml of the sample was taken in a conical flask. Added 1 ml potassium chromate indicator and titrated against standard silver nitrate solution, at the end of the reaction colour changed from yellow to reddish-orange.

Calculation

$$\text{Chloride, mg/L} = \frac{((A - B) \times N \times 35.45 \times 1000)}{50}$$

A - mL titration for sample

B – mL titration for blank

N – Normality of $AgNO_3$

j) Salinity (APHA 2520B)

The salinity of the water is the measurement of the number of dissolved salts in it. It is usually expressed in parts per million (ppm) or percentage (%).

Reagents

10 ppm Sodium chloride (NaCl): 10 mg NaCl in 1 L distilled water

Procedure

Calibrated the Salt meter according to the manufacturer's instructions using the 10 ppm NaCl standard solutions. For calibration, connected the salinity/ salt cell to the input socket. Put the cell in 10 ppm NaCl solution. Switched on the instrument. Measured the temperature of the solution. Calibrated the instrument to the proper value with the CALIB knob according to the calibration table. After calibration, washed the salt cell with distilled water. Put the salt cell into the unknown solution and took readings. Table 1.2 is the calibration table.

Table 1.2 Calibration table

Temperature	Calibration value	Temperature	Calibration value
10	7.2	28	10.5
20	9.0	29	10.7
21	9.2	30	10.9
22	9.4	31	11.1
23	9.6	32	11.2
24	9.8	33	11.4
25	10.0	34	11.6
26	10.1	35	11.7
27	10.3		

1.3 Result and Discussion

1.3.1 Diversity

1.3.1.1 Result

Altogether 34801 mosquito samples were collected from the study area during the collection period (June 2016- May 2018). Identified samples were from 20 species belonging to 5 genera displayed in Table 1.3. The samples were high in Site 1 Adat Kole compared to the other two sites (Adat Kole- 13746, Palakkal Kole- 9446, Muriyad Kole- 11609). *Anopheles barbirostris* 322 (0.93%), *Anopheles*

nigerrimus 365 (1.05%), *Anopheles peditaeniatus* 107 (0.31%), *Anopheles stephensi* 348 (1%), *Anopheles subpictus* 762 (2.2%), *Anopheles vagus* 371 (1.07%), *Aedes aegypti* 3353 (9.64%), *Aedes albopictus* 5149 (14.8%), *Aedes vittatus* 489 (1.41%), *Phagomyia cogilli* 15 (0.05%), *Armigeres subalbatus* 8954 (25.73%), *Culex bitaeniorhynchus* 31 (0.09%), *Culex gelidus* 4484 (12.89%), *Culex pipiens* 2882 (8.29%), *Culex quinquefasciatus* 2923 (8.4%), *Culex tritaeniorhynchus* 2080 (5.98%), *Lutzia fuscanus* 1025 (2.96%), *Mansonia bonneae* 37 (0.12%), *Mansonia indiana* 763 (2.19%), *Mansonia uniformis* 341 (0.99%) were the identified species from the study area. Figure 1.2 illustrates the species abundance graph of collected mosquito species. Identification characteristics of identified mosquito species are illustrated below.

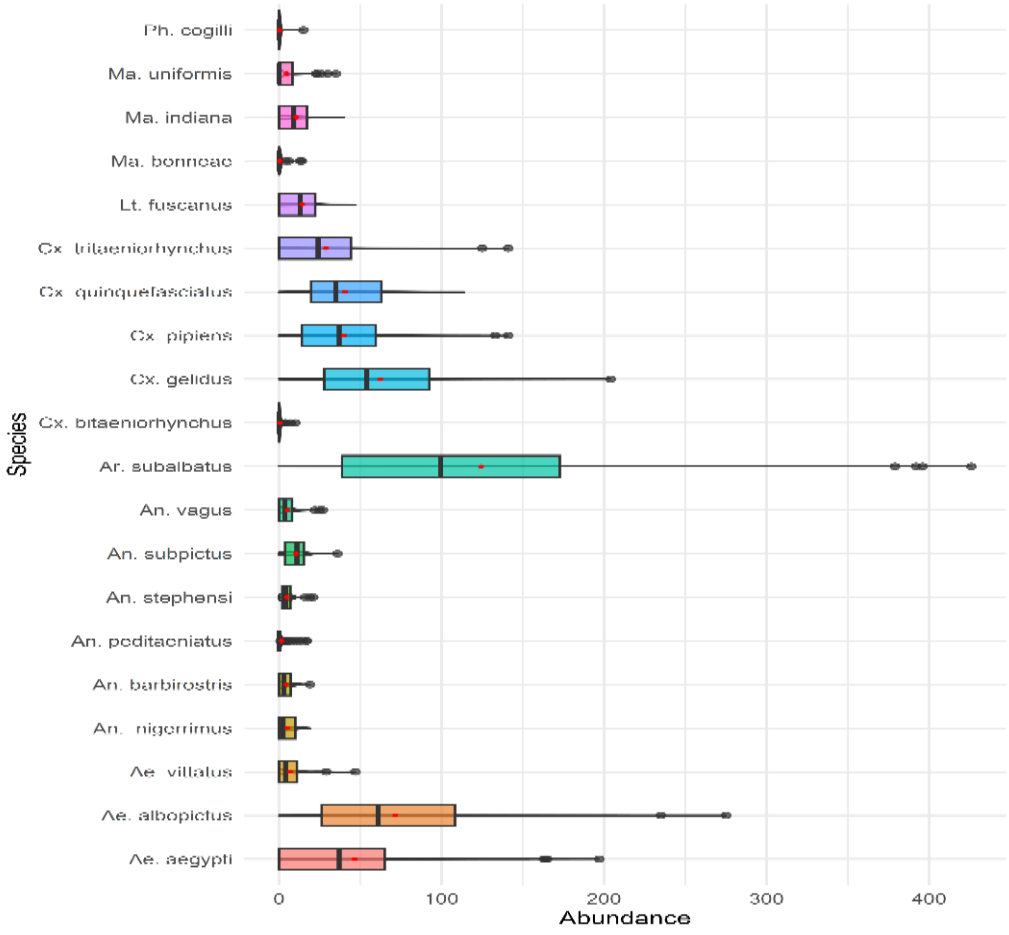

Figure 1.2 Abundance Graph of Captured Mosquito Species

1.3.1.1.1 Identification Characteristics

Phylum Arthropoda

Arthropods have segmented bodies and paired-joined appendages. Arthropods have a chitinous exoskeleton covering their bodies.

Class Insecta

The body of insects is distinctly divided into the head, thorax, and abdomen. Thorax comprises three segments, and each segment bears paired legs.

Order Diptera

Insects with single pair of membranous wings, the hind pair modified into halters are coming under Order Diptera. This order is also known as two-winged insects or true flies, which comprises mosquitoes, house flies, fruit flies, midges, etc. Larvae of Diptera are actively moving, free-living, legless organisms seen in terrestrial and aquatic habitats.

Suborder Nematocera

Segmented antennae are more prolonged than the head and thorax of the insect. Maxillary palpi usually have 4-5 segments.

Family Culicidae

A long proboscis is present. Stiff maxillary palpi are located on both sides of this proboscis. Long segmented antennae plumose in adult males and pilose in adult females. Larval and pupal forms are aquatic and active.

Mosquitoes

Mosquitoes are true dipterans with two real wings and a pair of primitive halters. Adult mosquitoes are terrestrial flying organisms whose juvenile forms are associated with stagnant aquatic habitats. These habitats spread all around the globe, distributed from desert pools to melted snow and from below sea level to higher altitudes. Mosquitoes are essential in public health zone because of the blood-feeding nature of mature female flies. Proteins from vertebrate blood are crucial for egg development in adult females. This link between mosquitoes and human beings will circulate pathogenic organisms, resulting in epidemic diseases (Resh and Carde, 2003).

Subfamily Toxorychitinae

Thick proboscis curved downwards and backwards.

Subfamily Anophelinae

Maxillary Palpi of both sexes are as long as the proboscis. The scutellum is rounded or semilunar. In males, the last two segments of the palpi appeared like a flattened and bulbous structure. The proboscis, head and rest of the body are arranged in a straight line and formed at a right angle with the surface during resting. The head-end is strongly tilted downwards, and the abdomen is pointed markedly away from the surface. Membranous wings are spotted in nature. Wing veins are alternately covered with dark and pale scales, which give rise to linear dark and pale spots. The median acinus of the salivary gland is saccular. Single spermatheca present in female Anophelinae. Eggs are boat-shaped with a well-defined upper surface. Eggs have lateral floats, which help to float on water. In larval forms of Anophelinae supporting tube of spiracular apparatus, called siphon, is absent. Owing to this

feature, larvae of Anophelinae keep a horizontal posture while resting or moving on the water surface with the help of floating abdominal hairs. During feeding, the floating larvae can rotate the head from 180° on for a better result.

Genus: *Anopheles*

The basal area of the male foreleg claw is composed of a median spur-like structure, and this claw-bearing segment is more extended than proceeding ones. Vein 5.1 is not curved beyond the cross vein.

Subgenus: *Anopheles*

Inner and outer parabasal spines are present on coxite. The inner spine is smaller and firmer than the outer spine arising from the tubercle. If only one spine is present, it will be inner. Usually, the propleural tuft has 4-5 hairs. Peculiar scale arrangements are seen in this subgenus; large black scales are distributed on divisions and crossing of veins - the presence of vast scales on the base of the 4th vein. On wings, different pale and dark scales can be seen in clearly defined distributions. In larvae, the inner side of the shaft is where the antennal hairs grow. There are also simple, long pleural hairs and weakly differentiated palmate hairs can be seen.

Group: *Anopheles*

Various wing ornamentation patterns are present. A higher degree of patterns is seen in wings, but these arrangements are moderate in legs. Pale tarsal tips are scarce. Pharynx with Long thin lateral flanges. Dorsal papillae 8-12 in number, larger second pair followed by 2-4 tiny hairs on each side of the anterior palate. The posterior palate is cone-shaped with a median linear pigmented area. Dorsal posterior prothoracic pleural hairs are simple in larval forms; the rest are similar to other groups.

Specimen Studied

a. *Anopheles barbirostris* van der Wulp, 1884 Figure 1.3

BIOLOGY: *Anopheles barbirostris* is a black mosquito with a considerably large size. Wing length varies from 3.8 - 4.6 mm. broad short, downwardly extending scales are present on occiput. The interocular vertex is narrow and straight. Dark stout vertical chaetae grow together, which forms a linear arrangement associated with ocular scales. Antennae have tiny dark scales on the torus and a large number of dark and a few pale scales present on flagellar segments. Palpi is shaggy and is also covered by exceptionally long, broad, dark scales. The apical area of the wings is completely dark, although two milk-white spots are present on veins 1 and 3. Numerous pale scales spread over the costa, but significant white spots are only present on the basal region. Scattered dark scales are present on the basal half of vein 6. An unmarkable fringe spot is present on veins 2,5; dark scales on the humeral cross vein. The basal half of the front femora is substantially bulbous. Three legs have ornamentation on their femora. With a few light-colored scales on the VIII segment, the abdomen is dark. Larvae range in colour from yellow-green to dark brown when fully mature. The Head is dark, and several silvery grey spots are present on the thorax and abdomen. An interesting habit of these larvae is that they lie on water surfaces as the body is apparently distorted.

DISTRIBUTION: Australia, Bangladesh, Cambodia, Guam, India, Indonesia, Laos, Malaysia, Myanmar, Nepal, Papua New Guinea, Pakistan, People's Republic of China, Philippines, Sri Lanka, Thailand, Vietnam.

ASSOCIATED DISEASES: Malaria, Japanese encephalitis, Lymphatic Filariasis

b. *Anopheles nigerrimus* Giles, 1900 Figure 1.4

BIOLOGY: Very dark large *Anopheline* having wing length of 3.5-5 mm. Regular scales are present on the occiput. Vertex has an expressive white spot and a tuft of chaetae present on it. The torus of the antennae has some small pale scales; five or more flagellar segments have broad white scales. Palpi are shaggy with long stiff black scales all over this structure with a pale apical band. A large tuft of dark scales presents laterally towards the base of the clypeus. Prothoracic lobe having a tuft of black scales; 4 or more propleural hairs present. Mesonotum is almost black in colour. Scutellum contains long dark scales. Dark pleurae with white horizontal lines. Spiracular hair-5, prealar-15, uppermesepidermal-10. Wings are three times longer than thorax. A combination of dark and pale scales is present on veins which create some interesting patterns. Pale scales are seen in the middle of the vein; dark scales are arranged towards the lateral side. Certain areas of the lower wing have dark scale, and the light scales seen on the upper wing surface. The proximal vein 1 and the costa junction have an apical pale spot but are not continuous with a pale apical fringe area. Beyond vein 3, the apex of the wing carries significant broad continuous areas. Black abdomen; margin of tergite VIII has some scale, tuft of black scales towards the apex of segment VII, rest of the area bare. Larva has dense, evenly pigmented antennae and long normal clypeal and frontal hairs - a Very small mentum with four teeth on either side. Tergite plates are short and wide - scantly modified spiracular chitinization.

DISTRIBUTION: Bangladesh, Brunei, Cambodia, India, Indonesia, Malaysia, Myanmar, Nepal, Pakistan, China, Philippines, Sri Lanka, Thailand, Vietnam.

ASSOCIATED DISEASES: Malaria. Lymphatic Filariasis

c. *Anopheles peditaeniatus* (Leicester, 1908) Figure 1.5

BIOLOGY: Maxillary palpi with pale apical bands; dark scales on palps are shining as purple. The number of propleural setae varies from 2-6. Intense longitudinal dark bands are seen on the mesonotum. A few white scales are present on the coxae. Tarsal segments contain a variety of arrangements of white scales; in the fore tarsi, the second band, and mid tarsi third band is more prominent; the hind tarsal segment with the broad white band which spreads towards the joints, midtarsal bands are prominent. Black scales cover the costal area between the base and the subcostal pale spot, and the humeral cross-vein area is not covered by any scales - no scales on the hind corners of the abdomen.

DISTRIBUTION: Afghanistan, Bangladesh, Bhutan, Cambodia, India, Indonesia, Iran, Malaysia, Myanmar, Nepal, Pakistan, People's Republic of China, Philippines, Sri Lanka, Thailand, Vietnam.

ASSOCIATED DISEASES: Malaria, Japanese encephalitis

Subgenus: Myzomyia

Adult coxite is comprised of 5 parabasal spines, which originated as a bunch. Basal spines are curved structures, and apical ones are longer. Dorsal papillae 6 in number posterior pair entirely different from others. They are completely lacking or a few numbers of propleural hairs. Significant wing ornamentation, veins 2 and 4 divided into two; junctions of most veins and cross veins bear a pale marking; costa having 4 black spots; edge area of costa which vein 2 arising extended is a pale area. Antennal hairs of larvae are simple, unbranched, and emerge from the outer region of the shaft. Leaflets of palmate hair have filamentous terminals. Most pleural hairs are simple, but some long hairs show a feathery appearance.

Specimen Studied

d. *Anopheles stephensi* Liston, 1901 Figure 1.6

BIOLOGY: Medium-sized mosquito with 2.5- 4.0 mm average wing length. The vertical area of the head pale in colour. Nine white flattened ventral chaetae bunched together to form a frontal tuft. Anteriorly, a linear arrangement of ocular scales is present. The torus and first 2.4 flagellar segments comprised pale scales - the apical segment of palpi having black and white interceding bands. The prothoracic lobe contains several pale scales. A median strip of mesonotum is composed of white scales, which are separated markedly from the darker lateral sides. The Wings have prehumeral continuous dark costal spots. A bulbous structure is present on the front leg's basal portion of the femora. Many significant spots are present on the legs; white spots on the hind femora and tibiae; some spots on the first tarsal segment; dark bands on 1-2 2-3 joining of the fore leg; dark bands on the mid tarsus. Dense dark and pale scales are present on the abdomen's II-VIII tergite, which also contain posterior lateral dark bands. Outer clypeal hairs are 2/3 the length of inner hairs; posterior ditto and outer clypeal hairs are the same. The end of the maxillary palp is divided into two.

DISTRIBUTION: Afghanistan, Bahrain, Bangladesh, Djibouti, Ethiopia, India, Iran, Iraq, Kuwait, Myanmar, Nepal, Oman, Pakistan, People's Republic of China, Philippines, Qatar, Saudi Arabia, Somaiia, Sri Lanka, Sudan, Thailand, United Arab Emirates, Vietnam.

ASSOCIATED DISEASES: Malaria, Chikungunya.

e. *Anopheles subpictus* Grassi, 1899 Figure 1.7

BIOLOGY: Medium-sized mosquito. The length of the wing varies from 2.3-4 mm. Pale vertical chaetae anteriorly clustered together to form a frontal tuft. Occular scales are narrow. The torus of the antennae with a few scales; the first flagellar segment has white scales. The apical segment of the palpi has a black-and-white arrangement of scales; the rest of the area is only covered by hairs in the thorax chaetae, only present on the prothoracic lobe. The median area of the mesonotum comprises tiny yellow hooked hairs and lateral areas having black hairs. The base of the costa contains three dark spots; the middle one is twice larger than the other two. Most of the areas of the femora are composed of pale scales except the basal dark rings. The tip of the tibiae is pale in colour - usually, light abdomen with golden yellow bands; the posterior end of VII and VIII have dark scales. Typically, cerci are covered by some dark scales, but sometimes pale scales are also present on them. The antennae of the larvae are slender. Well-developed palmate hair. Anal papillae are stout, which is twice longer as the anal segment.

DISTRIBUTION: Afghanistan, Australia, Bangladesh, Bhutan, Cambodia, India, Indonesia, Iran, Laos, Malaysia, Maldives, Mariana Islands, Myanmar, Nepal, Papua New Guinea, Pakistan, People's Republic of China, Philippines, Saudi Arabia, Solomon Islands, Sri Lanka, Taiwan, Thailand, Timor, Vietnam.

ASSOCIATED DISEASES: Malaria, Japanese encephalitis, Lymphatic Filariasis, west Nile Fever

f. *Anopheles vagus* Donitz, 1902 Figure 1.8

BIOLOGY: looks like *An. subpictus* with several characters; the Subapical area, which is 1/4th to 1/5th length of the pale apical region of the female palp, covers a

dark band. At the base of the costa, a spot is either wholly black or anterior pale and inner border dark scale combination. Legs are not spotted. In larvae, outer clypeal hairs are very short compared to inner hairs and originate very close to these inner hairs. Pleural hair 1 is simple but not divided into more than 2 as in *An. subpictus.* Pleural hair 2 rarely diverges. Only one hair on the metathorax is divided into many branches; other hairs are laterally divided into 4-6 on the distal end.

DISTRIBUTION: Afghanistan, Bangladesh, Bhutan, Cambodia, Guam, India, Indonesia, Laos, Malaysia, Mariana Isl., Myanmar, Nepal, People's Republic of China, Philippines, Singapore, Sri Lanka, Thailand, Vietnam.

ASSOCIATED DISEASE: Malaria.

Subfamily Culicinae

The proboscis is somewhat flexible and usually has even thickness, but a swollen tip may be present in some species. The tip of the proboscis does not have a hooked-like structure. Maxillary palpi shorter than proboscis. The posterior lining of the scutellum is trilobed, and three continuous rows of bristles are present. The abdomen is covered with broad scales and makes various patterns on it. The clypeus appears rounded at the top and the anterior end, and the length is greater than the broad. Body of a resting adult form 45° angles with the surface. The median acinus of the salivary gland is tubular with a narrow duct. The number of spermatheca in females varies from 1 to 3. Mouth brushes of larvae comprised of several hairs. Larvae of culicine have large tubular breathing air tubes called a siphon. viiith abdominal segment has a comb with or without chitinous plates.

Genus *Aedes*

Adult *Aedes* mosquitoes differ in size and scale patterns. *Aedes*'s 6th wing vein does not end at the 5th fork level; it ends close to the apex. Marginal hairs are seen on the squama of the wing. Lack of spiracular bristles as well as the occurrence of postspiracular bristles. Underneath, hairs on the wing base are absent. Pulvilli is absent. No bulbous structure appears at the tip of the proboscis. Proboscis is straight and somewhat bent upward in dried specimen. Prothoracic lobes look normal and are placed behind the head. Postnotum is a bare area; 4th tarsal segment of the front leg is long; small and symmetrical wing scales. Length of maxillary palpi differs in male and 1/4th of antennae in female mosquitoes; bushy antennal hairs seen in male mosquitoes, last two segments are long in male and, similar segments in females. Well-advanced bristles are seen on the mesonotum. In larvae, moderately hooked median hairs are seen in mouth brushes with apical serration. Four pairs of frontal hairs are seen in the head of the larvae. Thorax has modestly developed hairs; some species have large spines on the dorsum. I- VIII abdominal segments lack chitinized plates except lateral plates on VIII in some *Stegomyia* species. The VIIIth abdominal segment possesses comb scales that follow different numbers and patterns. The length of the siphon is 2-3.5 times its base diameter, but it varies in some species. The Siphon tube has only one pair of hair tufts nearer to the posterior margin. Pecten teeth that differ in shape and number according to species are seen in the siphon tube. The anal segment occupies a chitinous saddle and moderately developed anal fan - long lateral hairs. Long single outer short and bifurcate inner subdorsal hairs.

Subgenus: *Stegomyia*

Black-coloured mosquitoes with medium to small in size. The arrangement of pale scales creates some dots, patches, and stripes, which give demarcated ornamentation to the mosquito body parts. The basal region of the tarsal segment possesses white bands. In some species, only one pair of legs has bands on the tarsi, with some having more than one leg having these characteristics. Usually, tarsi are neither entirely black nor with apical and basal white scales. Black scales cover the proboscis; the vertex and scutellum have broad and flat scales. Instead of a group of hairs, only one hair is present on the larval antennal shaft. Frontal hairs B and C are arranged one by one in a line. Sometimes stellate hairs are seen on the thoracic dorsum and abdomen; spines are absent. Only one layer of comb scale is present, which occupies a serrated structure on its posterior end. Long siphon with 1-3 times the length of its base diameter with highly differentiated similar, small-sized pecten teeth. A pair of hair tufts are seen in the middle of the respiratory tube. The small anal segment with a chitinized saddle and a small anal fan.

Specimen Studied

g. *Aedes aegypti* (Linnaeus, 1762) Figure 1.9

BIOLOGY: The arrangement of silvery-white scales of distinctive body parts is one of the significant identifying characteristics of *Ae. aegypti.* A pale scale arose on the central vertical area and extended through amongst eyes; this white ornamentation is also seen in the tori, clypeus, and palpi tips. Flat pale scale patterns are also seen in scutellum. A long pale band extended almost the entire length of the mid-femur on its frontal view, but this band does not last to the knee. White scales that run nearly the whole length of the front portion of the hind femur extend as a strip to the knee

region. Mid and hind leg tarsi have a thin white band on 1-2 or 1-3 segments; on hind legs, these bands are present on segments 1-4, and entirely white 5th segment. Abdominal segments are brownish to black. The basal region of II-IV or V tergites possess lite pale scales, sometimes with bright dots. Lateral white patches are visible on the dorsal view of I- VII tergites. Larvae bearing 8-12 comb scales with highly differentiated lateral teeth. Single antennal hair arising near the base of the shaft. Moderately developed undivided pleural hairs are present in the meso and metathoracic segments. Long siphon (more than double the basal diameter length) has 3-5 hair sets beyond the middle area from the base. Anal segment entirely covered by chitin; papillae with rounded margins with double length fan.

DISTRIBUTION: Oriental area, Tropical, Subtropical, and temperate countries.

ASSOCIATED DISEASE: dengue vector, yellow fever, chikungunya, zika fever, yellow fever, West Nile fever, Malaria.

h. *Aedes albopictus* (Skuse, 1895) Figure 1.10

BIOLOGY: Black mosquito with white spots. The mesonotum of *Ae. albopictus* is almost entirely covered by a thin line of pale scales, which is a striking feature. A wide band of whitish scales may be seen on each of the hind tarsi segments. Bright flat scales cover every scutellar lobe. A strip of white scales is present on the margin of the mesonotum ahead of the wing root but not seen along with it. Every tergite base has a broad pale band. Larva has long antennae with 10 times longer shafts than its width. The centre of the antennae has one hair that emerges. The front of the head is covered with fine, visible hairs. In the thorax, lateral hairs are of medium length; in the abdomen, they are longer. 8-12 comb scales arranged in a single row; comb tooth without lateral denticles and have no chitinized base. The siphon is rather

twice longer as the diameter of its base. 7-14 pecten teeth with lateral denticles. The anal segment is covered by a chitinized ring, with some spines on the hind dorsal margin. A few long fan hairs arise from the fan-plate, long papillae with rounded tips.

DISTRIBUTION: Oriental area, Tropical, Subtropical, and temperate countries.

ASSOCIATED DISEASES: dengue vector, yellow fever, chikungunya, zika fever, yellow fever, West Nile fever, Malaria.

i. *Aedes vittatus* (Bigot, 1861) Figure 1.11

BIOLOGY: Particular scale arrangement is seen in *Aedes vittatus.* Distinct tiny white spots appeared on the thoracic mesonotum. White band on the preapical area of femora; white ring on the middle of tibiae. Vertex with upright scales; some yellow scales are present on the proboscis. Larva; various sets of frontal hairs present; A divided into 4 to 6 branches, B and C are simple; D and E are long hairs. 8 times longer shaft, when compared to its width, is a characteristic feature of antennae. The basal area of the antennae shows a hair tuft with 3 branches of hair. The median hair of mouth brushes bears tiny teeth on its apex. Lateral hairs; thoracic lateral hairs are moderately developed and originate from chitinised tubercles. A single row of comb scales with 6-9 simple teeth. Long siphon with 24-30 pecten teeth lying between hair tuft and apex. A chitinous saddle is present on the anal segment: long papillae, and a moderately developed fan with branch hairs that do not originate from the fan plate.

DISTRIBUTION: Algeria, Angola, Bangladesh, China, India, Iran, Italy, Kenya, Laos, Liberia, Madagascar, Malawi, Malaysia, Mali, Mozambique, Namibia, Nepal, Niger, Nigeria, Pakistan, Portugal, Republic of South Africa, Saudi Arabia, Sri

Lanka, Sudan, Tanzania, Thailand, Tunisia, Uganda, Vietnam, Yemen, Zambia, Zimbabwe.

ASSOCIATED DISEASES: Chikungunya, Dengue, Yellow fever, Zika, Japanese encephalitis.

Subgenus: *Phagomyia*

Vertical area of the head covered by wide accumbent median hairs. Several long hairs originate from the 3.4.5 segments of maxillary palpi. The thoracic scutum has a broad anterior area with a white scale pattern, sometimes incomplete or complete median strip, and different patterns. Postnotum with or without a patch of scales; sub and post-spiracular scales are absent. Legs are ornamented with patches of white scales. Apical dorsal area of fore tibiae, apical and basal region of hind tarsomere 1, basal area of tarsomere 2 or area between 2 and 3 have white patches of scales. Stiff broad scales cover the posterior area of the abdominal segment. Comb scales of larvae are uniformly serrated.

Specimen Studied

j. *Phagomyia cogilli* (Edwards, 1922) Figure 1.12

BIOLOGY: Medium-sized black and white mosquito species. A band of pale scales is absent on the head, but a narrow strip of white scales is seen on the eyes' margin. The pro-epimeron's posterior boarder comprises white patch rest of the bare area. White spot of large circular smooth located on the mesonotum of the thorax. The scutellar middle lobe contains a group of pale scales, but lateral lobes have dark scales.

DISTRIBUTION: Oriental area

Genus *Armigeres*

Members of Genus *Armigeres* vary in size from medium to large. This genus resembles *Aedes*, but they are not highly ornamented. Scales on the head and thorax wide and plane. Stiff proboscis with laterally compressed downwardly arched tip. In the male, the last two segments of the maxillary palpi are thin, elongated, and bent upward, making the palpi longer than the proboscis. Female palpi have ¼ to ¾ length of the proboscis. Females with pointed abdomen with incompletely retractile VIII segment and small cerci. The inner surface of the lobe contains a spine in male hypopygial coxite. Spines were seen on the style apex. No pecten teeth were seen on the larvae siphon, which differs from *Aedes*. An irregular line of comb scales. Short siphon and poorly developed fan and semi-circular papillae. The ventral tuft on the last segment prior to the fan is absent.

Specimen studied

k. *Armigeres subalbatus* (Coquillett, 1898) Figure 1.13

BIOLOGY: Maxillary palpi are 1/3th length of the proboscis. Boarder areas of mesonotum consist of yellowish scales. III- IV sternite apex bears a dark band.

DISTRIBUTION: Bangladesh, Cambodia, India, Indonesia, Japan, Laos, Malaysia, Myanmar, Nepal, Pakistan, People's Republic of China, Philippines, South Korea, Sri Lanka, Taiwan, Thailand, Vietnam.

ASSOCIATED DISEASES: Japanese encephalitis, Lymphatic Filariasis

Genus *Culex*

Highly evolved pulvilli, visible pale in the dark background is an identifying feature of the genus *Culex*. Buccopharyngeal armature present. Spiracular and post-

spiracular hairs are absent, but mesonotal hairs exist. In larval forms numerous sets of split hairs are seen on the larval siphon. Comb scales are arranged in a triangular pattern. The chitinous plate is absent in the abdomen.

Subgenus *Lutzia*

Large mosquitoes with more than 4 mesepimeral hairs and distinct pulvilli. In males, bushy, upwardly curved palpi are longer than the proboscis, but their length is more than one-fourth in females. White patches of scales are situated on the fore and middle femora and tibiae. Hypopygial coxite is bare, but 3.5 small stiff spines arise from its subapical lobe. The small style, which is naive and bent, usually ends in tiny projections. Apically pointed lateral plate with tooth-like structures. The mouth's teeth are typically large and broad, but occasionally they can be narrow and spoon-shaped. Larval mouth parts have evolved to fit their predatory nature. The apical half of a few hooked solid mouth brushes have hairs, but the frontal portion only has one hair. Pectin teeth and a line of posteroventral hairs are distributed throughout the air tube. The enlarged anal segment with the elongated dorsal area.

Specimen studied

I. *Lutzia fuscanus* (Wiedemann, 1820) Figure 1.14

BIOLOGY: The adult head consists of vertical dark, a median strip of light-yellow coloured, and white lateral scales. Antennae are brown, and pale scales are present on the tori and first flagellar segment. Palpi and clypeus are comprised of a brown and yellow combination of scale arrangements. The palpi have one-fourth length of the proboscis, which has median linear yellow and dark brown lateral scales. The mesonotum is dark brown and has unspecified patches and stripes. A pair of white spots is seen on the prespiracle region, dorsal area of the wing root, and linear light

scales on either side of the scutellum. The anterior prothoracic lobe has brown scales. Wings covered with dark scales. The femur of the front and mid-leg shows some peculiar ornamentation; the front view seems like scattered pale scales on the brown background, but the back view only occupies light yellow scales. The inner side of the hind femora consists of pale scales, however apical half of the outer side is occupied by pale scales, excluding the dorsum - these pale outer scales forming a narrow vague strip towards the tip. Tibiae is also freckled with white scales. Tarsi appears brown to yellow according to the angle of view, devoid of the white ring. Abdominal segment II-IV is either covered by deeply brownish scales or an apical narrow yellow band, and the presence or absence of a yellow spot in sub median area. V has a broad yellow band, and VI-VIII is completely yellow-scaled.

Larvae have moderately large heads and short antennae with a single hair on the base and other hairs on the tip. Hooked and strong mouth brushes are well adapted for predation, with tiny hairs associated on the apical half. Frontal hairs usually are long and simple, but sometimes they are bifurcated. Normal preclypeal hairs originated apart and extended. Thoracic lateral hairs are tapering spines originating from more prominent tubercles, and abdominal lateral hairs have split. A set of 35-45 serrated comb scales are situated on the VIII abdominal segment of larvae. Small wide siphon tube, distributed with toothed and simple pecten laterally and apical long irregular hairs posteriorly. The anal segment is larger than the siphon. 14 divided hairs originated from the fan plate.

DISTRIBUTION: India, Malaya. China, Philippines

ASSOCIATED DISEASES: Japanese encephalitis, Filariasis

Subgenus *Culex*

Subgenus *Culex* is comprised of tiny to medium-sized adult mosquitoes. The Head is composed of narrow, erect scales on the vertex, broad hairs on the sides, and thin hairs on the scutellum. Shorter proboscis than palpi in males with furry upturned last two segments. Arrangements of scales are different in every species. In males, antennae and hypopygium coxite are bare. Male hypopygium is devoid of the apico-ventral lobe, but the subapical lobe bears spines on its segments. A spiny crest is absent in style. The female buccopharyngeal structure has sharp or blunt teeth. Mouthparts of larvae are normal, not modified for predation.

Specimen studied

m. *Culex bitaeniorhynchus* Giles, 1901 Figure 1.15

BIOLOGY: The wing length of adult mosquitoes is 5 mm. significant characteristics of *Culex bitaeniorhynchus* are spotted wings, apically banded by yellow scales on tergites. The vertical area of the head is covered by thin golden yellow and brown and yellow erect scales; either side of the vertex consist of tiny dark spot and broad pale scales dispersed over the lateral area. Dark maxillary palpi with apical light scales. A wide creamy strip on the middle and pale scale along the tip of the proboscis, the rest of the area is dark. Sometimes pale spots may also be present on the proboscis' base. Thorax; thin golden-brown scales occupy the area between the front and wing base; the rest of the thorax contains dark brown scales, with few golden scales present on the anterior region of scutellum with a pair of submedian dark spots. The remnant area of the scutellum contains thin dark brown scales on the base of the mid-lobe and golden scales on the apical area of the lateral lobe. Dark and light-yellow speckles are noted in wing veins. Legs are ornamented with several

pale scales on the femur, tibiae and first segment of tarsi. Tarsi are dark-coloured except for pale bands on the basal apical area. Black-scaled tergite with scattered white scales with a relatively wide apical yellow band. Larvae have a length of 7 mm and a 2mm long siphon. Bright green body with relatively small, chitinized, pale Head. Antennae have a lighter base and a darker tip. The hairs closest to the shaft apex are called subapical hairs. Long solitary prothoracic hairs and moderately developed lateral hairs are present on the thorax. Additionally, there are a few lateral hairs on the abdomen. 5-6 enlarged sharp comb scales are present, which are unevenly arranged with basal hairs and without lateral serration. Narrow light siphon with 6-8 times longer than basal diameter. 3-6 tiny translucent pecten teeth seen on siphon hard to visible. Anal segments with hairs and papillae.

DISTRIBUTION: Australia, Bangladesh, Cambodia, Cameroon, Egypt, Ethiopia, Gabon, Gambia, Ghana, Hong Kong, India, Indonesia, Iran, Japan, Kenya, Laos, Liberia, Madagascar, Malaysia, Mali, Mozambique, Myanmar, Namibia, Nepal, Nigeria, Pakistan, Papua New Guinea, People's China, Philippines, Russia, Saudi Arabia, Senegal, Singapore, Somalia, South Africa, South Korea, Sri Lanka, Sudan & South Sudan, Taiwan, Tanzania, Thailand, Uganda, Vietnam, Yemen, Zambia, Zimbabwe.

ASSOCIATED DISEASES: Japanese encephalitis, Lymphatic Filariasis, Rift Valley fever, Dengue, Malaria.

n. *Culex gelidus* Theobald, 1901 Figure 1.16

BIOLOGY: Anterior mesonotum of the thorax is completely covered by white scales. The area beyond the wing root is completely covered by brownish black. The female proboscis bears a thin pale strip away from the base. Tiny thin median scales

are present on wings. The apical and basal regions of the tarsal segment exhibit a strip of white scales. The femur and tibiae are completely dark-coloured. Relatively short antennae with hair tuft at 2/3 length from base. The nearest area of the shaft apex possesses subapical hairs. 9- 10 small teeth occupied on the mentum. A triangular pattern of com scale composed of 35 tiny teeth in larvae. The Middle area of the siphon is broad and narrow towards the apex. The long siphon has 3–4-time length of its base diameter with four pairs of 4 -5 forked hairs on the posterior border. 9-11 comparatively large pecten teeth have pinpointed 4-5 projections on the lateral side. A soft chitinized ring covers the anal segment. Dorsal papillae are the same length as fan hairs, and ventral papillae are small.

DISTRIBUTION: Bangladesh, Cambodia, India, Indonesia, Japan, Laos, Malaysia, Myanmar, Nepal, Pakistan, Papua New Guinea, People's Republic of China (includes Hong Kong), Philippines, Singapore, Sri Lanka, Taiwan, Thailand, Vietnam.

ASSOCIATED DISEASES: Japanese encephalitis, Chikungunya, Ross River fever, West Nile fever.

o. *Culex tritaeniorhynchus* Giles, 1901 Figure 1.17

BIOLOGY: Mesonotum of the adult thorax is entirely coated by dark brown scales. Tibiae are completely dark-scaled, devoid of any white bands. The ventral area of the femur has pale scales rather dorsal area, clothed by relatively darker ones. Male palpi with black tip deprived of a stout. A transparent row of scales on the lower side of the longer segment. The female proboscis has a pale strip commonly seen on the underside but occasionally present on the dorsal surface. Anterior fork-cell

ending on wing base rather than next posterior segment. The siphon of the larvae has five pairs of posterior hair tuft and one pair of lateral hairs.

DISTRIBUTION: Afghanistan, Albania, Angola, Azerbaijan, Bangladesh, Cambodia, Cameroon, Central African Republic, Egypt, Ethiopia, Fiji, Gabon, Gambia, Georgia, Ghana, Greece, Guam, Hong Kong, India, Indonesia, Iran, Iraq, Israel, Japan, Jordan, Kenya, Kuwait, Laos, Lebanon, Liberia, Madagascar, Malaysia, Maldives, Mariana Islands, Mauritius, Mozambique, Myanmar, Nepal, Nigeria, Oman, Pakistan, Papua New Guinea, China, Philippines, Russia, Saudi Arabia, Senegal, Singapore, Somalia, South Korea, Sri Lanka, Syria, Taiwan, Tanzania, Thailand, Timor, Togo, Turkey, Turkmenistan, Uzbekistan, Vietnam, Yemen.

ASSOCIATED DISEASES: Japanese encephalitis, Lymphatic Filariasis, Dengue, Rift Valley fever, West Nile fever.

p. *Culex pipiens* Linnaeus,1758 Figure 1.18

BIOLOGY: Short proboscis with golden yellow scales. Thin scales are seen on the dorsal side of the occiput. Scutum concealed with golden yellow scales; a linear array of setae is presented on the middorsal region without any white dot. White and elongated scales are situated on the posterior border of the scutum and scutellum. A dull white scale saw on the basal area of the abdominal tergites. This strip appeared as a broad concave patch at the posterior border of tergite and well-defined basolateral mark at the lateral side. The legs are entirely black. Wing veins are evenly distributed with dark scales. Antennae of larvae are compressed in the distal portion with setae on the outer third of the shaft. 5th and 6th setae have 5 more splits. The siphon is small and somewhat concave shaped. Uniformly arranged

pecten teeth are present on the siphon, and three or more branched setae are located after this pecten.

DISTRIBUTION: Afghanistan, Albania, Algeria, Angola, Argentina, Armenia, Australia, Austria, Azerbaijan, Belgium, Bulgaria, Cameroon, Canada, Canary Islands, Central African Republic, Chad, Chile, Comoros, Cote d'Ivoire, Croatia, Denmark, Congo, Djibouti, Egypt, Ethiopia, Finland, France, Georgia, Germany, Ghana, Greece, Guam, Guinea, Guyana, Honduras, Hungary, India, Iran, Iraq, Ireland, Israel, Italy, Japan, Jordan, Kazakhstan, Kenya, Kosovo, Kuwait, Latvia, Lebanon, Liberia, Libya, Madagascar, Malaysia, Mali, Malta, Mauritania, Mexico, Moldova, Morocco, Netherlands, Nigeria, Norway, Pakistan, China, Philippines, Poland, Portugal, Qatar, South Africa, Romania, Russia, Saudi Arabia, Serbia, Slovakia, Slovenia, South Korea, Spain, Sudan & South Sudan, Sweden, Switzerland, Tajikistan, Tanzania, Thailand, Tunisia, Turkey, Turkmenistan, Uganda, Ukraine, United Kingdom, United States, Uruguay, Uzbekistan, Vietnam, Yemen, Zimbabwe.

ASSOCIATED DISEASES: Japanese Encephalitis, Lymphatic Filariasis, Rift Valle fever, West Nile fever.

q. *Culex quinquefasciatus* Say, 1823 Figure 1.19

BIOLOGY: Medium-sized adult mosquitoes. A significant characteristic of *Culex quinquefasciatus* is the absence of a definite white ring on the proboscis. Mesonotum enveloped with thin forked pale brown scales. Abdominal tergites have a yellowish strip on the dorsal base, and the ventral side has pale scales. Light brown and one or two white patches of scales are seen on pleurae. Tarsi are devoid of white rings. Post-spiracular scales are absent. Hairs on larval head tapering and

filamentous at distal region. The siphon is short and bulb-shaped. 30-50 comb scales arranged in a triangular pattern with fan-shaped fringed teeth.

DISTRIBUTION: Afghanistan, Algeria, Angola, Anguilla, Argentina, Australia, Bahrain, Bangladesh, Barbados, Belize, Bolivia, Bonaire, Brazil, British Indian Ocean Territory, Borneo, Burkina Faso, Cambodia, Cameroon, Cayman Islands, Central African Republic, Chile, Colombia, Comoros, Congo, Costa Rica, Croatia, Cuba, Curacao, Djibouti, Dominica, Ecuador, Egypt, El Salvador, Equatorial Guinea, Ethiopia, Fiji, France, French Guiana, French Polynesia, Gabon, Gambia, Ghana, Greece, Grenada, Guadeloupe, Guam, Guatemala, Guyana, Haiti, Honduras, Hong Kong, India, Indonesia, Iran, Iraq, Israel, Jamaica, Japan, Jordan, Kenya, Kiribati, Kuwait, Laos, Liberia, Madagascar, Malaysia, Maldives, Mali, Mariana Islands, Marshall Islands, Martinique, Mauritania, Mauritius, Mexico, Micronesia, Montserrat, Morocco, Mozambique, Myanmar, Nauru, Nepal, New Zealand, Nicaragua, Niger, Nigeria, Oman, Pakistan, Palau, Panama, Papua New Guinea, Paraguay, China, Peru, Philippines, Puerto Rico, South Africa, Russia, Saint Lucia, Saudi Arabia, Senegal, Singapore, Solomon Islands, Somalia, South Korea, Sri Lanka, Sudan & South Sudan, Suriname, Taiwan, Tanzania, Thailand, Timor, Togo, Tonga, Turkey, Tuvalu, Uganda, United States, Uruguay, Vanuatu, Venezuela, Vietnam, Virgin Islands, Yemen, Zambia.

ASSOCIATED DISEASES: Japanese Encephalitis, Lymphatic Filariasis, Rift Valley fever, West Nile fever, Malaria.

Genus *Mansonia*

Long antennae. The Squama of the wing is fringed, and the 6th wing vein ends further than the fork of 5th one at the wing margin. Spiracular area without any

scales. Post-notum and scutum without any hairs. A combination of dark and white scale patterns is seen on wings with broad, irregular scales. Solid even scales cover abdominal sternite and tergite. Palmate hairs are absent in the abdominal segments of *Mansonia* larvae. Siphon is present and adapted for piercing the tissues of aquatic plants

Subgenus *Mansonioides*

Mansonioides consist of medium-sized mosquitoes with yellowish-brown body. Legs have several white patches and pale rings on the tarsi. Wings are ornamented with wide black and white scales, and most are unequal. Male maxillary palpi longer than proboscis. The second last segment of the palpi has an upward bend and is devoid of hair. The tiny last segment is curved downwards. Significantly modified pleural hairs; 10-20 postspiracular, 9-15 prothoracic ppn, 12-18 upper mesepimeral, 4-9 lower mesepimeral. Larva; antennal shaft stiff and split into three equal parts. Simple elongated hairs make up mouth brushes. There are conspicuous and long preclypeal bristles. At the centre of the clypeus, a curved structure is formed by the frontal hairs A, B, and C. Large central teeth are covered by smaller ones on either side, presented in small mentum and reasonably modified thoracic and abdominal hairs. Comb with elongated broad teeth in larvae. A small siphon with modified valves for piercing plant tissue is associated with 2 pairs of long spines. Anal segment elongated than siphon and entirely protected by a sheath. Highly modified fan plate with 4 branched hairs between plate and segment.

Specimen studied

r. *Mansonia uniformis* (Theobald, 1901) Figure 1.20

BIOLOGY: Brownish species with a wing length of 4.5 mm. horizontal yellow and erect, vertical brown scales were noticed on the vertex. Broad pale scales are present on the lower sides of the eyes. An indefinite ring appeared on the femur, and the tarsi were yellow. Antennal flagellum brown with a slightly developed pale ring. Light brown clypeus. Palpi are 3 times shorter than proboscis with yellow and brown scales and pale but not precisely white tips. Yellow proboscis shows dark brown scales at the 1/3 apical area. Greenish and pale brown scales appeared on the mesonotum. A band made on the sublateral side starts from the frontal area and ends up above the wing root. Thin and light yellow scutellar scales are present. Some blotches of pale scales are distributed over brown pleurae and thin white scales on the prothoracic lobe. Yellowish-dark brown wide irregular scales are evenly arranged in the wings. Legs are yellow with bands on many segments; the hind femur has 4-5 tilted white marks on the outer region, the fore and mid pair have relatively faint marks; tibiae with pale rings, and tarsi with only one white ring. The outer area of the abdomen is composed of brown scales. White scales are mainly arranged as a lateral spot at the apex of every segment. Uniformly arranged chitinized fork on the lateral side of the tergite VIII. larval antennae with a dark ring at the base.

DISTRIBUTION: Angola, Australia, Bangladesh, Benin, Botswana, Burkina Faso, Cambodia, Central African Republic, Comoros, Ethiopia, Gabon, Gambia, Ghana, Guam, Hong Kong, India, Indonesia, Japan, Kenya, Liberia, Madagascar, Malawi, Malaysia, Mali, Mozambique, Myanmar, Nepal, Niger, Nigeria, Papua New Guinea, Pakistan, People's Republic of China, Philippines, Senegal, Sierra Leone, Solomon

Islands, Somalia, South Africa, South Korea, Sri Lanka, Sudan & South Sudan, Taiwan, Tanzania, Thailand, Timor, Uganda, Vietnam, Zambia.

ASSOCIATED DISEASES: Lymphatic Filariasis, Japanese Encephalitis, Ross River fever, Rift Valley fever, Malaria.

s. *Mansonia indiana* Edwards, 1930 Figure 1.21

BIOLOGY: Adult ones are very similar to *Ma. uniformis* but varies from this lack of greenish patch on mesonotum. Instead of this, dark brown scales are completely covered on the mesonotum. Some lateral white scales are also distributed on the mesonotum of some species. Chitinized forks are present on tergite VIII, which are set apart. VIII sternite lobes are slightly emarginate and smaller than *Ma. uniformis.* Larvae are the same as *Ma. uniformis.*

DISTRIBUTION: Bangladesh, Cambodia, India, Indonesia, Laos, Malaysia, Myanmar, Nepal, Papua New Guinea, Philippines, Singapore, Sri Lanka, Thailand, Vietnam.

ASSOCIATED DISEASES: Japanese Encephalitis, Lymphatic Filariasis.

t. *Mansonia bonneae* Edwards, 1930 Figure 1.22

BIOLOGY: Mesonotum diversly marked with two definite white rounded scales. Pleurae covered by dark scales. Five strips of pale scales are noticed on the hind femur. The mid lobe of the scutellum with thin scales. Small area of white scales with supra-alar bristles over the wing root is absent.

DISTRIBUTION: India, Burma, Sri Lanka, Africa, Japan, Australia.

ASSOCIATED DISEASES: Japanese Encephalitis, Lymphatic Filariasis.

Table 1.3 Total number of mosquitoes collected during the study period

Sl No	Mosquito Species	2016-2017	2007-2018	Total
1	*An. barbirostris*	161	161	332
2	*An. nigerrimus*	188	177	365
3	*An. peditaeniatus*	57	50	107
4	*An. stephensi*	184	164	348
5	*An. subpictus*	392	370	762
6	*An. vagus*	193	178	371
7	*Ae. aegypti*	1848	1505	3353
8	*Ae. albopictus*	2392	2757	5149
9	*Ae. vittatus*	239	250	489
10	*Ph. cogilli*	15	0	15
11	*Ar. subalbatus*	4639	4315	8954
12	*Cx. bitaeniorhynchus*	25	6	31
13	*Cx. gelidus*	2294	2190	4484
14	*Cx. pipiens*	1670	1212	2882
15	*Cx. quinquefasciatus*	1449	1474	2923
16	*Cx. tritaeniorhynchus*	973	1107	2080
17	*Lt. fuscanus*	572	453	1025
18	*Ma. bonneae*	10	27	37
19	*Ma. indiana*	364	399	763
20	*Ma. uniformis*	170	171	341
TOTAL				**34801**

1.3.1.1.2 Diversity Analysis

The dominance index (D) explains the abundance of the most common species and diversity. D value of Adat Kole 0.1376, Palakkal Kole 0.127, and Muriyad Kole 0.1389. D values of all study sites were low and close to 0, which indicated evenly distributed species were present. Simpson's indices (1-D) were estimated as Adat Kole- 0.8624, Palakkal Kole- 0.873, and Muriyad Kole- 0.8611; these high values illustrated the three sites' diversity was high.

The greatest species richness 'S' recorded from Adat Kole was 19, followed by Palakkal Kole 18 and Muriyad Kole 17. Shannon index values of all sites are almost

in the same range Site 1 - 2.311, Site 2 - 2.326, and Site 3 - 2.24. Shannon's index value of 1.5 to 3.5 indicates moderate diversity. The evenness index showed that each site has a medium amount of evenness Site 1 - 0.5309, Site 2 - 0.5688, and Site 3 - 0.5526. Berger Parker index of Adat Kole - 0.2729, Palakkal Kole - 0.2347, Muriyad Kole - 0.2572. Margalef's index of Adat Kole - 1.889, Palakkal Kole - 1.857, Muriyad Kole - 1.709. All calculated alpha diversity indices were included in Table 1.4.

Jaccard index between sites 1 and 2 was 0.6, between site 1 and site 3 was 0.57 and between site2 and site 3 was 0.62. Sorenson index of site 1 and site 2 combinations was 0.75, site 1 and site 3 was 0.73, site 2 and site 3 was 0.76. Gamma diversity was calculated as 20.

Table 1.4 Different diversity indices of mosquito collected from three different sampling sites of Kole wetlands of Thrissur

ALPHA DIVERSITY INDICES				
SL NO		Adat Kole	Paakkal Kole	Muriyad Kole
1	Species Richness	19	18	17
2	Dominance - D	0.1376	0.127	0.1389
3	Simpson- 1-D	0.8624	0.873	0.8611
4	Shannon - H	2.311	2.326	2.24
5	Evenness H/S	0.5309	0.5688	0.5526
6	Berger-Parker	0.2729	0.2347	0.2572
7	Margalef's index	1.889	1.857	1.709
BETA DIVERSITY INDICES				
		Adat-Palakkal	Palakkal-Muriyad	Adat-Muriyad
1	Jaccard index	0.6	0.62	0.57
2	Sorenson index	0.75	0.76	0.73
GAMMA DIVERSITY 20				

1.3.1.2 Discussion

1.3.1.2.1 Wetlands

Wetlands are permanent or temporary shallow water bodies (less than 6m in depth) with lentic or lotic freshwater, brackish or marine water. Various marshes, fen, peatlands, and water lands are considered wetlands (Barbier et al., 1997). Wetlands are highly productive ecosystems on earth and rich in faunal and floral diversity. They can manage the cleaning of the environment through the functioning of biogeochemical cycles and are considered the 'kidneys of the earth. They have also been involved in flood control, deposition of sediments, water preservation, groundwater reloading, shoreline maintenance, biogeochemical cycle regulation, and pollution control (Sujana and Sivaperuman, 2008). Mosquitoes are Dipteran (two-winged flies) insects coming under the Family Culicidae, which have a substantial role in many epidemiology of human beings and are a noticeable part of wetland ecosystems (Muesebeck, 1952; Goma, 1966; Schafer, 2004). Only a restricted number of mosquitoes have vectorial capacity among the Culicidae members. So spatial distribution of disease-causing microorganisms generally depends on the dispersion of these vector mosquito species (Ostfeld et al., 2005).

Wetland ecosystems of Kerala are well known for the presence of disease-spreading vector mosquitoes, and various epidemic outbreaks were reported from accompanying areas of these water bodies (Jomon et al., 2009). The present study investigates the diversity and spatial distribution of different mosquito species in the Kole wetlands of Thrissur, Kerala. It discusses factors like seasonal variation and physicochemical parameters of breeding water on the diversity pattern. 20 different mosquito species belonging to 5 genera were identified during the study period.

1.3.1.2.2 Diversity Analysis

A mosquito diversity study in Thrissur Kole wetlands concluded with 20 available mosquito species. These mosquitoes were collected from three sampling sites: Adat Kole, Palakkal Kole, and Muriyad Kole. There have already been similar diversification studies on marsh mosquitoes from other countries. In 2015 Medlock and Vaux studied habitat preferences and seasonal dynamics of wetland mosquitoes in the UK and listed almost 15 mosquito species. Another study was conducted by Dash and Hazra in and around the internationally recognized Chilika Lake, Orissa, India. Their survey identified 22 mosquito species of 6 genera, collected from 8 villages in and around Chilika lake. Out of 22, 10 species were included in Genus *Anopheles*; the present study in Thrissur Kole lands also revealed that more species were from *Anopheles* (6) and *Culex* (6) Genera. Cardo talked about some findings from their study on the community structure of ground-water mosquitoes in wetlands in Argentina. They discovered 23 mosquito species from 7 different genera. They stated that the genera *Culex* and *Ochlerotatus* accounted for 87% of the sample. (Cardo et al., 2011).

Alpha Beta and Gamma diversity indices were used to explain the mosquito diversity in the study area. This diversity analysis can help study the factors influencing biodiversities like climate, biotic and abiotic factors, and anthropological activities (Halffter, 1998;). Bernues and Jimenez conducted a mosquito diversity study in protected natural parks in Valencian Autonomous Region (Eastern Spain) from 2008 to 2011. They selected entirely different habitats, such as Inland Mountain areas (IMA), Coastal Mountains areas (CMA), and Coastal wetland marshes (CWM) as their sampling sites. Bernues and Jimenez reported maximum species richness from IMA (21), followed by CMA (8) and CWM (7).

The present study described slight variation in the species richness, with Adat Kole having 19, followed by Palakkal Kole 18 and Muriyad Kole 17. Simpson and Shannon's indices also follow this variation pattern, which has more remarkable dissimilarities in Bernues and Jimenez's study. Three study sites selected were part of the Thrissur Kole wetland ecosystem; environmental factors and breeding habitats were almost similar. These low variations in alpha diversity indices may be because of this similarity.

Kerala state is particularly susceptible to diseases spread by vectors due to its favourable year-round temperatures, substantial yearly rainfall, and plenty of mosquito breeding grounds. Environmental changes have significantly impacted the variety and abundance of mosquito fauna. Kerala has experienced numerous ecological changes over the past forty years, including water extraction, changes to water courses, the building of irrigation canals, and habitat modification for the growth of agriculture, which has resulted to a remarkable increase in the number of water bodies that support mosquito breeding (Jomon et al., 2009). Balasubramanian and Nikhil (2013) studied the mosquito fauna in several locations in the Alappuzha and Kottayam districts of Kerala state to gather data on the diversity of mosquitoes. Alappuzha district recorded a total of 44 species of mosquitoes, of which 21 subgenera and 11 genera, and Kottayam district, 21 species of mosquitoes, of which 14 subgenera and 9 genera, respectively. Radhakrishnan (2019) conducted a study to learn more about the current situation regarding the dispersion and species variety of vector mosquitoes in the Ernakulam district from October 2017 to August 2018. A survey of mosquito larvae and adults was conducted to gather data on the current species variety of mosquitoes in the district. In the study, 26 species of mosquitoes from 6 genera were identified. The most common genus was *Anopheles* (11), which

was followed by *Culex* (6), *Aedes* (5), *Mansonia* (2), *Armigeres* (1) and *Toxorhynchites* (1). Throughout the investigation, *Ae. albopictus, Cx. tritaeniorhynchus, Cx. gelidus, Cx. quinquefasciatus,* and *Ma. uniformis* were observed. There were found filariasis, dengue, chikungunya, Japanese encephalitis, and dengue fever vector mosquitoes from the study site.

Even though wetlands achieve much international importance, wetlands remain highly vulnerable landscapes because of several anthropological activities. These activities may lead to the habitat reduction of many organisms, including mosquitoes (Willott, 2004; Schafer et al., 2008). Francis and George 2013 were also discussing such human practices in Thrissur Kole lands. This habitat destruction and fluctuating climatic conditions significantly reflect the mosquito diversity in the study area. However, wetlands are biodiversity hotspot areas; only 20 different mosquito species could collect from the study area. Mari and Jimenez summarized their result on the 'difference in mosquito biodiversity across varying climates and land-use categories in Eastern Spain' in 2011. They identified 29 mosquito species from their 11,279 total collection and reported higher biodiversity in the wettest and non-anthropized areas (Mari and Jimenez, 2011). Thrissur Kole lands were periodically used for paddy cultivation. Organic and inorganic fertilizers and pesticides were regularly applied to these lands to improve the yield (Tessy and Sreekumar, 2008). Such chemical treatment diminished the faunal and floral diversity of Kole wetlands.

1.3.2. GIS

1.3.2.1 Result

The integration of maps showing the numbering system with those showing 12 different sampling locations in the Kole wetlands of Thrissur, Kerala (Figure 1.23,

1.24, 1.25). These sampling locations were distributed in 3 different sites (Adat Kole, Palakkal Kole, and Muriyad Kole). Location 1 (10º 33′ 01.2″ N 76º 09′ 10.2″ E) was the Ambakkad area of site 1 Adat Kole. 17 mosquito species were collected from there during the study period. They were *An. barbirostris, An. nigerrimus, An. peditaeniatus, An. stephensi, An. subpictus, An. vagus, Ae. aegypti, Ae. albopictus, Ae. vittatus, Ar. subalbatus, Cx. bitaeniorynchus, Cx. gelidus, Cx. pipiens, Cx. tritaeniorhynchus, Lt. fuscanus, Ma. indiana, Ma. uniformis.* Location 2 was Ambathonnu Area of Adat Kole. GPS location of Ambathonnu was10º 31′ 29.7″ N 76º 07′ 18.0″ E. 18 mosquito species were identified from Location 2, *An. barbirostris, An. nigerrimus, An. peditaeniatus, An. stephensi, An. subpictus, An. vagus, Ae. aegypti, Ae. albopictus, Ae. vittatus, Ar. subalbatus, Cx. gelidus, Cx. pipiens, Cx. quinquefasciatus, Cx. tritaeniorhynchus, Lt. fuscanus, Ma. bonneae, Ma. indiana, Ma. uniformis.* Location 3 was Krurpara region (10º 32′ 27.5″ N 76º 08′ 35.0″ E) of Site1. 19 mosquito species were collected from there during the study period. *An. barbirostris, An. nigerrimus, An. peditaeniatus, An. stephensi, An. subpictus, An. vagus, Ae. aegypti, Ae. albopictus, Ae. vittatus, Ph. cogilli, Ar. subalbatus, Cx. gelidus, Cx. pipiens, Cx. quinquefasciatus, Cx. tritaeniorhynchus, Lt. fuscanus, Ma. bonneae, Ma. indiana, Ma. uniformis* were the collected mosquito species from Location 3. Location 4 was the Puranattukara area of Adat Kole. GPS location of Puranattukara is 10º 30′ 08.5″ N 76º 09′ 48.2″ E. 18 mosquito species were collected and identified from Location 4 *An. barbirostris, An. nigerrimus, An. peditaeniatus, An. stephensi, An. subpictus, An. vagus, Ae. aegypti, Ae. albopictus, Ae. vittatus, Ar. subalbatus, Cx. gelidus, Cx. pipiens, Cx. quinquefasciatus, Cx. tritaeniorhynchus, Lt. fuscanus, Ma. bonneae, Ma. indiana, Ma. uniformis.*

Location 5 was part of Palakkal Kole, that was Chiyyaram region (10º 28′ 51.3″ N 76º 13′ 14.1″ E) of Palakkal Kole. 16 mosquito species were collected from Chiyyaram area. *An. barbirostris, An. nigerrimus, An. stehensi, An. subpictus, An. vagus, Ae. aegypti, Ae. albopictus, Ae. vittatus, Ar. subalbatus, Cx. gelidus, Cx. pipiens, Cx. quinquefasciatus, Cx. tritaeniorhynchus, Lt. fuscanus, Ma. indiana, Ma. uniformis.* Location 6 (10º 28′ 13.1″ N 76º 12′ 20.6″ E) was distributed in Site 2, and 17 mosquitoes identified from there. *An. barbirostris, An. nigerrimus, An. peditaeniatus, An. stephensi, An. subpictus, An. vagus, Ae. aegypti, Ae. albopictus, Ae. vittatus, Ar. subalbatus, Cx.bitaeniorynchus, Cx. gelidus, Cx. pipiens, Cx. quinquefasciatus, Cx. tritaeniorhynchus, Lt. fuscanus, Ma. indiana.* Location 7 was Nedupuzha (10º 28′ 16.7″ N 76º 12′ 16.7″ E) region of Palakkal Kole. 17 mosquitoes were identified from Location 7. They were *An. barbirostris, An. nigerrimus, An. peditaeniatus, An. stephensi, An. subpictus, An. vagus, Ae. aegypti, Ae. albopictus, Ae. vittatus, Ar. subalbatus, Cx. gelidus, Cx. pipiens, Cx. quinquefasciatus, Cx. tritaeniorhynchus, Lt. fuscanus, Ma. indiana, Ma. uniformis.* Location 8 was Palakkal (10º 28′ 34.2″ N 76º 12′ 49.3″ E) region of Site 2 with 17 mosquito collections. The collected mosquito species were *An. barbirostris, An. nigerrimus, An. stephensi, An. subpictus, An. vagus, Ae. aegypti, Ae. albopictus, Ae. vittatus. Ar. subalbatus, Cx. bitaeniorynchus, Cx. gelidus, Cx. pipiens, Cx. quinquefasciatus, Cx. tritaeniorhynchus, Lt. fuscanus, Ma. indiana, Ma. uniformis.*

Location 9 Ananthapuram relies on Muriyad Kole lands 10º 21′ 56.9″ N 76º 15′ 15.3″ E. Total 16 mosquitoes identify from Location 9. They were *An. barbirostris, An. nigerrimus, An. stephensi, An. subpictus, An. vagus, Ae. aegypti, Ae. albopictus, Ae. vittatus, Ar. subalbatus, Cx. gelidus, Cx. pipiens, Cx. quinquefasciatus, Cx. tritaeniorhynchus, Lt. fuscanus, Ma. indiana, Ma. uniformis.* Location 10

Konthipulam (10º 22′ 37.4″ N 76º 13′ 16.7″ E) located in Site 3 with 16 species of Mosquito collection (*An. barbirostris, An. nigerrimus, An. stephensi, An. subpictus, An. vagus, Ae. aegypti, Ae. albopictus, Ae. vittatus, Ar. subalbatus, Cx. gelidus, Cx. pipiens, Cx. quinquefasciatus, Cx. tritaeniorhynchus, Lt. fuscanus, Ma. indiana, Ma. uniformis*). Location 11 Nambyamkavu is a part of Muriyad Kole (10º 20′ 42.6″ N 76º 13′ 14.1″ E). Location 11 supports 16 mosquito species, *An. barbirostris, An. nigerrimus, An. stephensi, An. subpictus, An. vagus, Ae. aegypti, Ae. albopictus, Ae. vittatus, Ar. subalbatus, Cx. gelidus, Cx. pipiens, Cx. quinquefasciatus, Cx. tritaeniorhynchus, Lt. fuscanus, Ma. indiana, Ma. uniformis*. Location 12 was Nandipulam (10º 23′ 05.6″ N 76º 14′ 08.8″ E) distributed in Site 3 Muriyad Kole, and 17 mosquito species were collected from Location12, *An. barbirostris, An. nigerrimus, An. stephensi, An. subpictus, An. vagus, Ae. aegypti, Ae. albopictus, Ae. vittatus, Ar. subalbatus, Cx. bitaeniorynchus, Cx. gelidus, Cx. pipiens, Cx. quinquefasciatus, Cx. tritaeniorhynchus, Lt. fuscanus, Ma. indiana, Ma. uniformis*.

1.3.2.2 Discussion

Landscape changes impact mosquito diversity and disease transmission, i.e., vectors and their associated disease that were earlier exclusively found in forests are now located near the human environment due to human activities (Pereira et al., 2021). Recent developments clearly show that GIS has become an indispensable tool for processing, analysing, and visualizing spatial data within ecological research, disease ecology, and environmental and public health concerns (Graham et al., 2004; Kistemann et al., 2002). GIS application allows users to create interactive queries, analyse spatial information, edit data and maps, and help define the habitats of nuisance and vector mosquito populations, which are influenced by environmental conditions. It not only enables the creation of maps based on field monitoring but

also allows the production of forecasting maps for planning control strategies on small and large scales (Tourre et al., 2008; Dongus et al., 2009; Stensgaard et al., 2009). Improved knowledge and progressive adoption of new technologies improve prospects for efficient pest control (Lonc et al., 2004).

In contemporary mosquito control programs, accurate mapping of breeding sites is crucial for achieving acceptable results (Becker et al., 2010). The site's characteristics and application of environmentally friendly approaches to controlling mosquitoes would also evaluate different larvicidal formulations. Mosquito control includes employing various methods, according to various programs. However, appropriately targeted larvicidal treatments using advanced equipment and technology (i.e., GPS/GIS technology) are significantly more efficient than traditional mosquito control methods. As pointed out by Becker (2008), new strategies based on environmental approaches will be effective in vector control and preventing potential risks of human diseases being transmitted by mosquitoes. Properly planned mosquito control projects based on cost-effective strategies are more efficient, both from cost and disease prevention standpoints (Becker, 2008). In this study, GIS were prepared with identified mosquito species from 12 locations of 3 study sites. Figure 1.23, 1.24, and 1.25show the GIS map of Site 1, Site 2, and Site 3 correspondingly. A list of identified mosquito species from each Location is also attached to this site map. This GIS map may help to easily predict any mosquito-borne disease outbreak associated with these prevailing mosquito species in every Location. Mosquito control tactics can also take this GIS data for accurate and specific mosquito eradication programs in these localities.

Kumari and Kant designed a study to prepare thematic maps of six selected villages in Rohtak and Mewat districts for the years 2008 and 2013 by using RS & GIS

technology. High Annual Parasite Incidence (API) of the village Kalanaur (3.48) in Rohtak district and village Ujina (5.1) of Mewat district was directly correlated to the number of water bodies with an indication of favourable conditions for the breeding of *Anopheline* mosquitoes in the respective villages their study identified the risk factors associated with high malaria transmission in six selected Haryana PHCs of Rohtak and Mewat districts and helps to plan focused malaria control interventions to control malaria transmission (Kumari, and Kant., 2016). The present study provides similar data on mosquito diversity in the Kole wetlands of Thrissur, Kerala. All mosquito species collected from Thrissur Kole lands were either primary or potential vectors of various mosquito-borne diseases, which are very common in India. Awareness and spotting vector mosquitoes were the preliminary steps in mosquito control programs.

GIS PREPARATION

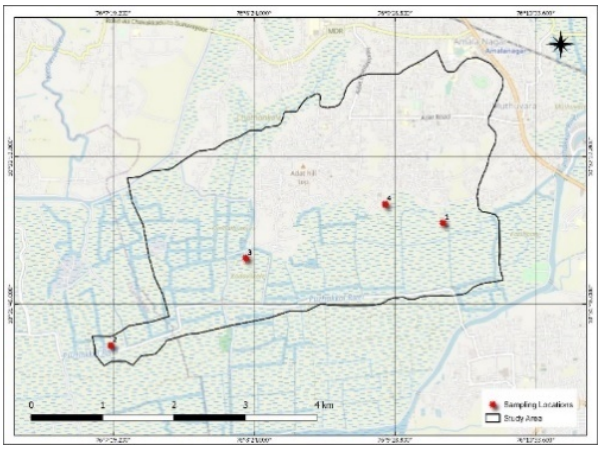

Figure 1.23 Site 1 Adat Kole

LOCATION 1 ADAT AMBAKKAD 10º 33′ 01.2″ N 76º 09′ 10.2″ E
1. An. barbirostris
2. An. nigerrimus
3. An. peditaeniatus
4. An. stephensi
5. An. subpictus
6. An. vagus
7. Ae. aegypti
8. Ae. albopictus
9. Ae. vittatus
10. Ar. subalbatus
11. Cx. bitaeniorynchus
12. Cx. gelidus
13. Cx. pipiens
14. Cx. tritaeniorhynchus
15. Lt. fuscanus
16. Ma. indiana
17. Ma. uniformis

LOCATION 2 ADAT AMBATHONNU 10º 31′ 29.7″ N 76º 07′ 18.0″ E	LOCATION 3 ADAT KURURPARA 10º 32′ 27.5″ N 76º 08′ 35.0″ E	LOCATION 4 ADAT PURANATTUKARA 10º 30′ 08.5″ N 76º 09′ 48.2″ E
1. An. barbirostris	*1. An. barbirostris*	*1. An. barbirostri*
2. An. nigerrimus	*2. An. nigerrimus*	*2. An. nigerrimus*
3. An. peditaeniatus	*3. An. peditaeniatus*	*3. An. peditaeniatus*
4. An. stephens,	*4. An. stephensi*	*4. An. stephensi*
5. An. subpictus	*5. An. subpictus*	*5. An. subpictus*
6. An. vagus	*6. An. vagus*	*6. An. vagus*
7. Ae. aegypti	*7. Ae. aegypti*	*7. Ae. aegypti*
8. Ae. albopictus	*8. Ae. albopictus*	*8. Ae. albopictus*
9. Ae. vittatus	*9. Ae. vittatus*	*9. Ae. vittatus*
10. Ar. subalbatus	*10. Ph. cogilli*	*10. Ar. subalbatus*
11. Cx. gelidus	*11. Ar. subalbatus*	*11. Cx. gelidus*
12. Cx. pipiens	*12. Cx. gelidus*	*12. Cx. pipiens*
13. Cx. quinquefasciatus	*13. Cx. pipiens*	*13. Cx. quinquefasciatus*
14. Cx. tritaeniorhynchus	*14. Cx. quinquefasciatus*	*14. Cx. tritaeniorhynchus*
15. Lt. fuscanus	*15. Cx. tritaeniorhynchus*	*15. Lt. fuscanus*
16. Ma. bonneae	*16. Lt. fuscanus*	*16. Ma. bonneae*
17. Ma. indiana	*17. Ma. bonneae*	*17. Ma. indiana*
18. Ma. uniformis	*18. Ma. indiana*	*18. Ma. uniformis*
	19. Ma. uniformis	

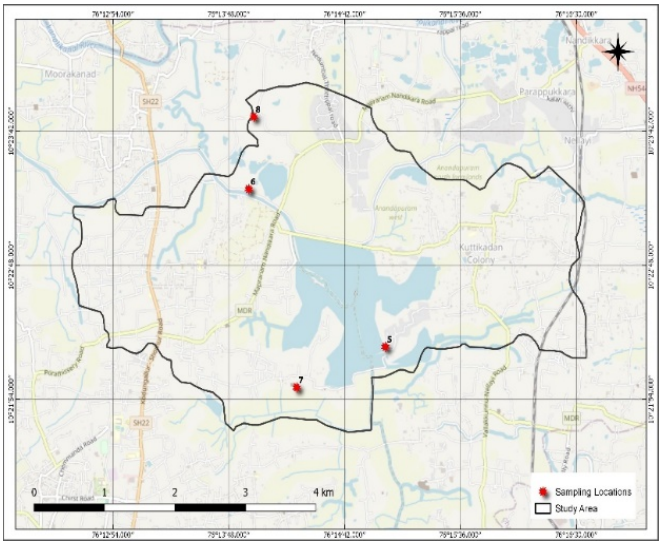

Figure 1.24 Site 2 Palakkal Kole

LOCATION 5 PALAKKAL CHIYYARAM 10º 28′ 51.3″ N 76º 13′ 14.1″ E
1. *An. barbirostris* 2. *An. nigerrimus* 3. *An. stehensi* 4. *An. subpictus* 5. *An. vagus* 6. *Ae. aegypti* 7. *Ae. albopictus* 8. *Ae. vittatus* 9. *Ar. subalbatus* 10. *Cx. gelidus* 11. *Cx. pipiens* 12. *Cx. quinquefasciatus* 13. *Cx. tritaeniorhynchus* 14. *Lt. fuscanus* 15. *Ma. indiana* 16. *Ma. uniformis*

LOCATION 6 PALAKKAL KANIMANGALAM 10º 28′ 13.1″ N 76º 12′ 20.6″ E	LOCATION 7 PALAKKAL NEDUPUZHA 10º 28′ 16.7″ N 76º 12′ 16.7″ E	LOCATION 8 PALAKKAL PALAKKAL 10º 28′ 34.2″ N 76º 12′ 49.3″ E
1. *An. barbirostris* 2. *An. nigerrimus* 3. *An. peditaeniatus* 4. *An. stephensi* 5. *An. subpictus* 6. *An. vagus* 7. *Ae. aegypti* 8. *Ae. albopictus* 9. *Ae. vittatus* 10. *Ar. subalbatus* 11. *Cx.bitaeniorynchus* 12. *Cx. gelidus* 13. *Cx. pipiens* 14. *Cx. quinquefasciatus* 15. *Cx. tritaeniorhynchus* 16. *Lt. fuscanus* 17. *Ma. indiana*	1. *An. barbirostris* 2. *An. nigerrimus* 3. *An. peditaeniatus* 4. *An. stephensi* 5. *An. subpictus* 6. *An. vagus* 7. *Ae. aegypti* 8. *Ae. albopictus* 9. *Ae. vittatus* 10. *Ar. subalbatus* 11. *Cx. gelidus* 12. *Cx. pipiens* 13. *Cx. quinquefasciatus* 14. *Cx. tritaeniorhynchus* 15. *Lt. fuscanus* 16. *Ma. indiana* 17. *Ma. uniformis*	1. *An. barbirostris* 2. *An. nigerrimus* 3. *An. stephensi* 4. *An. subpictus* 5. *An. vagus* 6. *Ae. aegypti* 7. *Ae. albopictus* 8. *Ae. vittatus* 9. *Ar. subalbatus* 10. *Cx. bitaeniorynchus* 11. *Cx. gelidus* 12. *Cx. pipiens* 13. *Cx. quinquefasciatus* 14. *Cx. tritaeniorhynchus* 15. *Lt. fuscanus* 16. *Ma. indiana* 17. *Ma. uniformis*

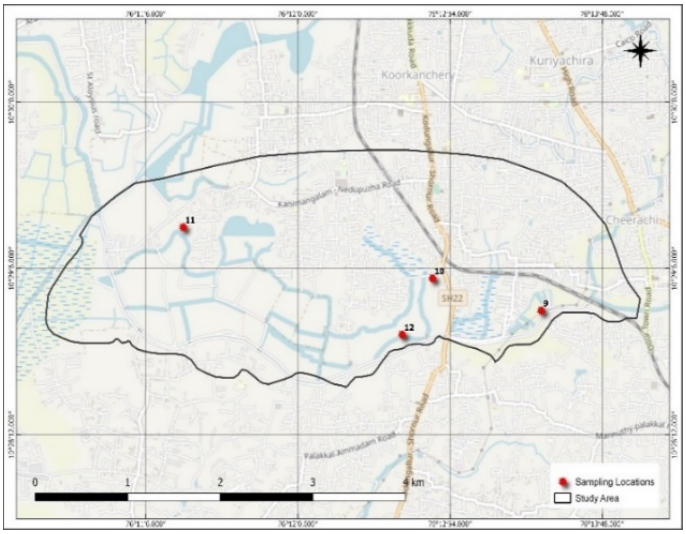

Figure 1.25 Site 3 Muriyad Kole

LOCATION 9 MURIYAD ANANTHAPURAM 10º 21′ 56.9″ N 76º 15′ 15.3″ E
1. *An. barbirostris* 2. *An. nigerrimus* 3. *An. stephensi* 4. *An. subpictus* 5. *An. vagus* 6. *Ae. aegypti* 7. *Ae. albopictus* 8. *Ae. vittatus* 9. *Ar. subalbatus* 10. *Cx. gelidus* 11. *Cx. pipiens* 12. *Cx. quinquefasciatus* 13. *Cx. tritaeniorhynchus* 14. *Lt. fuscanus* 15. *Ma. indiana* 16. *Ma. uniformis*

LOCATION 10 MURIYAD KONTHIPULAM 10º 22′ 37.4″ N 76º 13′ 16.7″ E	LOCATION 11 MURIYAD NAMBYAMKAVU 10º 20′ 42.6″ N 76º 13′ 14.1″ E	LOCATION 12 MURIYAD NANDIPULAM 10º 23′ 05.6″ N 76º 14′ 08.8″ E
1. *An. barbirostris,* 2. *An. nigerrimus* 3. *An. stephensi* 4. *An. subpictus* 5. *An. vagus* 6. *Ae. aegypti* 7. *Ae. albopictus* 8. *Ae. vittatus* 9. *Ar. subalbatus* 10. *Cx. gelidus* 11. *Cx. pipiens* 12. *Cx. quinquefasciatus* 13. *Cx. tritaeniorhynchus* 14. *Lt. fuscanus* 15. *Ma. indiana* 16. *Ma. uniformis*	1. *An. barbirostris* 2. *An. nigerrimus* 3. *An. stephensi* 4. *An. subpictus* 5. *An. vagus* 6. *Ae. aegypti* 7. *Ae. albopictus* 8. *Ae. vittatus* 9. *Ar. subalbatus* 10. *Cx. gelidus* 11. *Cx. pipiens* 12. *Cx. quinquefasciatus* 13. *Cx. tritaeniorhynchus* 14. *Lt. fuscanus* 15. *Ma. indiana* 16. *Ma. uniformis*	1. *An. barbirostris* 2. *An. nigerrimus* 3. *An. stephensi* 4. *An. subpictus* 5. *An. vagus* 6. *Ae. aegypti* 7. *Ae. albopictus* 8. *Ae. vittatus* 9. *Ar. subalbatus* 10. *Cx. bitaeniorynchus* 11. *Cx. gelidus* 12. *Cx. pipiens* 13. *Cx. quinquefasciatus* 14. *Cx. tritaeniorhynchus* 15. *Lt. fuscanus* 16. *Ma. indiana* 17. *Ma. uniformis*

1.3.3. Seasonal Variation on Mosquito Species Collected from Study Area

1.3.3.1 Result

34801 mosquito samples were collected from Thrissur Kole lands during the study period of two years. Out of the total collection, 8609 samples were obtained in the Monsoon season, and 14232, 11960 mosquitoes were collected in Post-monsoon and Pre-monsoon, respectively (Average number sample collection in Monsoon- 4304.5, Post-monsoon- 7116, Pre-monsoon- 5980). Almost all mosquitoes except *Ph. cogilli* and *Ma. bonneae* were collected in either two or three seasons in a year. More individuals obtained in the Monsoon season belonged to *Ae. aegypti,* and *Ae. albopictus* species and the rest of the two seasons enriched with *Ar. subalbatus*. According to our sampling, *Ph. cogilli* was reported only in the Monsoon season, and *Ma. bonneae* in the Post-monsoon season. In the case of other mosquitoes, the number of individuals collected in three seasons varied. The number of mosquitoes collected in three different seasons during the collection period (June- 2016 to May 2018) is displayed in Table 1.5.

Variation in the number of mosquitoes collected according to three distinct seasons was analysed with the one-way ANOVA test and displayed the consolidated result in table 1.6. ANOVA test reveals that almost all mosquitoes other than four species (*An. stephensi*, *Ph. cogilli*, *Cx. bitaeniorhynchus,* and *Ma. bonneae*) showed a significant variation in different seasons. Species with no variation were either present in only one season (*Ph. cogilli* and *Ma. bonneae*) or had a uniform distribution in two (*Cx. bitaeniorhynchus*) or three (*An. stephensi*) seasons.

Anopheles mosquitoes are seen in fresh, slightly polluted, and highly polluted water habitats. *An. barbirostris, An. nigerrimus* and *An. peditaeniatus* showed a similar

type of collection pattern according to season. A remarkable increase in the average number of individuals was observed during the Post-monsoon season compared to the other two (Figure 1.26, 1.27, 1.28 displayed graphical representation of these three *Anopheles* mosquitoes). *An. stephensi* collected from all 12 locations during every season. Average number of collections of *An. stephensi* is almost similar in Monsoon, Post-monsoon, and Pre-monsoon seasons, so there is no evident seasonal variation seen in abundance of this species. Graphical representation of collection of *An. stephensi* illustrated in the Figure 1.29. Collection result of *An. subpictus* revealed that all three seasons have evident differences, and its graphical representation showed in Figure 1.30. More collection was achieved during Post-monsoon, followed by the Pre-monsoon and Monsoon periods. Number of *An. vagus* obtained during Pre-monsoon is very high than during Monsoon and Post-monsoon. A demarcated deviation was observed between monsoon and Pre-monsoon as well as Post-monsoon and Pre-monsoon. Graphical representation of collection of *An. vagus* in different seasons represented in Figure 1.31.

Aedes species are freshwater inhabitants. *Aedes* mosquitoes are generally present in rainwater containing natural and artificial objects (tree holes, containers, tanks, coconut shells, tires, etc.). The presence of such mosquitoes is high in Monsoon and Pre-monsoon seasons. *Ae. aegypti* and *Ae. albopictus* had a larger number of collections during the Monsoon period than Pre-monsoon. Post-monsoon has a comparatively fewer number of individuals (Figure 1.32, 1.33 displayed graphical representation of seasonal variation of *Ae. aegypti* and *Ae. albopictus* respectively). In this *Ae. aegypti* exhibited a relevant differentiation seen in Monsoon - Post-monsoon, Monsoon - Pre-monsoon, And Post-monsoon - Pre-monsoon combinations. However, *Ae. albopictus* has significant variation in monsoon - Post-

monsoon and Monsoon - Pre-monsoon seasons. *Ae. vittatus* species presence was only reported in Monsoon and Pre-monsoon periods (Figure 1.34 displayed graphical representation of seasonal variation of *Ae. vittatus*). A considerable change in the number of *Ae. vittatus* collection between Monsoon and Post-monsoon, as well as Post-monsoon and Pre-monsoon. *Ph. cogilli* was rarest mosquito species collected during the entire study period. This species collected in only one season from Location 3. Figure 1.35 displayed graphical representation of seasonal change of *Ph. cogilli*

Ar. subalbatus is the most predominant mosquito species in Post-monsoon and Pre-monsoon periods and possesses the third prevalence position during Monsoon (Figure 1.36 displayed graphical representation of seasonal variation of *Ar. subalbatus*). The existing pattern of this mosquito followed dissentingly in the order of Post-monsoon, Pre-monsoon, and Monsoon. An evident variation was reported in the number of individuals collected from these species during all three seasons. *Culex* Genera inhabited freshwater to polluted water. These mosquito species were collected every season, but the number varies. Maximum *Culex* samples were collected during Post-monsoon, Pre-monsoon periods, and minimum in Monsoon except in the case of *Cx. bitaeniorhynchus*. *Cx. bitaeniorhynchus* collected during Post-monsoon and Pre-monsoon season from Location 6,8,10 (Figure 1.37 displayed graphical representation of variation of *Cx. bitaeniorhynchus)*. A significant difference in the collection was reported among Monsoon and Post-monsoon as well as Monsoon and pre-monsoon. *Cx. gelidus, Cx.pipiens, Cx. quinquefasciatus,* and *Lt. fuscanus* followed this manner of distribution pattern (Figure 1.38, 1.39,1.40, 1.41 displayed graphical representation of seasonal variation of *Cx. gelidus, Cx.pipiens, Cx. quinquefasciatus, and Lt.*

fuscanus respectively). Moreover, these patterns *Cx. tritaeniorhynchus* also exhibited Post- monsoon Pre-monsoon differences in the collection (Figure1.42). A significant difference, *Ma. indiana* collection re-recorded between Monsoon and Post-monsoon Monsoon and Pre-monsoon seasons (Figure 1.43). Monsoon and Post-monsoon collection of *Ma. uniformis* exhibited a relevant variation, and the same distribution was also reported between Post-monsoon and Pre-monsoon periods (Figure 1.44). *Ma. bonneae* only collected during Post-monsoon season and variation pattern represented in figure 1.45

1.3.3.2 Discussion

34801 mosquito samples were collected from Thrissur Kole lands during the study period two years. Out of the total collection, 8609 samples were obtained in the Monsoon season, and 14232, 11960 mosquitoes were collected in Post-monsoon and pre-monsoon, respectively (Average number sample collection in Monsoon- 4304.5, Post-monsoon- 7116, Pre-monsoon- 5980). Almost all mosquitoes except *Ph. cogilli,* and *Ma. bonneae* were collected in either two or three seasons in a year. More individuals obtained in the Monsoon season belonged to *Ae. aegypti,* and *Ae. albopictus* species and the rest of the two seasons enriched with *Ar*. *subalbatus*. According to our sampling, *Ph. cogilli* was reported only in the Monsoon season, and *Ma. bonneae* in the Post-monsoon season. In the case of other mosquitoes, the number of individuals collected in three seasons varied. Radhakrishnan, 2019 discussed seasonal variation of mosquito diversity in the Study on mosquito (Diptera: Culicidae) diversity in the Ernakulam district of Kerala. In that study, there was to be high species richness found during April 2018 with 16 species. Then July, 15 species were collected, January and March 2018 having 14 mosquito species each. Medium-level species richness was found during October 2017 (11 species),

December 2017 (10 species), February 2018 (13 species), May 2018(13 species), and August 2018(11 species). The least number of species was recorded during November 2017 and June 2018, with 7 species each.

Mosquito larvae were gathered in the pre-monsoon season from 12 sites representing five habitats and reared to adulthood before being identified at the species level by Sajith et.al; According to the findings, the Chavakkad municipal area had a greater larvae density than the Ponnani municipal region. During the pre-monsoon season, the variety of mosquito larvae was greater (4 genera, 9 species) in Ponnani municipal area and lower (5 genera, 8 species) in Chavakkad. In the post-monsoon season, diversity studies on mosquito larvae collected from 12 sites found that mosquito diversity was highest in Chavakkad municipal area (4 genera, 8 species), followed by Ponnani municipal area (4 genera, 8 species) (4 genera, 7 species). In comparison to Ponnani, the highest larval density was seen in Chavakkad (Sajith et al., 2016).

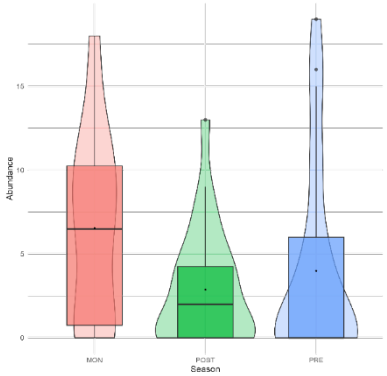

Figure 1.26 Seasonal variation of *An. barbirostris,*

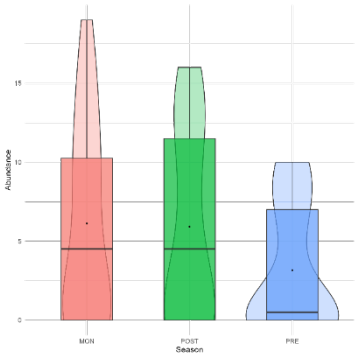

Figure 1.27 Seasonal variation of *An. nigerrimus*

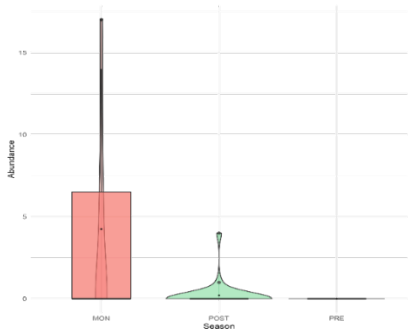

Figure 1.28 Seasonal variation of *An. peditaeniatus*

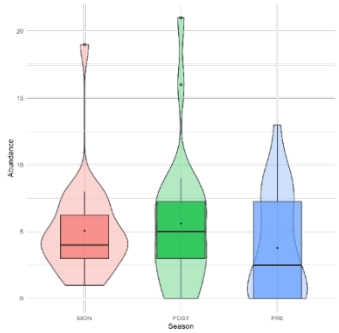

Figure 1.29 Seasonal variation of *An. stephensi*

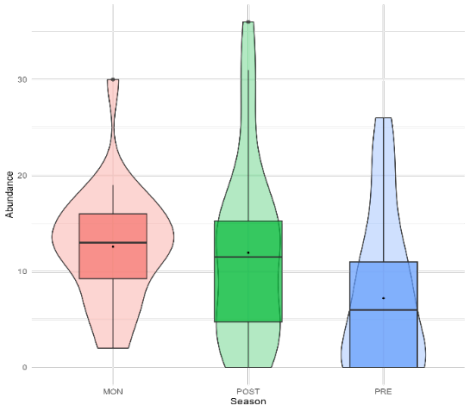

Figure 1.30 Seasonal variation of *An. subpictus*

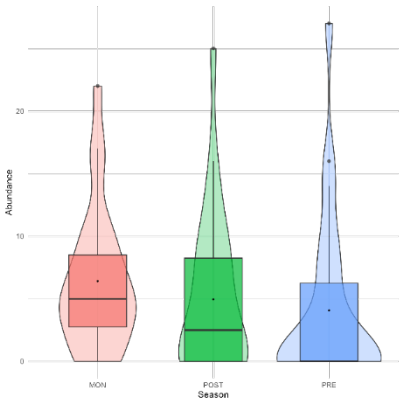

Figure 1.31Seasonal variation of *An. vagus*

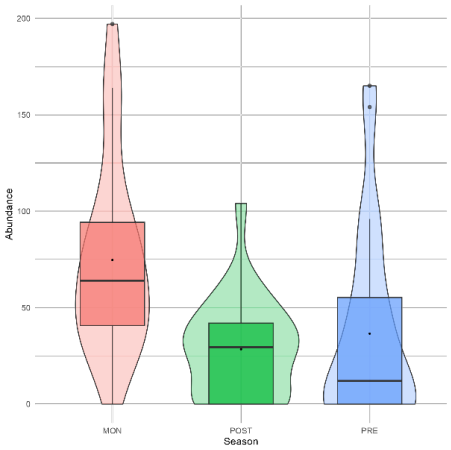

Figure 1.32 Seasonal variation of *Ae. aegypti*

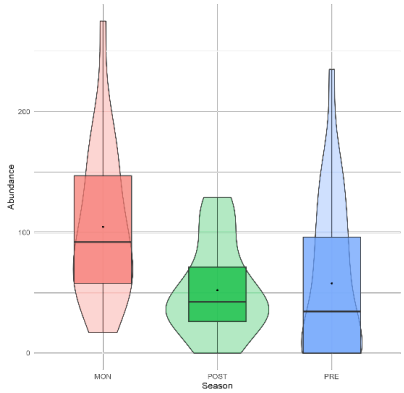

Figure 1.33 Seasonal variation of *Ae. albopictus*

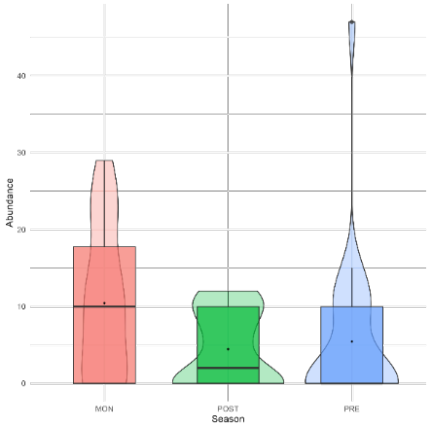

Figure 1.34 Seasonal variation of *Ae. vittatus*

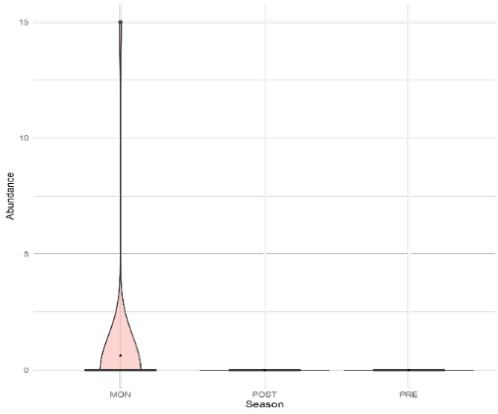

Figure 1.35 Seasonal variation of *Ph. cogilli*

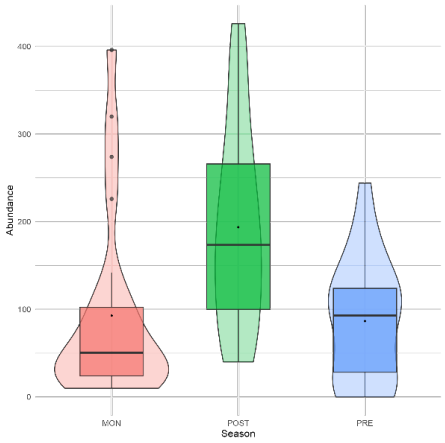

Figure 1.36 Seasonal variation of *Ar. subalbatus*

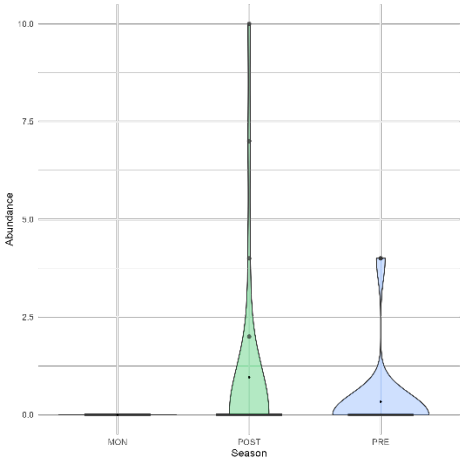

Figure 1.37 Seasonal variation of *Cx. bitaeniorhynchus*

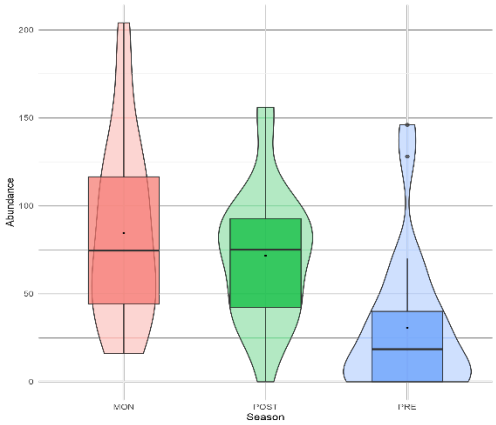

Figure 1.38Seasonal variation of *Cx. gelidus*

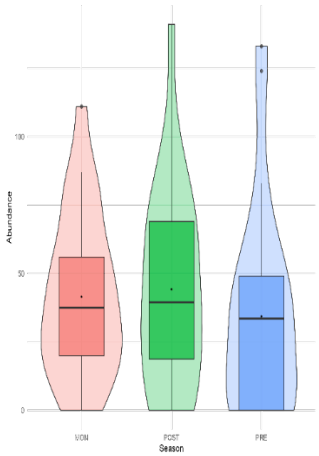

Figure 1.39Seasonal variation of *Cx. pipiens*

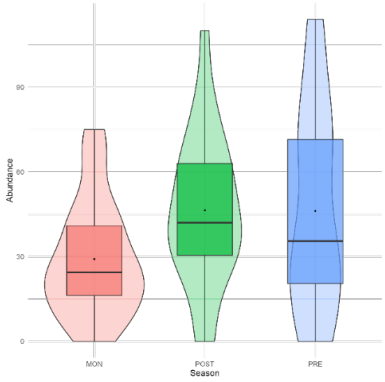

Figure 1.40 Seasonal variation of *Cx. quinquefasciatus*

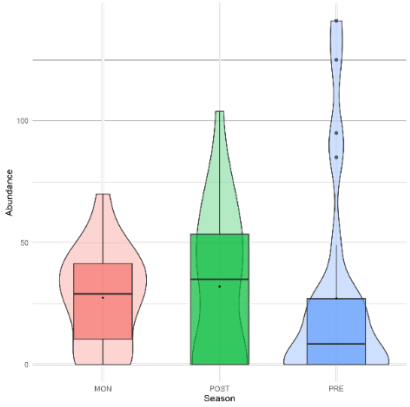

Figure 1.41 Seasonal variation of *Cx. tritaeniorhynchus*

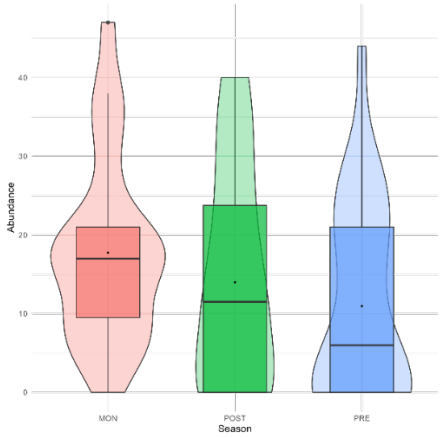

Figure 1.42 Seasonal variation of *Lt. fuscanus*

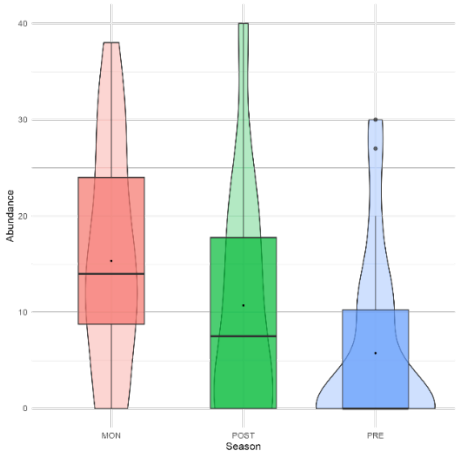

Figure 1.43Seasonal variation of *Ma. indiana*

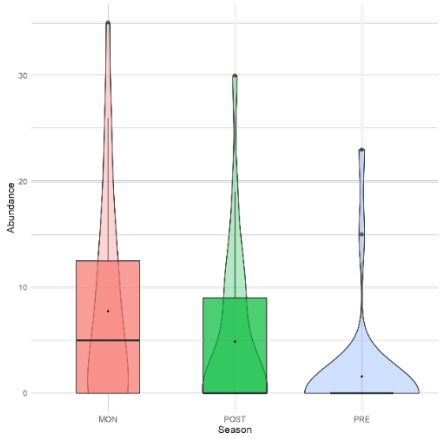

Figure 1.44 Seasonal variation of *Ma. uniformis*

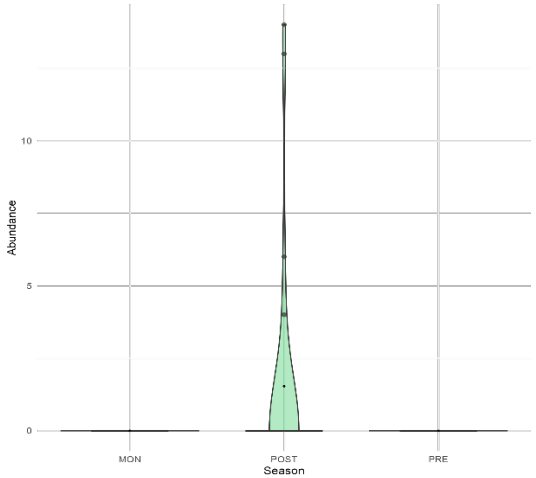

Figure 1.45 Seasonal variation of *Ma. bonneae*

Table 1.5 Seasonal variation displayed by collected mosquito species during the study period

SL NO	Mosquito Species	Season						P VALUE
		Monsoon		Post-monsoon		Pre-monsoon		
		2016	2017	2016	2017	2017	2018	
1	*An. barbirostris*	8	20	117	130	36	11	<0.001
2	*An. nigerrimus*	9	29	145	129	34	19	<0.001
3	*An. peditaeniatus*			52	50	5		0.004
4	*An. stephensi*	27	58	96	57	61	49	0.369
5	*An. subpictus*	31	64	213	188	148	118	<0.001
6	*An. vagus*	48	46	12		133	132	<0.001
7	*Ae. aegypti*	1146	1088	147		555	417	<0.001
8	*Ae. albopictus*	1403	1714	235	281	754	762	<0.001
9	*Ae. vittatus*	121	121			118	129	<0.001
10	*Ph. cogilli*	15						0.379
11	*Ar. subalbatus*	347	530	2916	2612	1376	1173	<0.001
12	*Cx. bitaeniorhynchus*			21	2	4	4	0.126
13	*Cx. gelidus*	191	269	1088	1141	1015	780	<0.001
14	*Cx. pipiens*	232	272	760	672	678	268	0.002
15	*Cx. quinquefasciatus*	280	382	562	639	607	453	0.009
16	*Cx. tritaeniorhynchus*		39	348	367	625	701	<0.001
17	*Lu. fuscanus*	21	22	284	217	267	214	<0.001
18	*Ma. bonneae*			10	27			0.056
19	*Ma. indiana*	20	38	193	214	151	147	0.003
20	*Ma. uniformis*		18	170	137		16	<0.001

*Statistical significance (P<0.05)

Table 1.6 ANOVA Results for Seasonal Data Analysis of collected mosquito species

Sl No	Mosquto Species	Monsoon Mean ± SD	Postmonsoon Mean ± SD	Premonsoon Mean ± SD	F Value	P Value
1	*An. barbirostris*	1.75 ± 2.379	10.29 ± 3.244	3.38 ± 2.638	32.007	<0.001
2	*An. nigerrimus*	1.75 ± 2.966	11.42 ± 3.295	3.33 ± 2.847	34.864	<0.001
3	*An. peditaeniatus*	0.00 ± .000	5.478 ± 5.478	0.42 ± 1.165	6.693	0.004
4	*An. stephensi*	4.79 ± 2.301	6.75 ± 2.360	6.17 ± 4.951	1.029	0.369
5	*An. subpictus*	6.58 ± 4.838	16.71 ± 4.624	11.67 ± 3.786	15.607	<0.001
6	*An. Vagus*	4.67 ± 3.725	1.00 ± 1.859	11.04 ± 5.614	19.026	<0.001
7	*Ae. aegypti*	93.08 ± 33.727	12.25 ± 17.571	41.79 ± 16.747	34.879	<0.001
8	*Ae. albopictus*	129.88 ± 47.150	33.33 ± 30.129	63.17 ± 26.003	23.105	<0.001
9	*Ae. vittatus*	11.50 ± 8.883	0.00 ± .000	12.21 ± 5.941	14.805	<0.001
10	*Ph. cogilli*	1.25 ± 4.330	0.00 ± .000	0.00 ± .000	1.000	0.379
11	*Ar. subalbatus*	43.13 ± 26.625	230.33 ± 82.851	113.54 ± 39.848	35.135	<0.001
12	*Cx. bitaeniorhynchus*	0.00 ± .000	1.67 ± 3.367	0.33 ± 1.155	2.211	0.126
13	*Cx. gelidus*	30.50 ± 18.792	92.88 ± 35.276	74.79 ± 25.039	16.667	<0.001
14	*Cx. pipiens*	24.75 ± 16.072	62.21 ± 17.253	52.71 ± 35.908	7.397	0.002
15	*Cx. quinquefasciatus*	29.00 ± 16.179	51.83 ± 16.057	48.29 ± 21.907	5.439	0.009
16	*Cx. tritaeniorhynchus*	3.25 ± 7.665	33.79 ± 19.076	58.08 ± 20.780	31.807	<0.001
17	*Lu. fuscanus*	3.00 ± 5.309	21.75 ± 7.599	20.04 ± 6.148	31.274	<0.001
18	*Ma. bonneae*	0.00 ± .000	2.38 ± 4.637	0.00 ± .000	3.147	0.056
19	*Ma. indiana*	4.00 ± 5.099	18.63 ± 10.488	14.96 ± 12.387	7.202	0.003
20	*Ma. uniformis*	1.50 ± 4.101	13.04 ± 7.803	1.33 ± 3.229	18.405	<0.001

*Statistical significance (P<0.05)

1.3.4 Breeding habitats of mosquito species collected

Mosquito larvae were collected from various breeding habitats. Breeding habitats may have changed according to seasons. In monsoon season the main breeding

habitat is flood plain because of Kole wetlands were completely submerged under flood water during this season. Other common habitats during monsoon season were rain water containing tanks, tyres, tree holes, containers, ditches, leaf axils leaf litters, leaf internodes etc. Paddy cultivation practices were started at Post-monsoon season. Rice fields, irrigation canals, ponds, marsh land, temporary pools, stagnant water bodies etc were the breeding habitats. Pre-monsoon season was harvesting period of rice cultivation in Kole lands. Summer rain made some temporary mosquito breeding grounds in Kole wetlands like rain water containing tanks, tyres, tree holes, containers, ditches. Irrigation canals, ponds, and rice field were dry or containing less water during Pre-monsoon season, because of this the water containing these habitats with high turbidity. Some regular breeding habitats and presence of mosquito species mentioned in table 1.7.

1.3.5 Correlation between physico-chemical parameters of the water and number of mosquitoes collected from different beeding habitats

1.3.5.1 Result

In this study, we tried to analyse the correlation between the physico-chemical parameters of water samples from different breeding habitats and mosquito diversity. Altogether 20 mosquito species were collected from different breeding habitats during the study period, and values of 10 water quality parameters were recorded. Habitat with sufficient amount of water for testing were considered for correlation analysis. pH, Turbidity, Conductivity, TDS, Hardness, Chloride exhibited significant correlation with a total number of mosquitoes collected. The rest of the parameters (Temperature, DO, Alkalinity, Salinity) did not correlate with the number of mosquitoes collected.

The pH of the water had a highly significant negative correlation with the total number of mosquitoes collected from various sampling sites (P value 0.007). Turbidity values of breeding water sample from various habitats and the number of mosquitoes collected showed a strong positive correlation (P value 0.000). Conductivity, TDS, and Hardness unveiled the similar relationship pattern of turbidity. These parameters strongly influenced the number of mosquitoes collected in a positive manner, and all of their P value is significant (<0.000). Chloride content of water was moderately affecting the number of mosquitoes in a positive direction with P value 0.011. Table 1.8 shows the correlation coefficient values of different physico-chemical parameters of water samples from various breeding habitats and number of mosquitoes collected from these habitats.

Out of 20 species identified, nine mosquitoes express some correlation between the physico-chemical parameters of water samples from their breeding habitats. Mosquito species that exhibit some correlation with different water quality parameters and their Pearson coefficient were listed in table 1.9. *An. barbirostris* display a highly significant positive association (P value was 0.009) with the hardness of the breeding water and mosquito collection. *An. nigerrimus* number of mosquitoes collected and salinity had a moderate negative correlation with a p-value of 0.010. Both Conductivity and TDS water quality parameters moderately influenced the total mosquito collection in *Ae. albopictus*. These correlations were instead a negative pattern having p-value 0.021 and 0.020 correspondingly. Water temperature of *Ae. vittatus* species modestly control many mosquitoes in the opposite direction. DO also followed the same pattern of positive association (p-value 0.014). *Ar. subalbatus* collection was directly proportional to

the increase in hardness of the water and inversely proportional to the increasing temperature (p-value temperature- 0.033, hardness- 0.010).

Six different water quality parameters significantly influence the collection of *Cx. gelidus* mosquitoes. Water temperature and mosquito collection had a moderate negative correlation with a significant value of 0.022. Turbidity showed the medium level of significant correlation (p-value 0.026). Conductivity and TDS also had a similar pattern with a p-value of 0.013 each. Alkalinity and hardness of *Cx. gelidus* breeding habitat had a slight positive correlation with mosquito collection with p-value of 0.042 and 0.023, respectively. DO, Chloride, and Salinity are negatively correlating with *Cx. quinquefasciatus* collection, their significant values were 0.010, 0.007, and 0.001, correspondingly. However, DO positively influenced mosquito collection in the case of *Cx. tritaeniorhynchus* (p-value 0.009). *Lt. fuscanus* sampling and conductivity and TDS values are directly proportional with a moderate significance level with a p value of 0.018 each. The breeding habitat's alkalinity also directly controls the number of mosquitoes having higher significant value 0.008.

Table 1.7 Breeding Habitat of Mosquito Species Collected from Kole Wetlands in Thrissur

Habitat	*An. barbirostris*	*An. nigerrimus*	*An. peditaeniatus*	*An. stephensi*	*An. subpictus*	*An. vagus*	*Ae. aegypti*	*Ae. albopictus*	*Ae. vittatus*	*Ph. cogilli*	*Ar. subalbatus*	*Cx. bitaeniorhynchus*	*Cx. gelidus*	*Cx. pipiens*	*Cx. quinquefasciatus*	*Cx. tritaeniorhynchus*	*Lu. fuscanus*	*Ma. bonneae*	*Ma. indiana*	*Ma. uniformis*
Flood Plain	+	+		+	+	+					+		+	+	+	+	+		+	+
Ditches				+	+	+					+		+	+	+	+	+			
Containers				+		+	+	+	+					+	+					
Tyres							+	+	+		+									
Cemented Tanks				+	+	+	+	+	+					+	+					
Rice Field	+	+	+	+	+			+			+		+	+	+	+	+	+	+	+
Irrigation Canal	+	+		+	+	+		+			+		+	+					+	+
Blocked Drainages	+	+	+	+	+						+		+	+	+	+	+			
Burrow Pit					+						+		+	+						
Canal Basin				+	+						+		+	+	+	+	+			
Temporary Pool		+		+	+						+		+	+	+	+	+			
Coconut Shell						+	+	+	+											
Tree Holes							+	+	+		+			+						
Leaf Litter							+	+	+											
Leaf Axils						+	+	+	+						+					
Clay Pot				+			+	+	+					+						
Marsh	+	+		+	+						+	+	+	+	+	+	+	+	+	+
Rocky Pool							+	+	+	+										
Stagnant Water Bodies	+	+		+	+						+		+	+	+	+	+			+
Hoof Prints											+			+						
Ponds	+	+			+						+	+			+					
Unused Wells	+	+		+							+		+		+	+				
Leaf Internodes							+	+												
Pods							+	+	+											
Flower Bracts								+												
Exoskelton Of Dead Organisims							+	+												

+ sign shows presence of mosquito species in Breeding Habit

Table 1.8 The correlation coefficient values between different physico-chemical parameters and number of collected mosquitoes

Correlations												
		Total No. of Mosquitoes	TEMPERATURE °C	pH	TURBIDITY (NTU)	CONDUCTIVITY (µS-cm)	TDS mg/L	DO mg/L	ALKALINITY mg/L	HARDNESS mg/L	CHLORIDE mg/L	SALINITY mg/L
Total No. of Mosquitoes	Pearson Correlation	1										
	Sig. (2-tailed)											
	N	336										
TEMPERATURE °C	Pearson Correlation	-.044	1									
	Sig. (2-tailed)	.426										
	N	336	336									
pH	Pearson Correlation	-.147**	.055	1								
	Sig. (2-tailed)	.007	.311									
	N	336	336	336								
TURBIDITY (NTU)	Pearson Correlation	.194**	-.019	-.067	1							
	Sig. (2-tailed)	.000	.735	.224								
	N	336	336	336	336							
CONDUCTIVITY (µS-cm)	Pearson Correlation	.232**	-.072	.013	.805**	1						
	Sig. (2-tailed)	.000	.189	.813	.000							
	N	336	336	336	336	336						
TDS mg/L	Pearson Correlation	.230**	-.072	.010	.806**	.999**	1					
	Sig. (2-tailed)	.000	.185	.862	.000	.000						
	N	336	336	336	336	336	336					
DO mg/L	Pearson Correlation	.006	-.095	.108*	-.041	.010	.010	1				
	Sig. (2-tailed)	.918	.082	.048	.451	.862	.860					
	N	336	336	336	336	336	336	336				
ALKALINITY mg/L	Pearson Correlation	-.011	-.046	.713**	.009	.182**	.179**	.085	1			
	Sig. (2-tailed)	.836	.400	.000	.875	.001	.001	.122				
	N	336	336	336	336	336	336	336	336			
HARDNESS mg/L	Pearson Correlation	.229**	-.133*	.182**	.256**	.368**	.365**	-.006	.246**	1		
	Sig. (2-tailed)	.000	.015	.001	.000	.000	.000	.919	.000			
	N	336	336	336	336	336	336	336	336	336		
CHLORIDE mg/L	Pearson Correlation	.138*	-.046	.180**	.106	.205**	.202**	.035	.193**	.391**	1	
	Sig. (2-tailed)	.011	.401	.001	.052	.000	.000	.522	.000	.000		
	N	336	336	336	336	336	336	336	336	336	336	
SALINITY mg/L	Pearson Correlation	.103	.035	.240**	.109*	.199**	.198**	.048	.280**	.303**	.689**	1
	Sig. (2-tailed)	.059	.523	.000	.046	.000	.000	.379	.000	.000	.000	
	N	336	336	336	336	336	336	336	336	336	336	336
**. Correlation is significant at the 0.01 level (2-tailed).												
*. Correlation is significant at the 0.05 level (2-tailed).												

Table 1.9 The correlation coefficient values between the different physico-chemical parameters of breeding water and the number of mosquitoes collected

PARAMETERS		*An. barbirostris*	*An. nigerrimus*	*Ae. albopictus*	*Ae. vittatus*	*Ar. subalbatus*	*Cx. gelidus*	*Cx. quinquefasciatus*	*Cx. tritaeniorhynchus*	*Lt. fuscanus*
Temperature	Pearson's Co-Efficient	-0.271	-0.207	0.150	-0.847*	-0.283*	-0.334*	-0.218	-0.016	-0.335
	Significant Value	0.147	0.292	0.327	0.016	0.033	0.022	0.156	.935	.095
pH	Pearson's Co-Efficient	0.017	0.111	0.255	-0.370	-0.203	-0.002	-0.239	0.153	0.298
	Significant Value	0.930	0.573	0.090	0.414	0.130	0.987	0.118	0.429	0.139
Turbidity	Pearson's Co-Efficient	-0.002	-0.021	-0.229	0.618	0.108	0.325*	-0.036	0.117	0.253
	Significant Value	0.992	0.915	0.129	0.139	0.425	0.026	0.816	0.545	0.212
Conductivity	Pearson's Co-Efficient	0.222	0.045	-0.344*	0.449	0.216	0.358*	-0.021	0.300	0.459*
	Significant Value	0.239	0.821	0.021	0.312	0.107	0.013	0.890	0.114	0.018
TDS	Pearson's Co-Efficient	0.234	0.053	-0.345*	0.447	0.215	0.358*	-0.021	0.299	0.459*
	Significant Value	0.214	0.790	0.020	0.315	0.108	0.013	0.891	0.115	0.018
DO	Pearson's Co-Efficient	-0.085	-0.086	-0.111	0.856*	0.121	0.016	-0.385**	0.473**	-0.077
	Significant Value	0.656	0.665	0.466	0.014	0.368	0.916	0.010	0.009	0.709
Alkalinity	Pearson's Co-Efficient	0.129	0.233	0.050	0-.329	0.096	0.298*	-0.130	0.319	0.511**
	Significant Value	0.495	0.233	0.745	0.471	0.476	0.042	0.400	0.091	0.008
Hardness	Pearson's Co-Efficient	0.468**	0.327	0.101	0.281	0.336*	0.332*	-0.039	0.073	0.331
	Significant Value	0.009	0.089	0.511	0.541	0.010	0.023	0.803	0.706	0.099
Chloride	Pearson's Co-Efficient	-0.315	-0.104	-0.253	0.262	0.186	-0.092	-0.399**	-0.039	0.202
	Significant Value	0.090	0.600	0.093	0.571	0.166	0.540	0.007	0.841	0.322
Salinity	Pearson's Co-Efficient	-0.243	-0.476*	-0.046	0.123	0.079	-0.203	-0.495**	-0.102	0.100
	Significant Value	0.196	0.010	0.762	0.792	0.561	0.170	0.001	0.597	0.626

** Correlation is significant at the 0.01 level (2-tailed)
* Correlation is significant at the 0.05 level (2-tailed)

1.3.5.2 Discussion

Mosquitoes are little insects, but they are a surprisingly well-evolved group of animals that can live in practically every aquatic habitat, including freshwater, salty water, and contaminated water (Webb and Russell, 2007). Water is an integral part of the environment, and its amount in the breeding location is a crucial factor in mosquito immature oviposition, development, and survival (Piyaratnea et al., 2005).

The physico-chemical and biological properties of mosquito larval habitats are critical in the zoning of breeding and dispersion zones. Entomological monitoring of mosquito vectors and the creation of tailored control strategies can benefit from the surveillance of these traits. Furthermore, more research should be conducted in a larger geographical region, considering the study area's physico-chemical and ecological parameters' complex features and their interactions with distinct mosquito species (Amini et al., 2020).

An. barbirostris displayed a highly significant positive association (P value 0.009) with the hardness of the breeding water and mosquito collection. *An. nigerrimus* number of mosquitoes collected and salinity has a moderate negative correlation with a p-value of 0.010. Both Conductivity and TDS water quality parameters moderately influence the total mosquito collection in *Ae. albopictus*. These correlations are rather a negative pattern having a p-value of 0.021 and 0.020. Water temperature of *Ae. vittatus* species modestly control some mosquitoes in the opposite direction. DO also follow the same pattern of positive association (p-value 0.014). *Ar. subalbatus* collection was directly proportional to the increase in hardness of the water and inversely proportional to the increasing Temperature (p-value temperature- 0.033. hardness- 0.010). Ghanbari et al., 2005, discussed the correlation between some *Anopheles* mosquitoes and the physico-chemical parameters of their breeding water. They reported that *An. culicufacies An. stephensi An. superpictus An. turkhudi An. multicolor*- Significant correlation with pH, Total hardness. Nitrate, calcium, EC, phosphate, sulphate. *Ae. vittatus* is one of the significant residents of the rocky pool, Temperature of 14-40, pH 5.8 - 9.8, a wide range of conductivity, and TDS (Adebote et al., 2008)

6 different water quality parameters significantly influence the collection of *Cx. gelidus* mosquitoes. Water temperature and mosquito collection have a moderate negative correlation with a significant value of 0.022. Turbidity shows a medium level of significant correlation (p-value 0.026). Conductivity and TDS also have a similar pattern of association with a p-value of 0.013 each. Alkalinity and hardness of *Cx. gelidus* breeding habitat has a slight positive correlation with mosquito collection with a p-value of 0.042 and 0.023, respectively. DO, Chloride, Salinity negatively correlate with *Cx. quinquefasciatus* collection and their significant values are 0.010, 0.007, 0.001, correspondingly. However, DO positively influence mosquito collection in the case of *Cx. tritaeniorhynchus* (p-value 0.009). *Lt. fuscanus* sampling and conductivity and TDS values are directly proportional with a moderate significance level with a p-value of 0.018 each. The breeding habitat's alkalinity also directly controls the number of mosquitoes having a higher significant value of 0.008.

Selection of oviposition sites by gravid mosquitoes principally depends on the availability of water resources and their physico-chemical parameters (Fillinger et al., 2004), and the presence of efficient predators (Shililu et al., 2003; Piyaratne et al., 2005). Oviposition, survival, and spatial distribution of mosquito larvae are affected by the presence of salts, dissolved solids, eutrophication level, turbidity, mud deposition, vegetation in the habitat, temperature, light, shade, pH (Tren and Bate, 2001). Physico-chemical factors of rice fields and nitrogen-based fertilizers used in agricultural development provide perfect requirements for the expansion and distribution of the mosquito larval population. The height of the paddy, aquatic temperature, dissolved oxygen, ammonia, and nitrogenous nitrogen hardly affect the richness of the larval mosquito population. The sprinkling of nitrogen base fertilizers

can elevate nitrogen and ammonia and eventually support the abundant larval inhabitants in the field (Sunish and Reuben, 2001; Muturi et al., 2008)

1.4 Conclusion

Mosquitoes are an integral component of wetland ecosystems, as they offer suitable conditions for their breeding and survival. The Kole wetlands of Thrissur, Kerala, are particularly rich in biodiversity and are frequently subjected to human activities like fishing, agriculture, and birdwatching. These regions serve as essential habitats for breeding multiple species of migratory birds. This makes the region a hub for disease transmission through mosquitoes, as the insects serve as vectors for human and animal pathogens. Understanding and documentation of the mosquito population and their determining factors is crucial for implementing effective vector control measures. This chapter focuses on the different species of mosquitoes found in the Thrissur Kole wetlands. During this study, 20 mosquito species belonging to 5 genera were identified in the Kole wetlands of Thrissur. The results of the study showed that, except for four species, the majority of mosquitoes showed significant variation in different seasons. Except for *Ph. cogilli* and *Ma. bonneae*, nearly all mosquitoes were collected in two or three seasons during the study period. Results from the ANOVA test showed that, apart from four species (*An. stephensi*, *Ph. cogilli*, *Cx. bitaeniorhynchus*, and *Ma. bonneae*), most mosquitoes showed a significant variation in their presence and abundance across different seasons. The species that showed no variation were either present in one season (*Ph. cogilli* and *Ma. bonneae*) or had a consistent distribution in two (*Cx. bitaeniorhynchus*) or three (*An. stephensi*) seasons. This study also aimed to examine the relationship between water quality and mosquito diversity by analysing the correlation between the physico-chemical parameters of water samples from different breeding habitats and

mosquito species diversity. GIS maps were created to show the presence of these species at different locations and their abundance, which varies with the seasons. Additionally, the study aimed to examine the relationship between water quality and mosquito diversity. Out of the ten water parameters recorded, only pH, Turbidity, Conductivity, TDS, Hardness, and Chloride showed a correlation with the number of mosquitoes collected. Nine out of the 20 species of mosquitoes discovered were found to have a correlation with the water parameters. Understanding the presence, abundance, distribution, and factors that influence the mosquito population in a given area is of utmost importance in designing and implementing effective vector control measures.

CHAPTER 2

Phylogenetic analysis of different mosquito species in Kole wetlands of Thrissur, Kerala

2.1 Introduction

Molecular identification is trending in the taxonomy field due to its accuracy and lesser time consumption than conventional morphological identification. It could also differentiate sibling species and identify immature life forms like eggs, larvae, and pupae (Bortolus, 2008). This technique extracts a small segment of DNA from the specimen and amplifies it. The amplified DNA is sequenced to get its base-pair composition and then compared to those already sequenced from the database. The area selected for DNA isolation contains a highly identical composition within the species and shows some difference even with closely related species. Generally, the mitochondrial COXI gene, having a minimum of 400 base pairs, is used in animal molecular identification (Hebert et al., 2003).

Mosquitoes have been major pests from ancient times, as they transmit disease to humans. The life cycle of mosquitoes is persistently reliant on humans and other animals as the egg development of female mosquito demand blood. The blood-feeding behaviour is the reason for the transmission of disease-causing pathogens between animals and humans, humans, and humans (Jang et al., 2002; Wilder et al., 2009; Mazzacano and Black, 2013). Proper vector identification has a significant role in vector control tactics. Therefore, intensive identification studies concentrate on vectors related to human diseases and other irrelevant species, getting insufficient consideration (Zavortink, 1990; Munstermann and Conn,1997; Krzywinski and Besansky, 2003).

Kole wetlands are the areas selected for the analysis as they are recognized as an exceedingly productive ecosystem. It involves various habitats that contrast vividly from each other in their physico-chemical properties offering suitable sites for the breeding and larval development of different mosquito species. Kole wetlands should also be marked as important mosquito surveillance sites as many anthropogenic activities like agriculture, fishing, and cattle feeding. Adult vector mosquitoes could spread pathogens between workers, native people, and other birds and animals (Sivaperuman and Jaison, 2000, Velayudhan et al., 2022).

Mosquitoes are an unavoidable part of the aquatic food chain, making the documentation of mosquitoes from Kole wetlands more crucial. The extensive use of chemical insecticides could eradicate the mosquitoes from the wetlands, causing direct damage to its ecological balance. Proper identification and knowledge about the existing species would help manage vector impact without damaging the environmental equilibrium.

2.2 Methodology

2.2.1 DNA Barcoding

2.2.1.1 Collection and Preservation

Mosquitoes were collected from different sampling locations using various sampling methods. Morphologically identified specimens were put into separate glassine ampules with 70% ethanol and stored at -20º C. Appropriate code numbers were assigned to these vessels and referred to as voucher specimens until further use.

2.2.1.2 Genomic DNA Extraction and PCR Amplification

The thoracic legs were used to extract the Genomic DNA of morphologically identified mosquito species. This procedure was carried out using ORIGIN Genomic

DNA isolation Kit per the manufacturer's instruction. DNA was extracted from the thoracic legs of the experimental sample (Shere-Kharwar et al., 2013). Agarose gel electrophoresis was conducted for the confirmation of the presence of DNA. The amplification reaction was performed by using a DNA thermal cycler (Takara). About 2 ng of genomic DNA was amplified for the mitochondrial cytochrome oxidase subunit I (COI) gene using forward and reverse primers. 2 ng (1 µl) of genomic DNA and a total volume of 50 µl were used for the PCR, 1 µl each forward and reverse primers (forward primer with DNA sequence 5'-GGTCAACAAATCATAAAGATA TTGG-3' and the DNA sequence of reverse primer 5'TAAACTTCAGGGTGACCAA AAAATCA-3') (Folmer et al., 1994), with at a concentration of 10 µM, 1 µl of dNTPS (2mM), 5 µl of 10X reaction buffer with MgCl2, 0.5 µl Taq polymerase (5 U/µl) and 41.5 µl of Water. amplification was done over 30 cycles, after an initial denaturation at 95 °C for 5 minutes. Each cycle lasts 10 seconds for denaturation at 95 °C, 1 minute for annealing at 50 °C, 45 seconds for the extension at 72 °C, and 3 minutes for the final extension at 72 °C.

2.2.1.3 Agarose Gel Electrophoresis

The PCR products were resolved on 2% TAE agarose gel (Mahesh et al., 2012), stained with EtBr (Sambrook and Russell, 2001), and photographed using a gel documentation system. A Gene Ruler (Thermo Scientific; GeneRuler 100bp DNA Ladder. #SM0242) was used to determine the size of the product. EtBr acts as an intercalating agent on the bases of DNA molecules and imparts an orange colour to DNA under ultraviolet light.

2.2.1.4 PCR Product Purification

After confirming the PCR amplification of the corresponding COI fragment, the remaining portion of the PCR product was column purified using Fermentas, GeneJET PCR purification kit. The GenElute TM PCR Clean-up Kit is designed to purify rapidly single-stranded or double-stranded PCR amplification products from other reactions such as excess primers, nucleotides, DNA polymerase, oils, and salts from the PCR products. The purified product was again resolved on 2% agarose gel check for the presence of DNA.

2.2.2 DNA sequencing and phylogenetic analysis

The purified PCR product was sequenced at IISc Bangulluru, and Sci Genom Labs Private Ltd. utilising Sanger's sequencing method and the PCR's forward and reverse primers with ABI 3730XL automated sequencer. The DNA sequence of mosquitoes from the Kole wetlands of Thrissur is presented here, along with phylogeny analysis. The trimmed COI sequences of forward and reverse obtained were multiply aligned using ClustralW (Thompson et al*.,* 1994). Take the aligned region as the final product sequence. The consensus sequence was searched for similarity using the BLAST n and BLAST p programs of NCBI (Altschul et al*.,* 1990). The partial COI gene sequence was deposited into GenBank (NCBI) for access by people worldwide. It may be used as a molecular barcode for the gathered insect pests. Using MEGAX, final nucleotide sequences were examined (Tamura et al*.,* 2013). The inter and intra-specific genetic diversity was generated using Kimura 2 parameter model, and a phylogenetic tree was generated using the neighbor-joining algorithm (Saitou and Nei, 1987). Percentage nucleotide distance calculations were performed using MEGAX.

2.3 Result and Discussion

20 mosquitoes were identified from the study site during the study period. DNA sequencing, molecular identification, and phylogenetic analysis were done with these collected species for species confirmation. Final nucleotide sequences of every collected species were deposited to NCBI GenBank and obtained Accession number. Table 2.1 included data of collected species, their location, Accession number obtained from NCBI etc.

Table 2.1: Accession number of collected mosquito species provided by NCBI GenBank

Sl No	Mosquito Species	Voucher Number	Sequence Length	Accession Number	Collection Area
1	*An. barbirostris*	Cdrl 49	605 bp	MW144288.1	Thrissur Kole Wetlands
2	*An. nigerrimus*	Cdrl 38	607 bp	MN700901.1	Thrissur Kole Wetlands
3	*An. peditaeniatus*	Cdrl 48	614 bp	MT345573.1	Thrissur Kole Wetlands
4	*An. stephensi*	Cdrl 39	552 bp	MN660044.1	Thrissur Kole Wetlands
5	*An. subpictus*	Cdrl 47	600 bp	MT258530.1	Thrissur Kole Wetlands
6	*An. vagus*	Cdrl 02	678 bp	MK628547.1	Thrissur Kole Wetlands
7	*Ae. aegypti*	Cdrl 12	679 bp	MK542379.1	Thrissur Kole Wetlands
8	*Ae. albopictus*	Cdrl 13	678 bp	MK542378.1	Thrissur Kole Wetlands
9	*Ae. vittatus*	Cdrl 33	679 bp	MK491498.1	Thrissur Kole Wetlands
10	*Ph. cogilli*	Cdrl 36	632 bp	MN700902.1	Thrissur Kole Wetlands
11	*Ar. subalbatus*	Cdrl 05	678 bp	MK644935.1	Thrissur Kole Wetlands
12	*Cx. bitaeniorhynchus*	Cdrl 43	587 bp	MT192883.1	Thrissur Kole Wetlands
13	*Cx. gelidus*	Cdrl 17	678 bp	MK724071.1	Thrissur Kole Wetlands
14	*Cx. pipiens*	Cdrl 04	678 bp	MK603829.1	Thrissur Kole Wetlands
15	*Cx. quinquefasciatus*	Cdrl 07	540 bp	MT895717.1	Thrissur Kole Wetlands
16	*Cx. tritaeniorhynchus*	Cdrl 03	679 bp	MK861440.1	Thrissur Kole Wetlands
17	*Lt. fuscanus*	Cdrl 25	678 bp	MK616587.1	Thrissur Kole Wetlands
18	*Ma. bonneae*	Cdrl 24	566 bp	MT177149.1	Thrissur Kole Wetlands
19	*Ma. indiana*	Cdrl 01	678 bp	MK637632.1	Thrissur Kole Wetlands
20	*Ma. uniformis*	Cdrl 22	682 bp	MK757484.1	Thrissur Kole Wetlands

2.3.1. Species Name: *Anopheles barbirostris*

GenBank Accession Number: MW144288.1

Voucher Number: CDRL49

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Anophelini
Genus	:	*Anopheles*
Subgenus	:	*Anopheles*
Species	:	*Anopheles barbirostris*

Description

An. barbirostris is a large, black-colored mosquito with specific white ornamentation on different body parts. Significant white spots are present on these mosquitoes' antennae, maxillary palpi, wings, legs, and abdomen. *An. barbirostris* were frequently collected from comparatively clear water, like Flood plain, Rice fields, Irrigation canals, Blocked drainages, Marshlands, and Stagnant water bodies.

2.3.1.2 Result

The forward and reverse primers were used to amplify the mitochondrial cytochrome oxidase subunit I gene of *An. barbirostris,* collected from Kole wetlands of Thrissur, Kerala, were 5'-GGTCAACAAATCATAAAGATATTGG-3' and 5'

TAAACTTCAG GGTGACCAAAAAATCA-3' respectively. A single product with a length of 605 bp was produced by PCR amplifying *An. barbirostris'* mitochondrial cytochrome oxidase subunit I (COI) gene fragment. Accession No. MW144288.1.was obtained from the NCBI GenBank, and Figures 2.1a- 2.1e displayed the DNA sequence, corresponding translation product, phylogenetic tree, electrophorogram, and molecular barcode.

> MW144288.1 *Anopheles barbirostris*|605bp

TGAGCCGGAATTGTAGGAACTTCTTGAAGAATTCTTATTCGAGCTGAATTAGGTCATCCAGGAGCTTTTATTG
GGGATGATCAAATTTATAATGTAATTGTTACAGCTCATGCTTTTATTATAATTTTCTTTATAGTTATACCTAT
TATAATTGGAGGATTTGGAAATTGATTAGTACCTTTACTATTAGGAGCTCCTGATATAGCATTTCCTCGAATA
AATAATATAAGATTTTGAATATTACCTCCTTCTCTTACTTTATTAATTTCTAGAAGTATAGTAGAAAATGGAG
CTGGAACTGGGTGAACGGTTTATCCTCCTTTATCTTCTGGGATTGCTCATGCAGGAGCTTCTGTTGATTTATC
AATTTATTCATTACATTTAGCAGGAATTTCTTCAATTTTAGGAGCAGTAAATTTTATTACTACTGTTATTAAT
ATACGTTCACCAGGTATTACTCTTGATCGAATACCTTTATTTGTTTGATCTGTAGTTATTACAGCAGTTCTTT
TATTATTATCTTTACCAGTATTAGCTCGTGCAATTACTATATTATTAACTGATCGAAATTTAAATACATCCTT
CTTGGACCCTGCAGGAGGAGC

Figure 2.1a: The DNA sequence of *Anopheles barbirostris* COI gene

> MW144288.1 *Anopheles barbirostris*

MVGTSWSILIRAELGHPGAFIGDDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLLLGAPDMAFPRMNNM
SFWMLPPSLTLLISSSMVENGAGTGWTVYPPLSSGIAHAGASVDLSIYSLHLAGISSILGAVNFITTVINMRS
PGITLDRMPLFVWSVVITAVLLLLSLPVLARAITMLLTDRNLNTSFLDPAGG

Figure 2.1b: The protein sequence of *Anopheles barbirostris* COI gene

The average nucleotide composition throughout the species was T=39.0%; C=15.5%; A=28.7%; G=16.8% (Table 2.2c). This outcome shows that evaluation situated on a mitochondrial gene will also be priceless for unravelling the phylogenetic relationships within *An. barbirostris*. The percentage of A+T was more than that of G+C, which reflected extra within the codon usage. The tree is depicted in scale, with branch lengths that match the evolutionary distances used to generate the phylogenetic tree and are within reasonable bounds.

Variable nucleotide composition is a fundamental feature of all living things, and this property can help in their phylogenetic analysis and species identification. The COI gene in the mitochondrial genome has been proven to be an excellent source of information for the set of closely related species belonging to the order Diptera. Variation in the nucleotide is the predominant property of all organisms, which could be utilised for its identification and phylogenetic study (Priya and Sebastian, 2015).

The BLAST analysis of 605 bp of the mosquito *An. barbirostris* showed significant homology with other *Anopheles* species. The genetic divergence analysis depicts the divergence of different geographically isolated species of *Anopheles* with various related species. *An. barbirostris* isolated from Kole wetlands of Thrissur, Kerala (GenBank Accession No. MW144288.1) showed 1.02% divergence, 1.36% divergence with *An. barbirostris* (GenBank Accession Nos. AB373943.1 and AB971312.1) (Table. 2.2c). *Anopheles* species were separated into related clades in the phylogenetic tree, and *Anopheles nitidus* and *Anopheles argyropus* species are in different clads. Table 2.2a contains the number of bases per position alterations among nucleotide sequences calculated by the Maximum Composite Likelihood model. The evaluation comprised of fourteen nucleotide sequences. Codon positions incorporated were 1st+2nd+3rd+Noncoding. There was a total of 595 positions in the final dataset.

Table 2.2a: The nucleotide substitution matrix estimate of COI gene sequence of *Anopheles barbirostris*

From\To	A	T	C	G
A	-	10.2029	4.0529	7.7406
T	7.5094	-	7.5919	4.3857
C	7.5094	19.1121	-	4.3857
G	13.2537	10.2029	4.0529	-

Table 2.2b: The Transition/Transversion bias estimate of mitochondrial COI gene sequence of *Anopheles barbirostris*

From\To	A	T	C	G
A	-	7.0431	7.0431	10.9138
T	7.0431	-	10.9138	7.0431
C	7.0431	10.9138	-	7.0431
G	10.9138	7.0431	7.0431	-

Table 2.2c: The nucleotide frequency comparison of *Anopheles barbirostris* COI gene sequence with its kin species

Accession Number and Name of the Species	**Nucleotide Frequencies (%)**																			
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MW144288.1 *Anopheles barbirostris*	40.5	14.8	28.4	16.3	595.0	27.1	14.6	28.6	29.6	199.0	42.4	27.8	13.1	16.7	198.0	52.0	2.0	43.4	2.5	198.0
AB373943.1 *Anopheles barbirostris*	40.8	14.5	28.7	16.0	595.0	27.1	13.6	29.1	30.2	199.0	42.9	27.8	13.1	16.2	198.0	52.5	2.0	43.9	1.5	198.0
AB971312.1 *Anopheles barbirostris*	40.8	14.6	28.6	16.0	595.0	26.6	14.1	29.1	30.2	199.0	43.4	27.8	12.6	16.2	198.0	52.5	2.0	43.9	1.5	198.0
LC333251.1 *Anopheles nitidus*	38.0	15.0	31.1	16.0	595.0	26.6	13.6	29.1	30.7	199.0	43.4	27.8	12.6	16.2	198.0	43.9	3.5	51.5	1.0	198.0
AB777810.1 *Anopheles nitidus*	38.0	15.0	31.4	15.6	595.0	26.6	13.6	29.1	30.7	199.0	43.4	27.8	12.6	16.2	198.0	43.9	3.5	52.5	0.0	198.0
AB826075.1 *Anopheles argyropus*	37.6	15.6	30.4	16.3	595.0	24.6	15.6	29.1	30.7	199.0	43.4	27.8	12.6	16.2	198.0	44.9	3.5	49.5	2.0	198.0
AB826080.1 *Anopheles argyropus*	37.1	16.1	30.4	16.3	595.0	24.6	15.6	29.1	30.7	199.0	43.4	27.8	12.6	16.2	198.0	43.4	5.1	49.5	2.0	198.0
MW542315.1 *Aedes albopictus*	38.3	17.1	28.4	16.1	595.0	23.6	17.6	30.2	28.6	199.0	44.4	27.3	12.6	15.7	198.0	47.0	6.6	42.4	4.0	198.0
MT890465.1 *Aedes albopictus*	38.3	17.1	28.4	16.1	595.0	23.6	17.6	30.2	28.6	199.0	44.4	27.3	12.6	15.7	198.0	47.0	6.6	42.4	4.0	198.0
MK713986.1 *Culex pipiens*	39.3	15.1	29.4	16.1	595.0	26.1	15.6	29.1	29.1	199.0	43.9	27.3	12.6	16.2	198.0	48.0	2.5	46.5	3.0	198.0
MK713985.1 *Culex pipiens*	39.3	15.1	29.4	16.1	595.0	26.1	15.6	29.1	29.1	199.0	43.9	27.3	12.6	16.2	198.0	48.0	2.5	46.5	3.0	198.0
KU578141.1 *Coccinella transversalis*	37.0	18.8	28.4	15.8	595.0	24.1	16.1	33.7	26.1	199.0	43.9	26.3	13.1	16.7	198.0	42.9	14.1	38.4	4.5	198.0
JF835944.1 *Nephila inaurata*	41.7	12.4	28.1	17.8	595.0	32.7	11.1	25.6	30.7	199.0	45.5	25.3	13.6	15.7	198.0	47.0	1.0	44.9	7.1	198.0
MN125137.1 *Pyganodon grandis*	39.3	15.6	20.8	24.2	595.0	28.6	15.1	25.1	31.2	199.0	44.4	22.2	13.6	19.7	198.0	44.9	9.6	23.7	21.7	198.0
Avg.	39.0	15.5	28.7	16.8	595.0	26.3	14.9	29.0	29.7	199.0	43.8	26.9	12.9	16.4	198.0	47.0	4.6	44.2	4.1	198.0

Table 2.2d: The evolutionary divergence percentage between *Anopheles barbirostris* and its closely related species

Sl. No.	Accession Number	Organism	Percentage of Divergence
1	MW144288.1	*Anopheles barbirostris*	0.00%
2	AB373943.1	*Anopheles barbirostris*	1.02%
3	AB971312.1	*Anopheles barbirostris*	1.36%
4	LC333251.1	*Anopheles nitidus*	12.16%
5	AB777810.1	*Anopheles nitidus*	12.16%
6	AB826075.1	*Anopheles argyropus*	12.13%
7	AB826080.1	*Anopheles argyropus*	12.52%
8	MW542315.1	*Aedes albopictus*	18.91%
9	MT890465.1	*Aedes albopictus*	18.91%
10	MK713986.1	*Culex pipiens*	17.34%
11	MK713985.1	*Culex pipiens*	17.34%
12	KU578141.1	*Coccinella transversalis*	27.51%
13	JF835944.1	*Nephila inaurata*	26.76%
14	MN125137.1	*Pyganodon grandis*	44.81%

2.3.1.2 Discussion

An. barbirostris is a considerably large-sized black mosquito that spreads over oriental areas, including New Guinea, Lesser Sunda Java Sumatra islands, China, and India. Morphological identification was done with available taxonomic keys and expert consultation and confirmed its species identity. Molecular identification was made using NCBI's cytochrome oxidase I gene analysis, ensuring its generic taxonomy. Phylogenetically *An. barbirostris* from Kerala is close to *An. barbirostris* (AB373943.1) isolated from Thailand by NCBI analysis. Wang et al., 2012 identified the main mosquito species in China based on DNA barcoding; they explained the detailed phylogeny of *An. barbirostris* collected from their study site. This study was a novel molecular work from Kerala, and the barcode generated could be used to spot the specimen easily and resolve its phylogeny. The above sequence with the NCBI BLAST tool revealed that no 100% sequence similarity for

the COI gene is available within the information base. It can be depicted that the resultant sequence obtained for the *An. barbirostris* Kerala is a novel.

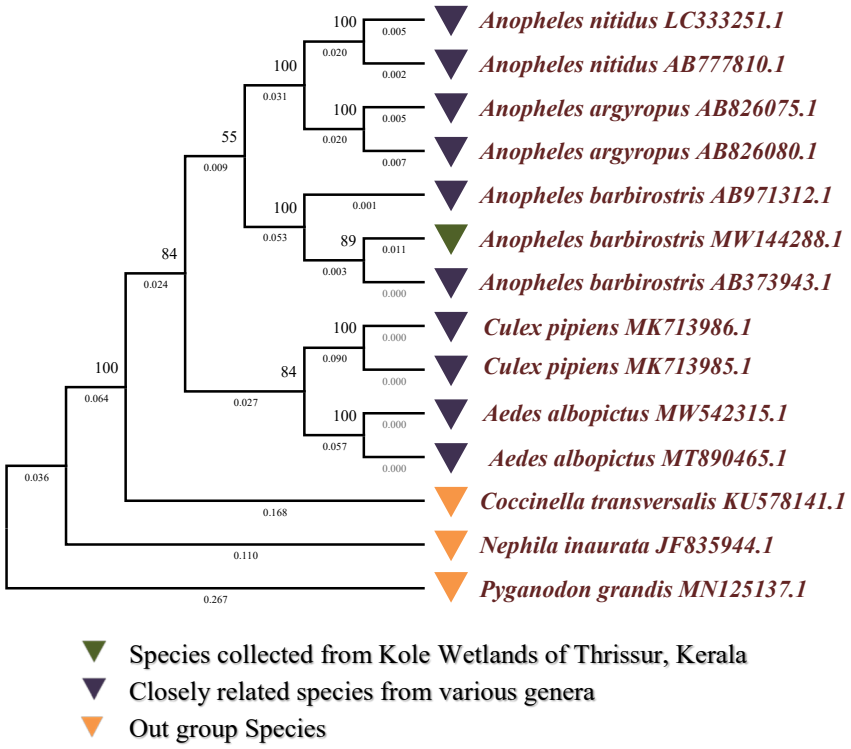

Figure 2.1c Phylogenetic tree of *Anopheles barbirostris*

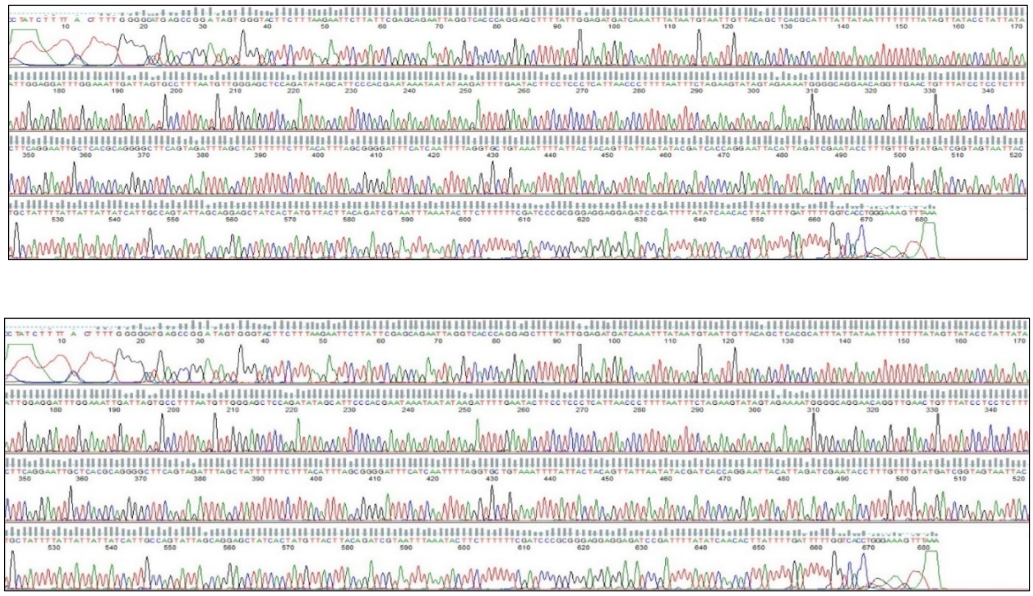

Figure 2.1d Electrophorogram showing the nucleotide sequence of mitochondrial COI gene of *Anopheles barbirostris*

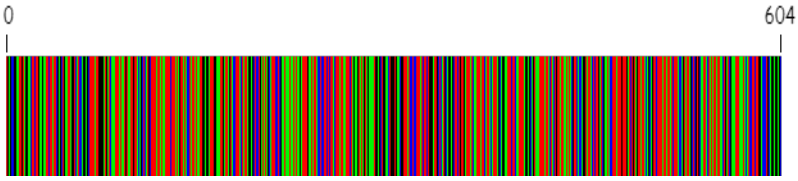

Figure 2.1e Molecular barcode of *Anopheles barbirostris*

2.3.2 Species Name: *Anopheles nigerrimus*

GenBank Accession Number: MN700901.1

Voucher Number: CDRL38

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Anophelini
Genus	:	*Anopheles*
Subgenus	:	*Anopheles*
Species	:	*Anopheles nigerrimus*

Description

Dark large-sized mosquitoes have pale spots on the head, body, and wings. Larval forms are commonly seen in freshwater habitats with moderate to dense vegetation. *An. nigerrimus* regularly collected from Flood plain, Rice fields, Irrigation canals, Blocked drainages, Temporary pools, Marshlands, and Stagnant water bodies.

2.3.2.1 Result

The forward and reverse primers were used to amplify the mitochondrial cytochrome oxidase subunit I gene of *An. nigerrimus,* collected from Kole wetlands of Thrissur, Kerala, were 5'-GGTCAACAAATCATAAAGATATTGG-3' and 5'TAAACTTCAG GGTGACCAAAAAATCA-3' respectively. A single product with a length of 607 bp was produced by PCR amplifying *An. nigerrimus'* mitochondrial cytochrome oxidase subunit I (COI) gene fragment. Accession No. MN700901.1 was obtained from the NCBI GenBank, and Figures 2.2a- 2.2e displayed the DNA sequence, corresponding translation product, phylogenetic tree, electrophorogram, and molecular barcode.

> MN700901.1 *Anopheles nigerrimus*| 607bp

```
ATAGTGGGAACTTCTTTAAGTATTCTAATTCGAGCTGAATTAGGTCATCCAGGAGCATTCATTGGAGATGATCA
AATTTATAATGTTATTGTAACAGCACATGCTTTTATTATAATTTTCTTTATAGTTATGCCTATTATAATTGGAG
GATTTGGAAATTGATTAGTTCCTTTAATACTAGGAGCCCCAGATATAGCATTCCCACGAATAAATAATATAAGT
TTTTGAATATTACCTCCTTCACTAACTTTATTAATTTCTAGAAGTATAGTAGAAAATGGAGCAGGAACAGGATG
AACTGTGTATCCACCACTTTCATCTGGAATTGCTCATGCTGGAGCATCAGTAGACTTAGCAATTTTTTCATTAC
ATTTAGCGGGGATTTCATCAATTTTAGGAGCAGTAAATTTTATTACTACTGTAATTAATATACGATCACCAGGA
ATTACATTAGATCGAATACCTTTATTTGTGTGATCAGTAGTAATTACAGCAGTATTATTATTATTATCTTTACC
CGTTTTAGCTGGAGCTATTACAATACTTTTAACAGATCGAAATTTAAATACTTCATTTTTTGACCCAGCTGGAG
GGGGAGACCCAATTT
```

Figure 2.2a: The DNA sequence of *Anopheles nigerrimus* COI gene

> MN700901.1 *Anopheles nigerrimus*

```
MVGTSLSILIRAELGHPGAFIGDDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLMLGAPDMAFPRMNNMS
FWMLPPSLTLLISSSMVENGAGTGWTVYPPLSSGIAHAGASVDLAIFSLHLAGISSILGAVNFITTVINMRSPG
ITLDRMPLFVWSVVITAVLLLLSLPVLAGAITMLLTDRNLNTSFFDPAGGGDPI
```

Figure 2.2b: The protein sequence of *Anopheles nigerrimus* COI gene

The nucleotide composition of *An. nigerrimus* showed T=36.1%, C=15.4%, A=31.6%, and G=16.9% nucleotides. The average nucleotide composition analysis

involved with 14 nucleotide sequences showed T=37.7%, C=15.8%, A=29.6%, and G=16.9% (Table 2.3c). The COI nucleotide composition analysis showed the variation in the nucleotide composition of *An. nigerrimus* isolated from the Kole wetlands of Thrissur, Kerala, and *An. nigerrimus* (MH330206.1) isolated from Srilanka 0.83%. The estimated Transition/Transversion bias (R) is 0.89. Rates and Substitution pattern were estimated under the Kimura (1980) 2-parameter model (Table. 2.3b). When performing a substitution matrix analysis, tree topology was automatically calculated to estimate ML values. In this analysis, 14 nucleotide sequences were considered, and the nucleotide frequencies calculated were A = 29.58%, T/U = 37.72%, C = 15.76%, and G = 16.93%. The final dataset contained 604 positions within the 1st+ 2nd+ 3rd noncoding codon positions. Rates and substitution pattern were estimated under the Tamura-Nei (1993) model (Table.2.3a).

The evolutionary divergence analysis of *An. nigerrimus* between 14 sequences revealed that *An. nigerrimus* showed 0.83% evolutionary divergence with *An. nigerrimus* (MH330206.1) isolated from Srilanka and 1.17% divergence with *An. nigerrimus* (AB778799.1) also isolated from Cambodia (Table.2.3d). The Neighbor-Joining approach was used to infer the evolutionary history. (Figure 2.2c). Phylogenetically, *An. nigerrimus* (MN700901.1) showed to be the closest relative of *An. nigerrimus* (MH330206.1) isolated from Sri Lanka and *An. nigerrimus* (AB778799.1) isolated from Cambodia. They seem to arose from the main clade and are divided into two sub-branches separately.

Table 2.3a: The nucleotide substitution matrix estimate of COI gene sequence of *Anopheles nigerrimus*

From\To	A	T	C	G
A	-	9.2792	3.8775	8.4572
T	7.2750	-	8.1277	4.1654
C	7.2750	19.4505	-	4.1654
G	14.7705	9.2792	3.8775	-

Table 2.3b: The Transition/Transversion bias estimate of mitochondrial COI gene sequence of *Anopheles nigerrimus*

From\To	A	T	C	G
A	-	6.6164	6.6164	11.7673
T	6.6164	-	11.7673	6.6164
C	6.6164	11.7673	-	6.6164
G	11.7673	6.6164	6.6164	-

Table 2.3c: The nucleotide frequency comparison of *Anopheles nigerrimus* COI gene sequence with its kin species

Accession Number and Name of the Species	**Nucleotide Frequencies (%)**																			
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MN700901.1 *Anopheles nigerrimus*	36.1	15.4	31.6	16.9	604.0	25.7	14.4	29.2	30.7	202.0	42.8	27.9	12.9	16.4	201.0	39.8	4.0	52.7	3.5	201.0
MH330206.1 *Anopheles nigerrimus*	36.1	15.4	32.0	16.6	604.0	26.2	13.9	29.2	30.7	202.0	42.8	27.9	12.9	16.4	201.0	39.3	4.5	53.7	2.5	201.0
AB778799.1 *Anopheles nigerrimus*	36.6	14.9	32.5	16.1	604.0	25.7	14.4	29.2	30.7	202.0	42.8	27.9	12.9	16.4	201.0	41.3	2.5	55.2	1.0	201.0
LC333251.1 *Anopheles nitidus*	37.6	15.1	31.3	16.1	604.0	26.2	13.9	29.2	30.7	202.0	42.8	27.9	12.9	16.4	201.0	43.8	3.5	51.7	1.0	201.0
AB777810.1 *Anopheles nitidus*	37.6	15.1	31.6	15.7	604.0	26.2	13.9	29.2	30.7	202.0	42.8	27.9	12.9	16.4	201.0	43.8	3.5	52.7	0.0	201.0
AB826075.1 *Anopheles argyropus*	37.1	15.9	30.6	16.4	604.0	24.3	15.8	29.2	30.7	202.0	42.8	27.9	12.9	16.4	201.0	44.3	4.0	49.8	2.0	201.0
AB826080.1 *Anopheles argyropus*	36.6	16.4	30.6	16.4	604.0	24.3	15.8	29.2	30.7	202.0	42.8	27.9	12.9	16.4	201.0	42.8	5.5	49.8	2.0	201.0
MW542315.1 *Aedes albopictus*	37.9	17.4	28.5	16.2	604.0	23.3	17.8	30.2	28.7	202.0	43.8	27.4	12.9	15.9	201.0	46.8	7.0	42.3	4.0	201.0
MT890465.1 *Aedes albopictus*	37.9	17.4	28.5	16.2	604.0	23.3	17.8	30.2	28.7	202.0	43.8	27.4	12.9	15.9	201.0	46.8	7.0	42.3	4.0	201.0
MK713986.1 *Culex pipiens*	38.9	15.2	29.6	16.2	604.0	25.7	15.8	29.2	29.2	202.0	43.3	27.4	12.9	16.4	201.0	47.8	2.5	46.8	3.0	201.0
MK713985.1 *Culex pipiens*	38.9	15.2	29.6	16.2	604.0	25.7	15.8	29.2	29.2	202.0	43.3	27.4	12.9	16.4	201.0	47.8	2.5	46.8	3.0	201.0
KU578141.1 *Coccinella transversalis*	36.4	19.0	28.6	15.9	604.0	23.8	16.3	33.7	26.2	202.0	43.3	26.4	13.4	16.9	201.0	42.3	14.4	38.8	4.5	201.0
JF835944.1 Nephila inaurata	41.4	12.6	28.1	17.9	604.0	32.2	11.4	25.7	30.7	202.0	44.8	25.4	13.9	15.9	201.0	47.3	1.0	44.8	7.0	201.0
MN125137.1 *Pyganodon grandis*	39.1	15.7	20.9	24.3	604.0	28.2	15.3	24.8	31.7	202.0	43.8	22.4	13.9	19.9	201.0	45.3	9.5	23.9	21.4	201.0
Avg.	37.7	15.8	29.6	16.9	604.0	25.8	15.2	29.1	30.0	202.0	43.2	27.0	13.1	16.6	201.0	44.2	5.1	46.5	4.2	201.0

Table 2.3d: The evolutionary divergence percentage between *Anopheles nigerrimus* and its closely related species

Sl. No.	Accession Number	Organism	Percentage of Divergence
1	MN700901.1	*Anopheles nigerrimus*	0.00%
2	MH330206.1	*Anopheles nigerrimus*	0.83%
3	AB778799.1	*Anopheles nigerrimus*	1.17%
4	LC333251.1	*Anopheles nitidus*	2.40%
5	AB777810.1	*Anopheles nitidus*	2.40%
6	AB826075.1	*Anopheles argyropus*	2.75%
7	AB826080.1	*Anopheles argyropus*	2.84%
8	MW542315.1	*Aedes albopictus*	8.88%
9	MT890465.1	*Aedes albopictus*	8.88%
10	MK713986.1	*Culex pipiens*	6.80%
11	MK713985.1	*Culex pipiens*	6.80%
12	KU578141.1	*Coccinella transversalis*	13.66%
13	JF835944.1	*Nephila inaurata*	14.20%
14	MN125137.1	*Pyganodon grandis*	22.96%

2.3.2.2 Discussion

An. nigerrimus is wide distribution in India, Burma, Ceylon, etc. Morphological identification was done with available keys and also with online photographs. Phylogenetically *An. nigerrimus* from the Kole wetlands of Thrissur, Kerala, is close to *An. nigerrimus* (Accession No. MH330206.1) isolated from Sri Lanka by NCBI analysis. Wijit et al., 2013 identified *An. nigerrimus* through COI sequencing. Their result said *An. nitidus* and *An. argyropus* were the closest species of *An. nigerrimus.* The present study also reveals the same nearest phylogenetic relationship of these Anopheline mosquitoes. The nucleotide frequencies were T=36.1%, C=15.4%, A=31.6% and G=16.9% indicated high AT content. The present study provided a novel report to all databases, and its unique barcode can easily spot and analyse the phylogenetic position of this species based on DNA

sequences. This is a pioneer molecular work from Kerala, and the barcode generated can easily spot the specimen and resolve its phylogeny.

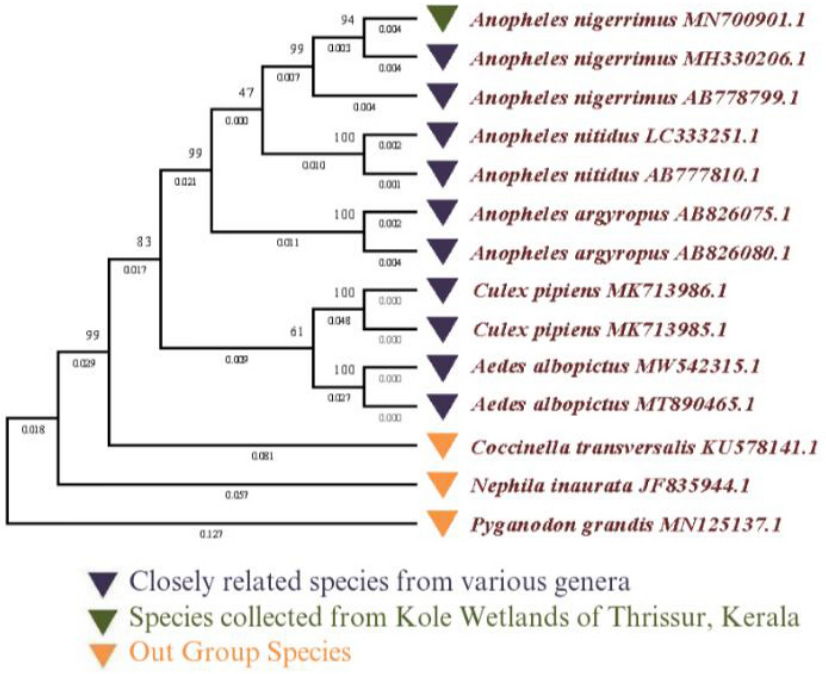

Figure 2.2c Phylogenetic tree of *Anopheles nigerrimus*

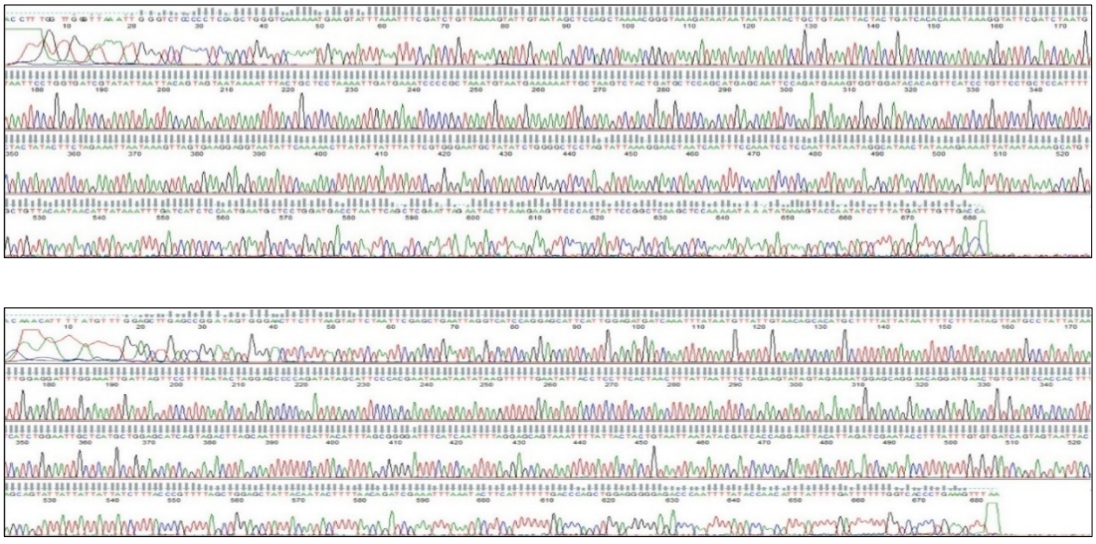

Figure 2.2d Electrophorogram showing the nucleotide sequence of mitochondrial COI gene of *Anopheles nigerrimus*

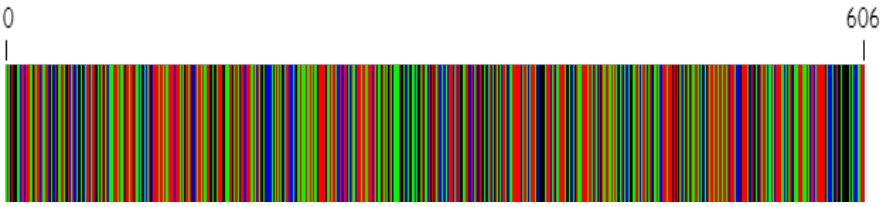

Figure 2.2e Molecular barcode of *Anopheles nigerrimus*

2.3.3 Species Name: *Anopheles peditaeniatus*

GenBank Accession Number: MT345573.1

Voucher Number: CDRL48

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Anophelini
Genus	:	*Anopheles*
Subgenus	:	*Anopheles*
Species	:	*Anopheles peditaeniatus*

Description

Large Anopheline has a dark body and spotted wings. The lower abdominal corners are bare areas. *An. peditaeniatus* larval forms were present in restricted habitats like rice filed and blocked drainages.

2.3.3.1 Result

The forward and reverse primers were used to amplify the mitochondrial cytochrome oxidase subunit I gene of *An. peditaeniatus,* collected from Kole wetlands of Thrissur, Kerala, were 5'-GGTCAACAAATCATAAAGATATTGG-3'

and 5'TAAACTTCAG GGTGACCAAAAAATCA-3' respectively. A single product with a length of 614 bp was produced by PCR amplifying *An. peditaeniatus'* mitochondrial cytochrome oxidase subunit I (COI) gene fragment. Accession No. MT345573.1 was obtained from the NCBI GenBank, and Figures 2.3a- 2.3e displayed the DNA sequence, corresponding translation product, phylogenetic tree, electrophorogram, and molecular barcode.

> MT345573.1 *Anopheles peditaeniatus*| 614bp

GGAGCTTGAGCCGGAATAGTAGGAACTTCTTTAAGTATTCTAATTCGAGCTGAATTAGGTCATCCTGGTGCT
TTTATTGGAGATGATCAAATTTATAATGTTATTGTAACAGCACATGCTTTTATTATAATTTTTTTTATAGTT
ATACCTATTATAATTGGAGGATTTGGAAATTGATTAGTTCCTTTAATATTAGGAGCCCCTGATATAGCTTTC
CCTCGAATAAATAATATAAGTTTTTGAATATTACCCCCTTCTTTAACTCTTTTAATTTCTAGAAGTATAGTA
GAAAATGGAGCCGGAACAGGATGAACTGTTTACCCACCTCTTTCATCAGGAATTGCTCATGCTGGAGCATCA
GTAGATTTAGCTATTTTTTCATTACATTTAGCTGGAATTTCTTCAATTTTAGGAGCAGTAAATTTTATTACA
ACTGTTATTAATATACGATCTCCAGGAATTACATTAGATCGAATACCATTATTTGTTTGATCAGTAGTAATT
ACAGCAGTATTATTATTATTATCTTTACCAGTCTTAGCAGGAGCTATTACTATACTTTTAACAGATCGAAAT
TTAAATACTTCATTTTTTGATCCTGCTGGAGGAGGAGA

Figure 2.3a: The DNA sequence of *Anopheles peditaeniatus* COI gene

> MT345573.1 *Anopheles peditaeniatus*

MVGTSLSILIRAELGHPGAFIGDDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLMLGAPDMAFPRM
NNMSFWMLPPSLTLLISSSMVENGAGTGWTVYPPLSSGIAHAGASVDLAIFSLHLAGISSILGAVNFITT
VINMRSPGITLDRMPLFVWSVVITAVLLLLSLPVLAGAITMLLTDRNLNTSFFDPAGGG

Figure 2.3b: The protein sequence of *Anopheles peditaeniatus* COI gene

The nucleotide composition of *An. peditaeniatus* showed T=38.7%, C=14.9%, A=30.2%, and G=16.2% nucleotides. The average nucleotide composition analysis involved with 14 nucleotide sequences showed T=38.5%, C=15.4%, A=29.0%, and G=17.2% (Table 2.4c). The COI nucleotide composition analysis showed the variation in nucleotide composition of *An. peditaeniatus* isolated from the Kole wetlands of Thrissur, Kerala, and *An. peditaeniatus* (KF406666.1) isolated from Pakistan were very close relatives (0% divergence). The estimated value of

Transition/Transversion bias (R) is 0.79. The rates and substitution pattern were estimated under the Kimura (1980) 2-parameter model (Table.2.4b). The nucleotide frequencies were A = 28.98%, T/U = 38.49%, C = 15.36%, and G = 17.17%. Tree topology was automatically generated to estimate ML values. There was a maximum Log-likelihood of -2955.824 for this calculation. In this experiment, there were 14 different nucleotide sequences. The first, second, third, and noncoding codon locations were noncoding. The total dataset contained 610 positions.

An. peditaeniatus (MT345573.1) collected from Thrissur Kole lands showed 0% evolutionary divergence with *An. peditaeniatus* (KF406666.1) isolated from Pakistan and *An. peditaeniatus* (MH330207.1) isolated from SriLanka, according to the evolutionary divergence analysis of the species between 14 sequences (Table.2.4d). Inferring the evolutionary history was done using the neighbour-joining method. In geographically remote populations of *An. peditaeniatus* from Pakistan (KF406666.1) and SriLanka (MH330207.1) were genetically the closest relatives of *An. peditaeniatus* (MT345573.1). They emerged from a single clade and were separated into two sub-branches.

Table 2.4a: The nucleotide substitution matrix estimate of COI gene sequence of *Anopheles peditaeniatus*

From\To	A	T	C	G
A	-	9.9937	3.9890	7.5163
T	7.5249	-	7.9492	4.4572
C	7.5249	19.9154	-	4.4572
G	12.6895	9.9937	3.9890	-

Table 2.4b: The Transition/Transversion bias estimate of mitochondrial COI gene sequence of *Anopheles peditaeniatus*

From\To	A	T	C	G
A	-	6.9941	6.9941	11.0118
T	6.9941	-	11.0118	6.9941
C	6.9941	11.0118	-	6.9941
G	11.0118	6.9941	6.9941	-

Table 2.4c: The nucleotide frequency comparison of *Anopheles peditaeniatus* COI gene sequence with its kin species

Accession Number and Name of the Species	**Nucleotide Frequencies (%)**																		
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MT345573.1 *Anopheles peditaeniatus*	38.7	14.9	30.2	16.2	610.0	42.2	27.9	12.7	17.2	204.0	47.3	3.4	49.3	0.0	203.0	26.6	13.3	28.6	31.5	203.0
KF406666.1 *Anopheles peditaeniatus*	38.7	14.9	30.2	16.2	610.0	42.2	27.9	12.7	17.2	204.0	47.3	3.4	49.3	0.0	203.0	26.6	13.3	28.6	31.5	203.0
MH330207.1 *Anopheles peditaeniatus*	38.7	14.9	30.2	16.2	610.0	42.2	27.9	12.7	17.2	204.0	47.3	3.4	49.3	0.0	203.0	26.6	13.3	28.6	31.5	203.0
EU699048.1 *Anopheles lesteri*	38.2	14.8	30.7	16.4	610.0	42.2	27.9	12.7	17.2	204.0	45.8	3.0	50.7	0.5	203.0	26.6	13.3	28.6	31.5	203.0
AY768947.1 *Anopheles lesteri*	38.2	14.8	30.7	16.4	610.0	42.2	27.9	12.7	17.2	204.0	45.8	3.0	50.7	0.5	203.0	26.6	13.3	28.6	31.5	203.0
MG761806.1 *Anopheles Hyrcanus*	38.0	15.2	30.5	16.2	610.0	42.2	27.9	12.7	17.2	204.0	46.8	3.0	50.2	0.0	203.0	25.1	14.8	28.6	31.5	203.0
MG761805.1 *Anopheles Hyrcanus*	37.9	15.2	30.7	16.2	610.0	42.2	27.9	12.7	17.2	204.0	46.3	3.0	50.7	0.0	203.0	25.1	14.8	28.6	31.5	203.0
MW542315.1 *Aedes albopictus*	38.2	16.9	28.4	16.6	610.0	43.6	27.0	12.7	16.7	204.0	46.8	6.4	42.9	3.9	203.0	24.1	17.2	29.6	29.1	203.0
MT890465.1 *Aedes albopictus*	38.2	16.9	28.4	16.6	610.0	43.6	27.0	12.7	16.7	204.0	46.8	6.4	42.9	3.9	203.0	24.1	17.2	29.6	29.1	203.0
MK713986.1 *Culex pipiens*	38.9	15.1	29.3	16.7	610.0	42.6	27.5	12.7	17.2	204.0	47.8	2.5	46.8	3.0	203.0	26.1	15.3	28.6	30.0	203.0
MK713985.1 *Culex pipiens*	38.9	15.1	29.3	16.7	610.0	42.6	27.5	12.7	17.2	204.0	47.8	2.5	46.8	3.0	203.0	26.1	15.3	28.6	30.0	203.0
KU578141.1 *Coccinella transversalis*	36.4	18.5	28.5	16.6	610.0	43.1	26.0	13.2	17.6	204.0	41.9	13.8	39.4	4.9	203.0	24.1	15.8	33.0	27.1	203.0
JF835944.1 *Nephila inaurata*	41.1	12.6	28.0	18.2	610.0	44.1	26.0	13.7	16.2	204.0	46.8	1.0	45.3	6.9	203.0	32.5	10.8	25.1	31.5	203.0
MN125137.1 *Pyganodon grandis*	38.9	15.2	20.8	25.1	610.0	43.6	21.6	13.7	21.1	204.0	43.8	9.4	24.1	22.7	203.0	29.1	14.8	24.6	31.5	203.0
Avg.	38.5	15.4	29.0	17.2	610.0	42.8	27.0	12.9	17.3	204.0	46.3	4.6	45.6	3.5	203.0	26.4	14.5	28.5	30.6	203.0

Table 2.4d: The evolutionary divergence percentage between *Anopheles peditaeniatus* and its closely related species

Sl. No.	Accession Number	Organism	Percentage of Divergence
1	MT345573.1	*Anopheles peditaeniatus*	0.00%
2	KF406666.1	*Anopheles peditaeniatus*	0.00%
3	MH330207.1	*Anopheles peditaeniatus*	0.00%
4	EU699048.1	*Anopheles lesteri*	2.30%
5	AY768947.1	*Anopheles lesteri*	2.30%
6	MG761806.1	*Anopheles hyrcanus*	2.40%
7	MG761805.1	*Anopheles hyrcanus*	2.41%
8	MW542315.1	*Aedes albopictus*	11.28%
9	MT890465.1	*Aedes albopictus*	11.28%
10	MK713986.1	*Culex pipiens*	8.69%
11	MK713985.1	*Culex pipiens*	8.69%
12	KU578141.1	*Coccinella transversalis*	17.45%
13	JF835944.1	*Nephila inaurata*	18.62%
14	MN125137.1	*Pyganodon grandis*	32.82%

2.3.3.2 Discussion

Large Anopheline commonly breeds in fresh water to slightly polluted water like rice filed and blocked drainages. The partial coding sequence of COI was proved to be a powerful tool for identifying organisms (Hebert and Gregory, 2005). The partial COI sequence generated in this study revealed considerable variation with other species. Morphological identification of this species has been done with available keys and online photographs. The molecular identification method in NCBI and BOLD database showed the conformity of this species as *An. peditaeniatus*. The mosquitoes' genetic structure and evolutionary relationship can provide information about the nature of ecosystems, especially on pest and host interactions. This is a pioneer molecular work from Kerala, and the barcode generated can be used to spot the specimen easily and resolve its phylogeny. Singh and Vashist, 2017 sequenced a partial sequence of 183 bp of the COII gene to identify Anopheline mosquitoes. They also sequenced and identified *An. peditaeniatus*, however, in the current

investigation, 614 bp of the COI gene was utilised to identify the organism. Wijit et al., 2013 discussed the phylogenetic relationship between *An. peditaeniatus* isolated from Thailand phylogenetically nearest to *paraliae* species. Closest phylogenetic species of *An. peditaeniatus* isolated from Thrissur Kole Lands was *An. lesteri.*

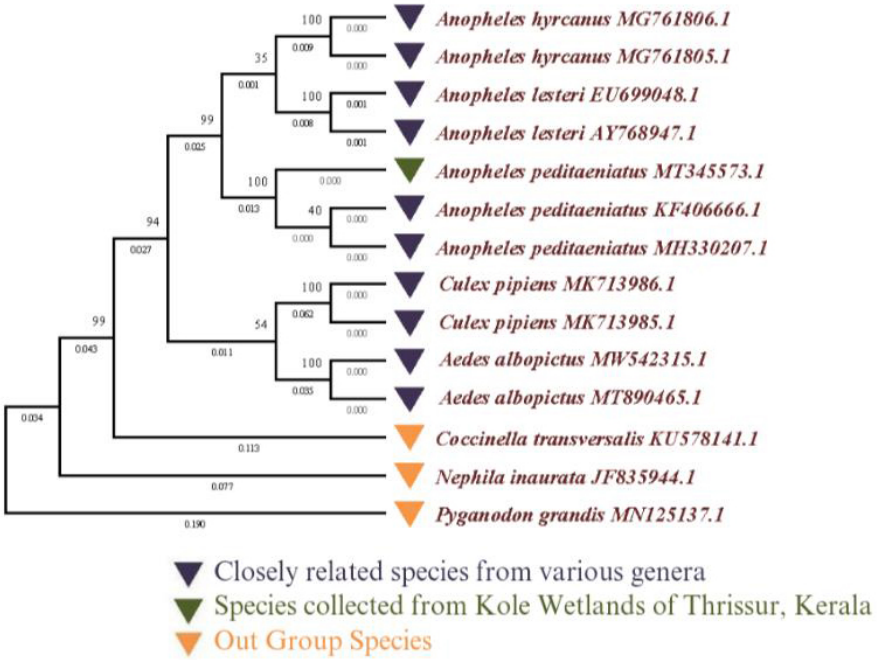

Figure 2.3c Phylognetic tree of *Anopheles peditaeniatus*

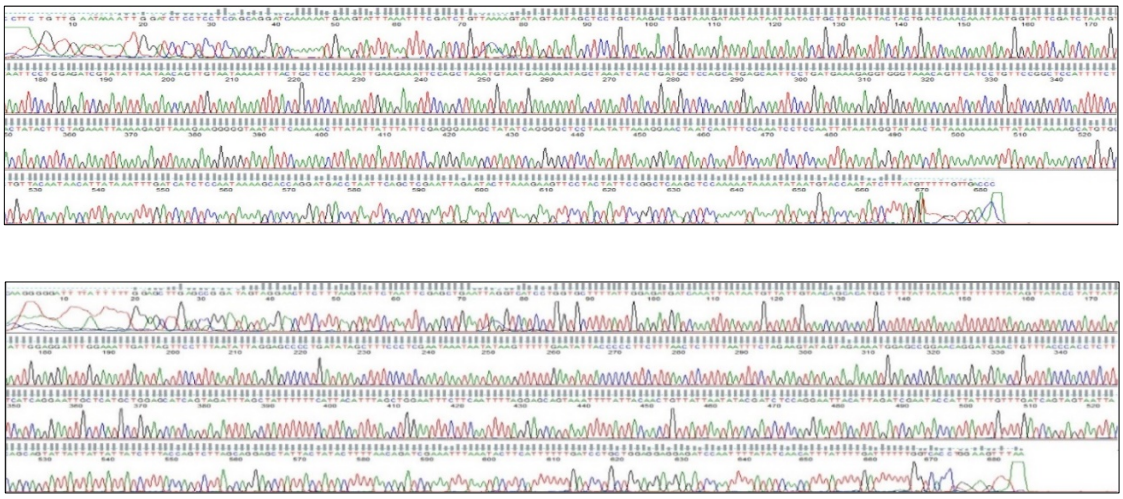

Figure 2.3d Electrophorogram showing the nucleotide sequence of mitochondrial COI gene of *Anopheles peditaeniatus*

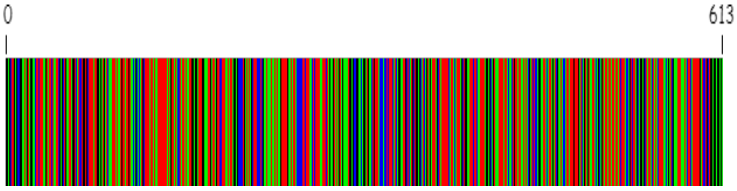

Figure 2.3e Molecular barcode of *Anopheles peditaeniatus*

2.3.4 Species Name: *Anopheles stephensi*

GenBank Accession Number: MN660044.1

Voucher Number: CDRL39

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	*Anophelini*
Genus	:	*Anopheles*
Subgenus	:	*Myzomyia*
Species	:	*Anopheles stephensi*

Description

A dark, medium-sized mosquito, one of the significant Malarial vectors, can breed in fresh and slightly polluted water. Dark and pale scale patterns are displayed on the thorax, abdomen, and legs of *Anopheles stephensi.* These mosquitoes are regularly collected from almost all habitats like rice fields, canals, flood plain, containers, temporary pools, etc.

2.3.4.1 Result

An. stephensi, found in the Kole wetlands of Thrissur, Kerala, had its mitochondrial cytochrome oxidase subunit I gene amplified using forward and reverse nucleotide primer sequences, were 5'-GGTCAACAAATCATAAAGATATTGG-3' and 5'TAAA CTTCAGGGTGACCAAAAAATCA-3' respectively. A single product with a length of 552 bp was produced by PCR amplifying *An. stephensi's* mitochondrial cytochrome oxidase subunit I (COI) gene fragment. Accession No. MN660044.1 was obtained from the NCBI GenBank, and Figures 2.4a- 2.4e displayed the DNA sequence, corresponding translation product, phylogenetic tree, electrophorogram, and molecular barcode.

> MN660044.1 *Anopheles stephensi*| 552bp

ATAGTAGGAACATCTTTAAGAATTCTTATTCGAGCTGAATTAGGACACCCAGGAGCATTTATTGGAGACGAT
CAAATTTATAATGTAATTGTAACTGCTCATGCTTTTATTATAATTTTCTTTATAGTTATACCTATTATAATT
GGGGGATTTGGAAATTGATTAGTTCCTTTAATATTAGGAGCACCAGATATAGCATTTCCTCGAATAAATAAT
ATAAGATTTTGAATATTACCCCCCTCATTAACTCTTTTAATTTCTAGAAGTATAGTAGAAAATGGAGCAGGA
ACAGGATGAACTGTTTATCCGCCTTTATCGTCTGGAATTGCTCACGCTGGGGCTTCAGTAGATTTAGCAATT
TTTTCATTACATTTAGCTGGAATTTCTTCAATTTTAGGAGCAGTTAATTTTATTACTACAGTAATTAATATA
CGATCGCCAGGAATTACGTTAGACCGAATACCTTTATTCGTTTGATCTGTTGTAATTACTGCTATTTTATTA
TTATTATCATTACCTGTATTAGCTGGAGCTATTACTATATTACTTACA

Figure 2.4a: The DNA sequence of *Anopheles stephensi* COI gene

> MN660044.1 *Anopheles stephensi*

MVGTSLSILIRAELGHPGAFIGDDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLMLGAPDMAFPRMNNM
SFWMLPPSLTLLISSSMVENGAGTGWTVYPPLSSGIAHAGASVDLAIFSLHLAGISSILGAVNFITTVINMRS
PGITLDRMPLFVWSVVITAILLLLSLPVLAGAITMLLT

Figure 2.4b: The protein sequence of *Anopheles stephensi* COI gene

The BLAST search revealed the partial COI nucleotide sequence of *An. stephensi,* isolated from the Kole wetlands of Thrissur, Kerala, is 100% similar to that isolated from Odisha, India (MN329060.1) and Madurai, India (LR736010.1). *An. stephensi* isolated from the Kole wetlands of Thrissur, Kerala, showed 9.07% evolutionary divergence with two *An. culicifascies* (KF406660.1,

KF406658.1) isolated from Pakistan. It showed a 9.03% divergence with *An. varuna* isolated from Vietnam (MT434331.1) (Table.2.5d).

The average nucleotide composition throughout the species was T=40.0%; C=15.3%; A=28.5%; G=16.5% (Table 2.5c). The estimate of the substitution matrix showed the probability of substitution from one base to another (Table 2.5a). The Tamura-Nei (1993) model (+G) was used to estimate substitution patterns and rates. The rate of different transitional substitutions and transversionsal substitutions were shown the Table 2.4b. A = 5.00%, T/U = 25.00%, C = 25.00%, and G = 25.00%. The analysis included 14 nucleotide sequences. The phylogeny tree was generated using the NJ method. Phylogenetic tree showing the phylogenetic position of *An. stephensi* isolated from the Kole wetlands of Thrissur, Kerala, is given in Figure. The phylogenetic tree showed the closest relatives of *An. stephensi,* which is isolated from Thrissur Kole lands (MN660044.1), Odisha, India (MN329060.1), and Madurai, India (LR736010.1). *An. stephensi's* closest relatives, which were separated from several populations, were organized into a single clade.

Table 2.5a: The nucleotide substitution matrix estimate of COI gene sequence of *Anopheles stephensi*

From\To	A	T	C	G
A	-	10.4164	3.9765	9.1177
T	7.3427	-	6.4237	4.3055
C	7.3427	16.8268	-	4.3055
G	15.5496	10.4164	3.9765	-

Table 2.5b: The Transition/Transversion bias estimate of mitochondrial COI gene sequence of *Anopheles stephensi*

From\To	A	T	C	G
A	-	7.0722	7.0722	10.8556
T	7.0722	-	10.8556	7.0722
C	7.0722	10.8556	-	7.0722
G	10.8556	7.0722	7.0722	-

Table 2.5c: The nucleotide frequency comparison of *Anopheles stephensi* COI gene sequence with its kin species

Accession Number and Name of the Species	**Nucleotide Frequencies (%)**																		
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MN660044.1 *Anopheles stephensi*	38.9	15.1	29.9	16.1	509.0	28.2	12.9	29.4	29.4	170.0	45.3	27.6	11.8	15.3	170.0	43.2	4.7	48.5	3.6	169.0
MN329060.1 *Anopheles stephensi*	38.9	15.1	29.9	16.1	509.0	28.2	12.9	29.4	29.4	170.0	45.3	27.6	11.8	15.3	170.0	43.2	4.7	48.5	3.6	169.0
LR736010.1 *Anopheles stephensi*	38.9	15.1	29.9	16.1	509.0	28.2	12.9	29.4	29.4	170.0	45.3	27.6	11.8	15.3	170.0	43.2	4.7	48.5	3.6	169.0
KF406660.1 *Anopheles culicifacies*	40.1	15.3	28.9	15.7	509.0	25.9	15.3	29.4	29.4	170.0	45.3	27.6	11.8	15.3	170.0	49.1	3.0	45.6	2.4	169.0
KF406658.1 *Anopheles culicifacies*	40.1	15.3	28.9	15.7	509.0	25.9	15.3	29.4	29.4	170.0	45.3	27.6	11.8	15.3	170.0	49.1	3.0	45.6	2.4	169.0
MT434331.1 *Anopheles varuna*	38.7	16.3	29.1	15.9	509.0	25.3	15.9	29.4	29.4	170.0	45.3	27.6	11.8	15.3	170.0	45.6	5.3	46.2	3.0	169.0
MT434330.1 *Anopheles varuna*	39.1	15.9	29.1	15.9	509.0	25.3	15.9	29.4	29.4	170.0	45.3	27.6	11.8	15.3	170.0	46.7	4.1	46.2	3.0	169.0
MT519730.1 *Aedes vittatus*	40.5	15.7	28.5	15.3	509.0	26.5	15.9	29.4	28.2	170.0	45.3	28.2	11.8	14.7	170.0	49.7	3.0	44.4	3.0	169.0
MT519729.1 *Aedes vittatus*	40.5	15.7	28.5	15.3	509.0	26.5	15.9	29.4	28.2	170.0	45.3	28.2	11.8	14.7	170.0	49.7	3.0	44.4	3.0	169.0
MH745093.1 *Culex tritaeniorhynchus*	39.7	15.5	29.3	15.5	509.0	24.7	17.6	28.8	28.8	170.0	45.3	27.6	11.8	15.3	170.0	49.1	1.2	47.3	2.4	169.0
MH330220.1 *Culex tritaeniorhynchus*	39.5	15.7	29.5	15.3	509.0	24.1	18.2	28.8	28.8	170.0	45.3	27.6	11.8	15.3	170.0	49.1	1.2	47.9	1.8	169.0
KY694466.1 *Afidenta misera*	38.1	18.7	27.7	15.5	509.0	24.7	16.5	33.5	25.3	170.0	45.3	26.5	12.4	15.9	170.0	44.4	13.0	37.3	5.3	169.0
KC849092.1 *Nephila sumptuosa*	41.9	12.2	28.0	17.9	508.0	34.1	11.2	25.3	29.4	170.0	47.6	24.7	12.9	14.7	170.0	44.0	0.6	45.8	9.5	168.0
EF033298.1 *Lampsilis hydiana*	45.2	12.0	17.9	25.0	509.0	34.7	11.8	24.7	28.8	170.0	45.9	20.0	13.5	20.6	170.0	55.0	4.1	15.4	25.4	169.0
Avg.	40.0	15.3	28.2	16.5	508.9	27.3	14.9	29.0	28.8	170.0	45.5	26.9	12.0	15.6	170.0	47.2	4.0	43.7	5.1	168.9

Table 2.5d: The evolutionary divergence percentage between *Anopheles stephensi* and its closely related species

Sl. No.	Accession Number	Organism	Percentage of Divergence
1	MN660044.1	*Anopheles stephensi*	0.00%
2	MN329060.1	*Anopheles stephensi*	0.00%
3	LR736010.1	*Anopheles stephensi*	0.00%
4	KF406660.1	*Anopheles culicifacies*	9.07%
5	KF406658.1	*Anopheles culicifacies*	9.07%
6	MT434331.1	*Anopheles varuna*	9.03%
7	MT434330.1	*Anopheles varuna*	9.05%
8	MT519730.1	*Aedes vittatus*	16.78%
9	MT519729.1	*Aedes vittatus*	16.78%
10	MH745093.1	*Culex tritaeniorhynchus*	16.72%
11	MH330220.1	*Culex tritaeniorhynchus*	16.76%
12	KY694466.1	*Afidenta misera*	35.49%
13	KC849092.1	*Nephila sumptuosa*	29.31%
14	EF033298.1	*Lampsilis hydiana*	67.90%

2.3.4.2 Discussion

An. stephensi is an urban mosquito that can spread malaria parasites, *Plasmodium falciparum* and *vivax.* In this study, sequencing and phylogenetic analysis of the COI gene of *An. stephensi* was done for the species identification. COII gene sequencing is also used in this molecular identification procedure. Singh and Vashist 2017 identified some Anopheline mosquitoes using the COII gene. Ashfaq et al., 2014 discussed baseline data on vector mosquitoes' composition and genetic diversity. In their study, *An. stephensi* showed some genetic similarities with *An. dravidicus,* apart from this study, revealed a 100% similarity to *An. stephensi* (Accession No. MN329060.1) Odisha, India (MN329060.1) and from Madurai, India (LR736010.1).

This variation in the nucleotide diversity may be due to the compulsion in the nucleotide changes in the different codon positions because of the degenerative

character of the triplet code second position of many codons, and the first position of some codons is less constrained. The amino acid sequence may change due to differences in the strong constraint sites. But the variations in the less constrained position do not affect the phenotype, and these less constrained codon positions evolved rapidly (Nei, 1987; Irwin et al., 1991). The COI sequence of *An. stephensi* showed significant diversity compared to the other species analysed in this study; therefore, it can be used as a DNA barcode for accurate identification. Morphological identification was made with available keys and also with online photographs. Phylogenetically *An. stephensi* from Kerala is 100% similar to *An. stephensi* (Accession No. MN329060.1) Odisha, India (LR736010.1) and from Madurai, India. This is a pioneer molecular work from Kerala, and the barcode generated can be used to spot the specimen easily and resolve its phylogeny.

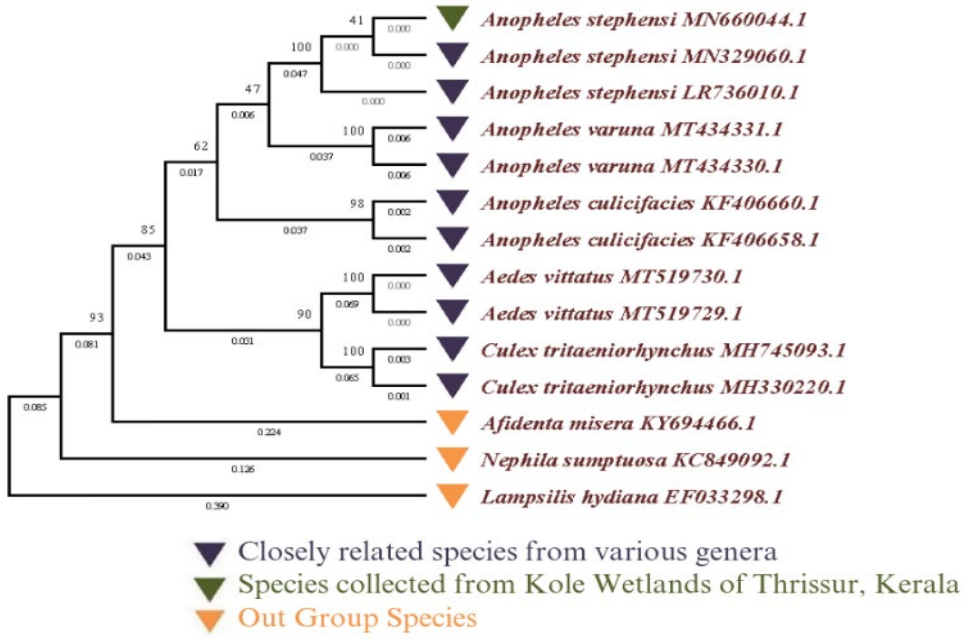

Figure 2.4c Phylogenetic tree of *Anopheles stephensi*

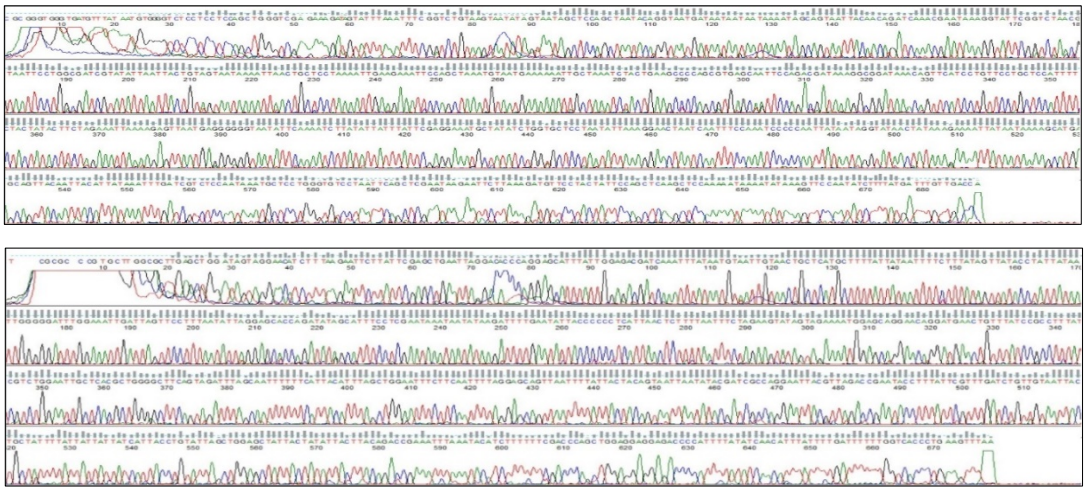

Figure 2.4d Electrophorogram showing the nucleotide sequence of mitochondrial COI gene of *Anopheles stephensi*

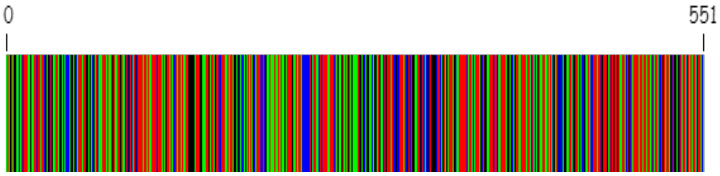

Figure 2.4e Molecular barcode of *Anopheles stephensi*

2.3.5 Species Name: *Anopheles subpictus*

GenBank Accession Number: MT258530.1

Voucher Number: CDRL47

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Anophelini
Genus	:	*Anopheles*
Subgenus	:	*Myzomyia*
Species	:	*Anopheles subpictus*

Description

The mosquito *An. subpictus* is of modest size. The antennae, maxillary palpi, thorax, and legs have many pale dots. Yellow bands are seen on the abdominal segments. Dark-coloured larvae are commonly found in various breeding habitats. Marshlands, temporary pools, canal basins, rice fields, blocked drainages, and canals are some of the larval collection points of these mosquitoes.

2.3.5.1 Result

The forward and reverse primers were used to amplify the mitochondrial cytochrome oxidase subunit I gene of *An. subpictus,* collected from Kole wetlands of Thrissur, Kerala, were 5'-GGTCAACAAATCATAAAGATATTGG-3' and 5'TAAACTTCAG GGTGACCAAAAAATCA-3' respectively. A single product with a length of 600 bp was produced by PCR amplifying *An. subpictus'* mitochondrial cytochrome oxidase subunit I (COI) gene fragment. Accession No. MT258530.1 was obtained from the NCBI GenBank, and Figures 2.5a- 2.5e displayed the DNA sequence, corresponding translation product, phylogenetic tree, electrophorogram, and molecular barcode.

> MT258530.1 *Anopheles subpictus*| 600bp

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TAGTGGGTACTTCTTTAAGAATTCTTATTCGAGCAGAATTAGGTCACCCAGGAGCTTTTATTGGAGATGATCAAA
TTTATAATGTAATTGTTACAGCTCACGCATTTATTATAATTTTTTTTATAGTTATACCTATTATAATTGGAGGAT
TTGGAAATTGATTAGTGCCTTTAATGTTGGGAGCTCCAGATATAGCATTCCCACGAATAAATAATATAAGATTTT
GAATACTTCCTCCCTCATTAACCCTTTTAATTTCTAGAAGTATAGTAGAAAATGGGGCAGGAACAGGTTGAACTG
TTTATCCCCCTCTTTCTTCAGGAATTGCTCACGCGGGGGCTTCAGTAGATTTAGCTATTTTTTCTTTACATTTAG
CAGGAATTTCATCAATTTTAGGTGCTGTAAATTTTATTACTACAGTTATTAATATACGATCACCAGGAATTACAT
TAGATCGAATACCTTTGTTTGTATGATCAGTAGTAATTACTGCTATTTTATTATTATTATCATTGCCAGTATTAG
CAGGAGCTATCACTATGTTACTTACAGATCGTAATTTAAATACTTCTTTTTTCGATCCCGCGGGAGGAGGAGATC
```

Figure 2.5a: The DNA sequence of *Anopheles subpictus* COI gene

> MT258530.1 *Anopheles subpictus*

```
VGTSLSILIRAELGHPGAFIGDDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLMLGAPDMAFPRMNNMS
FWMLPPSLTLLISSSMVENGAGTGWTVYPPLSSGIAHAGASVDLAIFSLHLAGISSILGAVNFITTVINMRSP
GITLDRMPLFVWSVVITAILLLLSLPVLAGAITMLLTDRNLNTSFFDPAGGGD
```

Figure 2.5b: The protein sequence of *Anopheles subpictus* COI gene

The COI sequence of *An. subpictus* showed bias to nucleotide AT, with the following nucleotide composition T=37.8%, C=15.7%, A=29.0%, and G=17.5%, and the average nucleotide composition value of fourteen nucleotides shows T=38.0%, C=15.8%, A=28.9% and G=17.3% (Table 2.6c). The Maximum Composite Likelihood model was used for the base substitution number per position analysis between nucleotide sequences is displayed in Table 2.6a. The Tamura-Nei (1993) model was used to estimate the substitution pattern and rates. A = 28.89%, T/U = 38.01%, C = 15.83%, and G = 17.26% were the estimated nucleotide frequencies from substitution matrix analysis. Tree topology was automatically computed for the calculation of ML values. The maximum Log-likelihood for this computation was -3472.349. This analysis involved fourteen nucleotide sequences. Codon positions comprised were 1st+2nd+3rd+Noncoding. There were a total of 600 positions in the final dataset.

Phylogenetic relationship of *An. subpictus* from Kerala revealed the evolutionary divergence with closely related species displayed in Table 2.6d. The table showed a 12.96% evolutionary divergence with *An. subpictus* from Kole lands of Kerala, and *An. subpictus* (KJ461784.1) isolated from Sri Lanka. Another close relative of *An. subpictus* from Kerala was *An. nigerrimus* (MH330206.1) was also isolated from Sri Lanka, which showed 13.56% evolutionary divergence.

The phylogenetic tree (Figure 2.5c) generated using the NJ method shows the phylogenetic position of *Anopheles subpictus* isolated from the Kole wetlands of Thrissur, Kerala. Phylogenetically, this *Anopheles subpictus* was the closest relative of *Anopheles subpictus* (KJ461784.1) isolated from SriLanka.

Table 2.6a: The nucleotide substitution matrix estimate of COI gene sequence of *Anopheles subpictus*

From\To	A	T	C	G
A	-	9.5144	3.9631	8.0483
T	7.2319	-	8.3571	4.3207
C	7.2319	20.0633	-	4.3207
G	13.4712	9.5144	3.9631	-

Table 2.6b: The Transition/Transversion bias estimate of mitochondrial COI gene sequence of *Anopheles subpictus*

From\To	A	T	C	G
A	-	6.7478	6.7478	11.5044
T	6.7478	-	11.5044	6.7478
C	6.7478	11.5044	-	6.7478
G	11.5044	6.7478	6.7478	-

Table 2.6c: The nucleotide frequency comparison of *Anopheles subpictus* COI gene sequence with its kin species

Accession Number and Name of the Species	Nucleotide Frequencies (%)																			
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MT258530.1 *Anopheles subpictus*	37.8	15.7	29.0	17.5	600.0	43.0	27.5	13.0	16.5	200.0	44.5	5.0	45.0	5.5	200.0	26.0	14.5	29.0	30.5	200.0
KJ461784.1 *Anopheles subpictus*	37.7	15.7	29.2	17.5	600.0	43.0	27.5	13.0	16.5	200.0	44.5	4.5	45.5	5.5	200.0	25.5	15.0	29.0	30.5	200.0
KJ461780.1 *Anopheles subpictus*	37.7	15.8	29.3	17.2	600.0	43.0	27.5	13.0	16.5	200.0	44.5	5.0	46.0	4.5	200.0	25.5	15.0	29.0	30.5	200.0
MH330206.1 *Anopheles nigerrimus*	36.3	15.3	31.7	16.7	600.0	43.0	27.5	13.0	16.5	200.0	39.5	4.5	53.5	2.5	200.0	26.5	14.0	28.5.	31.0	200.0
AB778799.1 *Anopheles nigerrimus*	36.8	14.8	32.2	16.2	600.0	43.0	27.5	13.0	16.5	200.0	41.5	2.5	55.0	1.0	200.0	26.0	14.5	28.5	31.0	200.0
AB826075.1 *Anopheles argyropus*	37.3	15.8	30.3	16.5	600.0	43.0	27.5	13.0	16.5	200.0	44.5	4.0	49.5	2.0	200.0	24.5	16.0	28.5	31.0	200.0
AB826080.1 *Anopheles argyropus*	36.8	16.3	30.3	16.5	600.0	43.0	27.5	13.0	16.5	200.0	43.0	5.5	49.5	2.0	200.0	24.5	16.0	28.5	31.0	200.0
MW542315.1 *Aedes albopictus*	38.0	17.3	28.3	16.3	600.0	44.0	27.0	13.0	16.0	200.0	46.5	7.0	42.5	4.0	200.0	23.5	18.0	29.5	29.0	200.0
MT890465.1 *Aedes albopictus*	38.0	17.3	28.3	16.3	600.0	44.0	27.0	13.0	16.0	200.0	46.5	7.0	42.5	4.0	200.0	23.5	18.0	29.5	29.0	200.0
MK713986.1 *Culex pipiens*	39.2	15.2	29.3	16.3	600.0	43.5	27.0	13.0	16.5	200.0	48.0	2.5	46.5	3.0	200.0	26.0	16.0	28.5	29.5.	200.0
MK713985.1 *Culex pipiens*	39.2	15.2	29.3	16.3	600.0	43.5	27.0	13.0	16.5	200.0	48.0	2.5	46.5	3.0	200.0	26.0	16.0	28.5	29.5	200.0
KU578141.1 *Coccinella transversalis*	36.7	19.0	28.3	16.0	600.0	43.5	26.0	13.5	17.0	200.0	42.5	14.5	38.5	4.5	200.0	24.0	16.5	33.0	26.5	200.0
JF835944.1 *Nephila inaurata*	41.7	12.5	27.8	18.0	600.0	45.0	25.0	14.0	16.0	200.0	47.5	1.0	44.5	7.0	200.0	32.5	11.5	25.0	31.0	200.0
MN125137.1 *Pyganodon grandis*	39.0	15.7	21.0	24.3	600.0	44.0	22.0	14.0	20.0	200.0	45.0	9.5	24.0	21.5	200.0	28.0	15.5	25.0	31.5	200.0
Avg.	38.0	15.8	28.9	17.3	600.0	43.5	27.0	13.0	17.0	200.0	44.7	5.4	44.9	5.0	200.0	25.9	15.5	28.6	30.1	200.0

Table 2.6d: The evolutionary divergence percentage between *Anopheles subpictus* and its closely related species

Sl. No.	Accession Number	Organism	Percentage of Divergence
1	MT258530.1	*Anopheles subpictus*	0.00%
2	KJ461784.1	*Anopheles subpictus*	12.96%
3	KJ461780.1	*Anopheles subpictus*	13.35%
4	MH330206.1	*Anopheles nigerrimus*	13.56%
5	AB778799.1	*Anopheles nigerrimus*	12.97%
6	AB826075.1	*Anopheles argyropus*	13.35%
7	AB826080.1	*Anopheles argyropus*	13.74%
8	MW542315.1	*Aedes albopictus*	20.51%
9	MT890465.1	*Aedes albopictus*	20.51%
10	MK713986.1	*Culex pipiens*	15.91%
11	MK713985.1	*Culex pipiens*	15.91%
12	KU578141.1	*Coccinella transversalis*	26.50%
13	JF835944.1	*Nephila inaurata*	29.16%
14	MN125137.1	*Pyganodon grandis*	48.20%

2.3.5.2 Discussion

An. subpictus is a medium-sized mosquito generally collected from slightly to highly polluted water bodies. *An. subpictus* distributed over the Oriental and Australian regions is known as a potential vector for malarial parasites.

Paul et al., 2013 analysed the morphological and molecular differences of *An. subpictus* in urban and rural areas of West Bengal, and reported a remarkable sequence variation in Urban and Rural species. Sindhania et al., (2020) discussed the molecular characterisation of *An. subpictus* and *An. sundaicus* mosquitoes in the Indian subcontinent. Maximum Likelihood (ML) rooted tree of different molecular forms of *An. subpictus* and *An. sundaicus* complex based upon 28S-D2-D3 sequences reveals the phylogenetic relationship of *An. subpictus* with *An. epiroticus* and *An. sundaicus*. COI sequencing of Anopheline mosquitoes in Sri Lanka reported that *An. vagus* and *An. subpictus* share a common clade in their

neighbour-joining tree. In present study *An. nigerrimus* and *An. agyropus* were the neighbouring Anopheline Anopheline species of *An. subpictus*.

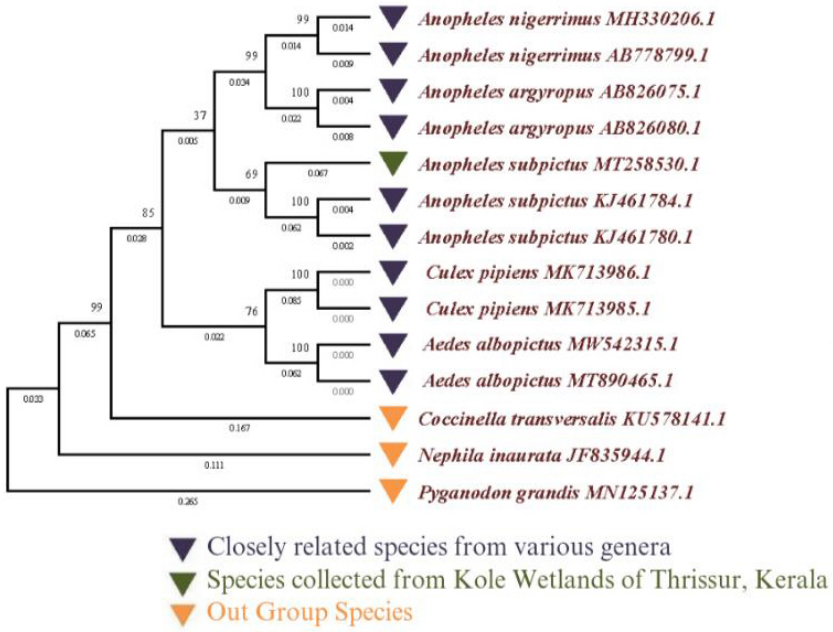

Figure 2.5c Phylogenetic tree of *Anopheles subpictus*

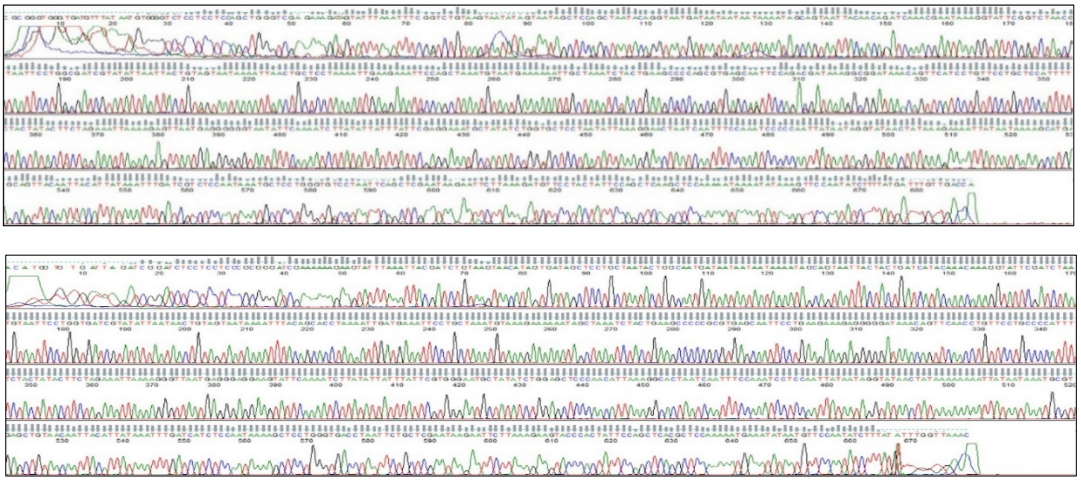

Figure 2.5d Electrophorogram showing the nucleotide sequence of mitochondrial COI gene of *Anopheles subpictus*

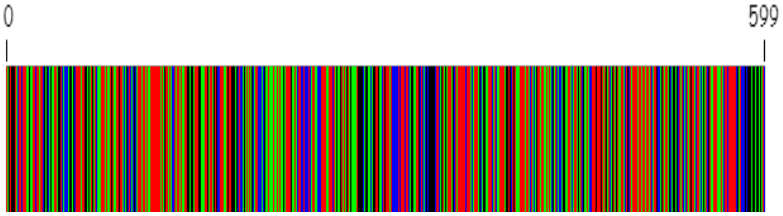

Figure 2.5e Molecular barcode of *Anopheles subpictus*

2.3.6 Species Name: *Anopheles vagus*

GenBank Accession Number: MK628547.1

Voucher Number: CDRL02

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Anophelini
Genus	:	*Anopheles*
Subgenus	:	*Myzomyia*
Species	:	*Anopheles vagus*

Description

An. vagus larvae are usually collected from freshwater habitats like rainwater containers, tanks, coconut shells, etc. It is a medium-sized Anopheline species with spotted wings and legs without pale scales.

2.3.6.1 Result

The forward and reverse primers were used to amplify the mitochondrial cytochrome oxidase subunit I gene of *An. vagus,* collected from Kole wetlands of Thrissur, Kerala, were 5'-GGTCAACAAATCATAAAGATATTGG-3' and 5'TAAACTTCAGGGTG ACCAAAAAATCA-3' respectively. A single product with a length of 678 bp was produced by PCR amplifying *An. vagus'* mitochondrial cytochrome oxidase subunit I (COI) gene fragment. Accession No. MK628547.1 was obtained from the NCBI GenBank, and Figures 2.6a- 2.6e displayed the DNA

sequence, corresponding translation product, phylogenetic tree, electrophorogram, and molecular barcode.

> MK628547.1 *Anopheles vagus*|678bp

AAGATATTGGAACATTATACTTTATTTTTGGAGCTTGAGCAGGAATAGTCGGAACATCTCTTAGAATTCTAA
TTCGAGCTGAACTAGGACATCCCGGAGCATTTATTGGGGATGATCAAATTTATAATGTAATTGTTACAGCCC
ACGCTTTTATTATAATTTTTTTTATAGTAATACCTATTATAATTGGAGGATTTGGAAACTGATTAGTTCCAC
TAATGCTAGGGGCTCCCGATATAGCATTCCCACGAATAAATAATATAAGTTTTTGAATATTACCCCCATCTC
TTACTCTTTTAATTTCTAGTAGTATAGTAGAAAATGGGGCAGGAACAGGTTGAACTGTATATCCCCCTCTTT
CATCGGGGATTGCTCACGCTGGGGCTTCAGTTGATTTAGCAATTTTCTCACTTCATTTAGCAGGAATTTCTT
CAATTTTAGGAGCAGTAAATTTTATTACTACAGTAATTAATATACGATCTCCAGGAATTACGCTAGATCGAA
TACCTTTATTTGTTTGATCAGTTGTAATTACTGCAGTCTTATTATTATTATCACTTCCAGTATTAGCAGGAG
CTATTACTATACTATTAACTGATCGAAATTTAAATACTTCGTTCTTTGACCCTGCGGGAGGAGGAGACCCTA
TTTTATATCAACACTTATTTTGATTTTTTG

Figure 2.6a: The DNA sequence of *Anopheles vagus* COI gene

> MK628547.1 *Anopheles vagus*

MGTLYFIFGAWAGMVGTSLSILIRAELGHPGAFIGDDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLM
LGAPDMAFPRMNNMSFWMLPPSLTLLISSSMVENGAGTGWTVYPPLSSGIAHAGASVDLAIFSLHLAGISSI
LGAVNFITTVINMRSPGITLDRMPLFVWSVVITAVLLLLSLPVLAGAITMLLTDRNLNTSFFDPAGGGDPIL
YQHLFWFF

Figure 2.6b: The protein sequence of *Anopheles vagus* COI gene

The BLAST search revealed the partial COI nucleotide sequence of *An. vagus* isolated from Thrissur Kole lands, Kerala, is 98.52% similar to *An. vagus* isolated from Vietnam (MT434323.1) and China (MF179260.1). *An. vagus* isolated from Thrissur Kole lands, Kerala showed 14.80% evolutionary divergence with *An. varuna* isolated from Vietnam (MT434331.1) and 17.37% evolutionary divergence with *An. culicifascies* isolated from Pakistan (KF406660.1) (Table. 2.7d). The average nucleotide composition throughout the species was T=38.9%; C=15.8%; A=28.5%; G=16.8% (Table 2.7c). The estimate of the Substitution Matrix showed the probability of substitution from one base to another (Table. 2.7a). Tamura-Nei (1993) model (+G) was used to calculate substitution patterns and rates. Different transitional and transversionsal substitutions rates were showed in Table 2.6b. A = 25.00%, T/U = 25.00%, C = 25.00%, and G = 25.00%

were the nucleotide frequencies from transition transversion bias analysis. Tree topology was automatically computed for the estimation of ML values. The maximum Log-likelihood for this computation was -3078.065. This analysis involved fourteen nucleotide sequences, and Codon positions included were 1st+2nd+3rd+Noncoding. The final dataset comprised a total of 548 positions. All these evolutionary analyses were done in MEGAX.

The phylogeny tree was generated using the NJ method. Phylogenetic tree showing the phylogenetic position of *An. vagus* isolated from Thrissur Kole lands, Kerala. The phylogenetic tree revealed the closest relationship of *An. vagus,* isolated from Thrissur Kole lands, and *An. vagus*, isolated from China (MF179260.1). *An. vagus* isolated from Vietnam (MT434323.1) showed another closest relative of *An. vagus.* These are isolated from different populations were arranged in a single branch.

Table 2.7a: The nucleotide substitution matrix estimate of COI gene sequence of *Anopheles vagus*

From\To	A	T	C	G
A	-	9.9053	4.0277	8.2906
T	7.2367	-	7.7388	4.2631
C	7.2367	19.0316	-	4.2631
G	14.0734	9.9053	4.0277	-

Table 2.7b: The Transition/Transversion bias estimate of mitochondrial COI gene sequence of *Anopheles vagus*

From\To	A	T	C	G
A	-	6.8145	6.8145	11.3710
T	6.8145	-	11.3710	6.8145
C	6.8145	11.3710	-	6.8145
G	11.3710	6.8145	6.8145	-

Table 2.7c: The nucleotide frequency comparison of *Anopheles vagus* COI gene sequence with its kin species

Accession Number and Name of the Species	Nucleotide Frequencies (%)																			
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS#1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MK628547.1 *Anopheles vagus*	36.7	17.3	29.2	16.8	548.0	22.4	18.0	29.5	30.1	183.0	45.4	27.3	11.5	15.8	183.0	42.3	6.6	46.7	4.4	182.0
MT434323.1 *Anopheles vagus*	36.7	17.7	29.0	16.6	548.0	22.4	18.0	29.5	30.1	183.0	45.4	27.3	11.5	15.8	183.0	42.3	7.7	46.2	3.8	182.0
MF179260.1 *Anopheles vagus*	36.7	17.5	29.0	16.8	548.0	22.4	18.0	29.5	30.1	183.0	45.4	27.3	11.5	15.8	183.0	42.3	7.1	46.2	4.4	182.0
KF406660.1 *Anopheles culicifacies*	39.6	15.1	29.2	16.1	548.0	25.1	15.3	30.1	29.5	183.0	45.4	27.3	11.5	15.8	183.0	48.4	2.7	46.2	2.7	182.0
KF406658.1 *Anopheles culicifacies*	39.6	15.1	29.2	16.1	548.0	25.1	15.3	30.1	29.5	183.0	45.4	27.3	11.5	15.8	183.0	48.4	2.7	46.2	2.7	182.0
MT434331.1 *Anopheles varuna*	38.3	16.1	29.4	16.2	548.0	24.6	15.8	30.1	29.5	183.0	45.4	27.3	11.5	15.8	183.0	45.1	4.9	46.7	3.3	182.0
MT434330.1 *Anopheles varuna*	38.7	15.7	29.4	16.2	548.0	24.6	15.8	30.1	29.5	183.0	45.4	27.3	11.5	15.8	183.0	46.2	3.8	46.7	3.3	182.0
MT519730.1 *Aedes vittatus*	40.3	15.3	28.8	15.5	548.0	26.2	15.3	30.1	28.4	183.0	45.4	27.9	11.5	15.3	183.0	49.5	2.7	45.1	2.7	182.0
MT519729.1 *Aedes vittatus*	40.3	15.3	28.8	15.5	548.0	26.2	15.3	30.1	28.4	183.0	45.4	27.9	11.5	15.3	183.0	49.5	2.7	45.1	2.7	182.0
MH745093.1 *Culex tritaeniorhynchus*	39.6	15.1	29.6	15.7	548.0	24.6	16.9	29.5	29.0	183.0	45.4	27.3	11.5	15.8	183.0	48.9	1.1	47.8	2.2	182.0
MH330220.1 *Culex tritaeniorhynchus*	39.4	15.3	29.7	15.5	548.0	24.0	17.5	29.5	29.0	183.0	45.4	27.3	11.5	15.8	183.0	48.9	1.1	48.4	1.6	182.0
KU578141.1 *Coccinella transversalis*	37.6	18.6	28.3	15.5	548.0	24.6	16.4	33.9	25.1	183.0	45.9	25.7	12.0	16.4	183.0	42.3	13.7	39.0	4.9	182.0
JF835944.1 *Nephila inaurata*	41.6	12.2	28.5	17.7	548.0	32.8	10.9	25.7	30.6	183.0	47.5	24.6	12.6	15.3	183.0	44.5	1.1	47.3	7.1	182.0
MN125137.1 *Pyganodon grandis*	40.1	15.1	20.3	24.5	548.0	29.0	15.3	24.6	31.1	183.0	46.4	21.9	12.6	19.1	183.0	45.1	8.2	23.6	23.1	182.0
Avg.	38.9	15.8	28.5	16.8	548.0	25.3	16.0	29.4	29.3	183.0	45.6	26.7	11.7	16.0	183.0	46.0	4.7	44.3	4.9	182.0

Table 2.7d: The evolutionary divergence percentage between *Anopheles vagus* and its closely related species

Sl. No.	Accession Number	Organism	Percentage of Divergence
1	MK628547.1	*Anopheles vagus*	0.00%
2	MT434323.1	*Anopheles vagus*	1.48%
3	MF179260.1	*Anopheles vagus*	1.48%
4	KF406660.1	*Anopheles culicifacies*	17.37%
5	KF406658.1	*Anopheles culicifacies*	17.13%
6	MT434331.1	*Anopheles varuna*	14.80%
7	MT434330.1	*Anopheles varuna*	15.31%
8	MT519730.1	*Aedes vittatus*	18.67%
9	MT519729.1	*Aedes vittatus*	18.67%
10	MH745093.1	*Culex tritaeniorhynchus*	18.45%
11	MH330220.1	*Culex tritaeniorhynchus*	18.49%
12	KU578141.1	*Coccinella transversalis*	31.81%
13	JF835944.1	*Nephila inaurata*	38.25%
14	MN125137.1	*Pyganodon grandis*	68.92%

2.3.6.2 Discussion

An. vagus is widely distributed in the Oriental region and is a potential vector for various pathogens, including malaria. It is a medium-sized Anopheline species with spotted wings and dark legs. Senjarini et al., 2021 identified and constructed a phylogenetic tree of *An. vagus* complex using based on their ITS2 sequencing. BLAST result showed that *An. vagus vagus* and *An. vagus* limosus were similar to *An. vagus* FJ654649.1 from East Java Indonesia and East Timor based on its 99% homology and molecular distance. This subspeciation might be due to the different rates of evolution. ITS2 sequences of *An. vagus vagus* and *An. vagus limosus* were submitted to GenBank with the accession numbers MW314227.1 and MW319822.1, respectively. COI gene PCR amplified of *An. vagus,* collected from the Kole wetlands of Thrissur, Kerala, was carried out, and a single product of 678bp sequence was obtained. The sequence has been deposited in the NCBI GenBank

with Accession No. MK628547.1. In 2016 Murugan et al., discussed the phylogenetic analysis result of some mosquitoes. In their result, the phylogenetic tree displays the relationship between *An. vagus* and *An. stephensi* as a neighbouring group sharing a common clade. In the present study, close relatives of *An. vagus* were *An. culicifascies* and *An. varuna.*

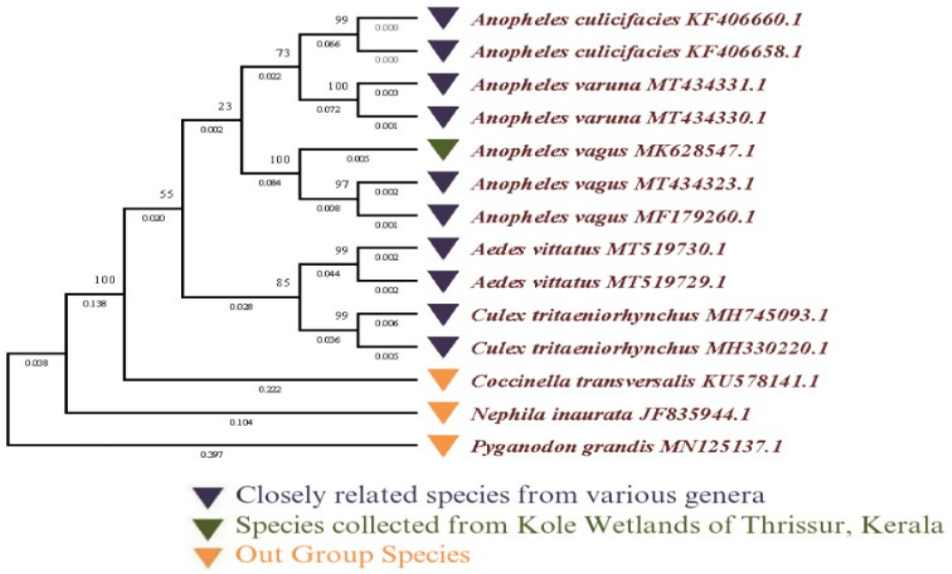

Figure 2.6c Phylogenetic tree of *Anopheles vagus*

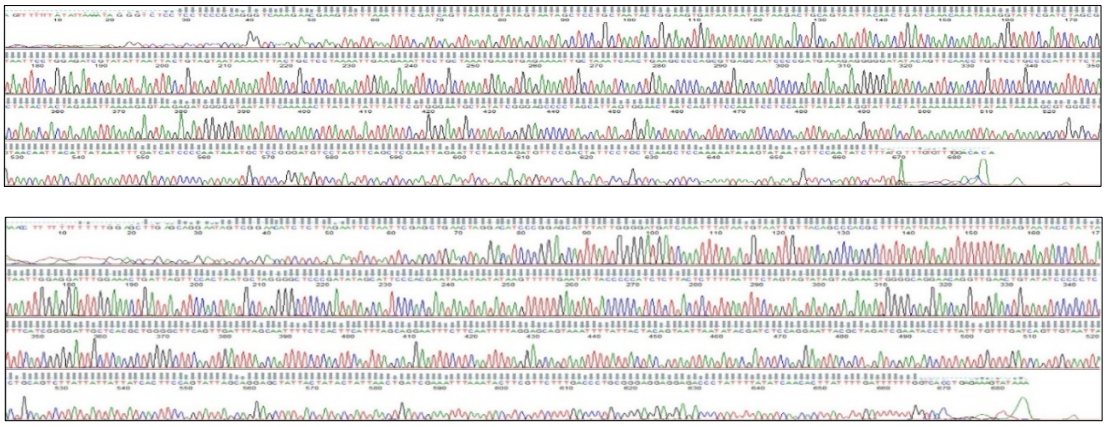

Figure 2.6d Electrophorogram showing the nucleotide sequence of mitochondrial COI gene of *Anopheles vagus*

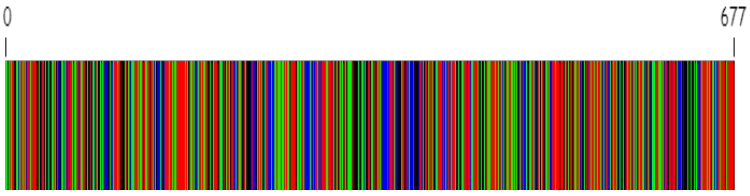

Figure 2.6e Molecular barcode of *Anopheles vagus*

2.3.7. Species Name: *Aedes aegypti*

GenBank Accession Number: MK542379.1

Voucher Number: CDRL12

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Culicini
Genus	:	*Aedes*
Subgenus	:	*Stegomyia*
Species	:	*Aedes aegypti*

Description

One of the most common tropical vector species which can transmit many arboviruses like dengue, zika, etc. The entire body and legs are covered with white and black scales. The pale scale arose on the central vertical area and extended through amongst eyes is the prominent identifying feature of these mosquitoes. They have usually collected from rainwater holding pots, containers, leaf axils, leaf internodes, etc.

2.3.7.1 Result

The forward and reverse primers were used to amplify mitochondrial cytochrome oxidase subunit I gene of *Ae. aegypti,* collected from Kole wetlands of Thrissur, Kerala, were 5'-GGTCAACAAATCATAAAGATATTGG-3' and 5'TAAACTTCAG GGTGACCAAAAAATCA-3' respectively. PCR amplified a mitochondrial cytochrome oxidase subunit I (COI) gene fragment from *Ae. aegypti*, and generated a single product with a length of 679 bp. Accession No. MK542379.1 was obtained from the NCBI GenBank, and Figures 2.7a- 2.7e displayed the DNA sequence, corresponding translation product, phylogenetic tree, electrophorogram, and molecular barcode.

> MK542379.1 *Aedes aegypti*|679bp

```
AAAGATATTGGAACTTTATATTTCATTTTTGGAGTATGATCCGGAATAGTCGGAACTTCTCTAAGAATTTTAA
TTCGTGCTGAACTTAGCCACCCTGGTATATTTATTGGGAATGACCAAATTTATAATGTAATTGTAACAGCTCA
TGCATTTATTATAATTTTCTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAATTGATTAGTTCCTTTA
ATATTAGGAGCCCCTGATATAGCCTTTCCTCGAATAAATAATATAAGTTTTTGAATACTACCTCCTTCATTGA
CTCTTCTATTATCAAGCTCAATAGTAGAAAATGGGGCAGGAACTGGGTGAACAGTTTATCCTCCTCTCTCTTC
AGGAACAGCTCATGCTGGAGCTTCTGTTGATTTAGCTATTTTTTCTCTTCATTTAGCTGGAATTTCCTCAATT
TTAGGGGCAGTAAATTTTATTACAACTGTAATTAATATACGATCGTCAGGAATTACTTTAGATCGACTACCTT
TATTTGTTTGATCTGTAGTTATTACAGCTATCTTATTACTTCTTTCTCTTCCTGTTTTAGCTGGAGCTATTAC
TATGTTATTAACAGACCGAAACTTAAATACATCTTTCTTTGATCCAATCGGAGGAGGAGACCCTATTTTATAC
CAACACTTATTCTGATTTTTTG
```

Figure 2.7a: The DNA sequence of *Aedes aegypti* COI gene

> MK542379.1 *Aedes aegypti*

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MGTLYFIFGVWSGMVGTSLSILIRAELSHPGMFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLM
LGAPDMAFPRMNNMSFWMLPPSLTLLLSSSMVENGAGTGWTVYPPLSSGTAHAGASVDLAIFSLHLAGISSI
LGAVNFITTVINMRSSGITLDRLPLFVWSVVITAILLLLSLPVLAGAITMLLTDRNLNTSFFDPIGGGDPIL
YQHLFWFF
```

Figure 2.7b: The protein sequence of *Aedes aegypti* COI gene

The BLAST search revealed the partial COI nucleotide sequence of *Ae. aegypti* isolated from Thrissur Kole lands, Kerala, is 100% similar to *Ae. aegypti* of isolated from Peru (MN299016.1) and England (MF043259.1). *Ae. aegypti* isolated from Thrissur Kole lands, Kerala showed 9.74% evolutionary divergence with *Ae. vexans* isolated from the USA (KP954638.1) and 11.06% evolutionary divergence

with *Ae. lineatopennis* isolated from Thailand (HQ398909.1). It showed a 15.51% divergence with *Ma. uniformis* isolated from Japan (LC473705.1) (Table.2.8d).

The average nucleotide composition throughout the species was T=40.1%; C=16.0%; A=28.0%; G=15.8% (Table.2.8c). The estimate of the Substitution Matrix showed the probability of substitution from one base to another base (Table.2.8a). The Tamura-Nei (1993) model was used to estimate substitution patterns and rates. Different transitional and transversionsal substitutions rates were shown in Table 2.8b. A = 25.00%, T/U = 25.00%, C = 25.00%, and G = 25.00% were the nucleotide frequencies estimated in transition transversion bias analysis. Tree topology was automatically calculated for the estimation of ML values. The maximum Log-likelihood for this evaluation was -3048.324. The fourteen nucleotide sequences used in this investigation have been organized into the 1st+2nd+3rd+Noncoding codon positions. The final dataset comprised a total of 556 positions. All these evolutionary analyses were conducted in MEGA X.

The phylogeny tree was generated using the NJ method. Phylogenetic tree showing the phylogenetic position of *Ae. aegypti* isolated from Thrissur Kole lands, Kerala. The phylogenetic tree revealed the closest relatives of *Ae. aegypti,* collected from Thrissur Kole lands, Kerala, and *Ae. aegypti* isolated from England (MF043259.1). The closest relatives of *Ae. aegypti* collected from different populations were arranged in a single clade.

Table 2.8a: The nucleotide substitution matrix estimate of COI gene sequence of *Aedes aegypti*

From\To	A	T	C	G
A	-	7.4966	7.4966	10.0067
T	7.4966	-	10.0067	7.4966
C	7.4966	10.0067	-	7.4966
G	10.0067	7.4966	7.4966	-

Table 2.8b: The Transition/Transversion bias estimate of mitochondrial COI gene sequence of *Aedes aegypti*

From\To	A	T	C	G
A	-	11.1748	4.4599	8.3459
T	7.8137	-	6.0330	4.4098
C	7.8137	15.1164	-	4.4098
G	14.7883	11.1748	4.4599	-

Table 2.8c: The nucleotide frequency comparison of *Aedes aegypti* COI gene sequence with its kin species

Accession Number and Name of the Species	**Nucleotide Frequencies (%)**																		
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MK542379.1 *Aedes aegypti*	39.4	17.6	27.7	15.3	556.0	43.5	28.5	13.4	14.5	186.0	48.6	7.0	40.5	3.8	185.0	25.9	17.3	29.2	27.6	185.0
MN299016.1 *Aedes aegypti*	39.4	17.6	27.7	15.3	556.0	43.5	28.5	13.4	14.5	186.0	48.6	7.0	40.5	3.8	185.0	25.9	17.3	29.2	27.6	185.0
MF043259.1 *Aedes aegypti*	39.4	17.6	27.7	15.3	556.0	43.5	28.5	13.4	14.5	186.0	48.6	7.0	40.5	3.8	185.0	25.9	17.3	29.2	27.6	185.0
KP954638.1 *Aedes vexans*	40.6	16.0	28.8	14.6	556.0	43.5	28.5	13.4	14.5	186.0	50.8	3.8	43.8	1.6	185.0	27.6	15.7	29.2	27.6	185.0
MK402823.1 *Aedes vexans*	39.9	16.0	29.3	14.7	556.0	43.5	28.5	13.4	14.5	186.0	48.6	3.8	45.4	2.2	185.0	27.6	15.7	29.2	27.6	185.0
AB738145.1 *Aedes lineatopennis*	40.3	16.2	28.8	14.7	556.0	43.5	28.0	13.4	15.1	186.0	51.4	3.8	43.8	1.1	185.0	25.9	16.8	29.2	28.1	185.0
HQ398909.1 *Aedes lineatopennis*	39.9	16.7	28.6	14.7	556.0	43.5	28.0	13.4	15.1	186.0	50.3	5.4	43.2	1.1	185.0	25.9	16.8	29.2	28.1	185.0
LC473705.1 *Mansonia uniformis*	40.1	14.9	30.0	14.9	556.0	44.1	27.4	14.0	14.5	186.0	49.2	2.7	45.9	2.2	185.0	27.0	14.6	30.3	28.1	185.0
LC517293.1 *Mansonia uniformis*	40.1	14.9	30.0	14.9	556.0	44.1	27.4	14.0	14.5	186.0	49.2	2.7	45.9	2.2	185.0	27.0	14.6	30.3	28.1	185.0
MH745093.1 *Culex tritaeniorhynchus*	38.8	16.2	29.7	15.3	556.0	43.5	28.0	13.4	15.1	186.0	48.6	2.2	47.0	2.2	185.0	24.3	18.4	28.6	28.6	185.0
MH330220.1 *Culex tritaeniorhynchus*	38.7	16.4	29.9	15.1	556.0	43.5	28.0	13.4	15.1	186.0	48.6	2.2	47.6	1.6	185.0	23.8	18.9	28.6	28.6	185.0
KY694466.1 *Afidenta misera*	38.1	18.7	28.1	15.1	556.0	44.1	26.3	14.0	15.6	186.0	45.4	13.0	36.8	4.9	185.0	24.9	16.8	33.5	24.9	185.0
KC849092.1 *Nephila sumptuosa*	42.0	12.6	27.9	17.5	555.0	45.7	25.3	14.5	14.5	186.0	46.7	0.5	43.5	9.2	184.0	33.5	11.9	25.9	28.6	185.0
EF033298.1 *Lampsilis hydiana*	44.8	12.6	18.5	24.1	556.0	44.6	20.4	15.1	19.9	186.0	55.7	4.9	15.1	24.3	185.0	34.1	12.4	25.4	28.1	185.0
Avg.	40.1	16.0	28.0	15.8	555.9	43.9	27.2	13.7	15.1	186.0	49.3	4.7	41.4	4.6	184.9	27.1	16.0	29.1	27.8	185.0

Table 2.8d: The evolutionary divergence percentage between *Aedes aegypti* and its closely related species

Sl. No.	Accession Number	Organism	Percentage of Divergence
1	MK542379.1	*Aedes aegypti*	0.00%
2	MN299016.1	*Aedes aegypti*	0.00%
3	MF043259.1	*Aedes aegypti*	0.00%
4	KP954638.1	*Aedes vexans*	9.74%
5	MK402823.1	*Aedes vexans*	10.39%
6	AB738145.1	*Aedes lineatopennis*	10.83%
7	HQ398909.1	*Aedes lineatopennis*	11.06%
8	LC473705.1	*Mansonia uniformis*	15.51%
9	LC517293.1	*Mansonia uniformis*	15.51%
10	MH745093.1	*Culex tritaeniorhynchus*	13.85%
11	MH330220.1	*Culex tritaeniorhynchus*	13.84%
12	KY694466.1	*Afidenta misera*	28.68%
13	KC849092.1	*Nephila sumptuosa*	31.07%
14	EF033298.1	*Lampsilis hydiana*	58.18%

2.3.7.2 Discussion

Aedes aegypti, commonly called yellow fever mosquito. Black-coloured medium to a small-sized mosquito with peculiar black and white ornamentation on the body and legs. It is a principal vector of Dengue fever and a potential vector of various arboviruses like chikungunya, yellow fever, Zika etc. Many identification and management studies have been done on this mosquito species. Accession number MK542379.1 was obtained from NCBI GenBank for the COI sequence of *Ae. aegypti* isolated from the Kole wetlands of Thrissur, Kerala. The COI sequence of *Ae. aegypti* showed significant diversity compared to the other species analysed in this study; therefore, it can be used as a DNA barcode for accurate identification. In the phylogenetic tree, the related species of *Ae. aegypti* were aligned as the nearest relative in the same clade. In the evolutionary process, some other species are there to connect the *Ae. vexans* and that found in another clade.

Makanda et al., 2019 employed PCR amplification and sequencing of COI gene mosquito species to identify and characterise mosquito species in Kenya's Nairobi, Kisumu, and Kilifi Countries. 14 haplotypes of *Aedes* mosquitoes were identified from the study area. Some Accession numbers of *Ae. aegypti* from the study site were MK300226, MK300227, MK300228, MK300229. COII gene sequencing is also used in *Ae. aegypti's* species identification and phylogenetic analysis. A ~500 to 550bp sequence of mitochondrial Cytochrome Oxidase II (COII) gene has been analysed to construct a molecular database and to establish the phylogenetic relationships among the five Culicinae mosquito species, including *Ae. aegypti* from the state of Punjab (India) and its adjoining areas (Singh et al., 2017). An earlier report of COI gene sequencing *of Ae. aegypti* was found in 2013. Accession number obtained for *Ae. aegypti* from NCBI was FJ372982 (Paramasivan et al., 2013).

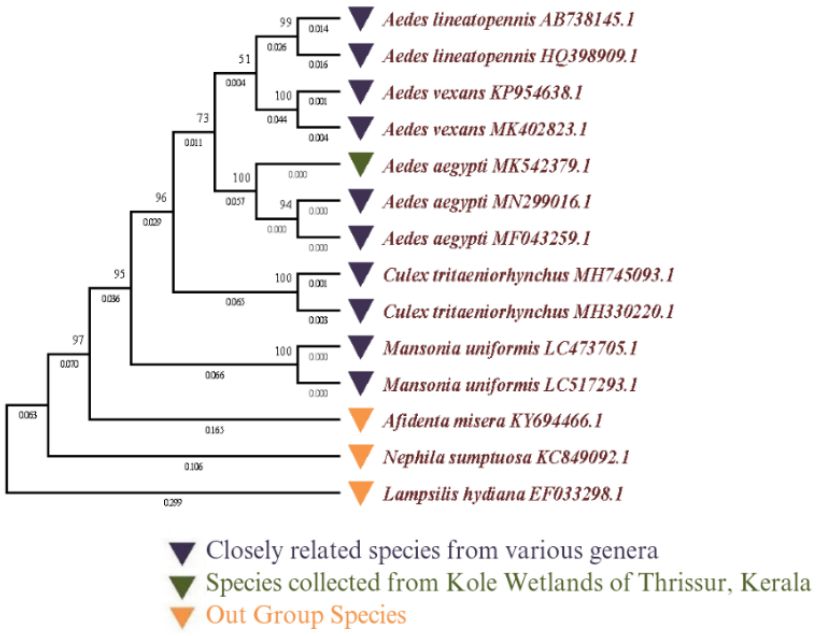

Figure 2.7c Phylogenetic tree of *Aedes aegypti*

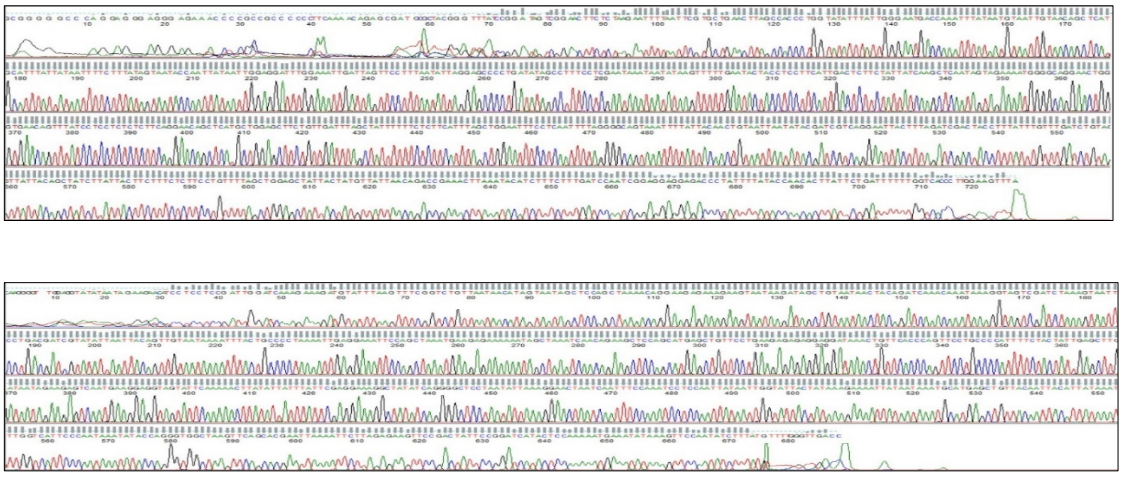

Figure 2.7d Electrophorogram showing the nucleotide sequence of mitochondrial CO I gene of *Aedes aegypti*

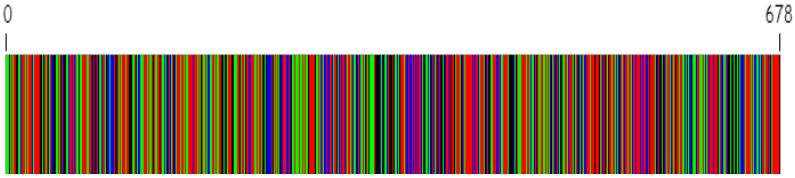

Figure 2.7e Molecular barcode of *Aedes aegypti*

2.3.8. Species Name: *Aedes albopictus*

GenBank Accession Number: MK542378.1

Voucher Number: CDRL13

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Culicini
Genus	:	*Aedes*
Subgenus	:	*Stegomyia*
Species	:	*Aedes albopictus*

Description

Ae. albopictus, commonly known as Asian tiger mosquito. Carriers of many arboviral diseases have a significant role in public health, and their breeding grounds are water bodies containing fresh water. The chief identifying character of this mosquito is a white patch of scales as a straight line at the middle of the mesonotum from top to bottom.

2.3.8.1 Result

The forward and reverse primers were used to amplify the mitochondrial cytochrome oxidase subunit I gene of *Ae. albopictus,* collected from Kole wetlands of Thrissur, Kerala, were 5'-GGTCAACAAATCATAAAGATATTGG-3' and 5'TAAACTTCAG GGTGACCAAAAAATCA-3' respectively. A single product with a length of 678 bp was produced by PCR amplifying *Ae. albopictus'* mitochondrial cytochrome oxidase subunit I (COI) gene fragment. Accession No. MK542378.1 was obtained from the NCBI GenBank, and Figures 2.8a- 2.8e displayed the DNA sequence, corresponding translation product, phylogenetic tree, electrophorogram, and molecular barcode.

> MK542378.1 *Aedes albopictus*|678bp

AAGATATTGGAACATTATACTTTATTTTCGGTATTTGATCTGGAATAGTCGGAACTTCACTAAGAGTTTTA
ATTCGTATTGAACTTAGACATCCTGGTATATTTATTGGAAATGATCAAATTTATAATGTAATTGTTACTGC
TCATGCTTTTATTATAATTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGAAACTGACTAGTAC
CCTTAATACTAGGAGCCCCTGATATAGCTTTTCCTCGAATAAATAATATAAGTTTTTGAATATTACCCCCC
TCTTTAACACTGCTGCTTTCTAGTTCTATAGTAGAAAACGGAGCTGGAACAGGGTGAACGGTTTATCCTCC
CCTTTCTTCTGGAACAGCTCATGCTGGGGCTTCAGTTGATTTAGCAATTTTTTCTTTACATTTAGCGGGAA
TCTCATCTATTTTAGGAGCAGTAAATTTTATTACAACTGTAATTAATATACGATCAGCTGGTATTACTCTT
GATCGACTACCTTTATTTGTATGATCAGTAGTAATTACAGCTATTTTATTACTTCTTTCTCTACCCGTATT
AGCCGGAGCTATTACTATATTATTAACAGACCGAAATTTAAATACATCTTTTTTTGATCCAATTGGAGGGG
GAGACCCTATTTTATATCAACATTTATTTTGATTTTTTG

Figure 2.8a: The DNA sequence of *Aedes albopictus* COI gene

> MK542378.1 *Aedes albopictus*

MGTLYFIFGIWSGMVGTSLSVLIRIELSHPGMFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLM
LGAPDMAFPRMNNMSFWMLPPSLTLLLSSSMVENGAGTGWTVYPPLSSGTAHAGASVDLAIFSLHLAGISSI
LGAVNFITTVINMRSAGITLDRLPLFVWSVVITAILLLLSLPVLAGAITMLLTDRNLNTSFFDPIGGGDPIL
YQHLFWFF

Figure 2.8b: The protein sequence of *Aedes albopictus* COI gene

The nucleotide composition of the COI sequence of *Ae. albopictus* showed bias to nucleotide AT was T=38.2%, C=17.5%, A=28.3%, and G=16.0% and T=38.7%, C=16.5%, A=28.1% and G=16.6% were the average value of nucleotide composition of 14 nucleotides (Table 2.9c). The number of base substitutions per position between nucleotide sequences is indicated in Table 2.9a using the Maximum Composite Likelihood model. The substitution model and rates were estimated under the Tamura-Nei (1993) model. The rates of different transitional substitutions and those of transversionsal substitutions were shown in Table.2.9b. A = 25.00%, T/U = 25.00%, C = 25.00%, and G = 25.00% were the nucleotide frequencies estimated in transition transversion bias analysis. Tree topology was automatically calculated for the estimation of ML values. The maximum Log-likelihood for this computation was -3195.995. The fourteen nucleotide sequences used in this investigation have been organized into the 1st+2nd+3rd+Noncoding codon positions. A total of 576 positions were present in the final dataset. All these evolutionary analyses were conducted in MEGA X. The data in Table 2.9d revealed that the *Ae. albopictus* shows 0.12% evolutionary divergence with *Ae. albopictus* (MW542315.1) isolated from Wayanad, Kerala, and (MT890465.1) from China. *Ae. albopictus* species showed 11.21% of evolutionary divergence, with *Ae. cinereus* isolated from the UK.

The phylogenetic tree generated using the NJ method shows the phylogenetic position of *Aedes albopictus* isolated from the Kole wetlands of Thrissur, Kerala. Phylogenetically, *Aedes albopictus* (MW542315.1) isolated from Wayanad, Kerala, and (MT890465.1) from China showed to be the closest relatives of *Aedes albopictus* (MK542378) of Kerala.

Table 2.9a: The nucleotide substitution matrix estimate of COI gene sequence of *Aedes albopictus*

From\To	A	T	C	G
A	-	10.0800	4.3025	8.1804
T	7.3139	-	7.7632	4.3315
C	7.3139	18.1880	-	4.3315
G	13.8128	10.0800	4.3025	-

Table 2.9b: The Transition/Transversion bias estimate of mitochondrial COI gene sequence of *Aedes albopictus*

From\To	A	T	C	G
A	-	6.9555	6.9555	11.0890
T	6.9555	-	11.0890	6.9555
C	6.9555	11.0890	-	6.9555
G	11.0890	6.9555	6.9555	-

Table 2.9c: The nucleotide frequency comparison of *Aedes albopictus* COI gene sequence with its kin species

Accession Number and Name of the Species	**Nucleotide Frequencies (%)**																		
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MK542378.1 *Aedes albopictus*	38.2	17.5	28.3	16.0	576.0	43.2	27.6	13.5	15.6	192.0	47.9	6.8	41.7	3.6	192.0	23.4	18.2	29.7	28.6	192.0
MW542315.1 *Aedes albopictus*	38.2	17.5	28.1	16.1	576.0	43.2	27.6	13.5	15.6	192.0	47.9	6.8	41.1	4.2	192.0	23.4	18.2	29.7	28.6	192.0
MT890465.1 *Aedes albopictus*	38.2	17.5	28.1	16.1	576.0	43.2	27.6	13.5	15.6	192.0	47.9	6.8	41.1	4.2	192.0	23.4	18.2	29.7	28.6	192.0
MK403296.1 *Aedes cinereus*	39.2	16.8	27.6	16.3	576.0	42.7	27.6	13.5	16.1	192.0	48.4	7.8	39.6	4.2	192.0	26.6	15.1	29.7	28.6	192.0
MK403230.1 *Aedes cinereus*	39.4	16.7	28.1	15.8	576.0	42.7	27.6	13.5	16.1	192.0	49.0	7.3	41.1	2.6	192.0	26.6	15.1	29.7	28.6	192.0
KU495081.1 *Aedes aegypti*	38.4	17.7	28.0	16.0	576.0	42.7	28.1	13.5	15.6	192.0	47.4	7.8	41.1	3.6	192.0	25.0	17.2	29.2	28.6	192.0
MF443395.1 *Aedes aegypti*	38.5	17.5	28.0	16.0	576.0	42.7	28.1	13.5	15.6	192.0	47.9	7.3	41.1	3.6	192.0	25.0	17.2	29.2	28.6	192.0
LC473705.1 *Mansonia uniformis*	39.4	14.8	30.2	15.6	576.0	43.2	27.1	14.1	15.6	192.0	49.0	2.6	46.4	2.1	192.0	26.0	14.6	30.2	29.2	192.0
LC517293.1 *Mansonia uniformis*	39.4	14.8	30.2	15.6	576.0	43.2	27.1	14.1	15.6	192.0	49.0	2.6	46.4	2.1	192.0	26.0	14.6	30.2	29.2	192.0
MH745093.1*Culex tritaeniorhynchus*	37.8	16.1	30.0	16.0	576.0	42.7	27.6	13.5	16.1	192.0	47.4	2.6	47.9	2.1	192.0	23.4	18.2	28.6	29.7	192.0
MH330220.1 *Culex tritaeniorhynchus*	37.7	16.3	30.2	15.8	576.0	42.7	27.6	13.5	16.1	192.0	47.4	2.6	48.4	1.6	192.0	22.9	18.8	28.6	29.7	192.0
KU578141.1 *Coccinella transversalis*	36.6	19.1	28.3	16.0	576.0	42.7	26.6	14.1	16.7	192.0	43.2	14.1	38.0	4.7	192.0	24.0	16.7	32.8	26.6	192.0
JF835944.1 *Nephila inaurata*	42.0	12.8	27.4	17.7	576.0	44.3	25.5	14.6	15.6	192.0	49.0	1.0	43.2	6.8	192.0	32.8	12.0	24.5	30.7	192.0
MN125137.1 *Pyganodon grandis*	39.1	16.1	20.8	24.0	576.0	42.7	22.9	14.6	19.8	192.0	46.4	9.9	24.0	19.8	192.0	28.1	15.6	24.0	32.3	192.0
Avg.	38.7	16.5	28.1	16.6	576.0	43.0	27.0	13.8	16.1	192.0	47.7	6.1	41.5	4.7	192.0	25.5	16.4	29.0	29.1	192.0

Table 2.9d: The evolutionary divergence percentage between *Aedes albopictus* and its closely related species

Sl. No.	Accession Number	Organism	Percentage of Divergence
1	MK542378.1	*Aedes albopictus*	0.00%
2	MW542315.1	*Aedes albopictus*	0.12%
3	MT890465.1	*Aedes albopictus*	0.12%
4	MK403296.1	*Aedes cinereus*	11.36%
5	MK403230.1	*Aedes cinereus*	11.21%
6	KU495081.1	*Aedes aegypti*	9.89%
7	MF443395.1	*Aedes aegypti*	10.05%
8	LC473705.1	*Mansonia uniformis*	13.87%
9	LC517293.1	*Mansonia uniformis*	13.87%
10	MH745093.1	*Culex tritaeniorhynchus*	11.55%
11	MH330220.1	*Culex tritaeniorhynchus*	11.22%
12	KU578141.1	*Coccinella transversalis*	22.42%
13	JF835944.1	*Nephila inaurata*	24.34%
14	MN125137.1	*Pyganodon grandis*	37.52%

2.3.8.2 Discussion

Aedes albopictus is one of the most offensive mosquitoes in the world that harbours and can transmit many arboviruses, most notably dengue and chikungunya virus (Ismail et al., 2016). *Ae. albopictus* is known as the "Asian tiger mosquito" about its geographical origin in the edge forest of Southeast Asia. It is preferentially found outdoors and has been considered a rural vector (Paupy et al., 2009). The sample were efficaciously sequenced utilising the forward and reverse primers to get sequences of 678bp. NCBI BLAST tool revealed the COI sequences of *Ae. albopictus* from Thrissur Kole lands of Kerala is closely related to *Ae. albopictus* isolated from various geographical areas like Malaysia and China. So, it could be interpreted that the resultant sequence obtained for the *Ae. albopictus* from Kole wetlands of Thrissur, Kerala, is novel. The sequence obtained in this study is used for phylogenetic analysis, revealing the evolutionary relationship of

various *Aedes* species. The partial coding sequence of COI proved a powerful tool for identifying organisms (Hebert et al., 2004). This investigation's partial mitochondrial DNA COI sequence showed significant variance with other species. Recent studies show insect interspecific genetic divergence is more significant than intraspecific. Taxonomic ambiguities have prevented a definitive species identification using morphological keys. Identifying these species was tedious without the help of a taxonomic specialist (Jung et al., 2011).

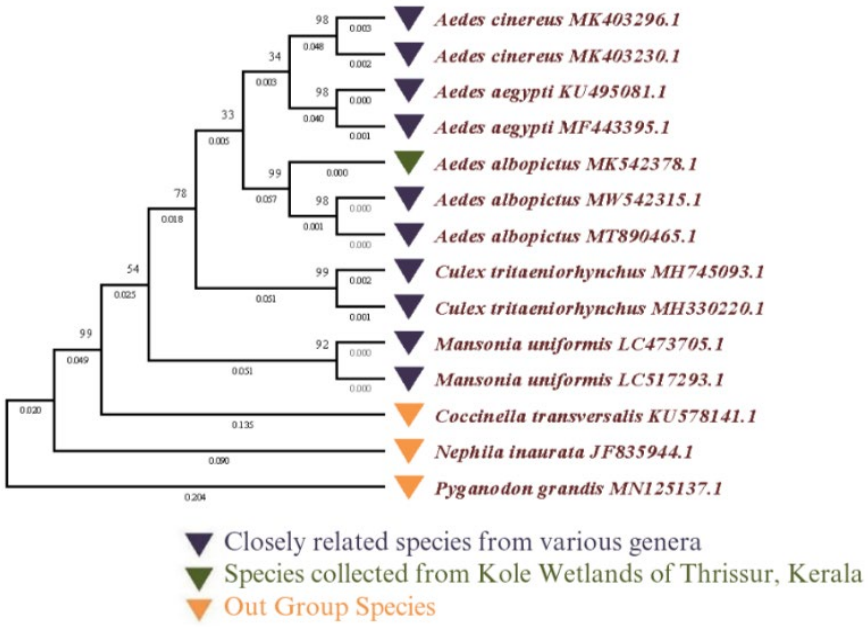

Figure 2.8c Phylogenetic tree of *Aedes albopictus*

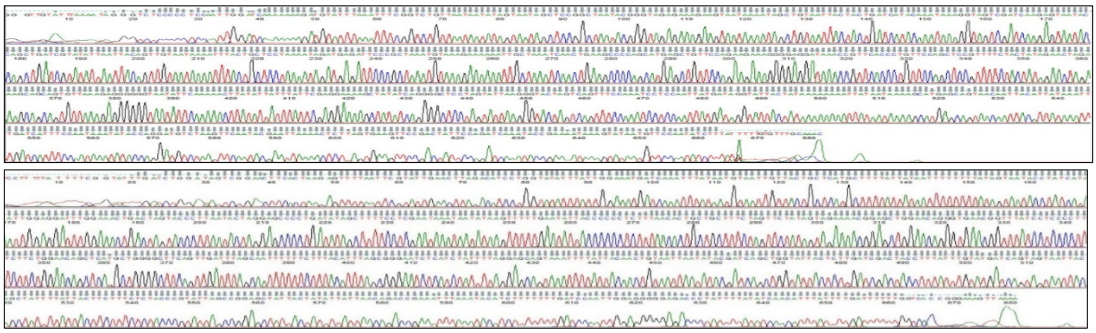

Figure 2.8d Electrophorogram showing the nucleotide sequence of mitochondrial COI gene of *Aedes albopictus*

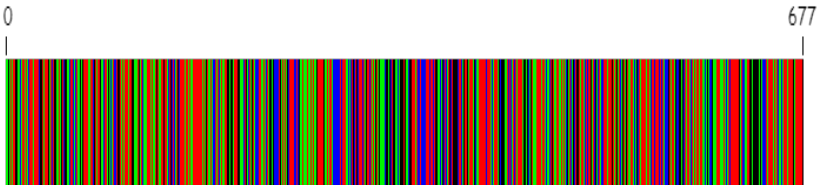

Figure 2.8e Molecular barcode of *Aedes abopictus*

2.3.9. Species Name: *Aedes vittatus*

GenBank Accession Number: MK491498.1

Voucher Number: CDRL31

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Culicini
Genus	:	*Aedes*
Subgenus	:	*Stegomyia*
Species	:	*Aedes vittatus*

Description

Black-coloured mosquito with specific ornamentation. Six distinct white spots on the mesonotum and several white patches on different parts of the legs are present. *Ae. vittatus* mosquitoes are usually collected from rainwater occupying habitats such as tyres, tanks, containers, tree holes, leaf axils, etc.

2.3.9.1 Result

The forward and reverse primers were used to amplify mitochondrial cytochrome oxidase subunit I gene of *Ae. vittatus,* collected from Kole wetlands of Thrissur, Kerala, were 5'-GGTCAACAAATCATAAAGATATTGG-3' and 5'TAAACTTCAG

GGTGACCAAAAAATCA-3' respectively. A single product with a length of 679 bp was produced by PCR amplifying *Ae. vittatus'* mitochondrial cytochrome oxidase subunit I (COI) gene fragment. Accession No. MK491498.1 was obtained from the NCBI GenBank, and Figures 2.9a- 2.9e displayed the DNA sequence, corresponding translation product, phylogenetic tree, electrophorogram, and molecular barcode.

> MK491498.1 *Aedes vittatus*|679bp

```
TAAAGATATTGGAACATTATATTTTATTTTTGGAGTTTGATCAGGAATAGTTGGAACTTCATTAAGTATATTA
ATTCGTGCTGAACTTAGTCATCCTGGGATATTTATTGGAAATGACCAAATTTATAATGTTATTGTAACAGCTC
ATGCATTTATTATAATTTTCTTTATAGTAATACCAATTATAATTGGTGGATTTGGAAATTGATTAGTTCCTTT
AATATTAGGAGCTCCTGATATAGCTTTCCCTCGAATAAATAATATAAGTTTTTGAATATTACCTCCTTCATTA
ACACTACTACTTTCTAGTTCTATAGTAGAAAACGGAGCAGGAACAGGTTGAACAGTTTATCCTCCTCTATCTT
CTGGGACTGCTCATGCTGGAGCATCAGTTGATTTAGCTATTTTTTCTCTTCATTTAGCAGGGATTTCTTCAAT
TTTAGGGGCAGTAAATTTTATTACTACTGTAATTAATATACGATCAGCAGGAATTACTTTAGATCGTTTACCT
TTATTTGTTTGATCTGTTGTAATTACAGCTATTCTATTACTTTTATCATTACCAGTATTAGCAGGGGCTATTA
CTATATTATTAACAGATCGAAATTTAAATACTTCATTCTTCGACCCAATTGGAGGAGGAGATCCTATTCTTTA
TCAACATTTATTTTGATTTTTG
```

Figure 2.9a: The DNA sequence of *Aedes vittatus* COI gene

> MK491498.1 *Aedes vittatus*

```
MGTLYFIFGVWSGMVGTSLSMLIRAELSHPGMFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLM
LGAPDMAFPRMNNMSFWMLPPSLTLLLSSSMVENGAGTGWTVYPPLSSGTAHAGASVDLAIFSLHLAGISSI
LGAVNFITTVINMRSAGITLDRLPLFVWSVVITAILLLLSLPVLAGAITMLLTDRNLNTSFFDPIGGGDPIL
YQHLFWFL
```

Figure 2.9b: The protein sequence of *Aedes vittatus* COI gene

The COI nucleotide sequence analysis revealed the composition of nucleotides in the COI gene of *Ae. vittatus* isolated from Thrissur Kole lands Kerala (Table 2.10c). The

COI sequence of *Ae. vittatus* showed bias to nucleotide AT, and estimated nucleotide composition was T=39.9%, C=16.0%, A=29.4%, and G=14.7%. The COI nucleotide composition analysis showed no variation in the composition of each nucleotide of *Ae. vittatus* isolated from Thrissur Kole lands Kerala with another *Ae. vittatus* (MH330197.1) species isolated from Sri Lanka. From the data given in

Table 2.10d, the *Ae. vittatus* showed 0% evolutionary divergence with *Ae. vittatus (MH330*197.1) isolated from Sri Lanka and 6.96% with *Ae. lineatopennis* (AB738145.1) from Japan. *Ae. vexans* (KP954638.1) isolated from the USA showed 9.62% evolutionary divergence with *Ae. vittatus* isolated from Kerala. The estimated value of Transition/Transversion bias (R) is 0.63. Kimura (1980) 2-parameter model was used for this substitution patterns, and rates were estimated (Table. 2.10b). The Maximum Composite Likelihood model was used to analyse the substitution matrix value, and the number of base substitutions in each position between sequences is displayed in Table 2.10a. The final dataset has 524 positions after all positions with gaps and missing data have been removed.

The phylogeny tree generated using the NJ method shows the phylogenetic position of *Aedes vittatus* isolated from Kerala. Phylogenetically *Ae. vittatus* species (MH330197.1) isolated from Sri Lanka showed to be the closest relatives of *Ae. vittatus* of Kerala, and they were in the same clade. *Ae. lineatopennis* (AB738145.1), isolated from Japan, and *Ae. vexans* (KP954638.1), isolated from the USA, are the other nearest relatives of *Ae. vittatus*, isolated from Kerala. They arose from a node and were divided into two sub-branches separately.

Table 2.10a: The nucleotide substitution matrix estimate of COI gene sequence of *Aedes vittatus*

From\To	A	T	C	G
A	-	11.5309	4.4296	8.2265
T	8.0770	-	5.4798	4.3638
C	8.0770	14.2646	-	4.3638
G	15.2266	11.5309	4.4296	-

Table 2.10b: The Transition/Transversion bias estimate of mitochondrial COI gene sequence of *Aedes vittatus*

From\To	A	T	C	G
A	-	7.6806	7.6806	9.6388
T	7.6806	-	9.6388	7.6806
C	7.6806	9.6388	-	7.6806
G	9.6388	7.6806	7.6806	-

Table 2.10c: The nucleotide frequency comparison of *Aedes vittatus* COI gene sequence with its kin species

Accession Number and Name of the Species	Nucleotide Frequencies (%)																			
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MK491498.1 *Aedes vittatus*	39.9	16.0	29.4	14.7	524.0	27.4	14.9	29.7	28.0	175.0	44.0	29.1	13.1	13.7	175.0	48.3	4.0	45.4	2.3	174.0
MH330198.1 *Aedes vittatus*	39.9	16.0	29.8	14.3	524.0	27.4	14.9	29.7	28.0	175.0	44.0	29.1	13.1	13.7	175.0	48.3	4.0	46.6	1.1	174.0
MH330197.1 *Aedes vittatus*	39.9	16.0	29.4	14.7	524.0	27.4	14.9	29.7	28.0	175.0	44.0	29.1	13.1	13.7	175.0	48.3	4.0	45.4	2.3	174.0
AB738145.1 *Aedes lineatopennis*	40.6	16.2	28.6	14.5	524.0	26.3	16.0	29.7	28.0	175.0	44.0	28.6	13.1	14.3	175.0	51.7	4.0	43.1	1.1	174.0
HQ398909.1 *Aedes lineatopennis*	40.3	16.8	28.4	14.5	524.0	26.3	16.0	29.7	28.0	175.0	44.0	28.6	13.1	14.3	175.0	50.6	5.7	42.5	1.1	174.0
KP954638.1 *Aedes vexans*	41.0	15.8	29.0	14.1	524.0	28.0	14.9	29.7	27.4	175.0	44.0	29.1	13.1	13.7	175.0	51.1	3.4	44.3	1.1	174.0
MK402823.1 *Aedes vexans*	40.5	15.8	29.4	14.3	524.0	28.0	14.9	29.7	27.4	175.0	44.0	29.1	13.1	13.7	175.0	49.4	3.4	45.4	1.7	174.0
LC473705.1 *Mansonia uniformis*	40.3	14.9	30.2	14.7	524.0	27.4	13.7	30.9	28.0	175.0	44.0	28.6	13.7	13.7	175.0	49.4	2.3	46.0	2.3	174.0
LC517293.1 *Mansonia uniformis*	40.3	14.9	30.2	14.7	524.0	27.4	13.7	30.9	28.0	175.0	44.0	28.6	13.7	13.7	175.0	49.4	2.3	46.0	2.3	174.0
MH745093.1 *Culex tritaeniorhynchus*	39.5	16.0	29.6	14.9	524.0	24.6	17.7	29.7	28.0	175.0	44.0	28.6	13.1	14.3	175.0	50.0	1.7	46.0	2.3	174.0
MH330220.1 *Culex tritaeniorhynchus*	39.3	16.2	29.8	14.7	524.0	24.0	18.3	29.7	28.0	175.0	44.0	28.6	13.1	14.3	175.0	50.0	1.7	46.6	1.7	174.0
KY694466.1 *Afidenta misera*	38.4	18.9	28.1	14.7	524.0	24.6	16.6	34.3	24.6	175.0	44.6	26.3	14.3	14.9	175.0	46.0	13.8	35.6	4.6	174.0
KC849092.1 *Nephila sumptuosa*	43.0	12.2	27.7	17.0	523.0	34.9	10.3	26.3	28.6	175.0	46.9	25.7	14.3	13.1	175.0	47.4	0.6	42.8	9.2	173.0
EF033298.1 *Lampsilis hydiana*	45.6	12.4	18.7	23.3	524.0	35.4	11.4	25.7	27.4	175.0	45.1	21.1	14.9	18.9	175.0	56.3	4.6	15.5	23.6	174.0
Avg.	40.6	15.6	28.4	15.4	523.9	27.8	14.9	29.7	27.7	175.0	44.3	27.9	13.5	14.3	175.0	49.7	4.0	42.2	4.1	173.9

Table 2.10d: The evolutionary divergence percentage between *Aedes vittatus* and its closely related species

Sl. No.	Accession Number	Organism	Percentage of Divergence
1	MK491498.1	*Aedes vittatus*	0.00%
2	MH330198.1	*Aedes vittatus*	0.38%
3	MH330197.1	*Aedes vittatus*	0.00%
4	AB738145.1	*Aedes lineatopennis*	6.96%
5	HQ398909.1	*Aedes lineatopennis*	7.42%
6	KP954638.1	*Aedes vexans*	9.62%
7	MK402823.1	*Aedes vexans*	8.66%
8	LC473705.1	*Mansonia uniformis*	15.38%
9	LC517293.1	*Mansonia uniformis*	15.38%
10	MH745093.1	*Culex tritaeniorhynchus*	12.24%
11	MH330220.1	*Culex tritaeniorhynchus*	12.27%
12	KY694466.1	*Afidenta misera*	30.79%
13	KC849092.1	*Nephila sumptuosa*	32.44%
14	EF033298.1	*Lampsilis hydiana*	63.69%

2.3.9.2 Discussion

Ae. vittatus is present in Africa, Asia, and Europe, where it serves as a vector of illnesses that cause diseases in animals and people (e.g., chikungunya, Zika, and dengue). *Ae. vittatus*, like other *Aedes* species, may reproduce in artificial containers (Díez-Fernández, 2018). A particular scale arrangement is seen in *Ae. vittatus* like thoracic mesonotum, leg, vertex, etc. CO1 gene fragment of*, Ae. vittatus* sequenced and yielded a single product of 679bp. The sequence has been deposited in the NCBI GenBank with Accession No. MK491498.1. According to the Phylogenetic tree, the nearby species of *Ae. vittatus* was *Ae. lineatopennis* and *Ae. vexans*. Molecular amplification of the COI gene used in the comparative study of mosquito sequences isolated from Spain was done in 2018. Phylogeny discloses the comparison of those deposited in public databases provided a ≥ 99% similarity with sequences for two *Aedes* mosquitoes, *Ae. vittatus* and *Ae. cogilli*, while similarities

with other *Aedes* species were ≤ 94%. There are no reports of *Aedes cogilli* in Europe, and it exclusively exists in India.

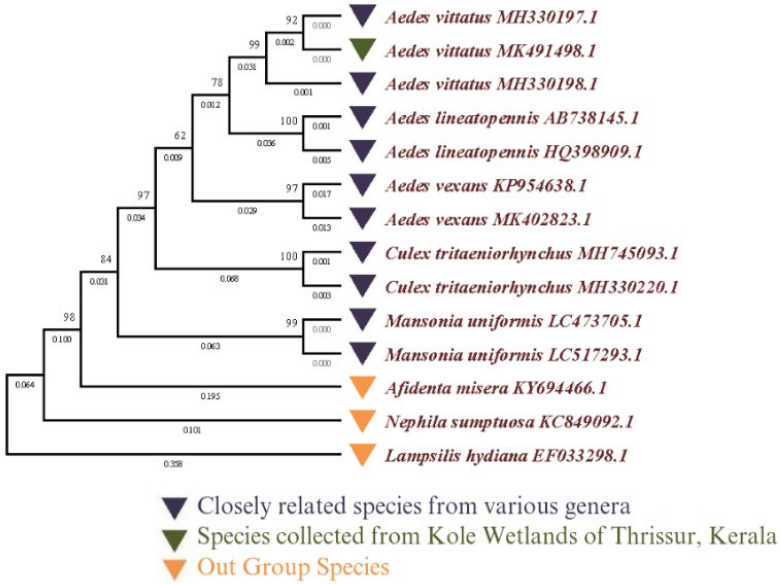

Figure 2.9c Phylogenetic tree of *Aedes vittatus*

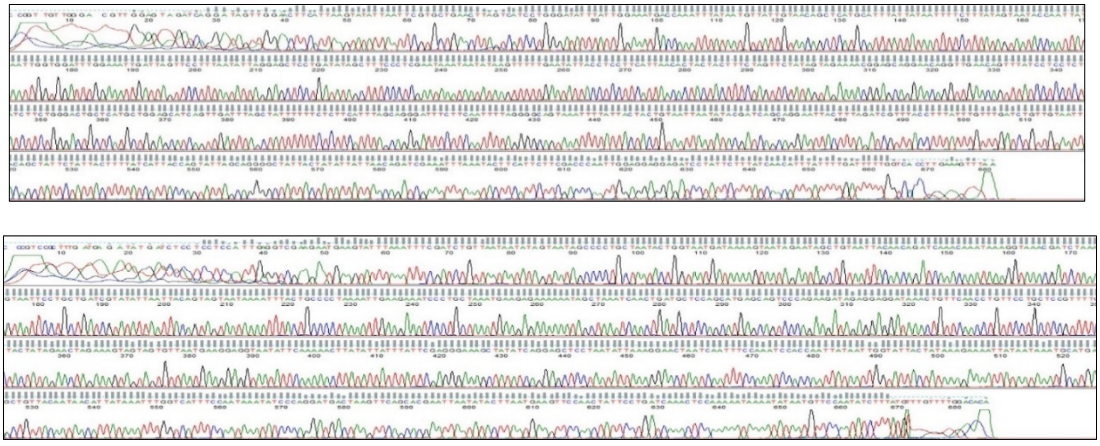

Figure 2.9d Electrophorogram showing the nucleotide sequence of mitochondrial COI gene of *Aedes vittatus*

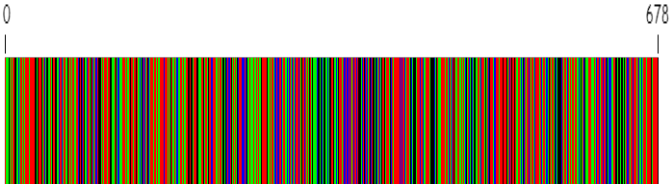

Figure 2.9e Molecular barcode of *Aedes vittatus*

2.3.10. Species Name: *Phagomyia cogilli*

GenBank Accession Number: MN700902.1

Voucher Number: CDRL36

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	*Culicini*
Genus	:	*Aedes*
Subgenus	:	*Phagomyia*
Species	:	*Phagomyia cogilli*

Description

Medium-sized mosquito. It looks like *Ae. vittatus*, but no white scales are present on the thoracic region. Rarely collected mosquito species occurred in rainwater-containing habitats. *Ph. cogilli* are occasionally collected from rocky pools and tree holes.

2.3.10.1 Result

The forward and reverse primers used to amplify the mitochondrial cytochrome oxidase subunit I gene of *Ph. cogilli,* collected from Kole wetlands of Thrissur, Kerala, were 5'-GGTCAACAAATCATAAAGATATTGG-3' and 5'TAAACTTCAG GGTGACCAAAAAATCA-3' respectively. A single product with a length of 632 bp was produced by PCR amplifying *Ph. cogilli's* mitochondrial cytochrome oxidase

subunit I (COI) gene fragment. Accession No. MN700902.1 was obtained from the NCBI GenBank, and Figures 2.10a- 2.10e displayed the DNA sequence, corresponding translation product, phylogenetic tree, electrophorogram, and molecular barcode.

> MN700902.1 *Phagomyia cogilli*|632bp

```
AAAGATATTGGAACATTATATTTTATTTTTGGAGTTTGATCAGGAATAGTTGGAACTTCATTAAGTATATTAA
TTCGTGCTGAACTTAGTCATCCTGGGATATTTATTGGAAATGACCAAATTTATAATGTTATTGTAACAGCTCA
TGCATTTATTATAATTTTCTTTATAGTAATACCAATTATAATTGGTGGATTTGGAAATTGATTAGTTCCTTTA
ATATTAGGAGCTCCTGATATAGCTTTCCCTCGAATAAATAATATAAGTTTTTGAATATTACCTCCTTCATTAA
CACTACTACTTTCTAGTTCTATAGTAGAAAACGGAGCAGGAACAGGTTGAACAGTTTATCCTCCTCTATCTTC
TGGGACTGCTCATGCTGGAGCATCAGTTGATTTAGCTATTTTTTCTCTTCATTTAGCAGGGATTTCTTCAATT
TTAGGGGCAGTAAATTTTATTACTACTGTAATTAATATACGATCAGCAGGAATTACTTTAGATCGTTTACCTT
TATTTGTTTGATCTGTTGTAATTACAGCTATTCTATTACTTTTATCATTACCAGTATTAGCAGGGGCTATTAC
TATATTATTAACAGATCGAAATTTAAATACTTCATTCTTCGACCCAAT
```

Figure 2.10a: The DNA sequence of *Phagomyia cogilli* COI gene

> MN700902.1 *Phagomyia cogilli*

```
MGTLYFIFGVWSGMVGTSLSMLIRAELSHPGMFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLML
GAPDMAFPRMNNMSFWMLPPSLTLLLSSSMVENGAGTGWTVYPPLSSGTAHAGASVDLAIFSLHLAGISSILG
AVNFITTVINMRSAGITLDRLPLFVWSVVITAILLLLSLPVLAGAITMLLTDRNLNTSFFDP
```

Figure 2.10b: The protein sequence of *Phagomyia cogilli* COI gene

The COI sequence of *Ph. cogilli* showed bias to nucleotide AT, and the nucleotide composition was T=39.9%, C=15.7%, A=29.5%, and G=15.0%. The average nucleotide composition value of fourteen nucleotides showed T=39.5%, C=15.6%, A=29.1% and G=15.8% (Table 2.11c). The Maximum Composite Likelihood model used for the substitution matrix analysis and the base substitutions number per position between involved nucleotide sequences was displayed in Table 2.11a using. Tamura-Nei's (1993) model was used to calculate substitution patterns and rates. A = 29.14%, T/U = 39.50%, C = 15.56%, and G = 15.79% were the computed nucleotide frequencies and tree topology was used in automatically computed ML value calculations. The value of the maximum Log-likelihood for this estimation

was -3133.843. The first, second, third, noncoding regions were covered in codon positions. Examining these 14 nucleotide sequences resulted in a final dataset with 587 locations. All these evolutionary analyses were conducted in MEGAX. The data in Table 2.11d reveals that the *Ph. cogilli* shows 0.53% evolutionary divergence with *Ph. cogilli* (KJ768136.1 & MK209633.1) isolated from Pakistan and Sri Lanka. *Ae. vexans* (KP954638.1) isolated from the USA shows 9.58% evolutionary divergence with *Ph. cogilli* isolated from Kerala.

The phylogenetic tree generated using the NJ method shows the phylogenetic position of *Ph. cogilli* isolated from the Kole wetlands of Thrissur, Kerala. Phylogenetically, *Ph. cogilli* (MN700902.1) was the closest relative of *Ph. cogilli* (KJ768136.1 & MK209633.1) of Pakistan and Sri Lanka. They are from the same clade. *Ae. vexans* (KP954638.1) isolated from the USA showed another closest relative of *Ph. cogilli.*

Table 2.11a: The nucleotide substitution matrix estimate of COI gene sequence of *Phagomyia cogilli*

From\To	A	T	C	G
A	-	10.4147	4.1036	7.7678
T	7.6843	-	7.1130	4.1646
C	7.6843	18.0521	-	4.1646
G	14.3328	10.4147	4.1036	-

Table 2.11b: The Transition/Transversion bias estimate of mitochondrial COI gene sequence of *Phagomyia cogilli*

From\To	A	T	C	G
A	-	7.1371	7.1371	10.7259
T	7.1371	-	10.7259	7.1371
C	7.1371	10.7259	-	7.1371
G	10.7259	7.1371	7.1371	-

Table 2.11c: The nucleotide frequency comparison of *Phagomyia cogilli* COI gene sequence with its kin species

Accession Number and Name of the Species	**Nucleotide Frequencies (%)**																		
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MN700902.1 *Phagomyia cogilli*	39.9	15.7	29.5	15.0	587.0	26.5	15.3	30.6	27.6	196.0	44.4	28.1	12.8	14.8	196.0	48.7	3.6	45.1	2.6	195.0
MK209633.1 *Phagomyia cogilli*	40.0	15.5	29.8	14.7	587.0	26.5	15.3	30.6	27.6	196.0	44.4	28.1	12.8	14.8	196.0	49.2	3.1	46.2	1.5	195.0
KJ768136.1 *Phagomyia cogilli*	40.0	15.5	29.8	14.7	587.0	26.5	15.3	30.6	27.6	196.0	44.4	28.1	12.8	14.8	196.0	49.2	3.1	46.2	1.5	195.0
KP954638.1 *Aedes vexans*	40.4	15.5	29.5	14.7	587.0	27.6	14.8	30.6	27.0	196.0	44.4	28.1	12.8	14.8	196.0	49.2	3.6	45.1	2.1	195.0
MK402823.1 *Aedes vexans*	39.7	15.5	30.2	14.7	587.0	27.6	14.8	30.6	27.0	196.0	44.4	28.1	12.8	14.8	196.0	47.2	3.6	47.2	2.1	195.0
AB738145.1 *Aedes lineatopennis*	40.0	15.7	29.5	14.8	587.0	26.0	15.8	30.6	27.6	196.0	44.4	27.6	12.8	15.3	196.0	49.7	3.6	45.1	1.5	195.0
HQ398909.1 *Aedes lineatopennis*	39.7	16.2	29.5	14.7	587.0	26.0	15.8	30.6	27.6	196.0	44.4	27.6	12.8	15.3	196.0	48.7	5.1	45.1	1.0	195.0
MH330206.1 *Anopheles nigerrimus*	37.0	15.3	31.9	15.8	587.0	27.0	13.8	29.6	29.6	196.0	43.9	28.1	12.8	15.3	196.0	40.0	4.1	53.3	2.6	195.0
AB778799.1 *Anopheles nigerrimus*	37.5	14.8	32.4	15.3	587.0	26.5	14.3	29.6	29.6	196.0	43.9	28.1	12.8	15.3	196.0	42.1	2.1	54.9	1.0	195.0
MK713986.1 *Culex pipiens*	39.7	15.3	29.5	15.5	587.0	26.5	15.8	29.6	28.1	196.0	44.4	27.6	12.8	15.3	196.0	48.2	2.6	46.2	3.1	195.0
MK713985.1 *Culex pipiens*	39.7	15.3	29.5	15.5	587.0	26.5	15.8	29.6	28.1	196.0	44.4	27.6	12.8	15.3	196.0	48.2	2.6	46.2	3.1	195.0
KU578141.1 *Coccinella transversalis*	37.5	19.1	28.3	15.2	587.0	24.5	16.3	34.2	25.0	196.0	44.4	26.5	13.3	15.8	196.0	43.6	14.4	37.4	4.6	195.0
JF835944.1 *Nephila inaurata*	42.1	12.6	28.1	17.2	587.0	33.2	11.2	26.0	29.6	196.0	45.9	25.5	13.8	14.8	196.0	47.2	1.0	44.6	7.2	195.0
MN125137.1 *Pyganodon grandis*	39.9	15.8	20.8	23.5	587.0	29.1	15.3	25.5	30.1	196.0	44.9	22.4	13.8	18.9	196.0	45.6	9.7	23.1	21.5	195.0
Avg.	39.5	15.6	29.1	15.8	587.0	27.2	15.0	29.9	28.0	196.0	44.5	27.2	12.9	15.4	196.0	46.9	4.4	44.7	4.0	195.0

Table 2.11d: The evolutionary divergence percentage between *Phagomyia cogilli* and its closely related species

Sl. No.	Accession Number	Organism	Percentage of Divergence
1	MN700902.1	*Phagomyia cogilli*	0.00%
2	MK209633.1	*Phagomyia cogilli*	0.51%
3	KJ768136.1	*Phagomyia cogilli*	0.51%
4	KP954638.1	*Aedes vexans*	9.58%
5	MK402823.1	*Aedes vexans*	8.98%
6	AB738145.1	*Aedes lineatopennis*	7.14%
7	HQ398909.1	*Aedes lineatopennis*	7.53%
8	MH330206.1	*Anopheles nigerrimus*	15.62%
9	AB778799.1	*Anopheles nigerrimus*	15.39%
10	MK713986.1	*Culex pipiens*	13.85%
11	MK713985.1	*Culex pipiens*	13.85%
12	KU578141.1	*Coccinella transversalis*	30.22%
13	JF835944.1	*Nephila inaurata*	29.18%
14	MN125137.1	*Pyganodon grandis*	53.46%

2.3.10.2 Discussion

Ph. cogilli is generally seen southern part of India (Bhat, 1975). Tyagi et al., 2015 in their work about a catalogue of Indian mosquitoes, discussed the presence of *Ph. cogilli* in the Indian subcontinent. Morphological identification of this species has been done with available keys and online photographs. Morphologically this species looks close to *Ae. vittatus*, and they were always seen flying together in grassland habitats. The NCBI database's molecular identification method showed this species' conformity as *Ph. cogilli.* The phylogenetic relationship also revealed that it was very close to *Ph. cogilli*. The phylogenetic tree interprets that all *Aedes* genera have a common ancestry and the same species are in one clade. It showed that the ancestor had been diverted into different clades earlier, with one clade containing *Ph. cogilli* and *Ae. vexans,* as sister taxa, while all other closely related *Ae. lineatopennis* on another clade. This result confirmed the *Aedes* genera

and also mosquito phylogeny. All the concerned species in the tree may have evolved from their common ancestor at different periods and were found in separate clades with little differences in the nucleotide sequences. Thus, both classical taxonomy and DNA barcoding techniques provided a better taxonomic tool for confirming the taxonomic identity and predicting evolutionary relationships.

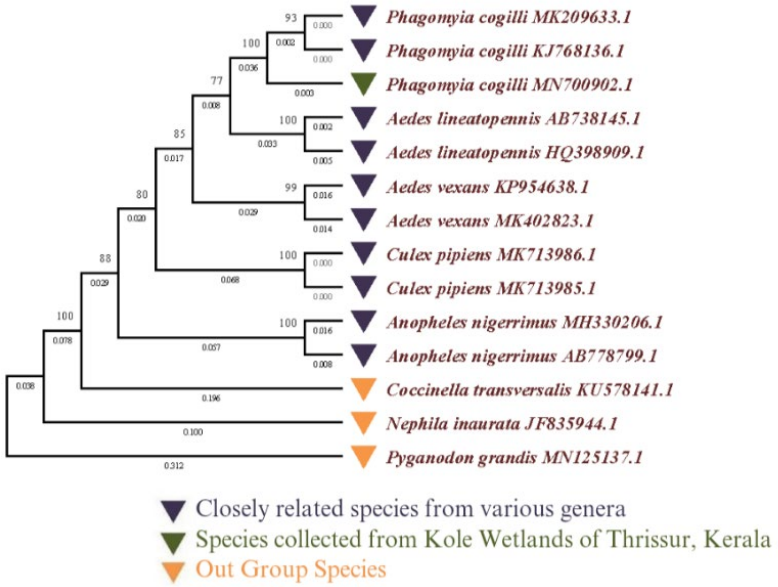

Figure 2.10c Phylogenetic tree of *Phagomyia cogilli*

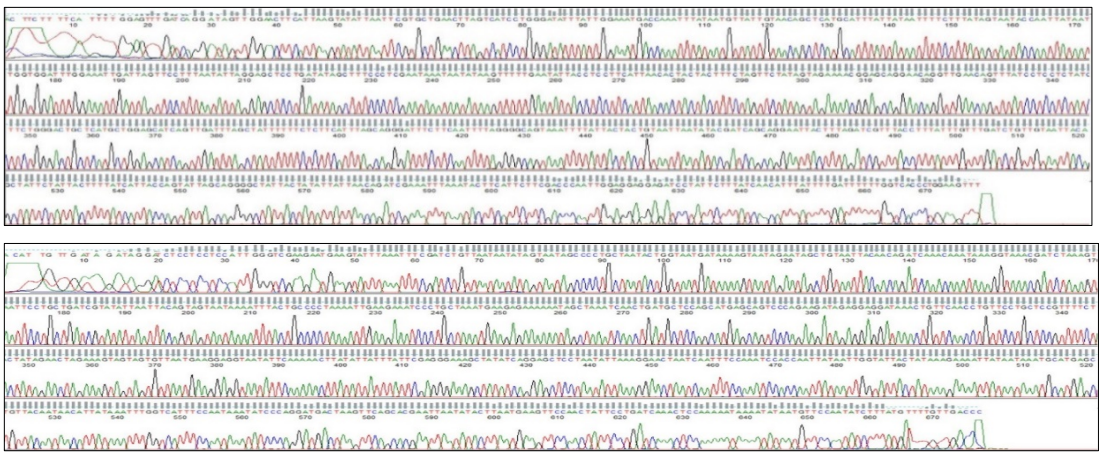

Figure 2.10d Electrophorogram showing the nucleotide sequence of mitochondrial COI gene of *Phagomyia cogilli*

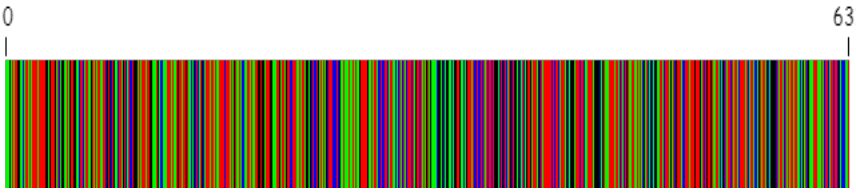

Figure 2.10e Molecular barcode of *Phagomyia cogilli*

2.3.11. Species Name: *Armigeres subalbatus*

GenBank Accession Number: MK644935.1

Voucher Number: CDRL05

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	*Culicini*
Genus	:	*Armigeres*
Species	:	*Armigeres subalbatus*

Description

Tiny pale-coloured mosquito with several yellow and black scales. Their breeding habitat varies from freshwater to polluted water.

2.3.11.1 Result

The forward and reverse primers were used to amplify Ar. *subalbatus'* mitochondrial cytochrome oxidase subunit I gene, collected from Kole wetlands of Thrissur, Kerala, were 5'-GGTCAACAAATCATAAAGATATTGG-3' and 5'TAAACTTCA GGGTGACCAAAAAATCA-3' respectively. A single product with a length of 678 bp was produced by PCR amplifying *Ar. subalbatus'* mitochondrial cytochrome

oxidase subunit I (COI) gene fragment. Accession No. MK644935.1 was obtained from the NCBI GenBank, and Figures 2.11a- 2.11e displayed the DNA sequence, corresponding translation product, phylogenetic tree, electrophorogram, and molecular barcode.

> MK644935 *Armigeres subalbatus*|678bp

AAGATATTGGAACTTTATATTTTATTTTTGGTGCTTGAGCTGGAATAGTGGGAACTTCTTTAAGTATTTTA
ATTCGAACAGAATTAAATCACCCTGGAATATTTATTGGAAATGATCAAATTTATAATGTAATTGTAACAGC
TCATGCTTTTATTATAATTTTTTTTATAGTTATACCAATTATAATTGGAGGATTTGGAAATTGATTAGTAC
CCCTTATACTTGGAGCTCCAGATATAGCCTTCCCTCGAATAAATAATATAAGTTTTTGAATATTACCCCCT
TCATTAACTCTACTAATTTCAAGTTCTTTAGTAGAAACAGGAGCTGGAACTGGATGAACCGTTTATCCTCC
TTTATCTTCTGGAACTGCCCATGCTGGAGCTTCTGTTGATTTAGCTATTTTCTCTCTTCATTTAGCAGGTA
TTTCTTCTATTTTGGGAGCAGTAAATTTTATTACAACTGTAATTAATATACGATCATCAGGGATTACTCTT
GATCGATTACCCTTATTTGTTTGATCTGTTGTTATTACAGCTATTTTACTTCTTCTTTCTTTACCAGTTTT
AGCAGGAGCTATTACTATACTATTAACTGATCGGAATTTAAATACCTCATTCTTTGACCCAATTGGAGGAG
GAGATCCGATCTTATACCAACATTTATTTTGATTTTTTG

Figure 2.11a: The DNA sequence of *Armigeres subalbatus* COI gene

> MK644935 *Armigeres subalbatus*

MGTLYFIFGAWAGMVGTSLSILIRTELNHPGMFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLM
LGAPDMAFPRMNNMSFWMLPPSLTLLISSSLVETGAGTGWTVYPPLSSGTAHAGASVDLAIFSLHLAGISSI
LGAVNFITTVINMRSSGITLDRLPLFVWSVVITAILLLLSLPVLAGAITMLLTDRNLNTSFFDPIGGGDPIL
YQHLFWFF

Figure 2.11b: The protein sequence of *Armigeres subalbatus* COI gene

T=38.8%; C=15.7%; A=28.7%; G=16.8% were the average composition of nucleotide throughout the species (Table 2.12c). This outcome exhibits that evaluating a mitochondrial gene will also be priceless for unravelling phylogenetic relationships within the *Ar. subalbatus*. The percentage of A+T was more than that of G+C, which reflected extra within the codon usage. The phylogenetic tree generated using Neighbour Joining (NJ) method reveals the phylogenetic status of *Ar. subalbatus* isolated from Kerala. Phylogenetically *Ar. subalbatus* is the closest relative of *Ar. subalbatus* of Calicut, Kerala, and China.

Table 2.12a: The nucleotide substitution matrix estimate of COI gene sequence of *Armigeres subalbatus*

From\To	A	T	C	G
A	-	10.6172	4.2808	7.5388
T	7.8445	-	7.1608	4.6010
C	7.8445	17.7600	-	4.6010
G	12.8532	10.6172	4.2808	-

Table 2.12b: The Transition/Transversion bias estimate of mitochondrial COI gene sequence of *Armigeres subalbatus*

From\To	A	T	C	G
A	-	7.3221	7.3221	10.3558
T	7.3221	-	10.3558	7.3221
C	7.3221	10.3558	-	7.3221
G	10.3558	7.3221	7.3221	-

Table 2.12c: The nucleotide frequency comparison of *Armigeres subalbatus* COI gene sequence with its kin species

Accession Number and Name of the Species	Nucleotide Frequencies (%)																			
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MK644935.1 *Armigeres subalbatus*	39.3	16.7	28.4	15.6	610.0	42.6	28.4	12.7	16.2	204.0	49.8	5.9	42.4	2.0	203.0	25.6	15.8	30.0	28.6	203.0
MW542319.1 *Armigeres subalbatus*	39.3	16.7	28.2	15.7	610.0	42.6	28.4	12.7	16.2	204.0	49.8	5.9	42.4	2.0	203.0	25.6	15.8	29.6	29.1	203.0
MW446159.1 *Armigeres subalbatus*	39.3	16.7	28.2	15.7	610.0	42.6	28.4	12.7	16.2	204.0	49.8	5.9	42.4	2.0	203.0	25.6	15.8	29.6	29.1	203.0
KP954638.1 *Aedes vexans*	39.7	15.1	29.3	15.9	610.0	43.1	27.5	12.7	16.7	204.0	48.3	3.4	45.8	2.5	203.0	27.6	14.3	29.6	28.6	203.0
MK402823.1 *Aedes vexans*	39.0	15.1	30.2	15.7	610.0	43.1	27.5	12.7	16.7	204.0	46.3	3.4	48.3	2.0	203.0	27.6	14.3	29.6	28.6	203.0
AB738145.1 *Aedes lineatopennis*	39.5	15.2	29.3	15.9	610.0	43.1	27.0	12.7	17.2	204.0	49.3	3.4	45.8	1.5	203.0	26.1	15.3	29.6	29.1	203.0
HQ398909.1 *Aedes lineatopennis*	39.2	15.7	29.3	15.7	610.0	43.1	27.0	12.7	17.2	204.0	48.3	4.9	45.8	1.0	203.0	26.1	15.3	29.6	29.1	203.0
FJ210896.1 *Anopheles pseudopictus*	37.7	15.2	30.7	16.4	610.0	42.2	27.9	12.7	17.2	204.0	45.3	3.4	50.7	0.5	203.0	25.6	14.3	28.6	31.5	203.0
MT993487.1 *Anopheles pseudopictus*	37.5	15.2	30.8	16.4	610.0	41.7	27.9	13.2	17.2	204.0	45.3	3.4	50.7	0.5	203.0	25.6	14.3	28.6	31.5	203.0
MH745093.1 *Culex tritaeniorhynchus*	38.4	15.4	29.8	16.4	610.0	42.6	27.5	12.7	17.2	204.0	48.3	2.0	47.8	2.0	203.0	24.1	16.7	29.1	30.0	203.0
MH330220.1 *Culex tritaeniorhynchus*	38.2	15.6	30.0	16.2	610.0	42.6	27.5	12.7	17.2	204.0	48.3	2.0	48.3	1.5	203.0	23.6	17.2	29.1	30.0	203.0
KU578141.1 *Coccinella transversalis*	36.4	18.5	28.5	16.6	610.0	43.1	26.0	13.2	17.6	204.0	41.9	13.8	39.4	4.9	203.0	24.1	15.8	33.0	27.1	203.0
JF835944.1 *Nephila inaurata*	41.1	12.6	28.0	18.2	610.0	44.1	26.0	13.7	16.2	204.0	46.8	1.0	45.3	6.9	203.0	32.5	10.8	25.1	31.5	203.0
MN125137.1 *Pyganodon grandis*	38.9	15.2	20.8	25.1	610.0	43.6	21.6	13.7	21.1	204.0	43.8	9.4	24.1	22.7	203.0	29.1	14.8	24.6	31.5	203.0
Avg.	38.8	15.7	28.7	16.8	610.0	42.9	27.0	13.0	17.1	204.0	47.2	4.9	44.2	3.7	203.0	26.4	15.0	29.0	29.7	203.0

Table 2.12d: The evolutionary divergence percentage between *Armigeres subalbatus* and its closely related species

Sl. No.	Accession Number	Organism	Percentage of Divergence
1	MK644935.1	*Armigeres subalbatus*	0.00%
2	MW542319.1	*Armigeres subalbatus*	0.16%
3	MW446159.1	*Armigeres subalbatus*	0.16%
4	KP954638.1	*Aedes vexans*	13.98%
5	MK402823.1	*Aedes vexans*	14.89%
6	AB738145.1	*Aedes lineatopennis*	9.78%
7	HQ398909.1	*Aedes lineatopennis*	10.60%
8	FJ210896.1	*Anopheles pseudopictus*	16.67%
9	MT993487.1	*Anopheles pseudopictus*	16.92%
10	MH745093.1	*Culex tritaeniorhynchus*	11.88%
11	MH330220.1	*Culex tritaeniorhynchus*	11.90%
12	KU578141.1	*Coccinella transversalis*	33.26%
13	JF835944.1	*Nephila inaurata*	33.94%
14	MN125137.1	*Pyganodon grandis*	61.40%

2.3.11.2 Discussion

The genetic constitution evaluation reveals the composition of the nucleotides within the codons of the COI sequence of *Ar. subalbatus* isolated from Thrissur Kole wetlands, Kerala. The variations in the nucleotides within the various positions of codons may result from the compulsion in the nucleotide changes in one of the codon positions. Within the nucleotide triplet code, there is a strong compulsion within the nucleotide alterations in the second and first positions of many codons. Because of the degenerative personality of the triplet code third position of many codons and the first position of some codons is much less constrained. The variants within the strong constraint positions lead to differences within the amino acid sequence. However, the variations in the much less constrained position will not impact (silent) the phenotype. These much less limited codon positions advanced excessively (Nei, 1987; Irwin et al., 1991).

The partial COI sequence generated in this study clearly showed considerable variation with other species. Nucleotide composition analysis of *Ar. subalbatus* reveals that they have no single variation in the codons of the first, second, and third positions. The BLAST analysis of 678 bp of the insect *Ar. subalbatus* showed significant homology with other *Ar. subalbatus*. The genetic divergence analysis depicts the divergence of different geographically isolated species of *Ar. subalbatus* with various related species. *Ar. subalbatus* isolated from Kerala shows 100% sequence similarity with *Ar. subalbatus* isolated from geographically distinct locations of Kerala and China (GenBank Accession No. MW542319.1, MW446159.1). They were in the same clade. This result indicates that the *Ar. subalbatus* isolated from Kerala is novel.

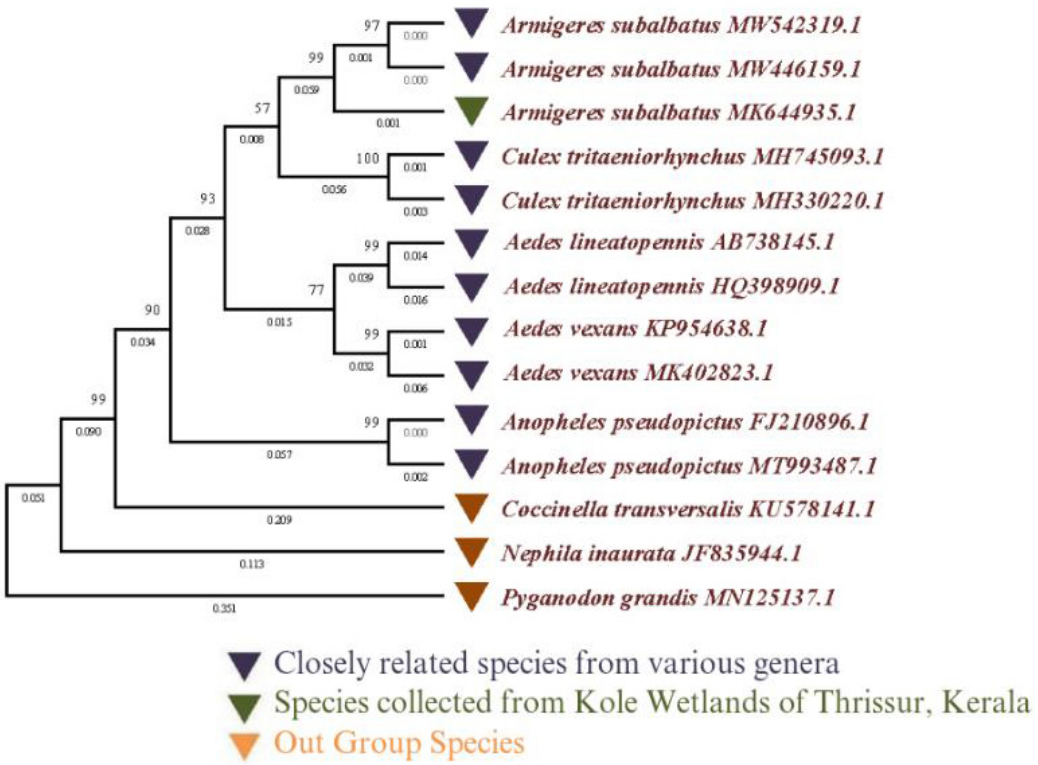

Figure 2.11c Phylogenetic tree of *Armigeres subalbatus*

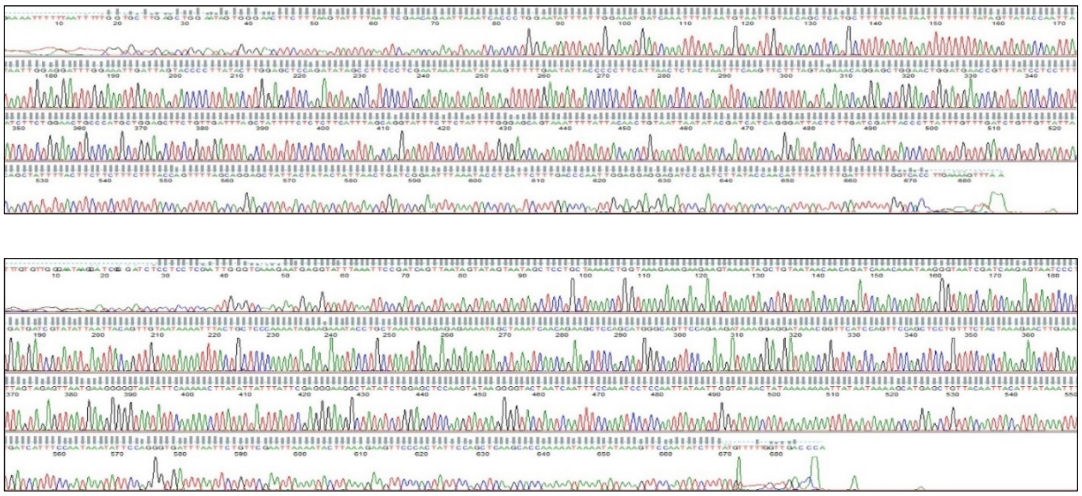

Figure 2.11d Electrophorogram showing the nucleotide sequence of mitochondrial COI gene of *Armigeres subalbatus*

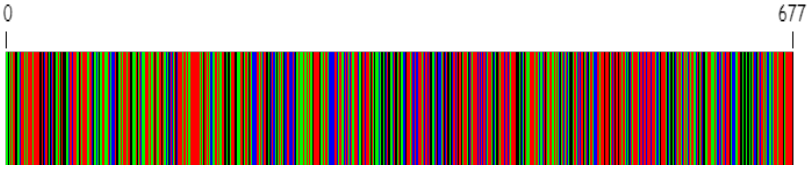

Figure 2.11e Molecular barcode of *Armigeres subalbatus*

2.3.12. Species Name: *Lutzia fuscanus*

GenBank Accession Number: MK616587.1

Voucher Number: CDRL25

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Culicini
Genus	:	*Culex*
Subgenus	:	*Lutzia*
Species	:	*Lutzia fuscanus*

Description

Lt. fuscanus is a large mosquito with combinations of yellow and dark patches of scales on its body. Larvae of these mosquitoes are large and show some predatory behaviour towards aquatic insects and worms, including mosquito larvae. These mosquitoes were regularly collected from polluted water.

2.3.12.1 Result

The forward and reverse primers used to amplify the mitochondrial cytochrome oxidase subunit I gene of *Lt. fuscanus,* collected from Kole wetlands of Thrissur, Kerala, were 5'-GGTCAACAAATCATAAAGATATTGG-3' and 5'TAAACTTCAG GGTGACCAAAAAATCA-3' respectively. A single product with a length of 678 bp was produced by PCR amplifying *Lt. fuscanus'* mitochondrial cytochrome oxidase subunit I (COI) gene fragment. Accession No. MK616587.1 was obtained from the NCBI GenBank, and Figures 2.12a- 2.12e displayed the DNA sequence, corresponding translation product, phylogenetic tree, electrophorogram, and molecular barcode.

> MK616587.1 *Lutzia fuscanus*|678bp

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AAAGATATTGGAACATTATATTTTATTTTTGGAGCTTGAGCAGGAATAATTGGAACTTCTTTAAGAATTCTTA
TTCGTGCAGAATTAAGTCAACCTGGAGTATTTATTGGAAATGATCAAATTTATAATGTTATTGTAACTGCACA
TGCTTTTATTATAATTTTTTTTATAGTTATACCAATTATAATTGGAGGATTTGGAAATTGATTAGTTCCTTTA
ATATTAGGAGCTCCTGATATAGCTTTTCCTCGAATAAATAATATAAGTTTCTGAATACTACCTCCCTCATTAA
CTTTACTCCTTTCAAGTAGTTTAGTAGAAAATGGAGCTGGAACTGGATGAACTGTTTACCCCCCTCTTTCATC
TGGAACTGCTCATGCAGGTGCATCAGTTGATTTAGCTATTTTTTCTTTACATTTAGCTGGTATTTCATCAATT
TTAGGAGCTGTTAATTTTATTACAACAGTTATTAATATACGATCTTCAGGAATTACTCTAGATCGAATACCTT
TATTTGTTTGATCAGTAGTAATTACTGCTGTTTTATTATTACTTTCTTTACCTGTATTAGCAGGAGCAATTAC
TATATTACTAACAGATCGAAATTTAAATACTTCATTTTTTGATCCTATTGGAGGGGGAGATCCAATTTTATAT
CAACATTTATTTTGATTTTTG
```

Figure 2.12a: The DNA sequence of *Lutzia fuscanus* COI gene

> MK616587.1 *Lutzia fuscanus*

MGTLYFIFGAWAGMIGTSLSILIRAELSQPGVFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLM
LGAPDMAFPRMNNMSFWMLPPSLTLLLSSSLVENGAGTGWTVYPPLSSGTAHAGASVDLAIFSLHLAGISSI
LGAVNFITTVINMRSSGITLDRMPLFVWSVVITAVLLLLSLPVLAGAITMLLTDRNLNTSFFDPIGGGDPIL
YQHLFWFL

Figure 2.12b: The protein sequence of *Lutzia fuscanus* COI gene

The COI nucleotide sequence analysis revealed the composition of nucleotides in the COI gene of *Lt. fuscanus* isolated from Thrissur Kole lands Kerala (Table 2.13c). T=40.5%, C=15.2%, A=29.5%, and G=14.8% were the nucleotide composition of The COI sequence of *Lt. fuscanus* showed bias to nucleotide AT. The COI nucleotide composition analysis showed slight variation in the composition of each nucleotide of *Lt. fuscanus* isolated from Kerala with other species.

From the data in Table 2.13d, it is clear that the *Lt. fuscanus* showed 0.17% evolutionary divergence with *Lt. fuscanus* (KF407917.1, HQ398896.1) isolated from Pakistan and Thailand. *Lt. chiangmaiensis* (MK271004.1, MK271005.1), isolated from Thailand, showed 1.36% of evolutionary divergence with *Lt. fuscanus,* isolated from Kerala. Table 2.13a, which uses the Maximum Composite Likelihood model, displays the number of base substitutions per site between sequences. A = 28.27%, T/U = 40.57%, C = 15.19%, and G = 15.96% were the nucleotide frequencies estimated through substitution matrix analysis. Tree topology was used in automatically computed ML value calculations. The value of the maximum Log-likelihood for this estimation was -2775.647. Codon positions covered the first, second, and third noncoding regions. The final dataset of these 14 nucleotide sequences analysis involved a total number of 593 positions. All these evolutionary analyses were conducted in MEGA X.

Table 2.13a: The nucleotide substitution matrix estimate of COI gene sequence of *Lutzia fuscanus*

From\To	A	T	C	G
A	-	11.3609	4.2536	8.0927
T	7.9168	-	5.8762	4.4695
C	7.9168	15.6948	-	4.4695
G	14.3348	11.3609	4.2536	-

Table 2.13b: The Transition/Transversion bias estimate of mitochondrial COI gene sequence of *Lutzia fuscanus*

From\To	A	T	C	G
A	-	7.5560	7.5560	9.8880
T	7.5560	-	9.8880	7.5560
C	7.5560	9.8880	-	7.5560
G	9.8880	7.5560	7.5560	-

Table 2.13c: The nucleotide frequency comparison of *Lutzia fuscanus* COI gene sequence with its kin species

Accession Number and Name of the Species	**Nucleotide Frequencies (%)**																			
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MK616587.1 *Lutzia fuscanus*	40.5	15.2	29.5	14.8	593.0	26.8	15.2	29.8	28.3	198.0	43.4	27.8	12.6	16.2	198.0	51.3	2.5	46.2	0.0	197.0
KF407917.1 *Lutzia fuscanus*	40.3	15.3	29.5	14.8	593.0	26.8	15.2	29.8	28.3	198.0	43.4	27.8	12.6	16.2	198.0	50.8	3.0	46.2	0.0	197.0
HQ398896.1 *Lutzia fuscanus*	40.5	15.2	29.3	15.0	593.0	26.8	15.2	29.8	28.3	198.0	43.4	27.8	12.6	16.2	198.0	51.3	2.5	45.7	0.5	197.0
MK271004.1 *Lutzia chiangmaiensis*	39.8	16.0	29.3	14.8	593.0	26.8	15.2	29.8	28.3	198.0	43.4	27.8	12.6	16.2	198.0	49.2	5.1	45.7	0.0	197.0
MK271005.1 *Lutzia chiangmaiensis*	39.8	16.0	29.3	14.8	593.0	26.8	15.2	29.8	28.3	198.0	43.4	27.8	12.6	16.2	198.0	49.2	5.1	45.7	0.0	197.0
LC507833.1 *Lutzia tigripes*	41.3	14.8	28.5	15.3	593.0	27.8	14.1	30.3	27.8	198.0	43.4	27.8	12.6	16.2	198.0	52.8	2.5	42.6	2.0	197.0
KU380351.1 *Lutzia tigripes*	41.0	15.2	28.5	15.3	593.0	27.8	14.1	30.3	27.8	198.0	43.4	27.8	12.6	16.2	198.0	51.8	3.6	42.6	2.0	197.0
KM593055.1 *Culex declarator*	41.1	15.0	29.0	14.8	593.0	25.8	15.7	30.3	28.3	198.0	43.4	27.8	12.6	16.2	198.0	54.3	1.5	44.2	0.0	197.0
KM593051.1 *Culex declarator*	41.1	15.0	29.0	14.8	593.0	25.8	15.7	30.3	28.3	198.0	43.4	27.8	12.6	16.2	198.0	54.3	1.5	44.2	0.0	197.0
AB738145.1 *Aedes lineatopennis*	40.0	15.7	29.2	15.2	593.0	26.8	15.7	29.8	27.8	198.0	43.4	27.8	12.6	16.2	198.0	49.7	3.6	45.2	1.5	197.0
HQ398909.1 *Aedes lineatopennis*	39.6	16.2	29.2	15.0	593.0	26.8	15.7	29.8	27.8	198.0	43.4	27.8	12.6	16.2	198.0	48.7	5.1	45.2	1.0	197.0
KY694466.1 *Afidenta misera*	37.4	18.5	28.3	15.7	593.0	24.7	16.2	33.8	25.3	198.0	43.9	26.3	13.1	16.7	198.0	43.7	13.2	38.1	5.1	197.0
KC849092.1 *Nephila sumptuosa*	41.0	12.5	28.7	17.7	592.0	32.8	11.1	26.8	29.3	198.0	45.5	25.8	13.6	15.2	198.0	44.9	0.5	45.9	8.7	196.0
EF033298.1 *Lampsilis hydiana*	44.5	12.0	18.4	25.1	593.0	35.4	11.6	24.7	28.3	198.0	44.9	19.7	14.1	21.2	198.0	53.3	4.6	16.2	25.9	197.0
Avg.	40.6	15.2	28.3	16.0	592.9	27.7	14.7	29.7	28.0	198.0	43.7	26.9	12.8	16.5	198.0	50.4	3.9	42.4	3.3	196.9

Table 2.13d: The evolutionary divergence percentage between *Lutzia fuscanus* and its closely related species

Sl. No.	Accession Number	Organism	Percentage of Divergence
1	MK616587.1	*Lutzia fuscanus*	0.00%
2	KF407917.1	*Lutzia fuscanus*	0.17%
3	HQ398896.1	*Lutzia fuscanus*	0.17%
4	MK271004.1	*Lutzia chiangmaiensis*	1.36%
5	MK271005.1	*Lutzia chiangmaiensis*	1.36%
6	LC507833.1	*Lutzia tigripes*	4.28%
7	KU380351.1	*Lutzia tigripes*	4.65%
8	KM593055.1	*Culex declarator*	6.73%
9	KJ461784.1	*Culex declarator*	6.73%
10	AB738145.1	*Aedes lineatopennis*	14.79%
11	HQ398909.1	*Aedes lineatopennis*	15.52%
12	KY694466.1	*Afidenta misera*	33.91%
13	KC849092.1	*Nephila sumptuosa*	35.19%
14	EF033298.1	*Lampsilis hydiana*	90.74%

2.3.12.2 Discussion

These mosquitoes were regularly collected from polluted water like drainages, blocked canals, canal basins, etc. The blast result showed 99.13% similarity with KF407917.1 isolated from Pakistan and HQ398896.1 from Thailand. The nearest species of the same genus is *Lt. chiangmaiensis* (MK271004.1, MK271005.1), isolated from Thailand. Wang identified many mosquito species, including *Culex fuscanus*, using the molecular tool. NCBI Accession number for identified *Cx. fuscanus* was JQ728037 (Wang et al., 2012). Usually, in the phylogeny tree, the related species were aligned as the nearest relative in the same clade. In the evolutionary process, some other species are there to connect the *Lt. fuscanus*. *Lt. fuscanus'* COI sequence demonstrated notable variability compared to the other species examined in this study; as a result, it may be utilised as a DNA barcode for precise identification.

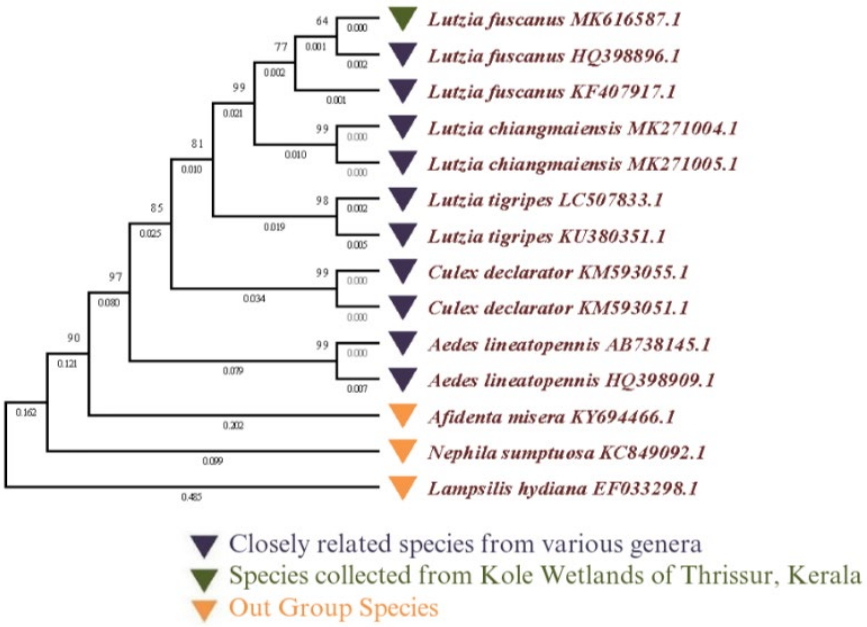

Figure 2.12c Phylogenetic tree of *Lutzia fuscanus*

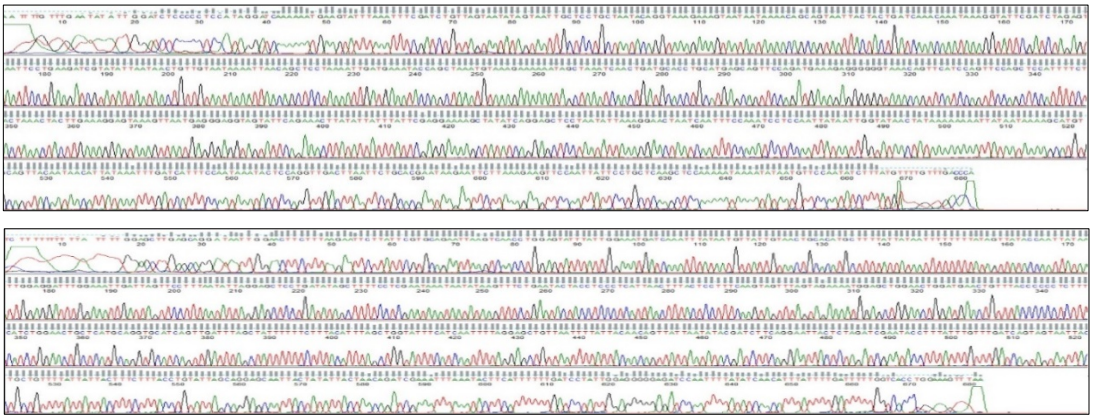

Figure 2.12d Electrophorogram showing the nucleotide sequence of mitochondrial CO I gene of *Lutzia fuscanus*

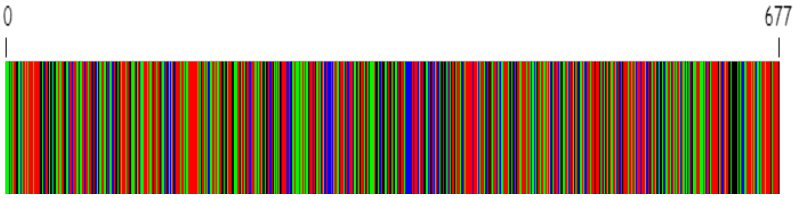

Figure 2.12e Molecular barcode of *Lutzia fuscanus*

2.3.13. Species Name: *Culex bitaeniorhynchus*

GenBank Accession Number: MT192883.1

Voucher Number: CDRL43

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Culicini
Genus	:	*Culex*
Subgenus	:	*Culex*
Species	:	*Culex bitaeniorhynchus*

Description

Cx. bitaeniorhynchus is a sizeable yellow mosquito whose body is covered with yellow and brown scales. Larvae of this mosquito were generally collected from marshlands with aquatic vegetation. Ornamented wings are one of the primary identifying characteristics of this species.

2.3.13.1 Result

The forward and reverse primers were used to amplify the Cx. *bitaeniorhynchus* mitochondrial cytochrome oxidase subunit I gene, collected from Kole wetlands of Thrissur, Kerala, were 5'-GGTCAACAAATCATAAAGA TATTGG-3' and 5'TAAACTTCAGGGTGACCAAAAAATCA-3' respectively. A single product with a length of 587 bp was produced by PCR amplifying *Cx. bitaeniorhynchus'* mitochondrial cytochrome oxidase subunit I (COI) gene

fragment. Accession No. MT192883.1 was obtained from the NCBI GenBank, and Figures 2.13a- 2.13e displayed the DNA sequence, corresponding translation product, phylogenetic tree, electrophorogram, and molecular barcode.

> MT192883.1 *Culex bitaeniorhynchus*|587bp

ATATTGGGAACTTCATTAAGTTTATTAATTCGAACTGAATTAAGTCATCCAGGAATATTCATTGGAAATGA
TCAAATTTATAATGTTATTGTAACTGCGCATGCATTTATTATAATTTTTTTTATAGTTATACCTATTATAA
TTGGAGGATTTGGAAATTGATTAGTTCCTCTAATATTAGGAGCCCCTGATATAGCATTCCCTCGAATAAAT
AATATAAGATTTTGAATACTTCCTCCTTCTTTAACTCTTCTTCTTTCTAGAAGAATGGTTGAAAATGGAGC
TGGTACTGGATGAACAGTTTACCCCCCTCTGTCATCTGGAACAGCACATGCAGGAGCTTCTGTTGATTTAG
CTATTTTTTCTCTTCATTTAGCTGGAATTTCTTCAATTCTTGGAGCTGTAAATTTTATTACTACAGTAATT
AATATACGATCTTCAGGAATTACTTTAGACCGAATACCTTTATTTGTTTGATCAGTAGTAATTACTGCTAT
TTTATTATTACTTTCGCTTCCAGTTTTAGCCGGGGCTATTACTATATTATTAACAGACCGAAATTTAAATA
CTTCATTCTTTGACCCAAT

Figure 2.13a: The DNA sequence of *Culex bitaeniorhynchus* COI gene

> MT192883.1 *Culex bitaeniorhynchus*

MLGTSLSLLIRTELSHPGMFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLMLGAPDMAFPRMN
NMSFWMLPPSLTLLLSSSMVENGAGTGWTVYPPLSSGTAHAGASVDLAIFSLHLAGISSILGAVNFITTVI
NMRSSGITLDRMPLFVWSVVITAILLLLSLPVLAGAITMLLTDRNLNTSFFDP

Figure 2.13b: The protein sequence of *Culex bitaeniorhynchus* COI gene

T=40.5%, C=14.9%, A=30.1%, and G=14.5% was the nucleotide composition of The COI sequence of *Cx. bitaeniorhynchus* showed bias to nucleotide AT. T=40.1%, C=15.7%, A=28.5% and G=15.6% were the average value of nucleotide composition of 14 nucleotides analysed (Table 2.14c). Base substitution rates between sequences, measured by the number of positions, were estimated using the maximum composite likelihood model and were shown in Table 2.14a. The patterns and rates substitution were computed under the Tamura-Nei model. A = 28.53%, T/U = 40.15%, C = 15.72%, and G = 15.60% were the nucleotide frequencies estimated through substitution matrix analysis. Tree topology was used in automatically computed ML value calculations. The value of maximum Log-likelihood for this estimation was -2725.601. Codon positions covered the first, second, and third noncoding regions. The final dataset of these 14 nucleotide

sequences analysis involved a total number of 538 positions. All these evolutionary analyses were conducted in MEGA X.

Table 2.14.d reveals the phylogenetic relationship of *Cx. bitaeniorhynchus*, with its closely related species showed 0.00% evolutionary divergence with *Cx. bitaeniorhynchus* (MT919715.1) isolated from Odisha, India, and 0.70% divergence with *Cx. bitaeniorhynchus* (MH881247.1) isolated from Thailand. The phylogenetic tree generated using the NJ method shows the phylogenetic position of *Cx. bitaeniorhynchus* isolated from Thrissur Kole lands, Kerala. Phylogenetically *Cx. bitaeniorhynchus* was the closest relative of *Cx. bitaeniorhynchus* (MT919715.1) isolated from Odisha, India. They are from the same clade.

Table 2.14a: The nucleotide substitution matrix estimate of COI gene sequence of *Culex bitaeniorhynchus*

From\To	A	T	C	G
A	-	10.4846	4.1051	7.9975
T	7.4509	-	7.0755	4.0739
C	7.4509	18.0712	-	4.0739
G	14.6269	10.4846	4.1051	-

Table 2.14b: The Transition/Transversion bias estimate of mitochondrial COI gene sequence of *Culex bitaeniorhynchus*

From\To	A	T	C	G
A	-	7.0729	7.0729	10.8542
T	7.0729	-	10.8542	7.0729
C	7.0729	10.8542	-	7.0729
G	10.8542	7.0729	7.0729	-

Table 2.14c: The nucleotide frequency comparison of *Culex bitaeniorhynchus* COI gene sequence with its kin species

Accession Number and Name of the Species	**Nucleotide Frequencies (%)**																		
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MT192883.1 *Culex bitaeniorhynchus*	40.5	14.9	30.1	14.5	538.0	44.4	28.3	12.8	14.4	180.0	49.7	2.2	46.9	1.1	179.0	27.4	14.0	30.7	27.9	179.0
MT919715.1 *Culex bitaeniorhynchus*	40.5	14.9	30.1	14.5	538.0	44.4	28.3	12.8	14.4	180.0	49.7	2.2	46.9	1.1	179.0	27.4	14.0	30.7	27.9	179.0
MH881247.1 *Culex bitaeniorhynchus*	40.0	15.4	29.9	14.7	538.0	44.4	28.3	12.8	14.4	180.0	47.5	3.9	46.9	1.7	179.0	27.9	14.0	30.2	27.9	179.0
MK713986.1 *Culex pipiens*	40.1	15.4	29.0	15.4	538.0	44.4	28.3	12.8	14.4	180.0	49.2	2.8	44.7	3.4	179.0	26.8	15.1	29.6	28.5	179.0
MK713985.1 *Culex pipiens*	40.1	15.4	29.0	15.4	538.0	44.4	28.3	12.8	14.4	180.0	49.2	2.8	44.7	3.4	179.0	26.8	15.1	29.6	28.5	179.0
MH745093.1 *Culex tritaeniorhynchus*	39.4	16.0	29.6	15.1	538.0	44.4	28.3	12.8	14.4	180.0	49.2	2.2	46.4	2.2	179.0	24.6	17.3	29.6	28.5	179.0
MH330220.1 *Culex tritaeniorhynchus*	39.2	16.2	29.7	14.9	538.0	44.4	28.3	12.8	14.4	180.0	49.2	2.2	46.9	1.7	179.0	24.0	17.9	29.6	28.5	179.0
KU495081.1 *Aedes aegypti*	39.8	16.9	28.3	15.1	538.0	44.4	28.9	12.8	13.9	180.0	48.0	6.1	41.9	3.9	179.0	26.8	15.6	30.2	27.4	179.0
MF443395.1 *Aedes aegypti*	39.8	16.9	28.3	15.1	538.0	44.4	28.9	12.8	13.9	180.0	48.0	6.1	41.9	3.9	179.0	26.8	15.6	30.2	27.4	179.0
LC473705.1 *Mansonia uniformis*	40.7	14.9	29.6	14.9	538.0	44.4	28.3	13.3	13.9	180.0	50.3	2.8	44.7	2.2	179.0	27.4	13.4	30.7	28.5	179.0
LC517293.1 *Mansonia uniformis*	40.7	14.9	29.6	14.9	538.0	44.4	28.3	13.3	13.9	180.0	50.3	2.8	44.7	2.2	179.0	27.4	13.4	30.7	28.5	179.0
KU578141.1 *Coccinella transversalis*	37.9	19.3	27.9	14.9	538.0	43.9	27.2	13.9	15.0	180.0	45.3	14.5	35.8	4.5	179.0	24.6	16.2	34.1	25.1	179.0
JF835944.1 *Nephila inaurata*	43.3	12.6	27.3	16.7	538.0	45.6	26.7	13.9	13.9	180.0	49.2	1.1	43.0	6.7	179.0	35.2	10.1	25.1	29.6	179.0
MN125137.1 *Pyganodon grandis*	40.0	16.4	21.2	22.5	538.0	43.9	23.9	14.4	17.8	180.0	46.4	10.6	24.0	19.0	179.0	29.6	14.5	25.1	30.7	179.0
Avg.	40.1	15.7	28.5	15.6	538.0	44.4	27.9	13.1	14.5	180.0	48.6	4.5	42.8	4.1	179.0	27.3	14.7	29.7	28.2	179.0

Table 2.14d: The evolutionary divergence percentage between *Culex bitaeniorhynchus* and its closely related species

Sl. No.	Accession Number	Organism	Percentage of Divergence
1	MT192883.1	*Culex bitaeniorhynchus*	0.00%
2	MT919715.1	*Culex bitaeniorhynchus*	0.00%
3	MH881247.1	*Culex bitaeniorhynchus*	0.70%
4	MK713986.1	*Culex pipiens*	5.06%
5	MK713985.1	*Culex pipiens*	5.06%
6	MH745093.1	*Culex tritaeniorhynchus*	4.52%
7	MH330220.1	*Culex tritaeniorhynchus*	4.52%
8	KU495081.1	*Aedes aegypti*	8.05%
9	MF443395.1	*Aedes aegypti*	8.05%
10	LC473705.1	*Mansonia uniformis*	9.37%
11	LC517293.1	*Mansonia uniformis*	9.37%
12	KU578141.1	*Coccinella transversalis*	16.52%
13	JF835944.1	*Nephila inaurata*	15.77%
14	MN125137.1	*Pyganodon grandis*	27.46%

2.3.13.2 Discussion

Cx. bitaeniorhynchusis a rare species occasionally collected from marshlands and water bodies with aquatic vegetation. This species is geographically distributed in India, Africa, Japan, and Australia. Morphological identification was made with available keys and online photographs as this species is a novel report in Kerala to the public databases. The present study provided a novel report to all databases, and its unique barcode can easily spot and analyse the phylogenetic position of this species based on DNA sequences. This study is a pioneer molecular work from Kerala, and the barcode generated can be used to spot the specimen easily and resolve its phylogeny. The mitochondrial genome is known to have evolved considerably faster than the nuclear gene; hence, it has many merits for predicting phylogenetic divergence. Also, nucleotide substitutions were lower in Nuclear DNA

than in mitochondrial DNA (Brown et al., 1979). The commonly used markers for resolving phylogeny from species to a family level were 16S and 28S and the COI gene. A nucleotide sequence with the Accession number KX524976 has been submitted to the NCBI GenBank. Phylogenetically related *Cx. bitaeniorhynchusis* (KF687363.1) isolated from China.

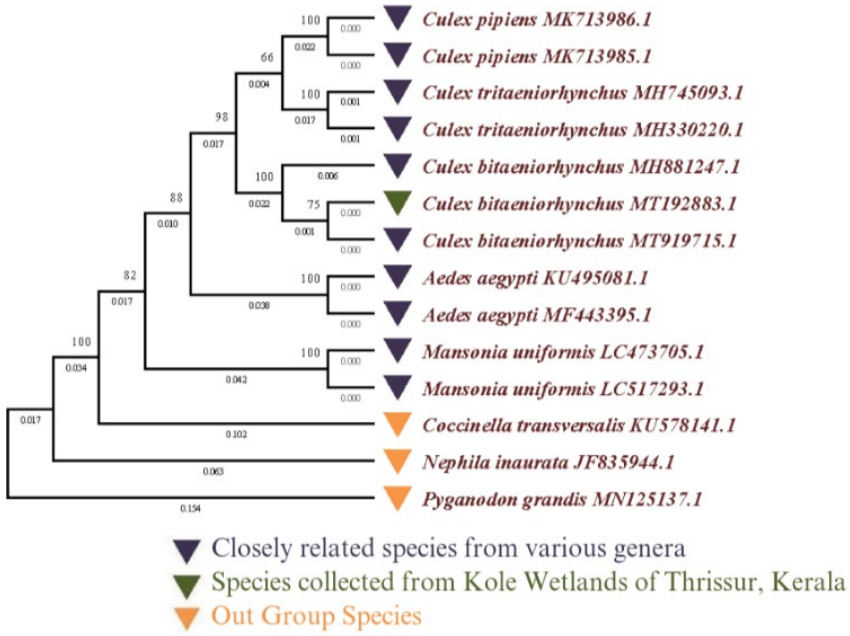

Figure 2.13c Phylogenetic tree of *Culex bitaeniorhynchus*

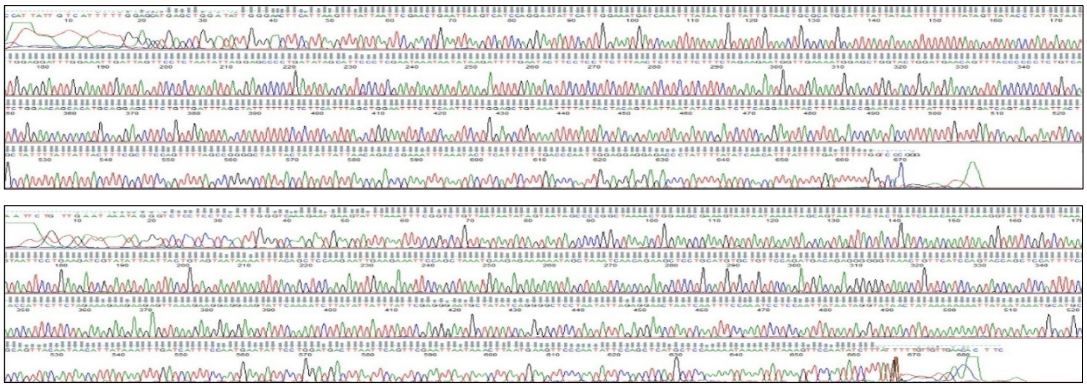

Figure 2.13d Electrophorogram showing the nucleotide sequence of mitochondrial COI gene of *Culex bitaeniorhynchus*

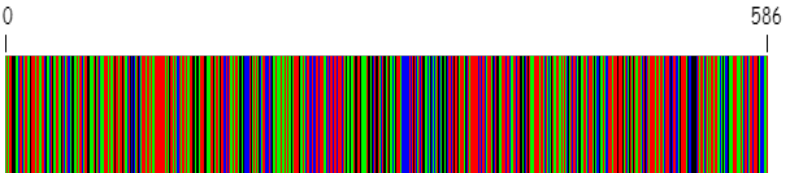

Figure 2.13e Molecular barcode of *Culex bitaeniorhynchus*

2.3.14. Species Name: *Culex gelidus*

GenBank Accession Number: MK724071.1

Voucher Number: CDRL17

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Culicini
Genus	:	*Culex*
Subgenus	:	*Culex*
Species	:	*Culex gelidus*

Description

Dark-coloured, medium-sized *Culex* mosquito. The white scale patches that cover the whole mesonotum region are this species' most distinctive characteristic. Breeding habitats of these mosquitoes vary from freshwater to polluted water bodies.

2.3.14.1 Result

The forward and reverse primers were used to amplify the mitochondrial cytochrome oxidase subunit I gene of *Cx. gelidus,* collected from Kole wetlands of

Thrissur, Kerala, were 5'-GGTCAACAAATCATAAAGATATTGG-3' and 5'TAAACTTCAG GGTGACCAAAAAATCA-3' respectively. A single product with a length of 678b bp was produced by PCR amplifying *Cx.*

> MK724071.1 *Culex gelidus*|678bp

AAGATATTGGAACATTATATTTTATTTTTGGGGCTTGAGCAGGAATAATTGGAACTTCATTAAGAATTCTAAT
TCGAGCAGAACTAAGTCAGCCTGGAGTATTTATTGGAAATGATCAAATTTATAATGTTATTGTAACTGCTCAC
GCTTTTATTATAATTTTTTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAATTGATTAGTTCCTTTAA
TACTAGGAGCTCCTGATATAGCATTTCCTCGAATAAATAATATAAGTTTTTGAATACTTCCTCCTTCATTAAC
TTTACTACTTTCAAGTAGTTTAGTTGAAAATGGGGCTGGAACTGGATGAACAGTTTATCCCCCTCTTTCATCA
GGTACAGCTCATGCTGGAGCTTCAGTTGATTTAGCTATTTTTTCTTTACATTTAGCTGGGATTTCATCAATTT
TAGGAGCAGTAAATTTTATTACAACAGTAATTAATATACGATCTTCAGGAATTACACTTGATCGAATACCTTT
ATTTGTTTGATCTGTAGTTATTACTGCTGTTTTATTACTCCTTTCATTACCCGTATTAGCCGGAGCTATTACA
ATATTATTAACTGATCGAAACCTAAATACTTCATTTTTTGACCCTATTGGAGGAGGAGATCCTATTTTATACC
AACATTTATTTTGATTTTTTG

Figure 2.14a: The DNA sequence of *Culex gelidus* COI gene

> MK724071.1 *Culex gelidus*

MGTLYFIFGAWAGMIGTSLSILIRAELSQPGVFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLML
GAPDMAFPRMNNMSFWMLPPSLTLLLSSSLVENGAGTGWTVYPPLSSGTAHAGASVDLAIFSLHLAGISSILG
AVNFITTVINMRSSGITLDRMPLFVWSVVITAVLLLLSLPVLAGAITMLLTDRNLNTSFFDPIGGGDPILYQH
LFWFF

Figure 2.14b: The protein sequence of *Culex gelidus* COI gene

The BLAST search revealed the partial COI nucleotide sequence of *Cx. gelidus* isolated from the Kole wetlands of Thrissur, Kerala, is 99.65% like *Cx. gelidus* isolated from Irinjalakuda, Thrissur, Kerala (MK630238.1). *Cx. gelidus* isolated from the Kole wetlands of Thrissur, Kerala, showed 0.70% evolutionary divergence with *Cx. gelidus* isolated from Sri Lanka (MH330217.1) and 5.34% evolutionary divergence with *Cx. declarator* isolated from Colombia (KM593051.1) (Table 2.15d). The average nucleotide composition throughout the species is T=39.3%; C=15.7%; A=28.3%; G=16.7% (Table 2.15c). The estimate of the Substitution Matrix showed the probability of substitution from one base to another base (Table 2.15a). The estimation of substitution patterns and rates was done with the Tamura-Nei model. The values of different transitional and transversional bias shown in Table 2.15b. A = 28.32%, T/U = 39.28%, C = 15.66%,

and G = 16.74% were the nucleotide frequencies estimated through transitional and transversionsal bias analysis. Tree topology was used in automatically computed ML value calculations. The value of the maximum Log-likelihood for this estimation was -3214.878. Codon positions covered the first, second, and third noncoding regions. The final dataset of these 14 nucleotide sequences analysis involved a total number of 573 positions. All these evolutionary analyses were conducted in MEGA X.

The phylogenetic tree was generated using the NJ method. The position of *Cx. gelidus*, a species isolated from the Kole wetlands in Thrissur, Kerala, is depicted on the evolutionary tree. The phylogenetic tree showed the closest relatives of *Cx. gelidus,* isolated from Irinjalakuda, Thrissur, Kerala (Accession No. MK630238.1). The closest relatives of *Cx. gelidus* isolated from different populations were arranged in a single clade.

Table 2.15a: The nucleotide substitution matrix estimate of COI gene sequence of *Culex gelidus*

From\To	A	T	C	G
A	-	10.1156	4.0321	7.8931
T	7.2938	-	7.7657	4.3114
C	7.2938	19.4824	-	4.3114
G	13.3530	10.1156	4.0321	-

Table 2.15b: The Transition/Transversion bias estimate of mitochondrial COI gene sequence of *Culex gelidus*

From\To	A	T	C	G
A	-	6.9246	6.9246	11.1509
T	6.9246	-	11.1509	6.9246
C	6.9246	11.1509	-	6.9246
G	11.1509	6.9246	6.9246	-

Table 2.15c: The nucleotide frequency comparison of *Culex gelidus* COI gene sequence with its kin species

Accession Number and Name of the Species	**Nucleotide Frequencies (%)**																		
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MK724071.1 *Culex gelidus*	39.4	16.1	28.6	15.9	573.0	42.9	27.2	13.6	16.2	191.0	50.8	3.7	44.0	1.6	191.0	24.6	17.3	28.3	29.8	191.0
MK630238.1 *Culex gelidus*	39.6	15.9	28.8	15.7	573.0	42.9	27.2	13.6	16.2	191.0	51.3	3.1	44.5	1.0	191.0	24.6	17.3	28.3	29.8	191.0
MH330217.1 *Culex gelidus*	39.6	15.9	28.8	15.7	573.0	42.9	27.2	13.6	16.2	191.0	51.3	3.1	44.5	1.0	191.0	24.6	17.3	28.3	29.8	191.0
KM593055.1 *Culex declarator*	40.5	15.0	29.1	15.4	573.0	42.9	27.2	13.6	16.2	191.0	53.4	1.6	45.0	0.0	191.0	25.1	16.2	28.8	29.8	191.0
KM593051.1 *Culex declarator*	40.5	15.0	29.1	15.4	573.0	42.9	27.2	13.6	16.2	191.0	53.4	1.6	45.0	0.0	191.0	25.1	16.2	28.8	29.8	191.0
MN793302.1 *Culex dolosus*	39.6	15.5	28.8	16.1	573.0	42.9	27.2	13.6	16.2	191.0	50.8	3.1	44.0	2.1	191.0	25.1	16.2	28.8	29.8	191.0
MN793283.1 *Culex dolosus*	39.4	15.5	29.1	15.9	573.0	42.9	27.2	13.6	16.2	191.0	50.3	3.1	45.0	1.6	191.0	25.1	16.2	28.8	29.8	191.0
KJ461792.1 *Anopheles subpictus*	37.7	15.7	28.8	17.8	573.0	42.4	27.7	13.6	16.2	191.0	44.5	4.7	44.5	6.3	191.0	26.2	14.7	28.3	30.9	191.0
KJ461784.1 *Anopheles subpictus*	37.3	16.1	29.1	17.5	573.0	42.4	27.7	13.6	16.2	191.0	44.5	4.7	45.5	5.2	191.0	25.1	15.7	28.3	30.9	191.0
KJ768160.1 *Mansonia uniformis*	38.6	16.1	29.7	15.7	573.0	43.5	26.7	14.1	15.7	191.0	46.6	6.3	45.0	2.1	191.0	25.7	15.2	29.8	29.3	191.0
LC517293.1 *Mansonia uniformis*	39.4	14.7	30.2	15.7	573.0	43.5	26.7	14.1	15.7	191.0	48.7	2.6	46.6	2.1	191.0	26.2	14.7	29.8	29.3	191.0
KU578141.1 *Coccinella transversalis*	36.8	19.0	28.1	16.1	573.0	42.9	26.2	14.1	16.8	191.0	43.5	14.1	37.7	4.7	191.0	24.1	16.8	32.5	26.7	191.0
JF835944.1 *Nephila inaurata*	42.2	12.7	27.2	17.8	573.0	44.5	25.1	14.7	15.7	191.0	49.2	1.0	42.9	6.8	191.0	33.0	12.0	24.1	30.9	191.0
MN125137.1 *Pyganodon grandis*	39.1	16.1	20.9	23.9	573.0	42.9	22.5	14.7	19.9	191.0	46.1	9.9	24.1	19.9	191.0	28.3	15.7	24.1	31.9	191.0
Avg.	39.3	15.7	28.3	16.7	573.0	43.0	26.7	13.9	16.4	191.0	48.9	4.5	42.7	3.9	191.0	25.9	15.8	28.3	29.9	191.0

Table 2.15d: The evolutionary divergence percentage between *Culex gelidus* and its closely related species

Sl. No.	Accession Number	Organism	Percentage of Divergence
1	MK724071.1	*Culex gelidus*	0.00%
2	MK630238.1	*Culex gelidus*	0.35%
3	MH330217.1	*Culex gelidus*	0.70%
4	KM593055.1	*Culex declarator*	5.34%
5	KM593051.1	*Culex declarator*	5.34%
6	MN793302.1	*Culex dolosus*	6.14%
7	MN793283.1	*Culex dolosus*	6.12%
8	KJ461792.1	*Anopheles subpictus*	16.79%
9	KJ461784.1	*Anopheles subpictus*	18.51%
10	KJ768160.1	*Mansonia uniformis*	15.73%
11	LC517293.1	*Mansonia uniformis*	15.36%
12	KU578141.1	*Coccinella transversalis*	32.62%
13	JF835944.1	*Nephila inaurata*	33.27%
14	MN125137.1	*Pyganodon grandis*	57.66%

2.3.14.2 Discussion

Cx. gelidus breeds in diverse aquatic habitats, varying from freshwater to polluted water. Wang *et al.,* in identified 122 mosquito species that were isolated from China. The sequence of *Cx. gelidus* submitted to NCBI GenBank and achieved Accession Number JQ728366. The accession number of *Cx. gelidus* isolated from the Kole wetlands of Thrissur, Kerala, is MK724071.1. It is a pioneer work in Kerala, so the study provided a novel report to all databases. Its unique barcode can easily spot and analyse the phylogenetic position of this species based on DNA sequences. Morphological identification was made with available keys and online photographs as this species is a novel report in Kerala to the public databases. Weeraratne et al., 2018 isolated and identified *Cx. gelidus* in Sri Lanka. The accession number obtained was KY053491. In their phylogenetic analysis *Cx. gelidus* showed some closest relation with *Cx. whitemorei*, and *Cx. quinquefasciatus*. In the present

phylogenetic analysis *Cx. gelidus* exhibit a neighbouring relationship with *Cx. declarator* and *Cx. dolosus*.

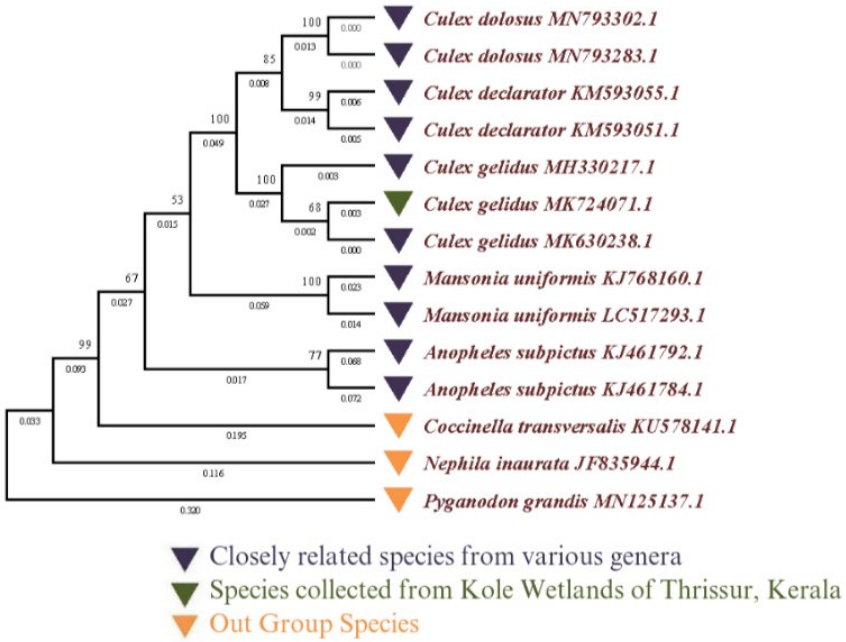

Figure 2.14c Phylogenetic tree of *Culex gelidus*

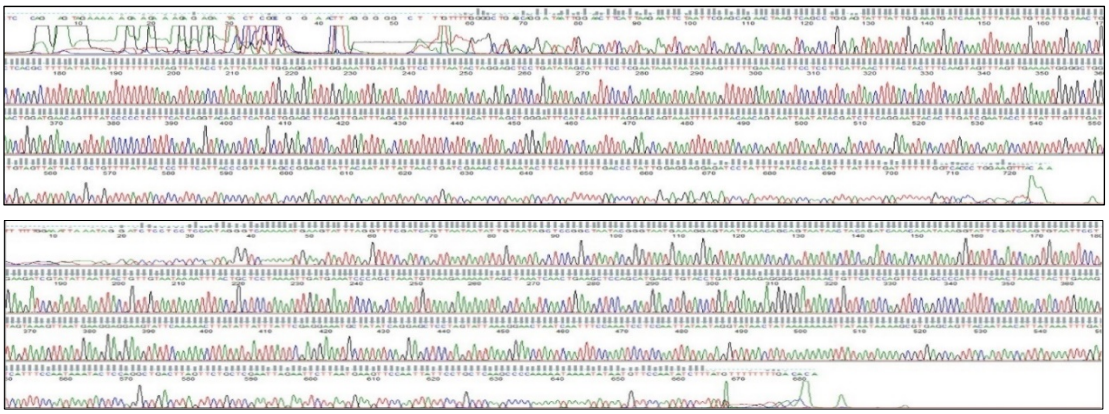

Figure 2.14d Electrophorogram showing the nucleotide sequence of mitochondrial COI gene of *Culex gelidus*

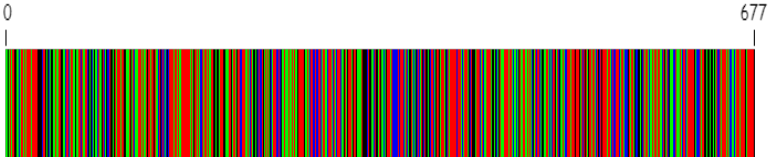

Figure 2.14e Molecular barcode of *Culex gelidus*

2.3.15. Species Name: *Culex tritaeniorhynchus*

GenBank Accession Number: MK861440.1

Voucher Number: CDRL03

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Culicini
Genus	:	*Culex*
Subgenus	:	*Culex*
Species	:	*Culex tritaeniorhynchus*

Description

The large, brown *Cx. tritaeniorhynchus* mosquito has black thoracic scales, and the ventral portion of the abdomen is covered with white scales. Larvae of these species are regularly collected from polluted water bodies like blocked drainages, canal basins, etc.

2.3.15.1 Result

The forward and reverse primers were used to amplify the mitochondrial cytochrome oxidase subunit I gene of *Cx. tritaeniorhynchus,* collected from Kole wetlands of Thrissur, Kerala, were 5'-GGTCAACAAATCATAAAGATATTGG-3' and 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' respectively. A single product with a length of 679 bp was produced by PCR amplifying *Cx. tritaeniorhynchus'* mitochondrial cytochrome oxidase subunit I (COI) gene

fragment. Accession No. MK861440.1 was obtained from the NCBI GenBank, and Figures 2.15a- 2.15e displayed the DNA sequence, corresponding translation product, phylogenetic tree, electrophorogram, and molecular barcode.

> MK861440.1 *Culex tritaeniorhynchus*|679bp

AAAGATATTGGAACATTATATTTTATTTTTGGGGCTTGAGCTGGAATAGTAGGTACTTCTTTAAGTATTTT
AATTCGAGCAGAATTAAGTCAACCCGGAGTATTTATTGGAAATGATCAAATTTATAATGTTATTGTAACTG
CTCATGCTTTTATTATAATTTTTTTTATAGTAATACCAATTATAATTGGTGGATTTGGAAATTGATTAGTT
CCTTTAATACTTGGAGCTCCTGATATAGCCTTTCCACGAATAAATAATATAAGTTTTTGAATACTACCTCC
TTCATTAACTCTACTACTTTCAAGTAGTTTAGTAGAAAATGGAGCTGGAACTGGATGAACAGTTTATCCAC
CTCTATCATCTGGAACAGCGCATGCTGGAGCTTCAGTTGATTTAGCTATTTTTTCTTTACATTTAGCTGGG
ATTTCATCAATTTTAGGGGCAGTAAATTTTATTACAACAGTAATTAATATACGATCTTCAGGAATTACACT
TGATCGAATACCTTTATTTGTTTGATCAGTAGTAATTACTGCTGTTTTATTACTTCTTTCACTACCAGTTT
TAGCAGGAGCTATTACTATACTATTAACAGATCGAAATCTTAATACTTCATTCTTTGACCCAATTGGAGGA
GGAGACCCAATTCTTTATCAACACTTATTCTGATTTTTTG

Figure 2.15a: The DNA sequence of *Culex tritaeniorhynchus* COI gene

> MK861440.1 *Culex tritaeniorhynchus*

MGTLYFIFGAWAGMVGTSLSILIRAELSQPGVFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPL
MLGAPDMAFPRMNNMSFWMLPPSLTLLLSSSLVENGAGTGWTVYPPLSSGTAHAGASVDLAIFSLHLAGIS
SILGAVNFITTVINMRSSGITLDRMPLFVWSVVITAVLLLLSLPVLAGAITMLLTDRNLNTSFFDPIGGGD
PILYQHLFWFF

Figure 2.15b: The protein sequence of *Culex tritaeniorhynchus* COI gene

T=38.8%, C=16.2%, A=29.9%, and G=15.1% was the nucleotide composition of The COI sequence of *Cx. tritaeniorhynchus* showed bias to nucleotide AT. T=40.0%, C=15.6%, A=28.4% and G=16.1% were the average value of nucleotide composition of 14 nucleotides analysed (Table 2.14c). Table 2.16b displayed the rates of various transitional substitutions as well as those of transitional replacements. Transition/Transversion bias (R) is calculated to be 0.74. When assessing instantaneous r, relative values should be taken into account. For the sake of simplicity, the sum of r values is set to 100, A = 28.38%, T/U = 39.98%, C = 15.57%, and G = 16.06% are the nucleotide frequencies. A tree topology was automatically generated to predict ML values (Table.2.16a). The contained codons were arranged in the following order: first, second, third, and noncoding. Data gaps

and missing areas have all been eliminated. The complete dataset has 556 locations in total.

Table 2.16d reveals that the *Cx. tritaeniorhynchus* shows 0.54% evolutionary divergence with *Cx. tritaeniorhynchus* (MH330220.1) isolated from Sri Lanka. *Cx. declarator* (KM593055.1) species showed 6.20% of evolutionary divergence with *Cx. tritaeniorhynchus* isolated from Kerala. The phylogenetic tree generated using the NJ method shows the phylogenetic position of *Cx. tritaeniorhynchus* isolated from Thrissur Kole lands, Kerala. Phylogenetically *Cx. tritaeniorhynchus* collected during the present study showed maximum similarity with the same species having Accession no: MH330220.1 isolated from Sri Lanka. *Cx. declarator* (KM593055.1) is the nearest relative of these two species, which arose from the same clade in the phylogenetic tree.

Table 2.16a: The nucleotide substitution matrix estimate of COI gene sequence of *Culex tritaeniorhynchus*

From\To	A	T	C	G
A	-	10.6633	4.1529	8.7411
T	7.5692	-	6.2995	4.2832
C	7.5692	16.1750	-	4.2832
G	15.4472	10.6633	4.1529	-

Table 2.16b: The Transition/Transversion bias estimate of mitochondrial COI gene sequence of *Culex tritaeniorhynchus*

From\To	A	T	C	G
A	-	7.2031	7.2031	10.5939
T	7.2031	-	10.5939	7.2031
C	7.2031	10.5939	-	7.2031
G	10.5939	7.2031	7.2031	-

Table 2.16c: The nucleotide frequency comparison of *Culex tritaeniorhynchus* COI gene sequence with its kin species

Accession Number and Name of the Species	**Nucleotide Frequencies (%)**																		
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MK861440.1 *Culex tritaeniorhynchus*	38.8	16.2	29.9	15.1	556.0	43.5	28.0	13.4	15.1	186.0	48.6	2.2	47.6	1.6	185.0	24.3	18.4	28.6	28.6	185.0
MH330220.1 *Culex tritaeniorhynchus*	38.7	16.4	29.9	15.1	556.0	43.5	28.0	13.4	15.1	186.0	48.6	2.2	47.6	1.6	185.0	23.8	18.9	28.6	28.6	185.0
MH330219.1 *Culex tritaeniorhynchus*	38.7	16.4	29.7	15.3	556.0	43.5	28.0	13.4	15.1	186.0	48.6	2.2	47.0	2.2	185.0	23.8	18.9	28.6	28.6	185.0
KM593055.1 *Culex declarator*	41.2	15.3	29.0	14.6	556.0	43.5	28.0	13.4	15.1	186.0	54.1	1.6	44.3	0.0	185.0	25.9	16.2	29.2	28.6	185.0
KM593051.1 *Culex declarator*	41.2	15.3	29.0	14.6	556.0	43.5	28.0	13.4	15.1	186.0	54.1	1.6	44.3	0.0	185.0	25.9	16.2	29.2	28.6	185.0
MN793302.1 *Culex dolosus*	40.1	15.8	28.8	15.3	556.0	43.5	28.0	13.4	15.1	186.0	50.8	3.2	43.8	2.2	185.0	25.9	16.2	29.2	28.6	185.0
MN793283.1 *Culex dolosus*	40.1	15.6	29.1	15.1	556.0	43.5	28.0	13.4	15.1	186.0	50.8	2.7	44.9	1.6	185.0	25.9	16.2	29.2	28.6	185.0
KJ461792.1 *Anopheles subpictus*	38.7	15.6	29.0	16.7	556.0	43.5	28.0	13.4	15.1	186.0	45.4	4.3	44.3	5.9	185.0	27.0	14.6	29.2	29.2	185.0
KJ461784.1 *Anopheles subpictus*	38.1	16.2	29.1	16.5	556.0	43.5	28.0	13.4	15.1	186.0	44.9	4.9	44.9	5.4	185.0	25.9	15.7	29.2	29.2	185.0
KJ768160.1 *Mansonia uniformis*	39.2	16.4	29.5	14.9	556.0	44.1	27.4	14.0	14.5	186.0	47.0	6.5	44.3	2.2	185.0	26.5	15.1	30.3	28.1	185.0
LC517293.1 *Mansonia uniformis*	40.1	14.9	30.0	14.9	556.0	44.1	27.4	14.0	14.5	186.0	49.2	2.7	45.9	2.2	185.0	27.0	14.6	30.3	28.1	185.0
KY694466.1 *Afidenta misera*	38.1	18.7	28.1	15.1	556.0	44.1	26.3	14.0	15.6	186.0	45.4	13.0	36.8	4.9	185.0	24.9	16.8	33.5	24.9	185.0
KC849092.1 *Nephila sumptuosa*	42.0	12.6	27.9	17.5	555.0	45.7	25.3	14.5	14.5	186.0	46.7	0.5	43.5	9.2	184.0	33.5	11.9	25.9	28.6	185.0
EF033298.1 Lampsilis hydiana	44.8	12.6	18.5	24.1	556.0	44.6	20.4	15.1	19.9	186.0	55.7	4.9	15.1	24.3	185.0	34.1	12.4	25.4	28.1	185.0
Avg.	40.0	15.6	28.4	16.1	555.9	43.9	27.0	13.7	15.3	186.0	49.3	3.7	42.4	4.5	184.9	26.8	15.9	29.0	28.3	185.0

Table 2.16d: The evolutionary divergence percentage between *Culex tritaeniorhynchus* and its closely related species

Sl. No.	Accession Number	Organism	Percentage of Divergence
1	MK861440.1	*Culex tritaeniorhynchus*	0.00%
2	MH330220.1	*Culex tritaeniorhynchu*	0.54%
3	MH330219.1	*Culex tritaeniorhynchus*	0.72%
4	KM593055.1	*Culex declarator*	6.20%
5	KM593051.1	*Culex declarator*	6.20%
6	MN793302.1	*Culex dolosus*	7.30%
7	MN793283.1	*Culex dolosus*	7.31%
8	KJ461792.1	*Anopheles subpictus*	18.88%
9	KJ461784.1	*Anopheles subpictus*	18.32%
10	KJ768160.1	*Mansonia uniformis*	18.68%
11	LC517293.1	*Mansonia uniformis*	16.88%
12	KY694466.1	*Afidenta misera*	37.90%
13	KC849092.1	*Nephila sumptuosa*	35.06%
14	EF033298.1	*Lampsilis hydiana*	77.39%

2.3.15.2 Discussion

Cx. tritaeniorhynchus is a sizeable brown mosquito with white scales on the ventral part of the abdomen. It is a significant vector of filariasis, JE, etc., commonly found in highly polluted water bodies. *Cx. tritaeniorhynchus*, isolated from Kole wetlands in Thrissur, Kerala, is a novel one and gets an Accession number from NCBI GenBank as MK861440.1. This identical database and molecular barcode may be helpful in future vector management programs in that area. In 11 Turkish provinces, a comprehensive systematic approach using morphological and genetic methods was used to study Turkish vector species. They selected a 658 bp length COI gene as a molecular marker. They sequence *Cx. tritaeniorhynchus*, along with 13 other mosquito species. Accession numbers of *Cx. tritaeniorhynchus* obtained from NCBI GenBank was KJ012243–250 and showed similarity with 99% *Cx. tritaeniorhynchus* AB738194, 99% *Cx. tritaeniorhynchus* AB738269 Japan (Gunay

et al., 2015). The present study discussed that *Cx. tritaeniorhynchus* isolated from Thrissur Kole lands shows 99.46% similarity with the same species isolated from Sri Lanka (MH330220.1).

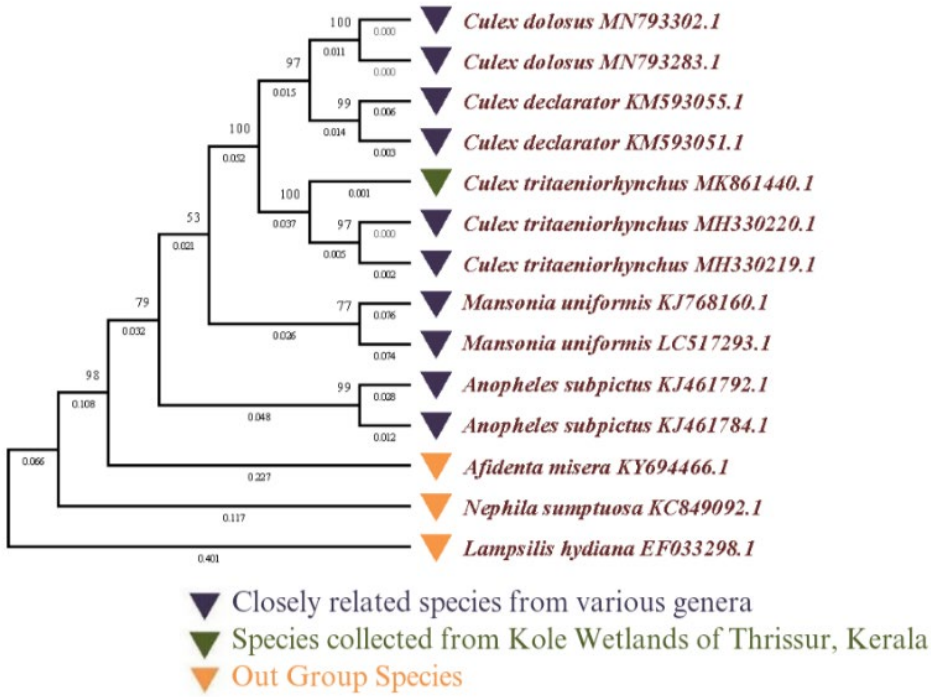

Figure 2.15c Phylogenetic tree of *Culex tritaeniorhynchus*

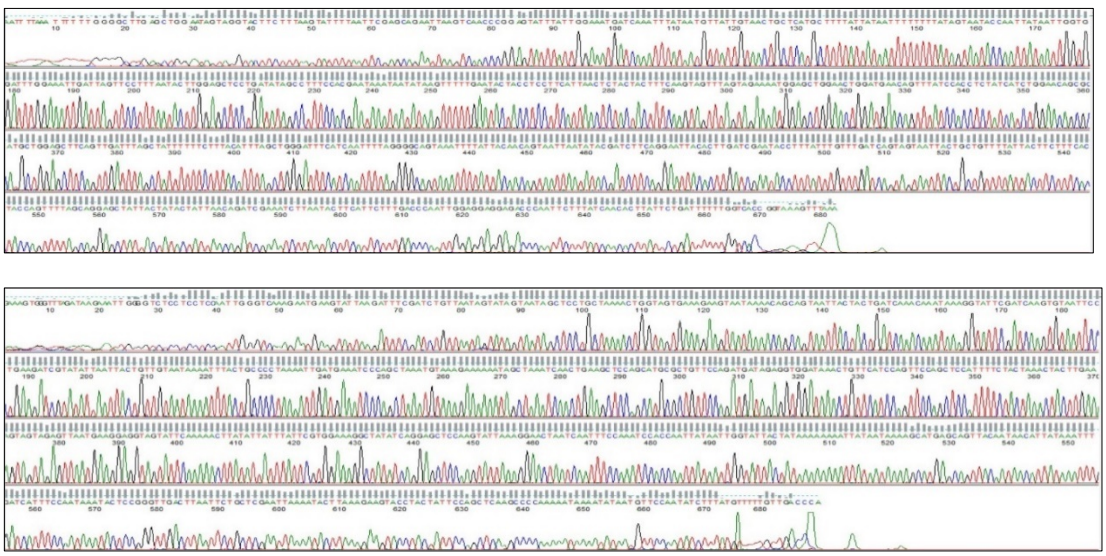

Figure 2.15d Electrophorogram showing the nucleotide sequence of mitochondrial COI gene of *Culex tritaeniorhynchus*

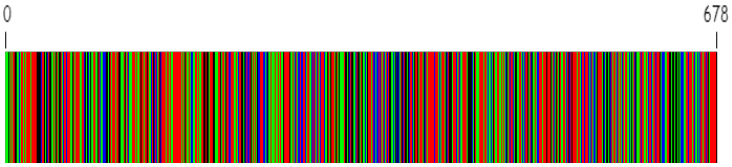

Figure 2.15e Molecular barcode of *Culex tritaeniorhynchus*

2.3.16. Species Name: *Culex pipiens*

GenBank Accession Number: MK603829.1

Voucher Number: CDRL04

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Culicini
Genus	:	*Culex*
Subgenus	:	*Culex*
Species	:	*Culex pipiens*

Description

Cx. pipiens is a medium-sized yellow mosquito. Golden yellow scales cover several parts of the body, and some white patches are also present in some areas, like the lower abdomen and posterior thoracic region. Legs and wings have no ornamentation; only dark scales are seen on them.

2.3.16.1 Result

The forward and reverse primers were used to amplify the mitochondrial cytochrome oxidase subunit I gene of *Cx. pipiens,* collected from Kole wetlands of Thrissur, Kerala, were 5'-GGTCAACAAATCATAAAGATATTGG-3' and 5'-

TAAACTTCA GGGTGACCAAAAAATCA-3' respectively. A single product with a length of 678 bp was produced by PCR amplifying *Cx. pipiens'* mitochondrial cytochrome oxidase subunit I (COI) gene fragment. Accession No. MK603829.1 was obtained from the NCBI GenBank, and Figures 2.16a- 2.16e displayed the DNA sequence, corresponding translation product, phylogenetic tree, electrophorogram, and molecular barcode.

> MK603829.1 *Culex pipiens*|678bp

AAGATATTGGAACATTATATTTTATTTTTGGAGCTTGAGCTGGAATAGTTGGGACTTCTTTAAGTTTACTAA
TTCGAGCAGAATTAAGTCAACCAGGTGTATTTATTGGAAATGATCAAATTTATAATGTTATTGTAACTGCTC
ATGCTTTTATTATAATTTTTTTTATAGTAATACCAATCATAATTGGAGGATTTGGAAATTGATTAGTTCCTT
TAATGTTAGGAGCTCCAGATATGGCCTTTCCTCGAATAAATAATATAAGTTTTTGAATACTACCTCCTTCAT
TGACACTACTACTTTCAAGTAGTTTAGTAGAAAATGGAGCTGGGACTGGATGAACAGTGTATCCCCCTCTTT
CATCTGGAACAGCTCATGCTGGAGCTTCAGTAGACTTAGCTATTTTTTCTTTACATTTAGCAGGAATTTCAT
CAATTTTAGGTGCAGTAAATTTTATTACAACAGTAATTAATATACGATCTTCAGGAATTACTCTTGATCGAA
TACCTTTATTTGTTTGATCAGTAGTAATTACTGCAGTTTTATTACTTCTTTCTTTACCTGTTTTAGCTGGTG
CTATTACTATGTTATTAACAGATCGAAATTTAAATACTTCATTCTTTGATCCAATTGGAGGAGGAGATCCAA
TTTTATATCAACATTTATTTTGATTTTTTG

Figure 2.16a: The DNA sequence of *Culex pipiens* COI gene

> MK603829.1 *Culex pipiens*

MGTLYFIFGAWAGMVGTSLSLLIRAELSQPGVFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLM
LGAPDMAFPRMNNMSFWMLPPSLTLLLSSSLVENGAGTGWTVYPPLSSGTAHAGASVDLAIFSLHLAGISSI
LGAVNFITTVINMRSSGITLDRMPLFVWSVVITAVLLLLSLPVLAGAITMLLTDRNLNTSFFDPIGGGDPIL
YQHLFWFF

Figure 2.16b: The protein sequence of *Culex pipiens* COI gene

T=39.5%, C=15.6%, A=29.3%, and G=15.6% was the nucleotide composition of The COI sequence of *Cx. pipiens* showed bias to nucleotide AT. T=39.5%, C=15.9%, A=28.5% and G=16% were the average value of nucleotide composition of 14 nucleotides analysed (Table 2.17c). Maximum Composite Likelihood model, used in estimating the frequency of base substitutions per site between sequences displayed in Table 2.17a. The patterns and rates substitution were computed under the Tamura-Nei model. A = 28.53%, T/U = 39.52%, C = 15.95%, and G = 16.00%

were the nucleotide frequencies estimated through substitution matrix analysis. Tree topology was used in automatically computed ML value calculations. The value of the maximum Log-likelihood for this estimation was -3193.712. Codon positions comprised of 1st+2nd+3rd+Noncoding. The final dataset of these 14 nucleotide sequences analysis involved a total number of 559 positions. All these evolutionary analyses were conducted in MEGA X.

Table 2.17d reveals that the *Cx. pipiens* from Kerala showed 0.00% evolutionary divergence with *Cx. pipiens* (MK714012.1, MK714001.1) isolated from Turkey and 8.36% evolutionary divergence with *Cx. corniger* (MT999314.1, MT999239.1) isolated from Mexico. The phylogenetic tree generated using the NJ method shows the phylogenetic position of *Cx. pipiens* isolated from the Kole wetlands of Thrissur, Kerala. Phylogenetically *Culex pipiens* showed to be the closest relatives of *Cx. pipiens* (MK714012.1, MK714001.1) of Turkey.

Table 2.17a: The nucleotide substitution matrix estimate of COI gene sequence of *Culex pipiens*

From\To	A	T	C	G
A	-	10.3883	4.1916	8.0862
T	7.4998	-	7.1650	4.2050
C	7.4998	17.7574	-	4.2050
G	14.4221	10.3883	4.1916	-

Table 2.17b: The Transition/Transversion bias estimate of mitochondrial COI gene sequence of *Culex pipiens*

From\To	A	T	C	G
A	-	7.0633	7.0633	10.8735
T	7.0633	-	10.8735	7.0633
C	7.0633	10.8735	-	7.0633
G	10.8735	7.0633	7.0633	-

Table 2.17c: The nucleotide frequency comparison of *Culex pipiens* COI gene sequence with its kin species

Accession Number and Name of the Species	**Nucleotide Frequencies (%)**																		
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MK603829.1 *Culex pipiens*	39.5	15.6	29.3	15.6	559.0	43.9	27.8	13.4	15.0	187.0	48.4	2.7	45.7	3.2	186.0	26.3	16.1	29.0	28.5	186.0
MK714012.1 *Culex pipiens*	39.5	15.6	29.3	15.6	559.0	43.9	27.8	13.4	15.0	187.0	48.4	2.7	45.7	3.2	186.0	26.3	16.1	29.0	28.5	186.0
MK714001.1 *Culex pipiens*	39.5	15.6	29.3	15.6	559.0	43.9	27.8	13.4	15.0	187.0	48.4	2.7	45.7	3.2	186.0	26.3	16.1	29.0	28.5	186.0
MT999314.1 *Culex corniger*	39.2	16.5	29.7	14.7	559.0	43.9	27.8	13.4	15.0	187.0	48.9	4.3	45.7	1.1	186.0	24.7	17.2	30.1	28.0	186.0
MT999239.1 *Culex corniger*	39.2	16.5	29.7	14.7	559.0	43.9	27.8	13.4	15.0	187.0	48.9	4.3	45.7	1.1	186.0	24.7	17.2	30.1	28.0	186.0
LC054470.1 *Culex orientalis*	39.9	16.1	29.2	14.8	559.0	43.9	27.8	13.4	15.0	187.0	50.5	3.2	45.2	1.1	186.0	25.3	17.2	29.0	28.5	186.0
LC054469.1 *Culex orientalis*	40.1	15.9	29.3	14.7	559.0	43.9	27.8	13.4	15.0	187.0	51.1	2.7	45.7	0.5	186.0	25.3	17.2	29.0	28.5	186.0
KJ461792.1 *Anopheles subpictus*	38.5	15.9	28.8	16.8	559.0	43.3	28.3	13.4	15.0	187.0	45.2	4.8	44.1	5.9	186.0	26.9	14.5	29.0	29.6	186.0
KJ461784.1 *Anopheles subpictus*	37.9	16.3	29.2	16.6	559.0	43.3	28.3	13.4	15.0	187.0	44.6	4.8	45.2	5.4	186.0	25.8	15.6	29.0	29.6	186.0
KJ768160.1 *Mansonia uniformis*	39.4	16.3	29.5	14.8	559.0	44.4	27.3	13.9	14.4	187.0	47.3	6.5	44.1	2.2	186.0	26.3	15.1	30.6	28.0	186.0
LC517293.1 *Mansonia uniformis*	40.3	14.8	30.1	14.8	559.0	44.4	27.3	13.9	14.4	187.0	49.5	2.7	45.7	2.2	186.0	26.9	14.5	30.6	28.0	186.0
KU578141.1 *Coccinella transversalis*	37.7	19.1	27.9	15.2	559.0	43.9	26.7	13.9	15.5	187.0	44.6	14.0	36.6	4.8	186.0	24.7	16.7	33.3	25.3	186.0
JF835944.1 *Nephila inaurata*	42.8	12.9	27.4	17.0	559.0	45.5	25.7	14.4	14.4	187.0	48.9	1.1	43.0	7.0	186.0	33.9	11.8	24.7	29.6	186.0
MN125137.1 *Pyganodon grandis*	39.9	16.3	20.8	23.1	559.0	43.9	23.0	14.4	18.7	187.0	46.8	10.2	23.1	19.9	186.0	29.0	15.6	24.7	30.6	186.0
Avg.	39.5	15.9	28.5	16.0	559.0	44.0	27.2	13.6	15.2	187.0	48.0	4.8	42.9	4.3	186.0	26.6	15.8	29.1	28.5	186.0

Table 2.17d: The evolutionary divergence percentage between *Culex pipiens* and its closely related species

Sl. No.	Accession Number	Organism	Percentage of Divergence
1	MK603829.1	*Culex pipiens*	0.00%
2	MK714012.1	*Culex pipiens*	0.00%
3	MK714001.1	*Culex pipiens*	0.00%
4	MT999314.1	*Culex corniger*	8.36%
5	MT999239.1	*Culex corniger*	8.36%
6	LC054470.1	*Culex orientalis*	7.16%
7	LC054469.1	*Culex orientalis*	6.96%
8	KJ461792.1	*Anopheles subpictus*	16.26%
9	KJ461784.1	*Anopheles subpictus*	17.62%
10	KJ768160.1	*Mansonia uniformis*	16.68%
11	LC517293.1	*Mansonia uniformis*	14.24%
12	KU578141.1	*Coccinella transversalis*	31.71%
13	JF835944.1	*Nephila inaurata*	27.90%
14	MN125137.1	*Pyganodon grandis*	47.19%

2.3.16.2 Discussion

Cx. pipiens is a small to a medium-sized golden yellow mosquito that breeds in fresh and slightly polluted water bodies. The genetic and phylogenetic relationships among the *Cx. pipiens* specimen isolated from Thrissur Kole lands, Kerala, was explored. The sequence identification with the NCBI database found that no sequences for the COI gene are available for the species *Cx. pipiens,* and maximum homology was shown with *Cx. pipiens us* (MK714012.1, MK714001.1) isolated from Turkey with an identity of 100%. This result reveals that the sequence generated is novel. The queried barcode sequence of *Cx. pipiens*, by the distance summary analysis and the phylogram constructed by MEGAX, revealed the same. In the phylogenetic tree drawn with the MEGAX software tool with NJ methodology. The phylogeny tree provided by MEGAX System depicts the phylogenetic position of the *Cx. pipiens* isolated from the Kole wetlands of Thrissur, Kerala. Accession

number obtained for *Cx. pipiens* isolated from Thrissur Kole lands was MK603829.1. Danabalan et al., 2012 in the UK and Koosha et al., 2017 in Iran sequenced and analysed molecular identification tools of *Cx. pipiens* with COI gene.

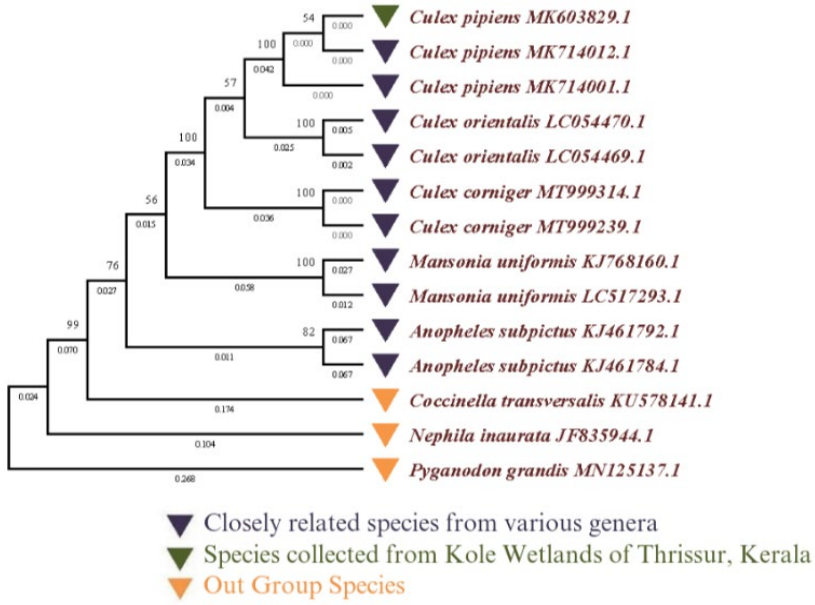

Figure 2.16c Phylogenetic tree of *Culex pipiens*

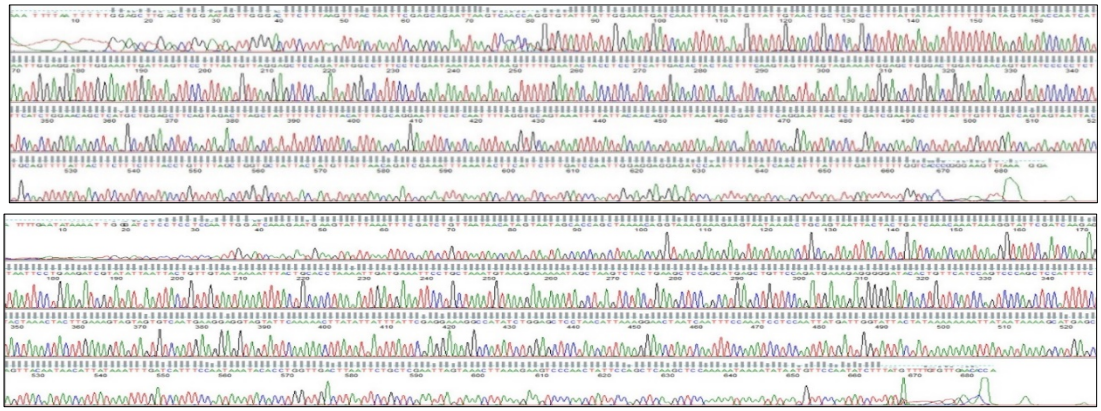

Figure 2.16d Electrophorogram showing the nucleotide sequence of mitochondrial COI gene of *Culex pipiens*

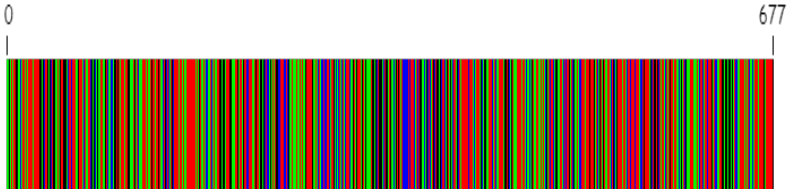

Figure 2.16e Molecular barcode of *Culex pipiens*

2.3.17. Species Name: *Culex quinquefasciatus*

GenBank Accession Number: MT895717.1

Voucher Number: CDRL07

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	*Culicini*
Genus	:	*Culex*
Subgenus	:	*Culex*
Species	:	*Culex quinquefasciatus*

Description

Cx. quinquefasciatus is a medium-sized mosquito with some similar characteristics to *Cx. pipiens.* The Mesonotum of the thorax have some pale scales, ventral region thorax and abdomen have a combination of white and yellow scale cluster. Larvae were collected from polluted waters like drainages, canal basins, etc.

2.3.17.1 Result

The forward and reverse primers were used to amplify the mitochondrial cytochrome oxidase subunit I gene of *Cx. quinquefasciatus,* collected from Kole wetlands of Thrissur, Kerala, were 5'-GGTCAACAAATCATAAAGATATTGG-3' and 5'-TAAA CTTCAGGGTGACCAAAAAATCA-3' respectively. A single product with a length of 540 bp was produced by PCR amplifying *Cx. quinquefasciatus'* mitochondrial cytochrome oxidase subunit I (COI) gene fragment. Accession No. MT895717.1 was obtained from the NCBI GenBank, and Figures

2.17a- 2.17e displayed the DNA sequence, corresponding translation product, phylogenetic tree, electrophorogram, and molecular barcode.

> MT895717.1 *Culex quinquefasciatus*|540bp

GAATAGTTGGAACTTCTTTAAGTTTACTAATTCGAGCAGAATTAAGTCAACCAGGTGTATTTATTGGAAAT
GATCAAATTTATAATGTTATTGTAACTGCTCATGCTTTTATTATAATTTTTTTTATAGTAATACCAATCAT
AATTGGAGGATTTGGAAATTGATTAGTTCCTTTAATGTTAGGAGCTCCAGATATGGCCTTTCCTCGAATAA
ATAATATAAGTTTTTGAATACTACCTCCTTCATTGACACTACTACTTTCAAGTAGTTTAGTAGAAAATGGA
GCTGGGACTGGATGAACAGTGTATCCCCCTCTTTCATCTGGAACAGCTCATGCTGGAGCTTCAGTAGACTT
AGCTATTTTTTCTTTACATTTAGCAGGAATTTCATCAATTTTAGGTGCAGTAAATTTTATTACAACAGTAA
TTAATATACGATCTTCAGGAATTACTCTTGATCGAATACCTTTATTTGTTTGATCAGTAGTAATTACTGCA
GTTTTATTACTTCTTTCTTTACCTGTTTTAGCTGGTGCTATTA

Figure 2.17a: The DNA sequence of *Culex quinquefasciatus* COI gene

> MT895717.1 *Culex quinquefasciatus*

MVGTSLSLLIRAELSQPGVFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLMLGAPDMAFPRMN
NMSFWMLPPSLTLLLSSSLVENGAGTGWTVYPPLSSGTAHAGASVDLAIFSLHLAGISSILGAVNFITTVI
NMRSSGIT

Figure 2.17b: The protein sequence of *Culex quinquefasciatus* COI gene

The COI nucleotide sequence analysis revealed the composition of nucleotides in the COI gene of *Cx. quinquefasciatus* isolated from the Kole wetlands of Thrissur, Kerala (Table 2.18c). T=39.4%, C=15.5%, A=28.8%, and G=16.3% were the nucleotide composition of the COI sequence of *Cx. quinquefasciatus* showed bias to nucleotide AT. T=39.3%, C=15.6%, A=28.3%, and G=16.8% was the average value of nucleotide composition of 14 nucleotides analysed. The evolutionary divergence analysis of *Cx. quinquefasciatus* conducted in MEGAX depicts the degree of divergence of different geographically isolated populations (Table 2.18d). The data demonstrates that the *Cx. quinquefasciatus* showed 0.00% evolutionary divergence with *Cx. quinquefasciatus* (MW509611.1, MW509610.1) geographically isolated from the USA and 9.21% with the species of *Cx. corniger* (MT999314.1, MT999239.1) isolated from Mexico. Using the Maximum Composite Likelihood

model, calculate the number of base substitutions between sequences at each place, as shown in Table 2.18a. A = 28.28%, T/U = 39.30%, C = 15.62%, and G = 16.81% were the nucleotide frequencies estimated through substitution matrix analysis. Tree topology was used in automatically computed ML value calculations. The value of the maximum Log-likelihood for this estimation was -2933.642. Codon positions comprised of 1st+2nd+3rd+Noncoding. The final dataset of this 14 nucleotide sequences analysis involved a total number of 510 positions. All these evolutionary analyses were conducted in MEGA X. The phylogeny tree generated using the NJ method shows the phylogenetic position of *Cx. quinquefasciatus* isolated from Kerala. *Cx. quinquefasciatus* (MW509611.1, MW509610.1) is geographically isolated from the USA and is the closest relative of *Cx. quinquefasciatus* of Kerala.

Table 2.18a: The nucleotide substitution matrix estimate of COI gene sequence of *Culex quinquefasciatus*

From\To	A	T	C	G
A	-	10.5026	4.1734	8.4695
T	7.5570	-	6.7769	4.4915
C	7.5570	17.0547	-	4.4915
G	14.2499	10.5026	4.1734	-

Table 2.18b: The Transition/Transversion bias estimate of mitochondrial COI gene sequence of *Culex quinquefasciatus*

From\To	A	T	C	G
A	-	7.1765	7.1765	10.6470
T	7.1765	-	10.6470	7.1765
C	7.1765	10.6470	-	7.1765
G	10.6470	7.1765	7.1765	-

Table 2.18c: The nucleotide frequency comparison of *Culex quinquefasciatus* COI gene sequence with its kin species

Accession Number and Name of the Species	**Nucleotide Frequencies (%)**																			
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MT895717.1 *Culex quinquefasciatus*	39.4	15.5	28.8	16.3	510.0	44.1	27.6	12.4	15.9	170.0	48.8	2.4	45.9	2.9	170.0	25.3	16.5	28.2	30.0	170.0
MW509611.1 *Culex quinquefasciatus*	39.4	15.5	28.8	16.3	510.0	44.1	27.6	12.4	15.9	170.0	48.8	2.4	45.9	2.9	170.0	25.3	16.5	28.2	30.0	170.0
MW509610.1 *Culex quinquefasciatus*	39.4	15.5	28.8	16.3	510.0	44.1	27.6	12.4	15.9	170.0	48.8	2.4	45.9	2.9	170.0	25.3	16.5	28.2	30.0	170.0
MT999314.1 *Culex corniger*	39.0	16.3	29.2	15.5	510.0	44.1	27.6	12.4	15.9	170.0	49.4	3.5	45.9	1.2	170.0	23.5	17.6	29.4	29.4	170.0
MT999239.1 *Culex corniger*	39.0	16.3	29.2	15.5	510.0	44.1	27.6	12.4	15.9	170.0	49.4	3.5	45.9	1.2	170.0	23.5	17.6	29.4	29.4	170.0
LC054470.1 *Culex orientalis*	39.8	15.9	28.6	15.7	510.0	44.1	27.6	12.4	15.9	170.0	51.2	2.4	45.3	1.2	170.0	24.1	17.6	28.2	30.0	170.0
LC054469.1 *Culex orientalis*	40.0	15.7	28.8	15.5	510.0	44.1	27.6	12.4	15.9	170.0	51.8	1.8	45.9	0.6	170.0	24.1	17.6	28.2	30.0	170.0
KJ461792.1 *Anopheles subpictus*	38.0	15.3	29.2	17.5	510.0	44.1	27.6	12.4	15.9	170.0	43.5	4.1	46.5	5.9	170.0	26.5	14.1	28.8	30.6	170.0
KJ461784.1 *Anopheles subpictus*	37.6	15.9	29.0	17.5	510.0	44.1	27.6	12.4	15.9	170.0	44.1	4.1	45.9	5.9	170.0	24.7	15.9	28.8	30.6	170.0
KJ768160.1 *Mansonia uniformis*	39.2	15.7	29.4	15.7	510.0	44.7	27.1	12.9	15.3	170.0	47.1	5.3	45.3	2.4	170.0	25.9	14.7	30.0	29.4	170.0
LC517293.1 *Mansonia uniformis*	40.0	14.3	30.0	15.7	510.0	44.7	27.1	12.9	15.3	170.0	48.8	1.8	47.1	2.4	170.0	26.5	14.1	30.0	29.4	170.0
KU578141.1 *Coccinella transversalis*	37.5	18.6	28.0	15.9	510.0	44.7	25.9	12.9	16.5	170.0	42.9	13.5	38.2	5.3	170.0	24.7	16.5	32.9	25.9	170.0
JF835944.1 *Nephila inaurata*	42.0	12.5	27.6	17.8	510.0	46.5	24.7	13.5	15.3	170.0	46.5	1.2	45.3	7.1	170.0	32.9	11.8	24.1	31.2	170.0
MN125137.1 *Pyganodon grandis*	39.8	15.7	20.2	24.3	510.0	44.7	22.4	13.5	19.4	170.0	46.5	8.8	23.5	21.2	170.0	28.2	15.9	23.5	32.4	170.0
Avg.	39.3	15.6	28.3	16.8	510.0	44.5	26.8	12.6	16.1	170.0	47.7	4.1	43.7	4.5	170.0	25.8	15.9	28.4	29.9	170.0

Table 2.18d: The evolutionary divergence percentage between *Culex quinquefasciatus* and its closely related species

Sl. No.	Accession Number	Organism	Percentage of Divergence
1	MT895717.1	*Culex quinquefasciatus*	0.00%
2	MW509611.1	*Culex quinquefasciatus*	0.00%
3	MW509610.1	*Culex quinquefasciatus*	0.00%
4	MT999314.1	*Culex corniger*	8.55%
5	MT999239.1	*Culex corniger*	8.55%
6	LC054470.1	*Culex orientalis*	7.23%
7	LC054469.1	*Culex orientalis*	7.02%
8	KJ461792.1	*Anopheles subpictus*	15.79%
9	KJ461784.1	*Anopheles subpictus*	18.04%
10	KJ768160.1	*Mansonia uniformis*	15.98%
11	LC517293.1	*Mansonia uniformis*	13.34%
12	KU578141.1	*Coccinella transversalis*	31.55%
13	JF835944.1	*Nephila inaurata*	28.51%
14	MN125137.1	*Pyganodon grandis*	49.38%

2.3.17.2 Discussion

The tropical regions are home to the medium-sized brown mosquito *Cx. quinquefasciatus*, a carrier for various parasitic illnesses. It is an opportunistic blood feeder often active at night, enabling the parasites to exploit humans as hosts. Therefore, it is imperative to give their control top attention. The nucleotide divergence study and the phylogeny analysis of *Cx. quinquefasciatus* revealed a new report in the database. The accession number of *Cx. quinquefasciatus* isolated from the Kole wetlands of Thrissur, Kerala, is MT895717.1. It is a pioneer work in Kerala, so the study provided novel reports to all databases. Its unique barcode can easily spot and analyse the phylogenetic position of this species based on DNA sequences. Morphological identification was made with available keys and online photographs as this species is a novel report in Kerala to the available databases. Gunay et al., isolated and sequenced *Cx. quinquefasciatus* from Turkey in 2015.

Accession numbers obtained from NCBI GenBank KJ012162–173. Daravath et al., 2015 studied the molecular characterisation of *Cx. quinquefasciatus* in Hyderabad. They checked the sequence similarity with other species from different geographical regions, and multiple sequence alignment of *Culex* species was accomplished. Sequence alignment suggests that Hyderabad species exhibited proper alignment with UK species.

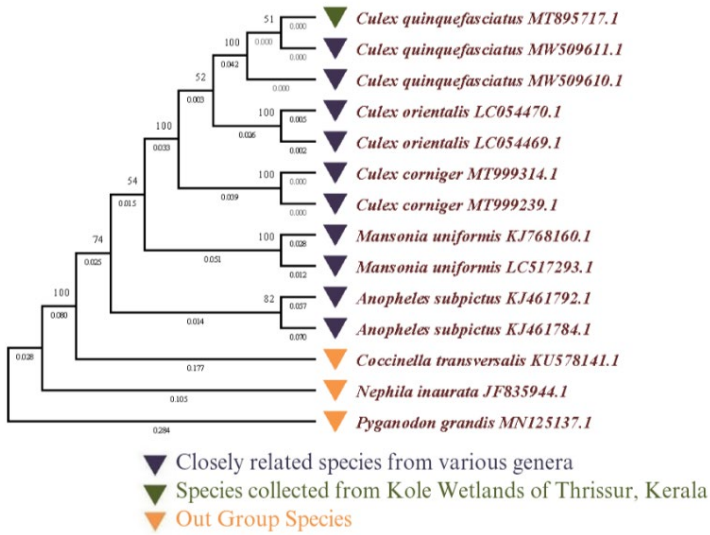

Figure 2.17c Phylogenetic tree of *Culex quinquefasciatus*

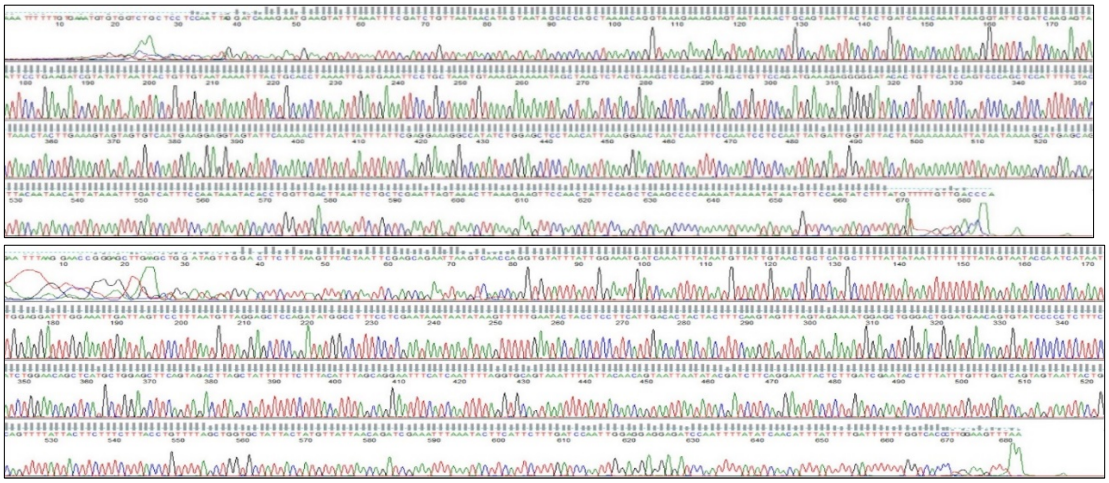

Figure 2.17d Electrophorogram showing the nucleotide sequence of mitochondrial COI gene of *Culex quinquefasciatus*

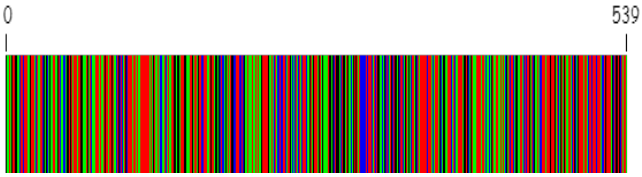

Figure 2.17e Molecular barcode of *Culex quinquefasciatus*

2.3.18. Species Name: *Mansonia uniformis*

GenBank Accession Number: MK757484.1

Voucher Number: CDRL22

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Culicini
Genus	:	*Mansonia*
Subgenus	:	*Mansonioides*
Species	:	*Mansonia uniformis*

Description

Brownish medium-sized mosquito. Yellow and brown scales are on the head, and white patches are on the eyes' lower side. Legs have several white spots. Larval forms were collected from habitats that have aquatic vegetation.

2.3.18.1 Result

The forward and reverse primers were used to amplify the mitochondrial cytochrome oxidase subunit I gene of *Ma. uniformis,* collected from Kole wetlands of Thrissur, Kerala, were 5'-GGTCAACAAATCATAAAGATATTGG-3' and 5'-TAAACTTCA GGGTGACCAAAAAATCA-3' respectively. A single product with

a length of 682 bp was produced by PCR amplifying *Ma. uniformis'* mitochondrial cytochrome oxidase subunit I (COI) gene fragment. Accession No. MK757484.1 was obtained from the NCBI GenBank, and Figures 2.18a- 2.18e displayed the DNA sequence, corresponding translation product, phylogenetic tree, electrophorogram, and molecular barcode.

> MK757484.1 *Mansonia uniformis*|682bp

GATATTGGAACTTTATATTTTATCTTTGGAGCATGATCTGGAATAATTGGAACTTCTCTAAGAATTTTAATT
CGAATAGAATTAAGTCAACCCGGAGTTTTCATTGGAAATGACCAAATTTATAATGTTATTGTTACAGCTCAT
GCATTTATTATAATTTTTTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAATTGATTAGTTCCATTA
ATATTAGGAGCTCCTGATATAGCATTTCCTCGAATAAATAATATAAGATTTTGACTTTTACCTCCATCATTA
ACATTATTAATTTCAGGAGGAATAGTAGAAAATGGGGCTGGAACTGGATGAACAGTTTATCCTCCTTTATCA
GCTAATACAGCTCATACTGGAGCATCTGTTGACTTAACAATTTTTTCTTTACATTTAGCCGGAGTTTCTTCA
ATTTTAGGAGCAGTAAATTTTATTACTACTGTTATTAATATACGATCTTCAGGAATTACTTTAGACCGAATA
CCTCTATTTGTATGATCTGTTGTAATTACAGCAATTTTGTTACTCCTTTCCCTTCCTGTTTTAGCTGGAGCT
ATTACAATACTTTTAACTGATCGTAATTTAAATACATCCTTCTTTGACCCTATAGGAGGAGGAGATCCTATT
CTTTATCAACACTTATTCTGATTTTTTGGTCACC

Figure 2.18a: The DNA sequence of *Mansonia uniformis* COI gene

> MK757484.1 *Mansonia uniformis*

MGTLYFIFGAWSGMIGTSLSILIRMELSQPGVFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLML
GAPDMAFPRMNNMSFWLLPPSLTLLISGGMVENGAGTGWTVYPPLSANTAHTGASVDLTIFSLHLAGVSSILG
AVNFITTVINMRSSGITLDRMPLFVWSVVITAILLLLSLPVLAGAITMLLTDRNLNTSFFDPMGGGDPILYQH
LFWFFGH

Figure 2.18b: The protein sequence of *Mansonia uniformis* COI gene

T=38.5%, C=15.8%, A=30.4%, and G=15.3% was the nucleotide composition of The COI sequence of *Ma. uniformis* showed bias to nucleotide AT. T=38.3%, C=16.2%, A=28.8% and G=16.7% was the average value of nucleotide composition of 14 nucleotides analysed (Table 2.19c). The maximum Composite Likelihood model, used in the base substitutions per site, was estimated between sequences displayed in Table 2.19a. The patterns and rates substitution were computed under the Tamura-Nei model. Substitution patterns and rates were estimated under the Tamura-Nei (1993) model. A = 25.00%, T/U = 25.00%, C = 25.00%, and G = 25.00% were the nucleotide frequencies estimated through Transition/Transversion bias estimation. Tree topology was used in automatically computed ML value

calculations. The value of the maximum Log-likelihood for this estimation was -3011.115. Codon positions comprised of 1st+2nd+3rd+Noncoding. The final dataset of these 14 nucleotide sequences analysis involved a total number of 510 positions. All these evolutionary analyses were conducted in MEGA X. The estimated Transition/Transversion bias (R) is 0.74 (Table 2.19b).

The data in Table 2.19d reveals that *Ma. uniformis* from Kerala shows 0.49% evolutionary divergence with *Ma. uniformis* (KT358411.1) isolated from Korea and 7.35% evolutionary divergence with *Ma. africana* (LC517270.1) isolated from Mozambique. The phylogenetic tree generated using the NJ method shows *Ma. uniformis'* phylogenetic position, isolated from the Kole wetlands of Thrissur, Kerala, is given in Figure 2.18d. Phylogenetically *Ma. uniformis* was the closest relative of *Ma. uniformis* (KT358411.1) isolated from Korea.

Table 2.19a: The nucleotide substitution matrix estimate of COI gene sequence of *Mansonia uniformis*

From\To	A	T	C	G
A	-	9.4701	4.0028	7.9279
T	7.1184	-	8.6064	4.1320
C	7.1184	20.3615	-	4.1320
G	13.6576	9.4701	4.0028	-

Table 2.19b: The Transition/Transversion bias estimate of mitochondrial COI gene sequence of *Mansonia uniformis*

From\To	A	T	C	G
A	-	6.6559	6.6559	11.6883
T	6.6559	-	11.6883	6.6559
C	6.6559	11.6883	-	6.6559
G	11.6883	6.6559	6.6559	-

Tble 2.19c: The nucleotide frequency comparison of *Mansonia uniformis* COI gene sequence with its kin species

Accession Number and Name of The Species	**Nucleotide Frequencies (%)**																		
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MK757484.1 *Mansonia uniformis*	38.5	15.8	30.4	15.3	615.0	42.9	27.3	13.2	16.6	205.0	46.8	5.4	46.8	1.0	205.0	25.9	14.6	31.2	28.3	205.0
KT358411.1 *Mansonia uniformis*	38.7	15.6	30.4	15.3	615.0	42.9	27.3	13.2	16.6	205.0	47.3	4.9	46.8	1.0	205.0	25.9	14.6	31.2	28.3	205.0
KJ768160.1 *Mansonia uniformis*	38.4	15.9	30.1	15.6	615.0	42.9	27.3	13.2	16.6	205.0	46.3	5.9	45.9	2.0	205.0	25.9	14.6	31.2	28.3	205.0
LC517280.1 *Mansonia africana*	36.6	17.6	29.9	15.9	615.0	42.9	27.3	13.2	16.6	205.0	42.9	8.8	45.4	2.9	205.0	23.9	16.6	31.2	28.3	205.0
LC517270.1 *Mansonia africana*	36.9	16.9	30.2	15.9	615.0	42.9	27.3	13.2	16.6	205.0	43.4	7.3	46.3	2.9	205.0	24.4	16.1	31.2	28.3	205.0
MW321855.1 *Mansonia dives*	38.7	16.6	29.6	15.1	615.0	42.9	27.3	13.2	16.6	205.0	48.3	6.8	44.4	0.5	205.0	24.9	15.6	31.2	28.3	205.0
MW321854.1 *Mansonia dives*	39.0	16.1	29.6	15.3	615.0	42.9	27.3	13.2	16.6	205.0	49.3	5.4	44.4	1.0	205.0	24.9	15.6	31.2	28.3	205.0
MW542315.1 *Aedes albopictus*	38.0	17.2	28.3	16.4	615.0	43.4	27.3	12.7	16.6	205.0	46.8	6.8	42.4	3.9	205.0	23.9	17.6	29.8	28.8	205.0
MT890465.1 *Aedes albopictus*	38.0	17.2	28.3	16.4	615.0	43.4	27.3	12.7	16.6	205.0	46.8	6.8	42.4	3.9	205.0	23.9	17.6	29.8	28.8	205.0
MK713986.1 *Culex pipiens*	38.7	15.3	29.4	16.6	615.0	42.4	27.8	12.7	17.1	205.0	47.8	2.4	46.8	2.9	205.0	25.9	15.6	28.8	29.8	205.0
MK713985.1 *Culex pipiens*	38.7	15.3	29.4	16.6	615.0	42.4	27.8	12.7	17.1	205.0	47.8	2.4	46.8	2.9	205.0	25.9	15.6	28.8	29.8	205.0
KU578141.1 *Coccinella transversalis*	36.1	18.9	28.6	16.4	615.0	42.9	26.3	13.2	17.6	205.0	41.5	14.1	39.5	4.9	205.0	23.9	16.1	33.2	26.8	205.0
JF835944.1 *Nephila inaurata*	41.0	12.8	28.1	18.0	615.0	43.9	26.3	13.7	16.1	205.0	46.8	1.0	45.4	6.8	205.0	32.2	11.2	25.4	31.2	205.0
MN125137.1 *Pyganodon grandis*	38.9	15.4	20.7	25.0	615.0	43.4	22.0	13.7	21.0	205.0	44.4	9.3	23.9	22.4	205.0	28.8	15.1	24.4	31.7	205.0
Avg.	38.3	16.2	28.8	16.7	615.0	43.0	26.9	13.1	17.0	205.0	46.2	6.2	43.4	4.2	205.0	25.7	15.5	29.9	28.9	205.0

Table 2.19d: The evolutionary divergence percentage between *Mansonia uniformis* and its closely related species

Sl. No.	Accession Number	Organism	Percentage of Divergence
1	MK757484.1	*Mansonia uniformis*	0.00%
2	KT358411.1	*Mansonia uniformis*	0.16%
3	KJ768160.1	*Mansonia uniformis*	0.49%
4	LC517280.1	*Mansonia africana*	7.73%
5	LC517270.1	*Mansonia africana*	7.35%
6	MW321855.1	*Mansonia dives*	10.23%
7	MW321854.1	*Mansonia dives*	10.70%
8	MW542315.1	*Aedes albopictus*	25.05%
9	MT890465.1	*Aedes albopictus*	25.05%
10	MK713986.1	*Culex pipiens*	20.50%
11	MK713985.1	*Culex pipiens*	20.50%
12	KU578141.1	*Coccinella transversalis*	34.96%
13	JF835944.1	*Nephila inaurata*	47.21%
14	MN125137.1	*Pyganodon grandis*	90.51%

2.3.18.2 Discussion

Mansonia uniformis is a medium-sized brown mosquito commonly seen in most parts of India, Burma, and Ceylon. Africa, Japan, and Australia. Morphological identification of this species has been made with available keys and online photographs. Morphologically this species seems very close to *Ma. uniformis*. The NCBI database's molecular identification method showed this species' conformity as *Ma. uniformis.* 682bp COI nucleotide was sequenced from *Ma. uniformis* isolated from Thrissur Kole lands. The accession number obtained from NCBI GenBank was MK757484.1. The phylogenetic relationship was studied with 14 mosquito species, and phylogenetically *Ma. uniformis* showed to be the closest relative of *Ma. uniformis* (KT358411.1) isolated from Korea. KU380460 and KU187175 were Accession numbers of two different *Ma. uniformis* mosquitoes isolated from Kenya (Ajamma et al., 2016). Weeraratne et al., 2018 identified 14 mosquito species from

Sri Lanka and discussed the integrated systematic approach and use of cox1 and ITS genetic markers in mosquito taxonomy. KY352264–KY352272 were the Accession numbers of *Ma. uniformis* received from NCBI GenBank.

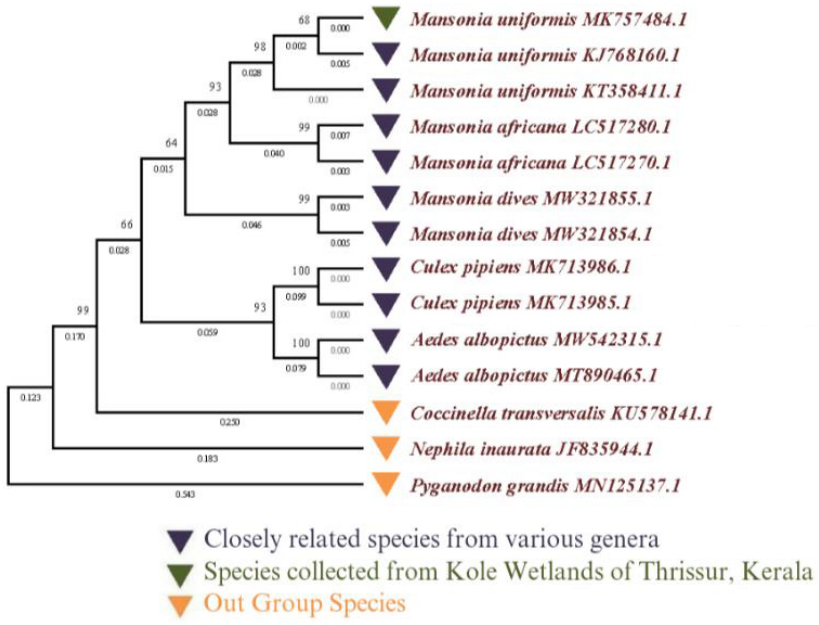

Figure 2.18c Phylogenetic tree of *Mansonia uniformis*

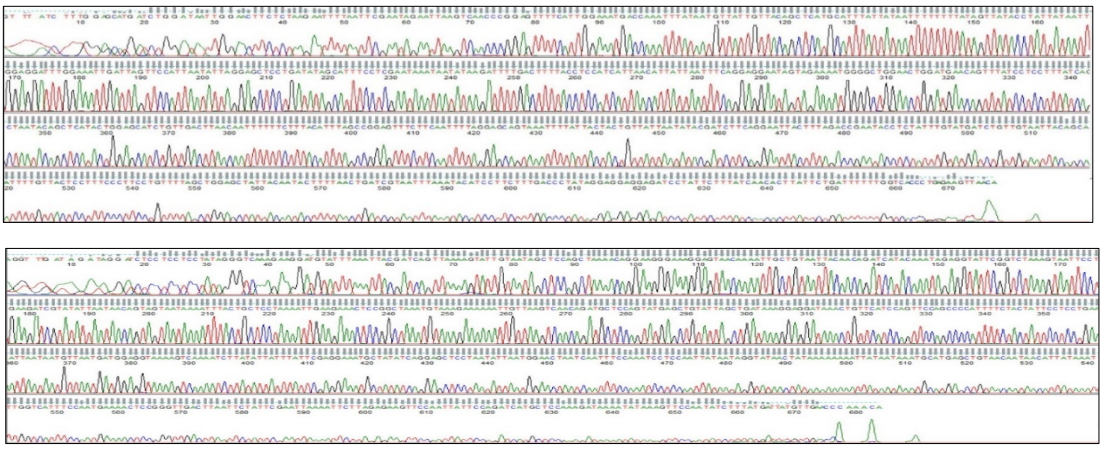

Figure 2.18d Electrophorogram showing the nucleotide sequence of mitochondrial COI gene of *Mansonia uniformis*

Figure 2.18e Molecular barcode of *Mansonia uniformis*

2.3.19. Species Name: *Mansonia indiana*

GenBank Accession Number: MK637632.1

Voucher Number: CDRL01

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Culicini
Genus	:	*Mansonia*
Subgenus	:	*Mansonioides*
Species	:	*Mansonia indiana*

Description

Adult ones are very similar to *Ma. uniformis* but varies from this lack of greenish patch on mesonotum. Instead of this, dark brown scales are entirely covered on the mesonotum. Larvae are the same as *Ma. uniformis* and inhabits waterbodies that have aquatic vegetation.

2.3.19.1 Result

The forward and reverse primers were used to amplify the mitochondrial cytochrome oxidase subunit I gene of *Ma. indiana,* collected from Kole wetlands of Thrissur, Kerala, were 5'-GGTCAACAAATCATAAAGATATTGG-3' and 5'-TAAACTTCA GGGTGACCAAAAAATCA-3' respectively. A single product with a length of 678 bp was produced by PCR amplifying *Ma. indiana's* mitochondrial cytochrome oxidase subunit I (COI) gene fragment. Accession No. MK637632.1.

was obtained from the NCBI GenBank, and Figures 2.19a- 2.19e displayed the DNA sequence, corresponding translation product, phylogenetic tree, electrophorogram, and molecular barcode.

> MK637632.1 *Mansonia indiana*|678bp

AAGATATTGGAACTTTATATTTTATTTTTGGAGCATGATCTGGAATAATCGGAACTTCTCTAAGAATTCTAA
TTCGAATAGAATTAAGACAACCTGGTGTATTCATTGGAAATGATCAAATTTATAATGTCATTGTTACTGCAC
ATGCATTTATTATAATTTTTTTTATAGTAATACCTATTATAATTGGAGGATTCGGAAATTGATTAGTTCCCC
TTATATTAGGAGCCCCTGATATAGCATTCCCTCGAATAAATAATATAAGATTTTGACTTTTACCCCCATCAT
TAACATTATTAATTTCAGGGGGTATAGTAGAAAACGGGGCTGGTACAGGTTGAACTGTTTACCCCCCTCTAT
CTGCCAACACTGCTCATACAGGAGCCTCAGTTGATTTAACAATTTTTTCTCTCCACTTAGCCGGAGTATCTT
CAATTTTAGGTGCAGTAAATTTTATTACTACTGTAATTAATATACGATCCTCAGGAATTACATTAGATCGAA
TACCATTATTTGTTTGATCAGTTGTAATTACAGCAATTTTATTACTCCTCTCCCTCCCCGTTTTAGCTGGAG
CTATTACTATACTTCTTACTGATCGTAATTTAAATACATCATTTTTTGATCCAATAGGAGGAGGAGACCCTA
TTTTATATCAACATCTCTTTTGATTTTTTG

Figure 2.19a: The DNA sequence of *Mansonia indiana* COI gene

> MK637632.1 *Mansonia indiana*

MGTLYFIFGAWSGMIGTSLSILIRMELSQPGVFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLM
LGAPDMAFPRMNNMSFWLLPPSLTLLISGGMVENGAGTGWTVYPPLSANTAHTGASVDLTIFSLHLAGVSSI
LGAVNFITTVINMRSSGITLDRMPLFVWSVVITAILLLLSLPVLAGAITMLLTDRNLNTSFFDPMGGGDPIL
YQHLFWFFGH

Figure 2.19b: The protein sequence of *Mansonia indiana* COI gene

The BLAST search revealed the partial COI nucleotide sequence of *Ma. indiana* isolated from Thrissur Kole lands, Kerala is 0.34% similar to that of two same species isolated from Singapore (MW321853.1, MW321852.1). *Ma. indiana* isolated from Thrissur Kole lands, Kerala showed 6.24% evolutionary divergence with *Ma. uniformis* isolated from Australia (MG712564.1) (Table.2.20d). The average nucleotide composition throughout the species is T=37.9%; C=16.6%; A=28.9%; G=16.7% (Table. 2.20c). The estimate of the Substitution Matrix showed the probability of substitution from one base to another base (Table. 2.20a). Substitution patterns and rates were estimated under the Tamura-Nei (1993) model. A = 25.00%, T/U = 25.00%, C = 25.00%, and G =

25.00% were the nucleotide frequencies estimated through Transition/Transversion bias estimation. Tree topology was used in automatically computed ML value calculations. The value of the maximum Log-likelihood for this estimation was -3530.177. Codon positions comprised of 1st+2nd+3rd+Noncoding. All these evolutionary analyses were conducted in MEGA X.

The phylogenetic tree was generated using the NJ method. Phylogenetic tree showing the phylogenetic position of *Ma. indiana wa*s isolated from Thrissur Kole lands, Kerala. The phylogenetic tree revealed the closest relatives of two same species *Ma. indiana,* isolated from Singapore. The closest relatives of *Ma. indiana* isolated from three populations were arranged in a single clade.

Table 2.20a: The nucleotide substitution matrix estimate of COI gene sequence of *Mansonia indiana*

From\To	A	T	C	G
A	-	8.9803	3.9267	8.1479
T	6.8475	-	9.2334	3.9707
C	6.8475	21.1170	-	3.9707
G	14.0511	8.9803	3.9267	-

Table 2.20b: The Transition/Transversion bias estimate of mitochondrial COI gene sequence of *Mansonia indiana*

From\To	A	T	C	G
A	-	6.3905	6.3905	12.2189
T	6.3905	-	12.2189	6.3905
C	6.3905	12.2189	-	6.3905
G	12.2189	6.3905	6.3905	-

Table 2.20c: The nucleotide frequency comparison of *Mansonia indiana* COI gene sequence with its kin species

Accession Number and Name of the Species	**Nucleotide Frequencies (%)**																			
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MK637632.1 *Mansonia indiana*	36.1	18.5	30.1	15.3	615.0	42.9	27.3	13.2	16.6	205.0	41.5	11.7	45.9	1.0	205.0	23.9	16.6	31.2	28.3	205.0
MW321853.1 *Mansonia indiana*	36.6	18.0	29.8	15.6	615.0	42.9	27.3	13.2	16.6	205.0	42.9	10.2	44.9	2.0	205.0	23.9	16.6	31.2	28.3	205.0
MW321852.1 *Mansonia indiana*	36.6	18.0	29.8	15.6	615.0	42.9	27.3	13.2	16.6	205.0	42.9	10.2	44.9	2.0	205.0	23.9	16.6	31.2	28.3	205.0
MK757484.1 *Mansonia uniformis*	38.5	15.8	30.4	15.3	615.0	42.9	27.3	13.2	16.6	205.0	46.8	5.4	46.8	1.0	205.0	25.9	14.6	31.2	28.3	205.0
MG712564.1 *Mansonia uniformis*	39.2	15.0	30.9	15.0	615.0	42.9	27.3	13.2	16.6	205.0	48.3	3.4	48.3	0.0	205.0	26.3	14.1	31.2	28.3	205.0
KU380393.1 *Mansonia africana*	36.7	17.1	30.1	16.1	615.0	42.9	27.3	13.2	16.6	205.0	42.9	7.8	45.9	3.4	205.0	24.4	16.1	31.2	28.3	205.0
KU380365.1 *Mansonia africana*	36.7	17.1	30.2	15.9	615.0	42.9	27.3	13.2	16.6	205.0	42.4	8.3	46.8	2.4	205.0	24.9	15.6	30.7	28.8	205.0
MW542315.1 *Aedes albopictus*	38.0	17.2	28.3	16.4	615.0	43.4	27.3	12.7	16.6	205.0	46.8	6.8	42.4	3.9	205.0	23.9	17.6	29.8	28.8	205.0
MT890465.1 *Aedes albopictus*	38.0	17.2	28.3	16.4	615.0	43.4	27.3	12.7	16.6	205.0	46.8	6.8	42.4	3.9	205.0	23.9	17.6	29.8	28.8	205.0
MK713986.1 *Culex pipiens*	38.7	15.3	29.4	16.6	615.0	42.4	27.8	12.7	17.1	205.0	47.8	2.4	46.8	2.9	205.0	25.9	15.6	28.8	29.8	205.0
MK713985.1 *Culex pipiens*	38.7	15.3	29.4	16.6	615.0	42.4	27.8	12.7	17.1	205.0	47.8	2.4	46.8	2.9	205.0	25.9	15.6	28.8	29.8	205.0
KU578141.1 *Coccinella transversalis*	36.1	18.9	28.6	16.4	615.0	42.9	26.3	13.2	17.6	205.0	41.5	14.1	39.5	4.9	205.0	23.9	16.1	33.2	26.8	205.0
JF835944.1 *Nephila inaurata*	41.0	12.8	28.1	18.0	615.0	43.9	26.3	13.7	16.1	205.0	46.8	1.0	45.4	6.8	205.0	32.2	11.2	25.4	31.2	205.0
MN125137.1 *Pyganodon grandis*	38.9	15.4	20.7	25.0	615.0	43.4	22.0	13.7	21.0	205.0	44.4	9.3	23.9	22.4	205.0	28.8	15.1	24.4	31.7	205.0
Avg.	37.9	16.6	28.9	16.7	615.0	43.0	26.9	13.1	17.0	205.0	45.0	7.1	43.6	4.3	205.0	25.5	15.6	29.9	29.0	205.0

Table 2.20d: The evolutionary divergence percentage between *Mansonia indiana* and its closely related species

Sl. No.	Accession Number	Organism	Percentage of Divergence
1	MK637632.1	*Mansonia indiana*	0.00%
2	MW321853.1	*Mansonia indiana*	0.66%
3	MW321852.1	*Mansonia indiana*	0.66%
4	MK757484.1	*Mansonia uniformis*	6.25%
5	MG712564.1	*Mansonia uniformis*	6.24%
6	KU380393.1	*Mansonia africana*	6.49%
7	KU380365.1	*Mansonia africana*	6.60%
8	MW542315.1	*Aedes albopictus*	10.69%
9	MT890465.1	*Aedes albopictus*	10.69%
10	MK713986.1	*Culex pipiens*	11.13%
11	MK713985.1	*Culex pipiens*	11.13%
12	KU578141.1	*Coccinella transversalis*	15.52%
13	JF835944.1	*Nephila inaurata*	19.34%
14	MN125137.1	*Pyganodon grandis*	27.09%

2.3.19.1 Discussion

The BLAST search revealed the partial COI nucleotide sequence of *Ma. indiana* isolated from Thrissur Kole lands, Kerala is 0.34% similar to that of two same species isolated from Singapore (MW321853.1, MW321852.1). *Ma. indiana*, isolated from Thrissur Kole lands, Kerala, showed 6.24% evolutionary divergence with *Ma. uniformis* isolated from Australia (MG712564.1). *Ma. uniformis*, isolated from Kole wetlands in Thrissur, Kerala, is a novel one and gets accession number from NCBI GenBank as MK637632.1, containing 678bp length of COI gene. This identical database and molecular barcode may be helpful in future vector management programs in that area. According to Afizah et al., 2019 the closely related species of *Ma. indiana* was *Ma. uniformis.* The present study also concluded the relationship between those two species.

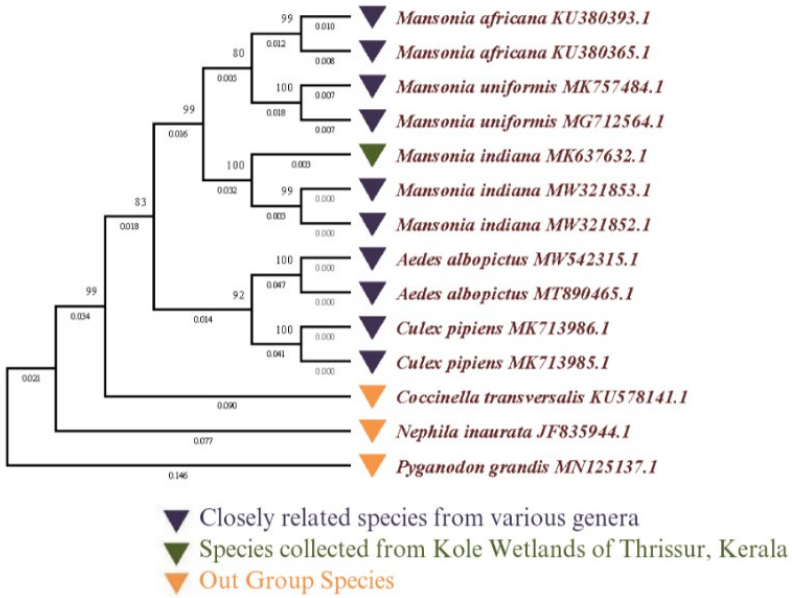

Figure 2.19c Phylognetic tree of *Mansonia indiana*

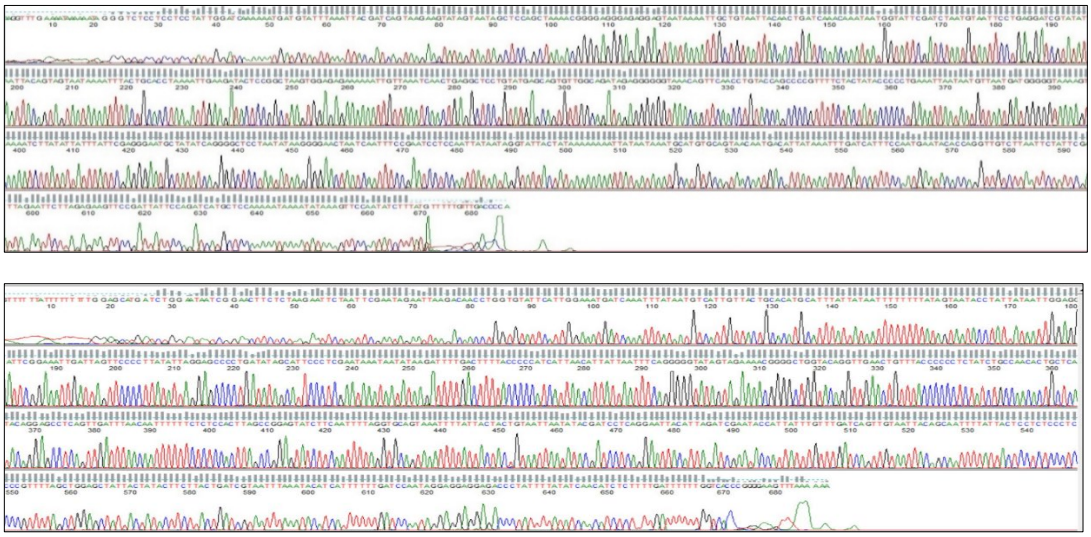

Figure 2.19d Electrophorogram showing the nucleotide sequence of mitochondrial COI gene of *Mansonia indiana*

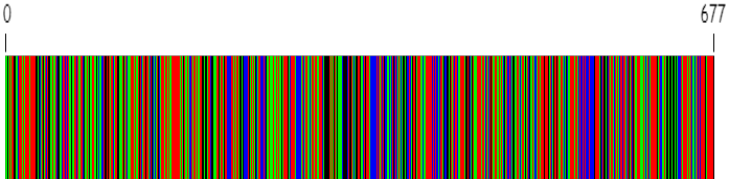

Figure 2.19e Molecular barcode of *Mansonia indiana*

2.3.20. Species Name: *Mansonia bonneae*

GenBank Accession Number: MT177149.1

Voucher Number: CDRL24

Systematic position

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Diptera
Suborder	:	Nematocera
Family	:	Culicidae
Subfamily	:	Culicini
Genus	:	*Mansonia*
Subgenus	:	*Mansonioides*
Species	:	*Mansonia bonneae*

Description

Mesonotum diversly marked with two definite white rounded scales. Pleurae covered by dark scales. Five strips of pale scales are noticed on the hind femur - the mid lobe of the scutellum with thin scales. A small area of white scales with supra-alar bristles over the wing root is absent.

2.3.20.1 Result

The forward and reverse primers were used to amplify Ma. *bonneae* mitochondrial cytochrome oxidase subunit I gene., collected from Kole wetlands of Thrissur, Kerala, were 5'-GGTCAACAAATCATAAAGATATTGG-3' and 5'-TAAACTTCAGGGTG ACCAAAAAATCA-3' respectively. A single product with a length of 566 bp was produced by PCR amplifying *Ma. bonneae's* mitochondrial cytochrome oxidase subunit I (COI) gene fragment. Accession No. MT177149.1 was obtained from the NCBI GenBank, and Figures 2.20a- 2.20e displayed the DNA

sequence, corresponding translation product, phylogenetic tree, electrophorogram, and molecular barcode.

> MT177149.1 *Mansonia bonneae*|566bp

```
ATAATTGGAACTTCTCTAAGAATTTTAATTCGAATAGAATTAAGTCAACCCGGAGTTTTCATTGGAAATGA
CCAAATTTATAATGTTATTGTTACAGCTCATGCATTTATTATAATTTTTTTTATAGTTATACCTATTATAA
TTGGAGGATTTGGAAATTGATTAGTTCCATTAATATTAGGAGCTCCTGATATAGCATTTCCTCGAATAAAT
AATATAAGATTTTGACTTTTACCTCCATCATTAACATTATTAATTTCAGGAGGAATAGTAGAAAATGGGGC
TGGAACTGGATGAACAGTTTATCCTCCTTTATCAGCTAATACAGCTCATACTGGAGCATCTGTTGACTTAA
CAATTTTTTCTTTACATTTAGCCGGAGTTTCTTCAATTTTAGGAGCAGTAAATTTTATTACTACTGTTATT
AATATACGATCTTCAGGAATTACTTTAGACCGAATACCTCTATTTGTATGATCTGTTGTAATTACAGCAAT
TTTGTTACTCCTTTCCCTTCCTGTTTTAGCTGGAGCTATTACAATACTTTTAACTGATCGTAATTTAAA
```

Figure 2.20a: The DNA sequence of *Mansonia bonneae* COI gene

> MT177149.1 *Mansonia bonneae*

```
MIGTSLSILIRMELSQPGVFIGNDQIYNVIVTAHAFIMIFFMVMPIMIGGFGNWLVPLMLGAPDMAFPRMN
NMSFWLLPPSLTLLISGGMVENGAGTGWTVYPPLSANTAHTGASVDLTIFSLHLAGVSSILGAVNFITTVI
NMRSSGITLDRMPLFVWSVVITAILLLLSLPVLAGAITMLLTDRNL
```

Figure 2.20b: The protein sequence of *Mansonia bonneae* COI gene

The average nucleotide composition throughout the species is T=39.1%; C=15.8%; A=29.2%; G=16.0% (Table.2.21c). This outcome exhibits that evaluating a mitochondrial gene will also be priceless for unravelling phylogenetic relationships within the *Ma. bonneae*. The percentage of A+T was more than that of G+C, which reflected extra within the codon usage. The tree is drawn to scale, and the length of branches in patterns equals the evolutionary distances used to deduce the phylogenetic tree. Variation in the nucleotide is a fundamental property of all living organisms, which can be used for their identification and phylogenetic status. The COI gene in the mitochondrial genome has been proven to be an excellent source of information for the closely related species belonging to the order Diptera. Variation

in the nucleotide is the predominant property of all organisms, which can be utilised for its identification and phylogenetic study.

The BLAST analysis of 566 bp of the insect *Ma. bonneae* showed significant homology with other *Mansonia* species. The genetic divergence analysis depicts the divergence of different geographically isolated species of *Ma. bonneae* with various related species. *Ma. bonneae* isolated from the Kole wetlands of Thrissur, Kerala (GenBank Accession No. MT177149.1) showed 100% sequence similarity to the same species reported from Pakistan (KF407919.1) (Table 2.21d). *Ma. bonneae* were separated into related clades in the phylogenetic tree. *Ma. africana* (LC517270.1) and *Ma. dive* (MW321855.1) species are in different clades. *Ma. bonneae* isolated from Kerala showed variations in the total nucleotide composition and composition of nucleotides in each position of codons with that of various species isolated from the different geographical areas. The maximum Composite Likelihood model was used to estimate the base differences per position between nucleotide sequences indicated in Table 2.21b. The analysis included 14 nucleotide sequences. The positions of the included Codons are 1st + 2nd + 3rd + Noncoding. After deleting gaps and missing in all positions, there were 566 positions in the final dataset. All these evolutionary analyses were conducted in MEGA X. The phylogenetic tree generated using Neighbour Joining (NJ) method reveals the phylogenetic status of *Ma. bonneae* isolated from Kerala. Phylogenetically *Ma. bonneae* was the closest relative of *Ma. bonneae* of Pakistan.

Table 2.21a: The nucleotide substitution matrix estimate of COI gene sequence of *Mansonia bonneae*

From\To	A	T	C	G
A	-	9.6578	3.9006	7.9502
T	7.2083	-	8.0911	3.9599
C	7.2083	20.0335	-	3.9599
G	14.4721	9.6578	3.9006	-

Table 2.21b: The Transition/Transversion bias estimate of mitochondrial COI gene sequence of *Mansonia bonneae*

From\To	A	T	C	G
A	-	6.7241	6.7241	11.5517
T	6.7241	-	11.5517	6.7241
C	6.7241	11.5517	-	6.7241
G	11.5517	6.7241	6.7241	-

Table 2.21c: The nucleotide frequency comparison of *Mansonia bonneae* COI gene sequence with its kin species

Accession Number and Name of the Species	**Nucleotide Frequencies (%)**																			
	T(U)	C	A	G	TOTAL	T-1	C-1	A-1	G-1	POS #1	T-2	C-2	A-2	G-2	POS #2	T-3	C-3	A-3	G-3	POS #3
MT177149.1 *Mansonia bonneae*	39.4	15.2	30.7	14.7	566.0	25.4	14.8	32.3	27.5	189.0	45.0	26.5	13.2	15.3	189.0	47.9	4.3	46.8	1.1	188.0
KF407919.1 *Mansonia bonneae*	39.4	15.2	30.7	14.7	566.0	25.4	14.8	32.3	27.5	189.0	45.0	26.5	13.2	15.3	189.0	47.9	4.3	46.8	1.1	188.0
MG816360.1 *Mansonia bonneae*	39.2	15.4	30.6	14.8	566.0	25.4	14.8	32.3	27.5	189.0	45.0	26.5	13.2	15.3	189.0	47.3	4.8	46.3	1.6	188.0
LC517270.1 *Mansonia africana*	37.8	16.3	30.6	15.4	566.0	23.8	16.4	32.3	27.5	189.0	45.0	26.5	13.2	15.3	189.0	44.7	5.9	46.3	3.2	188.0
LC517280.1 *Mansonia africana*	37.6	16.8	30.2	15.4	566.0	23.3	16.9	32.3	27.5	189.0	45.0	26.5	13.2	15.3	189.0	44.7	6.9	45.2	3.2	188.0
MW321855.1 *Mansonia dives*	39.8	15.9	29.9	14.5	566.0	24.3	15.9	32.3	27.5	189.0	45.0	26.5	13.2	15.3	189.0	50.0	5.3	44.1	0.5	188.0
MW321854.1 *Mansonia dives*	40.1	15.4	29.9	14.7	566.0	24.3	15.9	32.3	27.5	189.0	45.0	26.5	13.2	15.3	189.0	51.1	3.7	44.1	1.1	188.0
MW542315.1 *Aedes albopictus*	38.2	17.3	28.8	15.7	566.0	23.3	18.0	30.7	28.0	189.0	45.0	27.0	12.7	15.3	189.0	46.3	6.9	43.1	3.7	188.0
MT890465.1 *Aedes albopictus*	38.2	17.3	28.8	15.7	566.0	23.3	18.0	30.7	28.0	189.0	45.0	27.0	12.7	15.3	189.0	46.3	6.9	43.1	3.7	188.0
MK713986.1 *Culex pipiens*	39.4	15.0	29.7	15.9	566.0	25.9	15.9	29.6	28.6	189.0	44.4	27.0	12.7	15.9	189.0	47.9	2.1	46.8	3.2	188.0
MK713985.1 *Culex pipiens*	39.4	15.0	29.7	15.9	566.0	25.9	15.9	29.6	28.6	189.0	44.4	27.0	12.7	15.9	189.0	47.9	2.1	46.8	3.2	188.0
KU578141.1 *Coccinella transversalis*	37.1	18.6	29.0	15.4	566.0	23.8	16.4	34.9	24.9	189.0	45.0	25.4	13.2	16.4	189.0	42.6	13.8	38.8	4.8	188.0
JF835944.1 *Nephila inaurata*	41.5	12.2	28.8	17.5	566.0	32.3	11.1	26.5	30.2	189.0	46.6	24.3	13.8	15.3	189.0	45.7	1.1	46.3	6.9	188.0
MN125137.1 *Pyganodon grandis*	39.8	15.4	20.8	24.0	566.0	28.6	15.3	25.4	30.7	189.0	45.5	21.7	13.8	19.0	189.0	45.2	9.0	23.4	22.3	188.0
Avg.	39.1	15.8	29.2	16.0	566.0	25.4	15.7	31.0	28.0	189.0	45.0	26.0	13.2	15.8	189.0	46.8	5.5	43.4	4.3	188.0

Table 2.21d: The evolutionary divergence percentage between *Mansonia bonneae* and its closely related species

Sl. No.	Accession Number	Organism	Percentage of Divergence
1	MT177149.1	*Mansonia bonneae*	0.00%
2	KF407919.1	*Mansonia bonneae*	0.00%
3	MG816360.1	*Mansonia bonneae*	0.26%
4	LC517270.1	*Mansonia africana*	5.14%
5	LC517280.1	*Mansonia africana*	5.28%
6	MW321855.1	*Mansonia dives*	6.39%
7	MW321854.1	*Mansonia dives*	6.69%
8	MW542315.1	*Aedes albopictus*	14.77%
9	MT890465.1	*Aedes albopictus*	14.77%
10	MK713986.1	*Culex pipiens*	12.23%
11	MK713985.1	*Culex pipiens*	12.23%
12	KU578141.1	*Coccinella transversalis*	19.07%
13	JF835944.1	*Nephila inaurata*	21.41%
14	MN125137.1	*Pyganodon grandis*	34.32%

2.3.20.2 Discussion

The traditional classification of Culicidae is phenetic. Consequently, all classification levels are arbitrary groupings based on the subjective interpretation of anatomical similarity. As seen below, the formal acceptance of broad genus-group concepts has resulted in a classification of paraphyletic and polyphyletic taxa (Harbach, 2007). NCBI BLAST tool revealed the COI sequences of *Ma. bonneae* from Kerala are 100% sequence similar to *Ma. bonneae* isolated from Pakistan. So, it can be interpreted that the sequence result obtained for the *Ma. bonneae* from Kerala is novel. The sequence obtained in this study was used for phylogenetic analysis, revealing the evolutionary relationship of various *Mansonia* species.

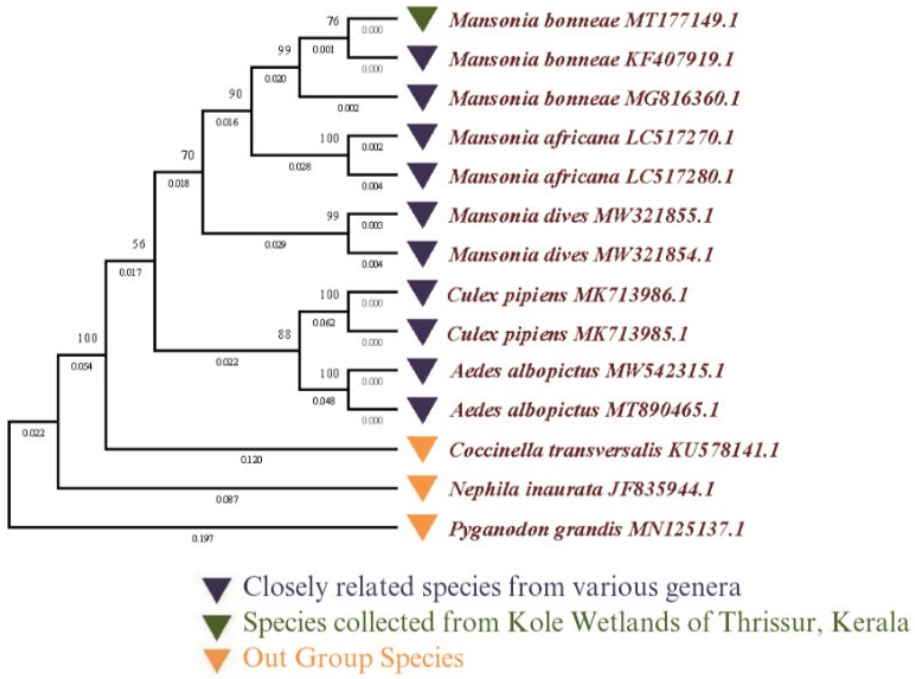

Figure 2.20c Phylogenetic tree of *Mansonia bonneae*

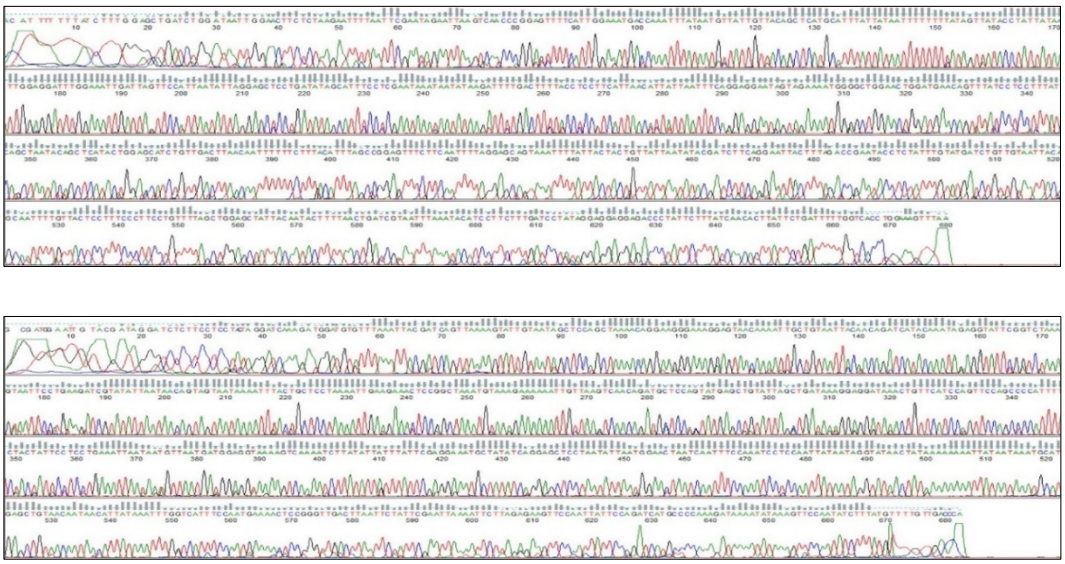

Figure 2.20d Electrophorogram showing the nucleotide sequence of mitochondrial COI gene of *Mansonia bonneae*

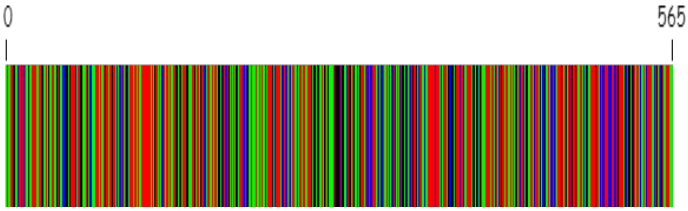

Figure 2.20e Molecular barcode of *Mansonia bonneae*

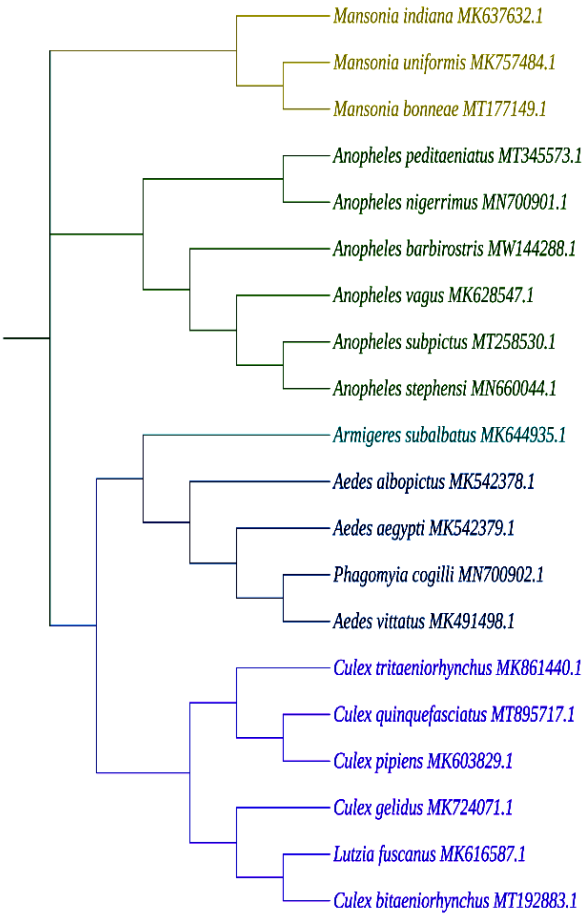

Figure 2.21 Phylogenetic tree of collected mosquito species from Kole wetlands of Thrissur, Kerala during the study period

2.4 Conclusion

The recognition of mosquito species existing in an area plays a vital role in maintaining biodiversity and ecological equilibrium. Mosquitoes are a significant concern in public health due to their ability to act as vectors. Molecular identification through DNA sequencing provides a more accurate and rapid identification process than conventional taxonomic methods. The results from DNA barcoding were found to be similar to those obtained through morphological identification, and importantly, it was able to accurately identify species in cases where morphological identification failed, for instance, in the presence of

indistinguishable characteristics in damaged specimens or the existence of subspecies and closely related species. Knowing the types of mosquitoes present in an area is important for maintaining the balance of the ecosystem and biodiversity. Mosquitoes play a significant role in public health because they can transmit diseases between humans and animals. DNA barcoding provides a more accurate and quicker method of identifying species than traditional taxonomic methods, which may not work if the specimen is damaged or if there are indistinguishable subspecies or sibling species. DNA barcoding can be used by non-ecologists to identify disease-carrying mosquitoes and study the diseases they spread, as well as to find treatments. A global effort to create a reference barcode library of mosquitoes can assist public health professionals in controlling these species more efficiently. In this study, COX1 gene sequencing of 20 morphologically identified species was done, and the results were confirmed through NCBI nucleotide blast. COX1 fragment sequences of the morphologically identified species of mosquitoes were deposited to the NCBI GenBank, obtaining unique accession numbers. Phylogenetic analysis was performed on the final sequences to understand their relationships with other neighbouring species. This study provides a new and valuable addition to existing databases. The molecular barcode generated can be useful in future vector management programs in identifying and analysing the species' phylogenetic position based on their DNA sequences.

CHAPTER 3

A Comparative study on susceptibility of *Aedes albopictus* and *Culex quinquefasciatus* against conventional insecticides

3.1 Introduction

Mosquitoes are considered to be important Dipterans because of their vectorial capacity. Different species of mosquitoes carry various pathogens ranging from viruses to nematodes. Numerous cases of mosquito-borne diseases are reported worldwide and even cause death. Most disease-carrying mosquitoes belong to three genera, *Anopheles, Culex*, and *Aedes* (Lindsay et al., 1993; Jones et al., 2002; Samuel et al., 2004; Pitzer et al., 2009). Malaria, dengue, chikungunya, zika, filariasis, yellow fever, Japanese encephalitis, etc., are major diseases spread by mosquitoes. Mosquito-borne diseases are considered one of the prime reasons for mortality and illness around the globe, especially in tropical and subtropical countries. Besides this disease transmission, mosquitoes are the most annoying human pest by their irritating blood-feeding behaviour (Jang et al., 2002; Wilder et al., 2009). This study compared susceptibility of mosquito species from selected areas of Thrissur Kole wetlands, Kerala, and insecticide free laboratory strain mosquitoes against some conventional insecticides. Field collected and laboratory reared *Ae. albopictus* and *Cx. quinquefasciatus* were chosen to study the susceptibility status of mosquitoes against some traditional insecticides.

Ae. albopictus was an indigenous species in southeast Asia; hence the name Asian tiger mosquito; then, it spread worldwide. It has the potential vectorial capacity to transmit chikungunya, dengue and some other viruses like West Nile Virus (Aneesh et al., 2014). Freshwater ecosystems generally favour breeding of *Ae. albopictus*, and a peak in the emergence is frequently reported during rainy seasons. The female

mosquitoes are active all through the daytime, making an angle of forty-five degrees against the feeding surface with their abdomen during blood feeding. As most of the wetland ecosystem entails fresh water, it could provide a variety of breeding habitats for this species, like tree holes, containers, leaf axils, and leaf internodes (Yap et al., 1997; Senthamarai and Jebanesan, 2014).

Cx. quinquefasciatus is one of the most annoying mosquito species (Yap et al., 2000). The breeding habitats of *Cx. quinquefasciatus* mosquitoes varies from slightly polluted to highly polluted waterbodies. They are known to be evening feeders as they predominantly tend to feed during the late evening to early morning. The female members of this mosquito species make a forty-five-degree angle between the abdomen and the surface of the feeding object during blood feeding (Zuharah and Sumayyah, 2019). They belong to the typical inhabitants of the wetlands as the wetland ecosystem involves polluted water favouring their breeding (Owolabi and Bagbe, 2019).

Chemical control is one of the most successful mosquito control strategies in decades. In most areas, chemicals, including organophosphates, organochlorines, carbamates, and synthetic pyrethroids, control mosquitoes effectively (Ho and Zairi, 2013). Wetland ecosystems need special attention in mosquito control because the risk of disease transmission is high. It is considered a biodiversity hotspot marking the presence of significant inhabitants like migratory birds, rodents, cattle, etc. Furthermore, rice cultivation is practiced after every monsoon season, which raises the presence of workers and natives high during this cultivation period, enhancing the transmission of mosquito-borne diseases (Schafer, 2004; Schafer et al., 2008; Dian and Changxing, 2011; Srinivasan, 2012).

Wetlands are low-lying regions, which may result in the accumulation of insecticide residues applied in nearby areas. The Thrissur Kole wetlands are located around Thrissur Corporation, an urban area and the chemicals used for mosquito control are drained into the Kole wetlands through rainwater. This additional deposition of chemicals bigger than those other chemicals applied directly to this region (Srinivasan, 2010). An enormous quantity of chemical residues in the form of pesticides and fertilizers is accumulated in Kole wetlands through different pest management tactics practiced for pest control during rice cultivation in the post-monsoon season (Srinivasan, 2012; Joseph, 2016). In these areas, a wide array of chemical insecticides is applied to control vector rearing and spreading. This tactless application could lead to resistance development, in which insects alter their gene expression by a mutation in their gene in both targeted and non-targeted species. Resistance against some insecticides may cause tolerance against other insecticides resulting in cross-resistance (Suwanchaichinda and Brattsten, 2001; Riaz et al., 2009).

The pollution hazards created by the insensitive use of chemical insecticides are increasing exponentially and destroying wetland ecosystem's natural resources. A thorough study regarding the susceptibility status of the mosquito vector species is needed to control this situation. This result could help strategize an effective but adequate quantity of chemical insecticides for successful vector management without damaging the ecosystem. In this study, five commonly used chemical insecticides were selected, and their LC_{50} values against field-collected and laboratory-maintained major vectors like *Ae. albopictus* and *Cx. quinquefasciatus* were calculated. This comparison would provide a picture of the resistance

development in those species in the study area due to the extensive and continuous exposure to chemical insecticides.

3.2 Methodology

3.2.1 Insecticide

Lambda-cyhalothrin, Deltamethrin, Malathion, Temephos, and Propoxur (Technical grade) were purchased from "New India Surgicals, Calicut, Kerala, India.

3.2.2 Mosquito Sampling and Colony Maintenance

Ae. albopictus and *Cx. quinquefasciatus* mosquito larvae were collected from different localities of Thrissur Kole wetlands and identified by classical taxonomic methods (Barraud,1934; Nagpal and Sharma 1995). Collected larvae were reared into adults in laboratory conditions (Temperature 26±2, larval food prepared by mixing yeast and dog biscuit). Adult mosquitoes were fed with 5% sucrose and provided a blood meal on the third day of emergence. F1 progeny larvae of field-collected mosquitoes were subjected to larval bioassay. Laboratory colonized insecticide-free *Ae. albopictus*, and *Cx. quinquefasciatus* were maintained in the Communicable Disease Research Laboratory at St. Joseph's College, Irinjlalakuda. These untreated larvae were also used in larval bioassay as laboratory strains.

3.2.3 Larval Bioassay

The method used to determine larval susceptibility follows WHO protocol (Brown, 1986). Consequently, the larvae were exposed to various insecticide concentrations prepared from stock solutions using distilled water as the solvent. 1mg/ml stock solution of lambda-cyhalothrin, deltamethrin, malathion, temephos, and propoxur was prepared in water. Test concentrations were prepared by adding 1ml insecticide-

containing solution to 249 ml of water in a 500 ml capacity beaker and stirring strenuously for 30 seconds with a glass rod. In place of pesticide, 249 ml of dechlorinated water was mixed with 1ml of distilled water or pure alcohol as necessary for the control. With a strainer, 25 late third or early fourth instar larvae were released into each of the beakers containing various tests and control. Six serial test concentrations of insecticides were prepared for larval bioassay. Mortality was recorded after 24 hours. Unmoved and moribund larvae were treated as dead. Using probit analysis, the dosage mortality regression line calculated the LC_{25}, LC_{50} and LC_{90} values for insecticides (Finney,1971).

3.2.4 Statistical Analysis

The lethal values of the pesticides that were tested were computed using SPSS version 22. LC_{25}, LC_{50}, and LC_{90} values were calculated to discuss the larval bioassay result with experimented insecticides. One way ANOVA analysis was done with the observed values of larval bioassay.

3.3 Result and Discussion

3.3.1 Result

Larvicidal efficacy of 5 different insecticides on field-collected and laboratory-reared *Ae. albopictus* and *Cx. quinquefasciatus* is discussed in this chapter. Lambda-cyhalothrin, deltamethrin, malathion, temephos, and propoxur, which come under 3 different categories, were selected for the larval bioassay. *Ae. albopictus* and *Cx. quinquefasciatus*, which were collected from the Kole wetlands of Thrissur, Kerala, were considered field-collected mosquito strains, and laboratory strains were reared in the Communicable Disease Research Laboratory, St. Joseph's College,

Irinjalakuda. LC_{25}, LC_{50} and LC_{90} values of all these insecticides are displayed in Table 3.1 and Table 3.2.

Malathion comes under the organophosphate group of insecticides, which is lethal to insects and less harmful to mammals. Only after DDT and dieldrin were outlawed in the USA in the 1970s were these pesticides used internationally to control mosquitoes. Results of malathion susceptibility of field-collected and laboratory-colonized *Ae. albopictus*, and *Cx. quinquefasciatus* larvae illustrated that the laboratory strain was more susceptible than the field-collected strain. LC_{50} value of field-collected *Ae. albopictus* was $1.4x10^{-2}$ ppm, about 6.66 times greater than the laboratory strain whose LC_{50} was $2.1x\ 10^{-3}$ ppm. LC_{50} value of field-collected *Cx. quinquefasciatus* $3.1x10^{-2}$ ppm and LC_{50} of laboratory strain was $8x10^{-3}$ ppm which is 3.87 times lesser than field strain. Figure 3.1 and Figure 3.2 illustrated the comparison of percentage mortality of Field and Laboratory strain of *Ae. albopictus*, and *Cx. quinquefasciatus* mosquitoes against malathion.

Temephos is also coming under the organophosphate group of insecticides. Larval bioassay result revealed that LC_{50} value of field-collected *Ae. albopictus* was $3.9x10^{-2}$ ppm, and laboratory strain *Ae. albopictus* was $1.7x10^{-3}$ ppm. LC_{50} value of field strain *Cx. quinquefasciatus* was $7.3\ x10^{-2}$ ppm, and that of laboratory strain was $6.4x10^{-3}$ ppm. Both field-collected and laboratory reared *Ae. albopictus* were more susceptible than *Cx. quinquefasciatus*. All the LC_{50} values indicated that field-collected mosquitoes had higher LC_{50} values than laboratory strains. Approximately 22 times and 11 times difference happened in these *Aedes* and *Culex* species, respectively. Figure 3.3 and Figure 3.4 illustrated the comparison of percentage mortality of field and laboratory strain of *Ae. albopictus*, and *Cx. quinquefasciatus* mosquitoes against temephos.

Carbamates are similar to organophosphate insecticides in their mode of action. Propoxur is a widely used chemical in integrated pest management programs. Susceptibility test results disclosed that laboratory strain *Ae. albopictus* had lesser LC_{50} value ($4.1x10^{-2}$ ppm) than field collected ($6.18x10^{-1}$ ppm). *Cx. quinquefasciatus* followed the same pattern as *Ae. albopictus.* LC_{50} of field collected *Cx. quinquefasciatus* was $2.28x10^{-1}$ ppm, and that of laboratory strain was $7.7x10^{-2}$ ppm. Figure 3.5 and Figure 3.6 illustrated the comparison of percentage mortality of field and laboratory strain of *Ae. albopictus*, and *Cx. quinquefasciatus* mosquitoes against propoxur.

Pyrethroids are highly effective, commonly used insecticides. Deltamethrin and lambda-cyhalothrin are two synthetic pyrethroids used in mosquito eradication programs. Deltamethrin susceptibility result displayed that LC_{50} value of field-collected *Ae. albopictus* was $6.7x10^{-4}$ ppm, and laboratory strain *Ae. albopictus* was $5.5\ x10^{-5}$ ppm. LC_{50} value of field strain *Cx. quinquefasciatus* was $9x10^{-4}$ ppm, and that of laboratory strain was $8.4x10^{-5}$. Deltamethrin LC_{50} values indicated that this pyrethroid is a more effective insecticide than organophosphate and carbamate. In the case of both tested species, laboratory-strain mosquitoes were more susceptible than field-collected ones. Figure 3.7 and Figure 3.8 illustrated the comparison of percentage mortality of field and laboratory strain of *Ae. albopictus*, and *Cx. quinquefasciatus* mosquitoes against deltamethrin.

The susceptibility results of another pyrethroid, lambda-cyhalothrin, denoted that the laboratory strain was more susceptible than the field strain. LC_{50} of field-collected *Ae. albopictus* $3.2x10^{-4}$ ppm, and LC_{50} of laboratory strain was $3.5x10^{-5}$ ppm. LC_{50} of field-collected *Cx. quinquefasciatus* is $7.4x10^{-4}$ ppm and LC_{50} of laboratory strain *Cx. quinquefasciatus* was $5.4x10^{-5}$ ppm. Among these group of insecticides, lambda-

cyhalothrin shows more efficacy than the other four insecticides. Figure 3.9 and Figure 3.10 illustrated the comparison of percentage mortality of field and laboratory strain of *Ae. albopictus*, and *Cx. quinquefasciatus* mosquitoes against lambda-cyhalothrin.

Ae. albopictus mosquitoes showed greater vulnerability when five different pesticides were applied to the field and laboratory strains of *Cx. quinquefasciatus* in all three groups. When compared to the other four insecticides used, lambda-cyhalothrin was the most effective, while propoxur had the lowest efficacy. All mosquitoes showed more susceptibility towards lambda-cyhalothrin and less towards propoxur. The susceptibility rate can be illustrated as Lambda-cyhalothrin > Deltamethrin > Temephos > Malathion > Propoxur.

3.3.2 Discussion

Over the past few year's unusual disease occurrences and severe annoyance have been frequent in infected areas; hence scientists found that mosquito control would be the most appropriate solution for this puzzle (Invest and Lucas, 2008). Chemical treatment leftovers as the primary indispensable tactic used in pest management because of their rapid result in the broad treatment area (Ho and Zairi, 2013). Good insecticide practices are regarded as an influential defence to achieve better public health manifestations and to increase agricultural yield in developing countries (Lengeler 2004; Mabaso et al., 2004; Karunamoorthi et al., 2012). WHO recommended 12 chemical insecticides to control the mosquito population in mosquito eradication strategies worldwide. These insecticides belonged to organochlorines, organophosphates, carbamates, and pyrethroids (Marcombe et al., 2009; WHO, 2009).

Malathion belongs to the organophosphate group of insecticides, lethal to insects and less harmful to mammals. After excluding DDT and dieldrin in the USA, this second-generation insecticide has been used worldwide for mosquito control since the 1970s. It is generally used in agricultural pest management and vector control plans in public health (Cox, 2003; Prato et al., 2012). Mixing this chemical into adjacent waterways would be harmful to nontargeted species. Larval mosquitoes in this environment were naturally exposed to the insecticides and their residues (Gratz and Jany, 1994; U.S. Department of Agriculture, 1997; Walker, 2000; Relyea, 2004). The present study reveals that laboratory strain *Ae. albopictus* and *Cx. quinquefasciatus* is more susceptible than the field stains collected from Thrissur Kole wetlands. Malathion resistance studies have been reported since the 1990s. Bisset et al., 1990; Bracco et al., 1997 said malathion and carbamate resistance in *Cx. quinquefasciatus* in Cuba and Brazil, respectively. In 2010, Selvi et al., discussed their result in the study on the susceptibility of *Ae. albopictus* against malathion. They discovered that field-strain mosquitoes were more resistant than laboratory-strain mosquitoes due to the frequent exposure to insecticides used in the area (Selvi et al., 2010).

Another second-generation insecticide used in pest management is temephos. World Health Organization recommended this organophosphate as a mosquito larvicide (WHO, 2006). Viana et al., spotted such international usage of temephos as a larvicide in their study in 2018 (Viana et al., 2018). Due to the high efficacy of this chemical, it was effusively used in almost all countries and led to resistance development in mosquitoes against them (Melo-Santos et al., 2010; Grisales et al., 2013; Koou et al., 2014; Prophiro et al., 2021). In 2014 Singh et al., assessed temephos LC_{50} values against *Ae. aegypti* obtained from several Delhi locations.

They provided LC_{50} values between 0.007 ppm and 0.018 ppm. According to the current investigation, temephos had a LC_{50} value of $3.9x10^{-2}$ ppm against *Ae. albopictus*, which was found in the Thrissur Kole wetlands. Field-collected mosquitoes exhibited more resistance than laboratory strains, both *Ae. albopictus*, and *Cx. quinquefasciatus* mosquitoes. The susceptibility status of *Aedes* and *Culex* mosquitoes against organophosphate was documented in the 1970's itself, and they observed LC_{50} values of temephos ranges from 0.001-0.01 ppm (Rettich, 1977).

Propoxur is a carbamate with a similar structure and mode of action to organophosphate. Carbamates can permanently block acetylcholine, called acetylcholine inhibitors (Prato et al., 2012). The present study showed that the laboratory strain of *Ae. albopictus* and *Cx. quinquefasciatus* mosquitoes were more susceptible than field-collected mosquitoes. Carbamates are weaker insecticides than organophosphates. In 1972 Georghiou published data in California that supported this statement. LC_{50} value of malathion is 0.085 ppm, and propoxur was 0.39 ppm. In Thailand, Jirakanjanakit et al., conducted a study during 2003-2005, and they estimated the LC_{50} value of propoxur in *Aedes* mosquitoes reported as 0.04 ppm. Organophosphate, carbamate, and pyrethroid resistance were also noted in *Cx. quinquefasciatus* mosquito by some studies conducted in America, Saudi Arabia, and northern Thailand (Georghiou et al., 1980; Amin and Pieris 1990; Somboon et al., 2003).

Synthetic pyrethroids are one of the newer insecticide groups, which evolved in the 1980's (Prato et al., 2012). Pyrethroid compounds are used for the control of agricultural pests and pathogen carriers. In 1993 Dorta et al., studied pyrethroid and organophosphorus susceptibility in six vector species. Their results indicated that synthetic pyrethroids were more efficient insecticides than organophosphates. They

recorded the LC_{50} values of deltamethrin against *Aedes albopictus* as 6.96 $x10^{-6}$ ppm, and that of *Culex quinquefasciatus* was $2.95x10^{-6}$ ppm. In India, Bansal and Singh 2007 recorded a similar deltamethrin susceptibility of 0.00004 ppm in *Ae. albopictus* and 0.00073 ppm in *Cx. quinquefasciatus*. Several national and international researchers conducted studies on mosquito susceptibility against different insecticides. Most of them concluded that synthetic pyrethroids were the most efficient group of insecticides when compared to other conventional insecticides (Nazni et al., 2005; Jahan and Shahid. 2013; Hariprasad and Arun, 2015; Hammad et al., 2015; Kopya et al., 2015; Meena, and Kachhwaha, 2016).

Kole Wetland is the direct rainfall inundation region of the Thrissur district's environs. Every year, the Karuvannur and Kecheri river's urban and industrial atrophies are transported to this delta by the monsoon floodwaters (Binil and Ramanathan, 2008). Tessy and Sreekumar, in 2008, reported almost 30 pollutant-tolerant algal species from Thrissur Kole lands. The paddy production methods occur from the northeast monsoon through the post-monsoon season at our research site, one of Kerala's key rice bowls (September to March). To safeguard their crops and achieve optimum output, the farmers have sprayed a tremendous quantity of insecticides, weedicides, and fertilizers in these paddy fields (Srinivasan, 2012; Joseph, 2016). These chemical combinations cause insecticidal tolerance in mosquito species and other non-targeted organisms. Two individual studies publicized such tolerance in *Ae. albopictus* and *Ae. aegypti* by Suwanchaichinda and Brattsten, 2001, Riaz et al., 2009 respectively.

In the current study, we compared the susceptibility status of field-collected and laboratory-colonized *Ae. albopictus* and *Cx. quinquefasciatus* mosquitoes against malathion, temephos, propoxur deltamethrin, and lambda-cyhalothrin. Laboratory

strains *Ae. albopictus* and *Cx. quinquefasciatus* were more susceptible than field strain. The outcome unveiled that both *Ae. albopictus* and *Cx. quinquefasciatus* exhibited more susceptibility towards lambda-cyhalothrin and is least susceptible to propoxur. Susceptibility rate in the order Lambda-cyhalothrin > Deltamethrin > Malathion > Temephos > Propoxur in these mosquitoes.

Hamdan et al., 2005 discovered some LC_{50} values in *Ae. albopictus* and *Cx. quinquefasciatus* mosquitoes. The result of *Ae. albopictus* susceptibility test is LC_{50} of malathion is 0.1972 ppm, temephos 0.0514 ppm, and permethrin 0.0022 ppm. LC_{50} values in *Cx. quinquefasciatus* against malathion is 0.0163 ppm, and permethrin is 0.00001 ppm. In our study *Ae. albopictus* shows more susceptibility towards malathion temephos propoxur, deltamethrin, and lambda-cyhalothrin than *Cx. quinquefasciatus* larvae. It may be because of larval habitats of *Culex* mosquitoes is more polluted when compared to *Aedes* and *Anopheles* species which breed in freshwater habitats.

Aedes mosquitoes typically live in freshwater habitats, while *Culex* prefers to grow in moderate to severely polluted water. The fact that both species were gathered from the same location, according to the current research, suggests that these mosquitoes had some resistance level. Susceptibility assays also support these findings against various widely used insecticides in the pest management study area. The difference between field and laboratory strains can be seen in the results of the quantitative susceptibility assays, where field strains displayed greater resistance to all of the tested insecticides. According to a study done in Thailand, *Cx. quinquefasciatus* exhibited this array of resistance to several insecticides, and the researchers hypothesised that this resistance may have developed due to the frequent contact between insecticides (Sathantriphop et al., 2006). The remarkable difference

in LC_{50} values between strains of the same species may be the product of the evolution of insecticide resistance. This trait may be essential as a biological indicator of insecticide pollution (Wielgolaski, 1975). There have been some early observations regarding mosquitoes as potential bioindicators of insecticide pollution, and some recent studies have supported the use of *Culex* mosquitoes as bioindicators of lead effluence (Kitvatanachai et al., 2011). The results of this study are more important because the study area is used for seasonal paddy cultivation and regular exposure to the aforementioned chemical insecticides for insect pest management (Srinivasan, 2012).

Table 3.1 Mosquito larvicidal effect of *Aedes albopictus* against Malathion, Temephos, Propoxur, Deltamethrin and Lambia-cyhalothrin

INSECTICIDE	LC_{25} (LCL-UCL)	LC_{50} (LCL-UCL)	LC_{90} (LCL-UCL)	P VALUE
FIELD STRAIN				
MALATHION	9×10^{-3} ppm (6×10^{-3} - 1.1×10^{-2})	1.4×10^{-2} ppm (1.2×10^{-2} - 1.6×10^{-2})	2.4×10^{-2} ppm (2.1×10^{-2} - 2.9×10^{-2})	<0.001
TEMEPHOS	3.1×10^{-2} ppm (2.7×10^{-2} - 3.4×10^{-2})	3.9×10^{-2} ppm (3.6×10^{-2} - 4.2×10^{-2})	5.9×10^{-2} ppm (5.2×10^{-2} - 7.3×10^{-2})	<0.001
PROPOXUR	5.19×10^{-1} ppm (4.69×10^{-1} – 5.56×10^{-1})	6.18×10^{-1} ppm (5.81×10^{-1}– 6.57×10^{-1})	8.60×10^{-1} ppm (7.86×10^{-1} - 1.002)	<0.001
DELTAMETHRIN	5.9×10^{-4} ppm (5.5×10^{-4} - 6.2×10^{-4})	6.7×10^{-4} ppm (6.4×10^{-4} - 7×10^{-4})	8.4×10^{-4} ppm (7.9×10^{-4} - 9.2×10^{-4})	<0.001
LAMBDA-CYHALOTHRIN	2.7×10^{-4} ppm (2.4×10^{-4} - 2.9×10^{-4})	3.2×10^{-4} ppm (3×10^{-4} - 3.4×10^{-4})	4.5×10^{-4} ppm (4.1×10^{-4} - 5.1×10^{-4})	<0.001
LABORATORY STRAIN				
MALATHION	1.3×10^{-3} ppm (8×10^{-4} - 1.6×10^{-3})	2.1×10^{-3} ppm (1.8×10^{-3} - 2.4×10^{-3})	3.6×10^{-3} ppm (3.2×10^{-3} - 4.3×10^{-3})	<0.001
TEMEPHOS	1×10^{-3} ppm (7×10^{-4} - 1.2×10^{-3})	1.7×10^{-3} ppm (1.4×10^{-3} - 2×10^{-3})	4.6×10^{-3} ppm (3.5×10^{-3} - 7.4×10^{-3})	<0.001
PROPOXUR	3.2×10^{-2} ppm (2.8×10^{-2} - 3.5×10^{-2})	4.1×10^{-2} ppm (3.7×10^{-2} - 4.4×10^{-2})	6.4×10^{-2} ppm (5.6×10^{-2} - 7.9×10^{-2})	<0.001
DELTAMETHRIN	4.8×10^{-5} ppm (4.4×10^{-5} - 5.1×10^{-5})	5.5×10^{-5} ppm (5.2×10^{-5} - 5.8×10^{-5})	7.2×10^{-5} ppm (6.7×10^{-5} - 8×10^{-5})	<0.001
LAMBDA-CYHALOTHRIN	2.7×10^{-5} ppm (2.3×10^{-5} - 3.1×10^{-5})	3.5×10^{-5} ppm (3.2×10^{-5} - 3.8×10^{-5})	5.7×10^{-5} ppm (5×10^{-5} - 6.9×10^{-5})	<0.001

*Statistical Significance ($P<0.05$)

Table 3.2 Mosquito larvicidal effect of C*ulex quinquefasciatus* against Malathion, Propoxur, Temephos, Deltamethrin, and Lambda-cyhalothrin

INSECTICIDE	LC25 (LCL-UCL)	LC50 (LCL-UCL)	LC90 (LCL-UCL)	P VALUE
FIELD STRAIN				
MALATHION	1.7×10^{-2} ppm (8×10^{-3} - 2.3×10^{-2})	3.1×10^{-2} ppm (2.6×10^{-2} - 3.6×10^{-2})	5.8×10^{-2} ppm (5.1×10^{-2} - 6.9×10^{-2})	<0.001
TEMEPHOS	6.7×10^{-2} ppm (6.4×10^{-2} - 7×10^{-2})	7.3×10^{-2} ppm (7.1×10^{-2} - 7.6×10^{-2})	8.6×10^{-2} ppm (8.2×10^{-2} - 9.3×10^{-2})	<0.001
PROPOXUR	1.46×10^{-1} ppm (1.09×10^{-1} - 1.75×10^{-1})	2.28×10^{-1} ppm (1.94×10^{-1} - 2.65×10^{-1})	5.32×10^{-1} ppm (4.23×10^{-1} - 7.96×10^{-1})	<0.001
DELTAMETHRIN	8×10^{-4} ppm (7.5×10^{-4} - 8.4×10^{-4})	9×10^{-4} ppm (8.6×10^{-4} - 9.4×10^{-4})	1.12×10^{-3} ppm (1.06×10^{-3} - 1.24×10^{-3})	<0.001
LAMBDA CYHALOTHRIN	6.8×10^{-4} ppm (6.4×10^{-4} - 7×10^{-4})	7.4×10^{-4} ppm (7.1×10^{-4} - 7.7×10^{-4})	8.8×10^{-4} ppm (8.5×10^{-4} - 9.5×10^{-4})	<0.001
LABORATORY STRAIN				
MALATHION	4×10^{-3} ppm (2×10^{-3} - 5×10^{-3})	8×10^{-3} ppm (6×10^{-3} - 9×10^{-3})	1.5×10^{-2} ppm (1.3×10^{-2} - 1.8×10^{-2})	<0.001
TEMEPHOS	5.5×10^{-3} ppm (5.1×10^{-3}- 5.8×10^{-3})	6.4×10^{-3} ppm (6×10^{-3} - 6.7×10^{-3})	8.3×10^{-3} ppm (7.7×10^{-3} - 9.4×10^{-3})	<0.001
PROPOXUR	6.8×10^{-2} ppm (6.4×10^{-2} - 7.2×10^{-2})	7.7×10^{-2} ppm (7.4×10^{-2} - 8×10^{-2})	9.6×10^{-2} ppm (9.1×10^{-2} - 1.07×10^{-1})	<0.001
DELTAMETHRIN	7.5×10^{-5} ppm (7×10^{-5} - 7.8×10^{-5})	8.4×10^{-5} ppm (8×10^{-5} - 8.7×10^{-5})	1.05×10^{-4} ppm (9.9×10^{-5} - 1.15×10^{-4})	<0.001
LAMBDA CYHALOTHRIN	4.6×10^{-5} ppm (4.1×10^{-5} - 4.9×10^{-5})	5.4×10^{-5} ppm (5.1×10^{-5} - 5.8×10^{-5})	7.6×10^{-5} ppm (7×10^{-5} - 8.8×10^{-5})	<0.001

*Statistical Significance ($P<0.05$)

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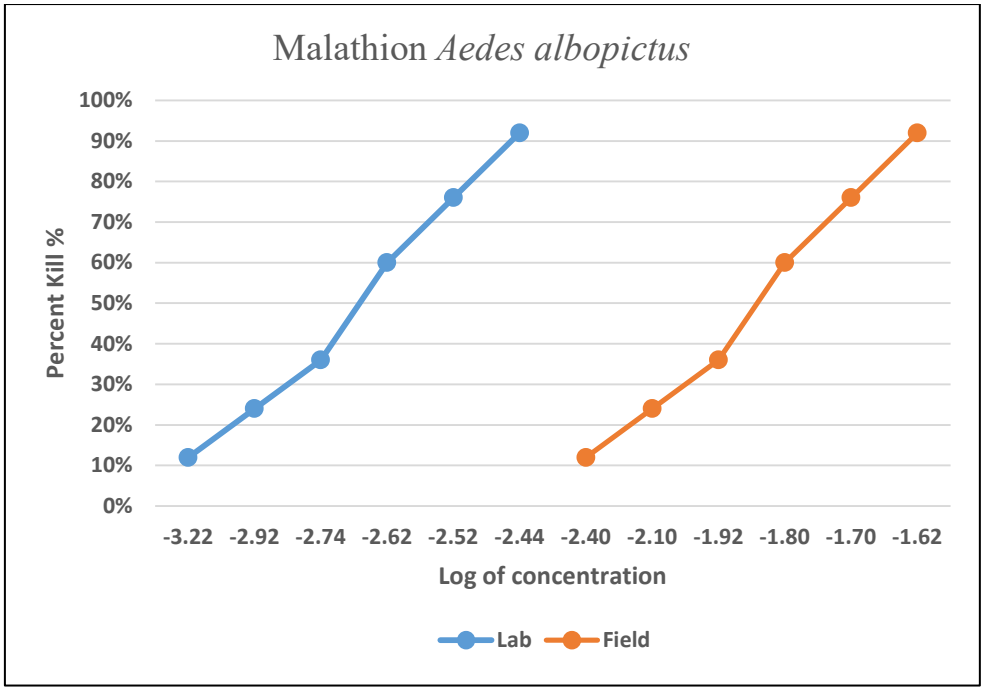

Figure 3.1 Comparison of percentage mortality of Field and Laboratory strain of *Ae. albopictus* against Malathion

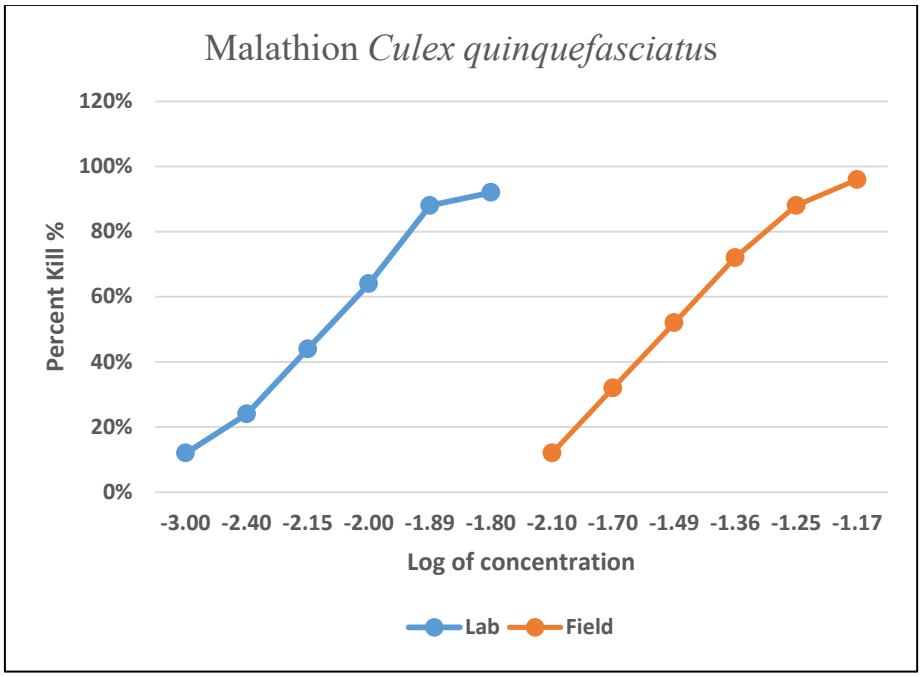

Figure 3.2 Comparison of percentage mortality of Field and Laboratory strain of *Cx. quinquefasciatus* against Malathion.

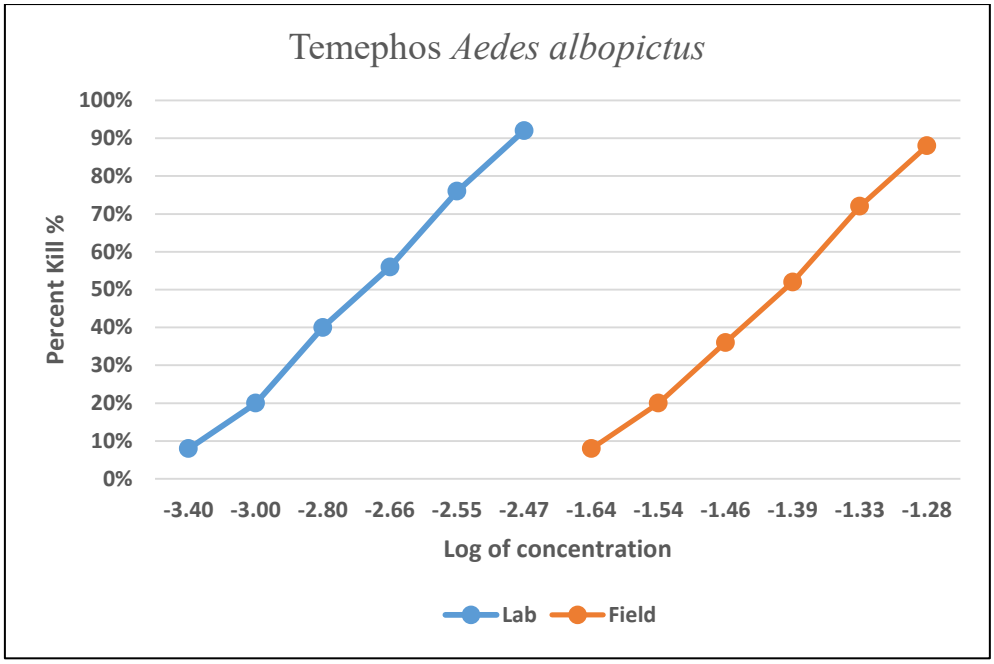

Figure 3.3 Comparison of percentage mortality of Field and Laboratory strain of *Ae. albopictus* against Temephos.

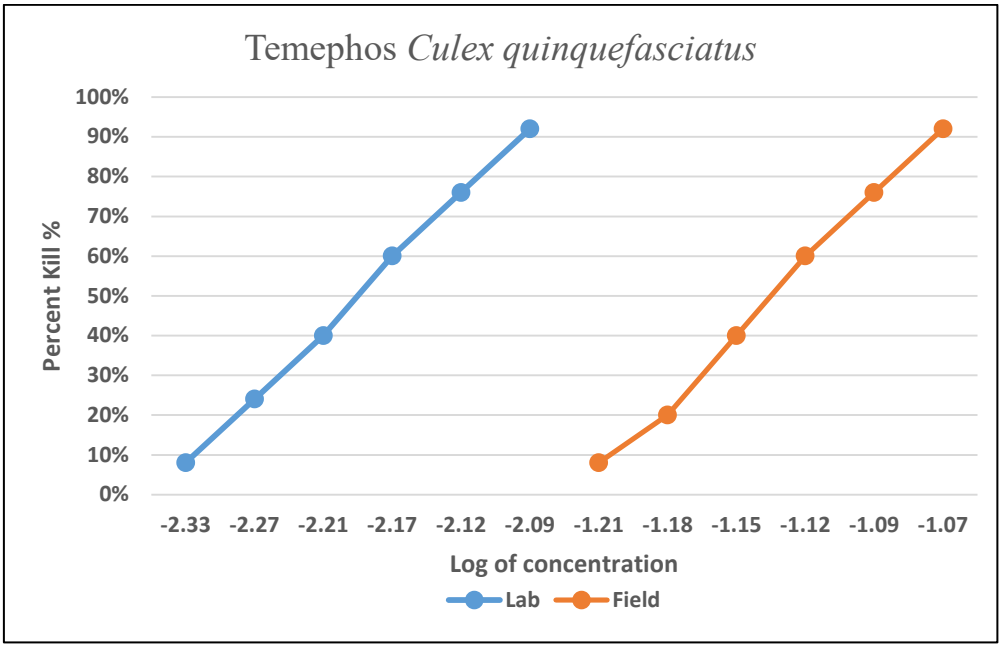

Figure 3.4 Comparison of percentage mortality of Field and Laboratory strain of *Cx. quinquefasciatus* against Temephos.

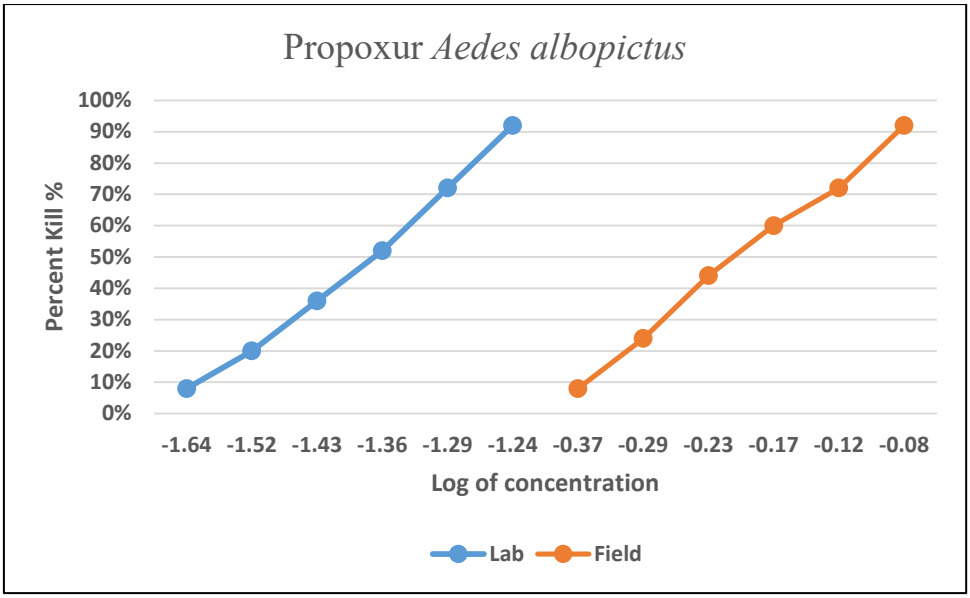

Figure 3.5 Comparison of percentage mortality of Field and Laboratory strain of *Ae. albopictus* against Propoxur.

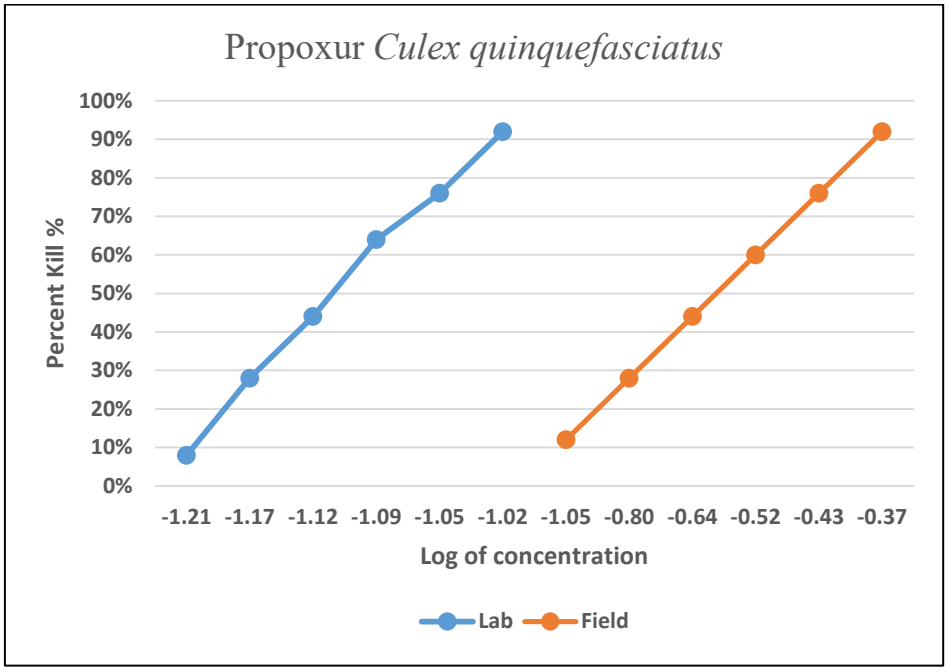

Figure 3.6 Comparison of percentage mortality of Field and Laboratory strain of *Cx. quinquefasciatus* against Propoxur.

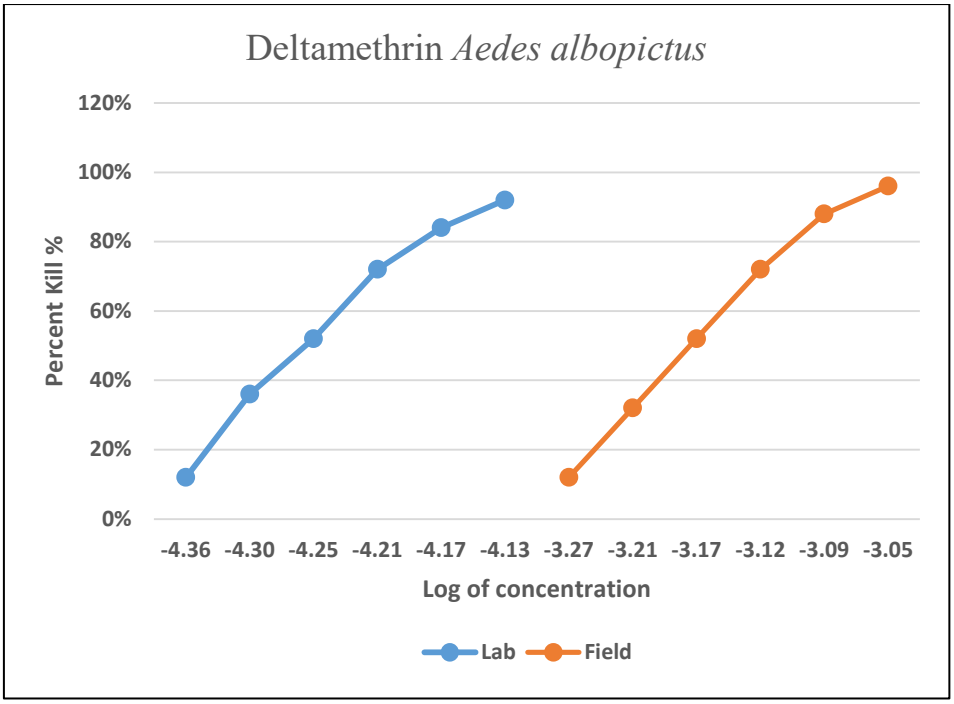

Figure 3.7 Comparison of percentage mortality of Field and Laboratory strain of *Ae. albopictus* against Deltamethrin.

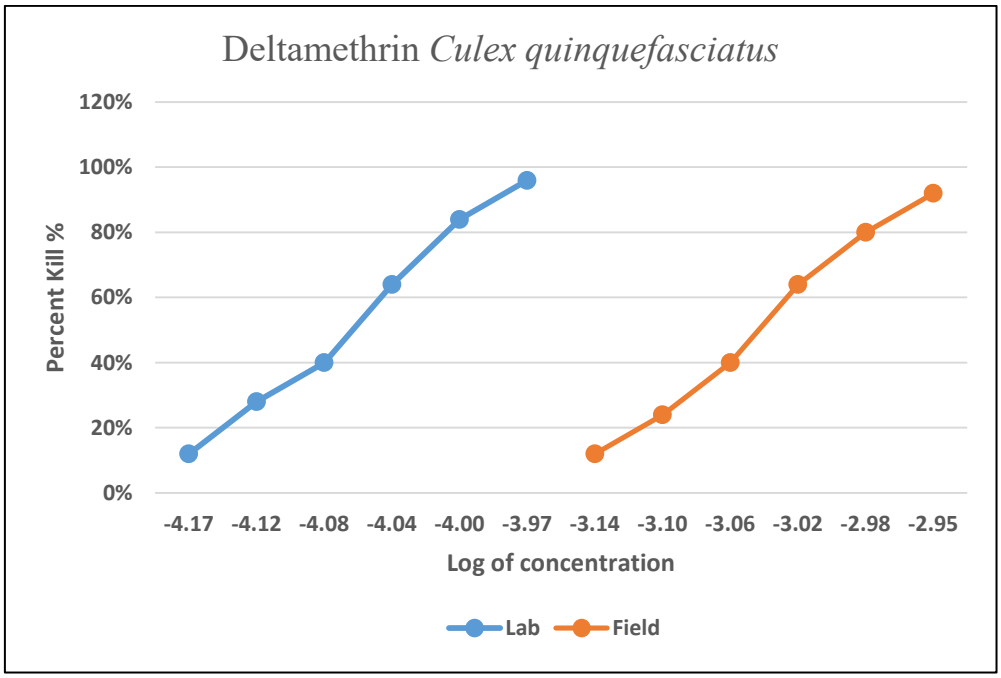

Figure 3.8 Comparison of percentage mortality of Field and Laboratory strain of *Cx. quinquefasciatus* against Deltamethrin.

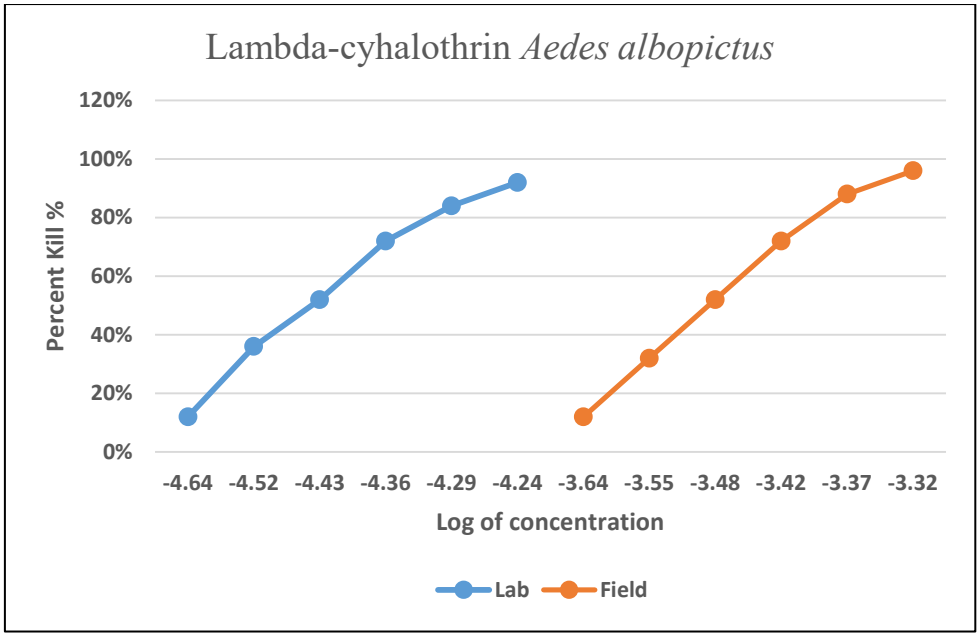

Figure 3.9 Comparison of percentage mortality of Field and Laboratory strain of *Ae. albopictus* against Lambda-cyhalothrin.

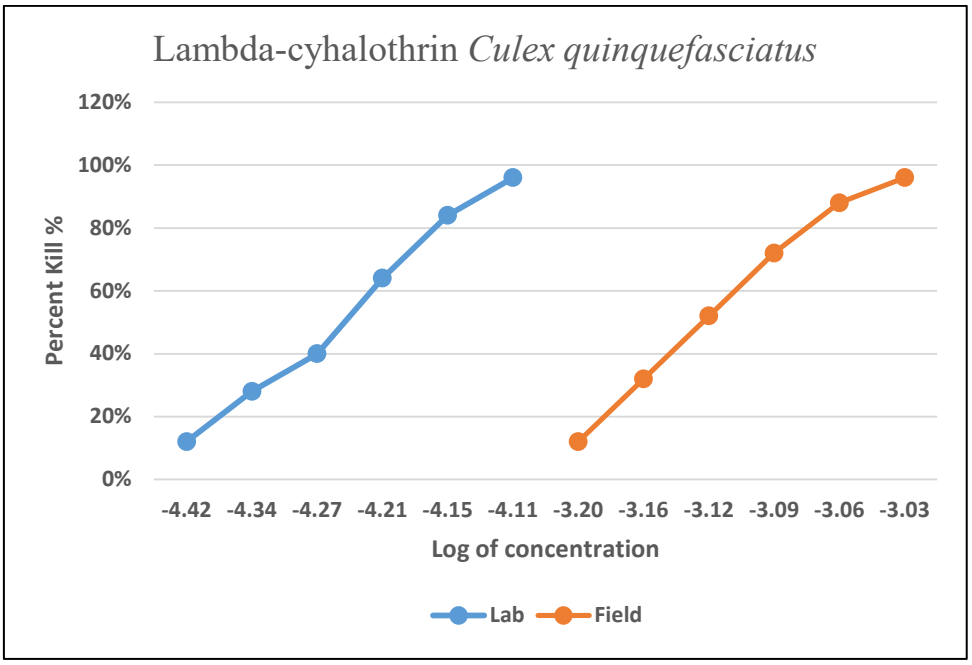

Figure 3.10 Comparison of percentage mortality of Field and Laboratory strain of *Cx. quinquefasciatus* against Lambda-cyhalothrin.

3.4 Conclusion

This chapter discussed the susceptibility comparison analysis of field-collected and laboratory-reared *Ae. albopictus* and *Cx. quinquefasciatus* mosquitoes against five conventional insecticides. Field strain mosquitoes were collected from various regions of the Kole wetlands of Thrissur, Kerala, which was considered an insecticide-exposed strain. Laboratory-reared mosquitoes were free from insecticide exposure and maintained in Communicable Disease Research Laboratory, St. Joseph's College, Irinjalakuda. Five insecticides belonging to three different classes were taken to prepare the test solution (malathion and temephos from organophosphate, propoxur from carbamate, and deltamethrin and lambda-cyhalothrin from synthetic pyrethroid). Comparison analysis of lethal concentration values of field and laboratory strain *Ae. albopictus* and *Cx. quinquefasciatus* revealed that laboratory strain was more susceptible than field strain mosquitoes in every insecticide. The remarkable differences in LC_{50} values between strains of the same species may be the product of the evolution of insecticide resistance. This trait may be essential as a biological indicator of insecticide pollution. In the context of tested insecticides, synthetic pyrethroids were the most influential group of insecticides, and carbamate was the least effective. The insecticide efficacy varies as Lambda-cyhalothrin > Deltamethrin > Temephos > Malathion > Propoxur. Since many years ago, several chemical pesticides have been employed in agricultural and public health contexts to increase crop yield and provide better mosquito management. However, resistance in the targeted organisms is developed because of overuse and excessive usage of these chemical pesticides. Mosquitoes gathered from locations with heavy chemical contamination displayed some insecticidal tolerance. Environmental effluence and negative impacts on organisms that are not the target

are minor components of this ad hoc usage. These principles might contribute to the effectiveness of mosquito-monitoring strategies with a minimum imbalance in the ecosystem.

SUMMARY

The Kole wetlands in Thrissur, Kerala, is a strip of land that transitions between land and permanent water systems. It is a central rice-growing area and is also recognized internationally as a Ramsar site, along with Vembanad Kole. Kole wetlands have their own unique ecological characteristics and are a hotspot for biodiversity, with a large number of birds and animals present. The relationship between wetlands and mosquitoes is well known, with wetlands providing the ideal conditions for the growth and development of mosquitoes, including shelter, food, a favourable environment, and host animals. Mosquitoes have a fundamental impact on public health because of their capability to spread diseases. Mosquito control is a crucial measure in preventing the spread of diseases transmitted by mosquitoes. The Kole wetlands in Thrissur, Kerala, have a high level of human activities such as agriculture, fishing, vegetable cultivation, construction, and research, making the wetlands a bustling environment. This increases the likelihood of the transmission of pathogens between humans and animals, as well as between humans themselves.

Documentation of the presence and influencing characters of available mosquito species in a particular area is a preliminary step in vector control tactics. Chapter 1 deals with collection and identification of mosquito species in selected areas of Kole wetlands of Thrissur Kerala, GIS mapping. Seasonal variation and physicochemical parameters of breeding water which are influencing the abundance of mosquito species during collection also discussed. 20 mosquito species belonging to 5 genera were collected, and identified from different locations during the study period. GIS map of 3 sampling sites prepared with these identified mosquito species. Presence

and abundance of mosquitoes varies in different seasons. Almost all mosquitoes except *Ph. cogilli* and *Ma. bonneae* were collected in either two or three seasons in a year. The results of the ANOVA test indicate that the majority of the mosquito species, except for four (*An. stephensi*, *Ph. cogilli*, *Cx. bitaeniorhynchus*, and *Ma. bonneae*), showed significant variations in their abundance across different seasons. The species that did not show any variation were either present only in one season (*Ph. cogilli* and *Ma. bonneae*) or had a consistent distribution throughout two seasons (*Cx. bitaeniorhynchus*) or three seasons (*An. stephensi*). This study aimed to examine the relationship between the physical and chemical characteristics of water samples from various breeding habitats and the diversity of mosquitoes. During the study period, 20 mosquito species were collected from different breeding habitats, and data was collected on ten water quality parameters. The results showed that pH, Turbidity, Conductivity, TDS, Hardness, and Chloride had a significant correlation with the total number of mosquitoes collected. The rest of the parameters (Temperature, DO, Alkalinity, Salinity) do not correlate with the number of mosquitoes collected. Out of 20 species discovered, 9 mosquitoes express some correlation between the physico-chemical parameters of water samples from their breeding habitats.

Chapter 2 describes the use of DNA sequencing and phylogenetic analysis to improve the accuracy of the identification of mosquito species, especially in cases where conventional taxonomic keys are insufficient for immature forms or sibling species. The chapter explains the molecular identification process through COX1 gene sequencing of morphologically identified mosquito species. The final sequences obtained from each collected species were deposited in the NCBI GenBank and assigned an Accession number. Phylogenetic analysis of collected

species with their related species done for the relationship study. Identical molecular barcode also created with the final sequences. The present study provided a novel report to all databases, and its unique barcode can easily spot and analyse the phylogenetic position of this species based on DNA sequences. This identical database and molecular barcode may be helpful in future vector management programs in that area.

Insecticide application is a widespread method for controlling vectors. Organochlorides, organophosphates, carbamates, and synthetic pyrethroids are the commonly used insecticides for mosquito control. Wetlands, as low-lying areas, may experience the accumulation of insecticide residues from nearby applications. The Thrissur Kole wetlands in Kerala are situated near the Thrissur corporation, an urban area, and chemicals used for mosquito control are carried into the wetlands through rainwater. Excessive chemical deposition in the form of pesticides and fertilizers from pest control practices during rice cultivation in the post-monsoon season in the Kole wetlands adds to the already high amount of chemical residues in the area. The heavy use of chemicals may alter the genetics of mosquito species, leading to a resistance to these chemicals. The study in Chapter 3 compared the susceptibility of mosquito species from Kole lands to insecticides with that of laboratory-reared mosquitoes without exposure to insecticides. Susceptibility status of *Ae. albopictus* and *Cx. quinquefasciatus* against five conventional insecticides were discussed in Chapter 3. Organophosphate, Carbamate, and Synthetic pyrethroids were used for the susceptibility analysis. In the context of tested insecticides synthetic pyrethroids were most effective group of insecticide and carbamate were least effective. The insecticide efficacy varies as Lambda-cyhalothrin>Deltamethrin>Temephos>Malathion> propoxur. Since many years ago,

several chemical pesticides have been employed in agricultural and public health contexts to increase crop yield and provide better mosquito management. However, the development of resistance in the targeted organisms is because of overuse and excessive usage of these chemical pesticides. Mosquitoes that were gathered from locations that had heavy chemical contamination displayed some insecticidal tolerance. Environmental effluence and negative impacts on organisms that are not the target are minor components of this ad hoc usage. These principles might contribute to the effectiveness of mosquito-monitoring strategies with a minimum imbalance in the ecosystem.

RECOMMENDATIONS

- The public health department can utilize documentation of Kole wetland mosquitoes and its GIS map to predict any mosquito-borne disease outbreak associated with these mosquitoes in future.
- Mosquitoes have a significant role in the food chain. Complete eradication or uncontrolled growth of any organism in an ecosystem adversely affects the ecosystem balance. Maintaining biodiversity in Kole wetlands is very important.
- Regulate anthropological activities like clay mining, building construction, and conversion of paddy fields to normal land. These practices will change the identical characteristics of Kole wetlands, and they will also influence the abiotic and biotic components of the wetland ecosystem. Conservation of the Kole wetland is essential.
- Reduce inorganic synthetic fertilizers and pesticides, and promote organic farming. Wide application of pesticides adversely affects the fauna and flora of Kole lands. Decreasing the usage of inorganic compounds helps to reduce chemical pollution. Chemical application rapidly removes pest populations, but in future, the traces of these chemicals cause gene mutation and resistance development in targeted and non-targeted organisms.
- Accurate application of insecticides in vector control programs. Information about mosquitoes' insecticide susceptibility helps determine the amount of insecticide used in vector control tactics. Inappropriate usage of pesticides can generate a resistant population. The resistant population demand more

and more insecticide in future control programs. Adequate and proper insecticide application is the most crucial stage in the vector control program.

- Mosquitoes can serve as signs of pesticide pollution. The difference in toxicity level between field and laboratory strains of the same species could be due to the development of insecticide resistance. This particular trait of mosquitoes found in the field could be crucial in identifying biological markers of pesticide contamination.
- The excessive and frequent use of chemical pesticides can cause non-targeted organisms to develop tolerance towards them. Mosquitoes collected from heavily contaminated areas have shown resistance to insecticides. In Addition, the unregulated use of pesticides can lead to environmental pollution and harmful effects on organisms present in the same environment, which were not the intended target.

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PUBLICATION & PARTICIPATION

PUBLICATION

"Mosquitoes as Pesticide Pollution Indicators- A Comparative Susceptibility Analysis of Field and Laboratory Strains of Mosquitoes Against Different Conventional Insecticides"

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Mosquitoes as pesticide pollution Indicators: A comparative susceptibility analysis of field and laboratory strains of mosquitoes against different conventional insecticides

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ABSTRACT

Thrissur Kole wetlands fall under the category of temporary wetlands that are exceedingly ideal procreation environments for mosquitoes. A broad array of insecticides is used as a competitive weapon in mosquito restriction tactics, including organochlorines, organophosphates, carbamates, and pyrethroids. Indiscriminate use of these chemicals could develop resistance in targeted and nontargeted species present in that environment. The present study is an assessment of the susceptibility status of laboratory and field strain *Aedes albopictus* and *Culex quinquefasciatus* mosquitoes against deltamethrin, lambda-cyhalothrin, and malathion employing the *World Health Organization* protocol. The results show that laboratory strain mosquitoes are more susceptible than the field-collected mosquitoes toward these insecticides. The field strain of *Ae. albopictus* showed 6.36, 11.74, and 18.36 times resistance than the laboratory strain against malathion, deltamethrin, and lambda-cyhalothrin, respectively. *Cx. quinquefasciatus* larvae also repeated this resistance pattern like 4.54 times resistance against malathion and 13.15 times and 12.62 times resistance against deltamethrin and lambda-cyhalothrin, correspondingly. The increased susceptibility of the field strain could also lead to a prospect of treating mosquitoes as an indicator species of pesticide contamination. Finding out the precise dosage of insecticide applications could furthermore help in the vector management program and diminish environmental pollution caused by these chemicals.

1. INTRODUCTION

Wetlands are inimitable waterlogged ecosystems with distinctive abiotic and biotic environmental characteristics. These aquatic systems might support the massive quantity of floral and faunal diversity concerning their origin, topographical position, aquatic organization, and interaction among them [1]. As stated by a public health organization, wetlands are considered favorable procreation grounds for vector mosquitoes transmitting arboviruses and parasites [2,3]. Thrissur Kole wetlands is a shallow-water low-lying strip of the aquatic system acting as an intermediary region between terrestrial and marine ecosystems, which is maintained at 0.5–1 m under sea level. These Ramsar sites are topographically located in the central region of Kerala, which overlays an area of 10,187 ha and spreads across Mukundapuram, Chavakad, and Thrissur Taluks of Thrissur district. Kole lands are inundated around half of the year with monsoon water dispensed by the two major rivers in Thrissur, and in the next half of the year, paddy as well as vegetable cultivation is practiced [4]. Byproduct deposition by agriculture could modify the depth and parameters of the water system, and these favorable changes might provide a reproducing environment for mosquitoes. For example, declining water temperature led to vector species development [5,6].

Many parasites and arboviruses that are causative for diseases like malaria, dengue, chikungunya, zika, filariasis, yellow fever, Japanese encephalitis, etc. have accomplished association with some mosquitoes to disperse their pathogenicity. Mosquito-borne diseases are considered one of the primary reasons for mortality

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and illness around the globe, especially in tropical and subtropical countries. Apart from the disease transmission, mosquitoes are the most annoying human pests with their irritating blood-feeding behavior [7,8]. *Anopheles, Aedes, and Culex* are the three major genera that comprise disease-spreading vector mosquitoes [9]. *Cx. quinquefasciatus* is one of the most annoying common mosquito species [10]. This mosquito can carry a nematode called *Wuchereria bancrofti* which cause filariasis, West Nile virus, Saint Luis encephalitis virus, Ross river virus, and Japanese encephalitis virus across the Earth [11–15]. *Ae. albopictus* is an indigenous species in Southeast Asia; hence, the name Asian tiger mosquito, which is spread all over the world. It has the potential vectorial capacity to transmit chikungunya, dengue, and some other west Nile viruses [16,17].

Mosquito-borne diseases substantially provide infection load, mortality, poverty, and devitalization to the society in tropical countries where the disease has been dispersed [9]. Over the past few years, unusual disease occurrence and severe annoyance were frequent in infected areas; hence, scientists have found out that mosquito control would be the most appropriate solution for this puzzle [18]. Chemical treatment leftovers as the primary indispensable tactic are used in pest management because of their rapid results in the broad treatment area [19]. Adequate insecticide practice is regarded as an influential defense to achieve better public health manifestations and increase agricultural yield in developing countries [20–22]. From the beginning of pest management programs, insecticide-based mosquito eradication operations could be assessed as the highly efficient approach, but only a limited number of less harmful and highly economical insecticides are used in these practices [23].

Malathion is a chemical compound in the organophosphate insecticide family, which is chemically formed by the esterification of thiophosphoric acid and phosphoric acid. These substances are regarded as neurotoxins due to their detrimental effect on a neurotransmitter called acetylcholine and are mainly used in the pest management program. A group of synthetic insecticides is also used in mosquito control, namely pyrethroids, which contains deltamethrin, lambda-cyhalothrin, permethrin, etc. Pyrethroids act on the nervous system of targeted organisms similar to organophosphate, but instead of the neurotransmitter, pyrethroids can influence the sodium channels of neurons [24,25]. Malathion and pyrethroids are widely used in mosquito management in the public health domain. However, its prolonged and excessive use led to a considerable degree of chemical resistance in the targeted organisms [26]. Screening of insecticide susceptibility and dosage estimation can help in vector control operations and the evaluation of insecticide influence on mosquito behavior [27].

2. METHODOLOGY

2.1. Study Site

Thrissur Kole wetlands is a 10,187 ha area located between 10° 20′ and 10° 40′ N latitude and between 75° 58′ and 76° 11′ E longitude in Kerala's central region. Thrissur Kole wetlands extend across Mukundapuram, Chavakad, and Thrissur Taluks of Thrissur district. This area spreads from Velukara in the south to the northern bank of Chalakudy river and Tholur and Kaiparambu areas of Thrissur Taluk in the north. The following wetland area of Thrissur Taluk is named Ponnani Kole. The significant breeding habitats of mosquitoes in Kole wetlands comprise paddy fields, rocky pools, tree holes, coconut shells, ditches, containers, irrigation canals, and ponds [28].

2.2. Insecticide

Lambda-cyhalothrin, deltamethrin, and malathion (technical grade) were purchased from "New India Surgicals", Calicut, Kerala, India.

2.3. Mosquito Sampling and Colony Maintenance

Ae. albopictus and *Cx. quinquefasciatus* mosquitoes were collected from different Thrissur Kole wetlands' localities and identified by classical taxonomic methods [29,30]. The collected larvae were reared into adults in laboratory conditions (temperature 26 ± 2°C, larval food was prepared by mixing yeast and dog biscuits). Adult mosquitoes were fed with 5% sucrose and blood meal was provided on the third day of emergence. F1 progeny larvae of field-collected mosquitoes were subjected to larval bioassay. Laboratory colonized insecticide-free *Ae. albopictus* and *Cx. quinquefasciatus* were maintained in the communicable disease research laboratory, St. Joseph's College, Irinjlalakuda. These untreated larvae were also used in larval bioassay as laboratory strain.

2.4. Larval Bioassay

The standard *World Health Organization* (WHO) procedure was followed for determining larval susceptibility [31]. Accordingly, the larvae were subjected to different concentrations of insecticides whose stock solutions were prepared using distilled water as the solvent. 1 mg/ml deltamethrin, lambda-cyhalothrin, and malathion stock solution was prepared in water. Test concentrations were prepared by adding 1 ml insecticide-containing solution to 249 ml of water in a 500 ml capacity beaker and stirred vigorously for 30 seconds with a glass stirrer. For the control, 1 ml of distilled water or acetone as required was added to 249 ml of dechlorinated water instead of insecticide. To each of the beakers containing different tests and control, 25 late third or early fourth instar larvae were released with the help of a glass strainer. Six serial test concentrations of insecticides were prepared for larval bioassay. Mortality was recorded after 24 hours. Unmoved and moribund larvae were treated as dead larvae. If 5%–20% mortality was obtained in the control experiment, it was corrected by using Abbott's formula [32]. The LC_{50} and LC_{90} values for insecticides were calculated using Probit analysis by the dosage mortality regression line [33].

3. RESULT

Larvicidal efficacy of malathion, deltamethrin, and lambda-cyhalothrin on field-collected and laboratory-reared *Ae. albopictus* and *Cx. quinquefasciatus* is given in Tables 1 and 2. The results of malathion susceptibility of field-collected and laboratory-colonized *Ae. albopictus* and *Cx. quinquefasciatus* larvae illustrated that the

Table 1: Mosquito larvicidal effect of malathion, deltamethrin, and lambda-cyhalothrin against *Aedes albopictus*.

Insecticide	LC25 (LCL-UCL)	LC50 (LCL-UCL)	LC90 (LCL-UCL)	CHI square value	*p* value
Field strain					
Malathion	7.4643×10^{-3} ppm ($3.4597 \times 10^{-3} - 1.0200 \times 10^{-2}$)	1.2398×10^{-2} ppm ($8.6815 \times 10^{-3} - 1.7250 \times 10^{-2}$)	3.2516×10^{-2} ppm ($2.1659 \times 10^{-2} - 1.0775 \times 10^{-1}$)	23.362	0.000107202[a]
Deltamethrin	6.4881×10^{-4} ppm ($4.5221 \times 10^{-4} - 8.0519 \times 10^{-4}$)	1.0249×10^{-3} ppm ($8.3082 \times 10^{-4} - 1.2185 \times 10^{-3}$)	2.4435×10^{-3} ppm ($1.9358 \times 10^{-3} - 3.6504 \times 10^{-3}$)	9.709	0.045634782[a]
Lambda-Cyhalothrin	5.3587×10^{-4} ppm ($3.2222 \times 10^{-4} - 7.0675 \times 10^{-4}$)	9.0259×10^{-4} ppm ($6.7797 \times 10^{-4} - 1.13.6 \times 10^{-3}$)	2.4305×10^{-3} ppm ($1.8091 \times 10^{-3} - 4.2521 \times 10^{-3}$)	12.671	0.013001896[a]
Laboratory strain					
Malathion	1.1442×10^{-3} ppm ($5.1524 \times 10^{-4} - 1.5676 \times 10^{-3}$)	1.8672×10^{-3} ppm ($1.2904 \times 10^{-3} - 2.6246 \times 10^{-3}$)	4.7353×10^{-3} ppm ($3.1612 \times 10^{-3} - 1.6322 \times 10^{-2}$)	25.735	0.00003579[a]
Deltamethrin	4.3115×10^{-5} ppm ($1.86603 \times 10^{-5} - 6.46454 \times 10^{-5}$)	8.7237×10^{-5} ppm ($5.5912 \times 10^{-5} - 1.2074 \times 10^{-4}$)	3.3284×10^{-4} ppm ($2.1763 \times 10^{-4} - 8.1799 \times 10^{-4}$)	14.853	0.005[a]
Lambda-Cyhalothrin	2.3138×10^{-5} ppm ($8.3473 \times 10^{-6} - 3.6602 \times 10^{-5}$)	4.9158×10^{-5} ppm ($2.8954 \times 10^{-5} - 7.1322 \times 10^{-5}$)	2.0578×10^{-4} ppm ($1.2750 \times 10^{-4} - 6.1128 \times 10^{-4}$)	17.003	0.002[a]

Table 2: Mosquito larvicidal effect of malathion, deltamethrin, and lambda-cyhalothrin against Culex quinquefasciatus.

Insecticide	LC25 (LCL-UCL)	LC50 (LCL-UCL)	LC90 (LCL-UCL)	CHI square value	*p* value
Field strain					
Malathion	1.5 × 10-2 ppm (8 × 10-3-2 × 0-1)	2.6×10^{-2} ppm ($1.9 \times 10^{-2} - 3.3 \times 10^{-2}$)	7.3×10^{-2} ppm ($5.3 \times 10^{-2} - 1.38 \times 10^{-1}$)	14.010	0.007[a]
Deltamethrin	1.2482 × 10-3 ppm (7.869 × 10-4–1.5796 × 10-3)	1.9125×10^{-3} ppm ($1.4928 \times 10^{-3} - 2.3853 \times 10^{-3}$)	4.3021×10^{-3} ppm ($3.2180 \times 10^{-3} - 8.1746 \times 10^{-3}$)	16.778	0.002134925[a]
Lambda-Cyhalothrin	1.2360 × 10-3 ppm (7.5425 × 10-4–1.5784 × 10-3)	1.9052×10^{-3} ppm ($1.4675 \times 10^{-3} - 2.4018 \times 10^{-3}$)	4.3345×10^{-3} ppm ($3.2060 \times 10^{-3} - 8.6456 \times 10^{-3}$)	17.828	0.00133342[a]
Laboratory strain					
Malathion	2.7717×10^{-3} ppm ($1.6451 \times 10^{-4} - 5.1244 \times 10^{-3}$)	5.7159×10^{-3} ppm ($1.8352 \times 10^{-3} - 1.1258 \times 10^{-2}$)	2.2612×10^{-2} ppm ($1.1416 \times 10^{-2} - 7.8949 \times 10^{-1}$)	44.394	0.000000005[a]
Deltamethrin	1.0272×10^{-4} ppm ($7.45144 \times 10^{-5} - 1.228286 \times 10^{-4}$)	1.4540×10^{-4} ppm ($1.2131 \times 10^{-4} - 1.7020 \times 10^{-4}$)	2.8138×10^{-4} ppm ($2.277889 \times 10^{-4} - 4.253177 \times 10^{-4}$)	13.518	0.009002003[a]
Lambda-Cyhalothrin	9.8709×10^{-5} ppm ($5.99641 \times 10^{-5} - 1.2622 \times 10^{-4}$)	1.5090×10^{-4} ppm ($1.1552 \times 10^{-4} - 1.884708 \times 10^{-4}$)	3.3806×10^{-4} ppm ($2.5384 \times 10^{-4} - 6.386195 \times 10^{-4}$)	17.740	0.001[a]

LCL = lower confidence limit; UCL = upper confidence limit.
p-value < 0.05 shows a significant difference at 5%.

laboratory strain was more susceptible than field-collected strain. The LC_{50} value of field-collected *Ae. albopictus* was 1.2398×10^{-2} ppm, which was about 6.64 times greater than the laboratory strain whose LC_{50} was 1.8672×10^{-3} ppm. LC_{50} value of field-collected *Cx. quinquefasciatus* 2.6×10^{-2} ppm and LC_{50} of laboratory strain was 5.7159×10^{-3} ppm.

Deltamethrin susceptibility result displayed that the LC_{50} value of field-collected *Ae. albopictus* was 1.0249×10^{-3} ppm and laboratory strain *Ae. albopictus* was 8.7237×10^{-5} ppm. LC_{50} value of field strain *Cx. quinquefasciatus* was 1.9125×10^{-3} ppm, and that of laboratory strain was 1.4540×10^{-4}. Susceptibility results of lambda-cyhalothrin indicated that the laboratory strain was more susceptible than field strain. LC_{50} of field-collected *Ae. albopictus* 9.0559×10^{-4} ppm and LC_{50} of laboratory strain was 4.91589×10^{-5} ppm. LC_{50} of field-collected *Cx. quinquefasciatus* was 1.9052×10^{-3} ppm and LC_{50} of laboratory strain *Cx. quinquefasciatus* was1.5090×10^{-4}

Ae. albopictus mosquitoes exhibited more susceptibility than *Cx. quinquefasciatus*, and both field and laboratory strains of all three groups were treated by three different insecticides.

Figures 1–3 show the comparison of susceptibility status of field and laboratory strains of *Ae. albopictus* and *Cx. quinquefasciatus* against three different insecticides. Lambda-cyhalothrin was the most efficient insecticide, and malathion showed the least efficacy when compared to the other two insecticides used. All mosquitoes showed more susceptibility toward lambda-cyhalothrin and less toward malathion. The susceptibility rate can be illustrated in the order of lambda-cyhalothrin > deltamethrin > malathion.

4. DISCUSSION

Organochlorines, organophosphates, carbamates, and pyrethroids are the widespread chemical compounds used in the pest control strategy. Altogether, 12 different insecticides belonging to these four classes were recommended by the WHO for mosquito

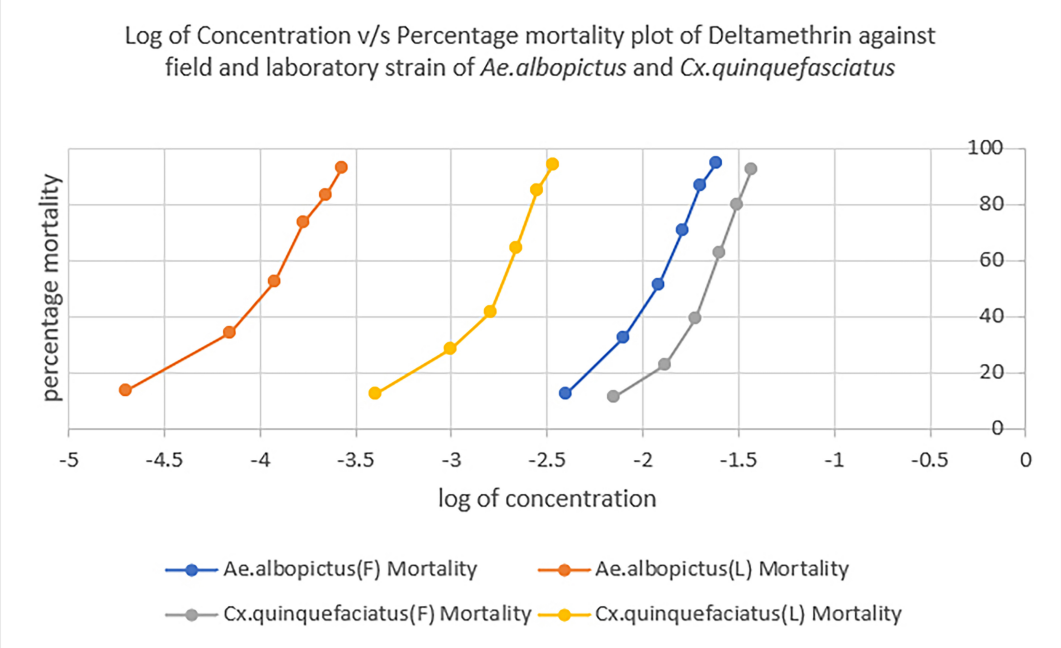

Figure 1: Comparison of susceptibility status of deltamethrin against laboratory and field strains of *Ae. albopictus* and *Cx. quinquefasciatus.*

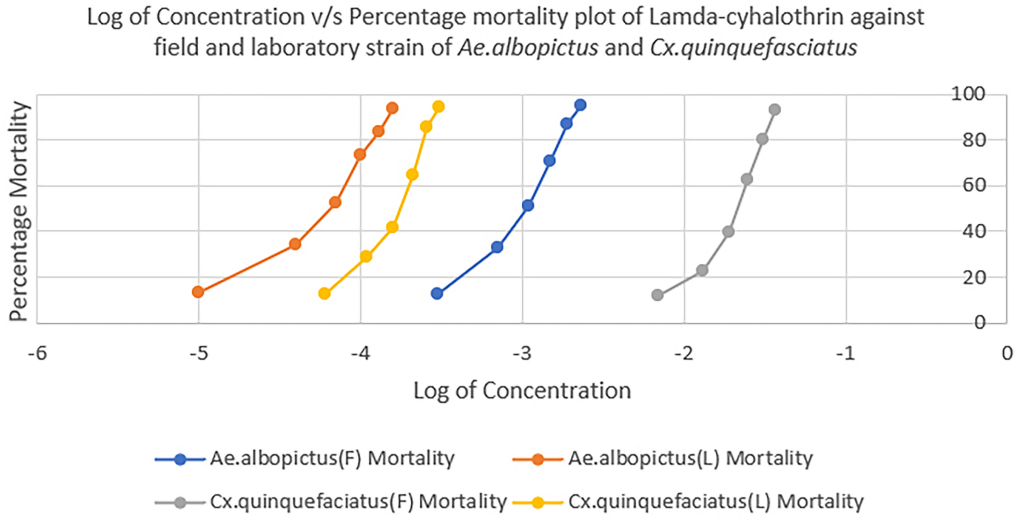

Figure 2: Comparison of susceptibility status of lambda-cyhalothrin against laboratory and field strains of *Ae. albopictus* and *Cx. quinquefasciatus*.

eradication [34,35]. Malathion comes under the organophosphate group of insecticides, is lethal to insects at the same time, and is less harmful to mammals. These second-generation insecticides are globally used for mosquito control only after the prohibition of Dichlorodiphenyltrichloroethane and dieldrin in the USA in the 1970s [24]. The extensive use of malathion arose from some resistance problems in different mosquito species worldwide, and several researchers also conducted many studies. Our present study discloses that laboratory strain *Ae. albopictus* and *Cx. quinquefasciatus* more susceptible than the field stain, which was collected from Thrissur Kole wetlands. Studies on malathion resistance against mosquitoes were reported in the 1990s itself. Bisset *et al.* [36] and Bracco *et al.* [37] reported on malathion and carbamate resistance in *Cx. quinquefasciatus* in Cuba And Brazil, respectively. In 2010, Selvi *et al.* [38] discussed their result in the study on the susceptibility of *Ae. albopictus* in against malathion. They discovered that the laboratory strain mosquitoes were more susceptible than field strain ones because of the recurrent contact with the insecticides used in the field. Organophosphate, carbamate, and pyrethroid resistance were also noted in *Cx. quinquefasciatus* mosquito by some studies conducted in America, Saudi Arabia, and northern Thailand [39–41].

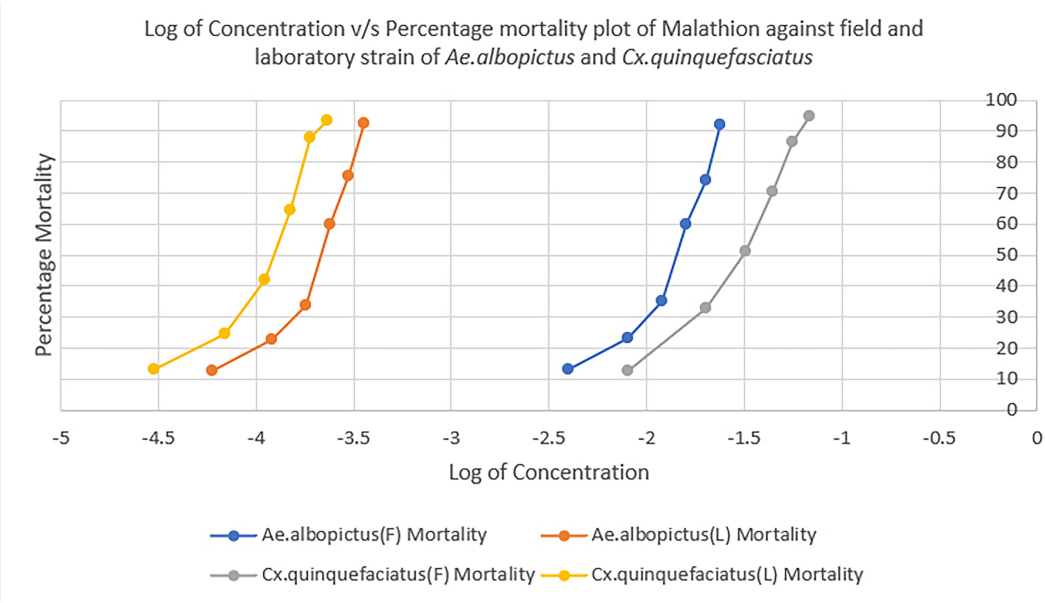

Figure 3: Comparison of susceptibility status of malathion against laboratory and field strains of *Ae. albopictus* and *Cx. quinquefasciatus*.

Kole wetlands is the major rainwater plunked ground of the surrounding area of the Thrissur district. Karuvannur and Kecheri river sediments urban and industrial atrophies with their monsoon flood watercourse to this delta every year [42]. Tessy and Sreekumar [43] reported almost 30 pollutant tolerant algal species from Thrissur Kole wetlands. Our study site is considered one of Kerala's major rice bowls, and the paddy cultivation practices take place during northeast monsoon season to post-monsoon season (September–March). An enormous number of pesticides, weedicides, and fertilizers have been applied by the farmers in these paddy fields to protect their crops and attain maximum yield [44,45]. These chemical combinations bring about some insecticidal tolerance in different mosquito species and other non-targeted organisms. Two individual studies publicized such tolerance in *Ae. albopictus* and *Ae. aegypti*, respectively [46,47].

In the current study, we made a comparative analysis of field-collected and laboratory-colonized *Ae. albopictus* and *Cx.quinquefasciatus* mosquitoes against malathion, deltamethrin, and lambda-cyhalothrin. Laboratory strains of *Ae. albopictus* and *Cx. quinquefasciatus* were more susceptible than the field strains. The outcome unveiled that both *Ae. albopictus* and *Cx. quinquefasciatus* exhibit more susceptibility toward lambda-cyhalothrin and least susceptible toward malathion. The susceptibility rate is in the order of lambda-cyhalothrin > deltamethrin > malathion in these mosquitoes. Hamdan *et al.* [48] discovered some LC_{50} values in *Ae. albopictus* and *Cx. quinquefasciatus* mosquitoes. The result of *Ae.albopictus* susceptibility test showed LC_{50} of malathion is 0.1972 ppm, temephos is 0.0514 ppm, and permethrin is 0.0022 ppm. The LC value in *Cx. quinquefasciatus* against malathion is 0.0163 ppm and permethrin is 0.00001 ppm. The result indicates that *Ae.albopictus* is more susceptible than *Cx. quinquefasciatus* toward all the insecticides tested and could explain the natural larval habitat preference of *Culex* mosquitoes is more polluted larval habitats when compared to *Aedes* species which breeds in freshwater.

Aedes mosquitoes generally adopt freshwater habitats and *Culex* prefers slightly to immensely polluted water for their development. The present study perceived that both species were collected from the same environment, which indicates that these mosquitoes had developed some resistance. The susceptibility assays conducted against different commonly used insecticides in the pest control study area also support these findings. Results from the quantitative susceptibility assays demonstrate the variance between the field and laboratory strains, in which field strains showed more resistance toward all the tested insecticides. The field strain of *Ae. albopictus* showed 6.36, 11.74, and 18.36 times more resistance than the laboratory strain against malathion, deltamethrin, and lambda-cyhalothrin, respectively. *Cx. quinquefasciatus* larvae followed this resistance pattern with 4.54 times resistance against malathion and 13.15 times and 12.62 times resistance against deltamethrin and lambda-cyhalothrin, correspondingly. A study conducted in Thailand observed such resistance array in *Cx. quinquefasciatus* against a group of insecticides, and they suggested that the prevalent interaction of insecticides might have resulted in some resistance in these mosquitoes [49]. The remarkable difference of LC_{50} values between the same species' strains might result from their resistance development to the insecticides, and this trait could be considered a crucial characteristic of a biological indicator of insecticide pollution. There are some early remarks on mosquitoes as possible bioindicators of insecticide pollution [50], and some recent surveys justified *Culex* mosquitoes as lead effluence bioindicators [51]. The utilization of the study area for seasonal paddy cultivation and periodical exposure to the mentioned chemical insecticides for insect pest management makes this study's findings more significant [45].

5. CONCLUSION

Various chemical insecticides have been used for decades in agricultural as well as public health indices to improve crop

productivity and accomplish better mosquito management. However, overdependence and disproportionate use of these chemical insecticides are primarily attributed to the development of resistance in targeted organisms. Mosquitoes collected from the areas contaminated with extensive chemical exposure showed some insecticidal tolerance. Subordinate part of this unsystematic usage is environmental effluence and harmful effects on non-targeted organisms. This study tried to discuss some lethal concentration values of two mosquitoes against three conventional insecticides. These values may help to contribute toward efficiency in mosquito-monitoring tactics with a minimal ecosystem imbalance.

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PLATES

Plate 1: Mosquito species collected from Kole wetlands of Thrissur

Figure 1.3

Figure 1.4

Figure 1.5

Figure 1.6

Figure 1.7

Figure 1.8

Figure 1.9

Figure 1.10

Figure 1.11

Figure 1.12

Fig 1.3 Anopheles barbirostris van der Wulp, 1884
Fig 1.4 Anopheles nigerrimus Giles, 1900
Fig 1.5 Anopheles peditaeniatus (Leicester, 1908)
Fig 1.6 Anopheles stephensi Liston, 1901
Fig 1.7 Anopheles subpictus Grassi, 1899
Fig 1.8 Anopheles vagus Donitz, 1902
Fig 1.9 Aedes aegypti (Linnaeus, 1762)
Fig 1.10Aedes albopictus (Skuse, 1895)
Fig 1.11 Aedes vittatus (Bigot, 1861)
Fig 1.12 Phagomyia cogilli (Edwards, 1922)

Plate 1: Mosquito species collected from Kole wetlands of Thrissur

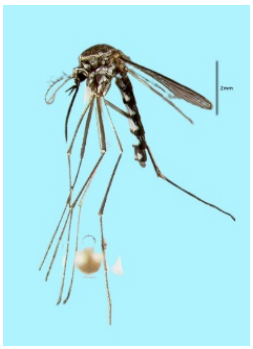


Figure 1.13

Figure 1.14

Figure 1.15

Figure 1.16

Figure 1.17

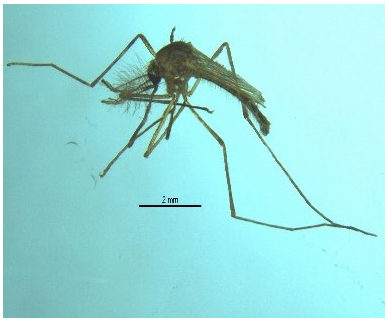


Figure 1.18

Figure 1.19

Figure 1.20

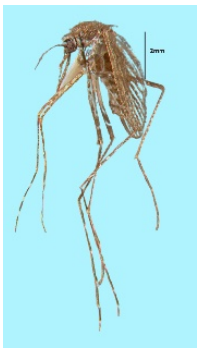


Figure 1.21

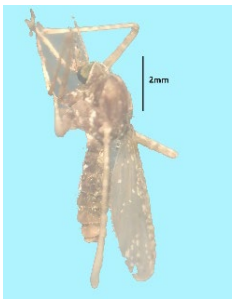


Figure 1.22

Fig 1.13 Armigeres subalbatus (Coquillett, 1898)
Fig 1.14 Lutzia fuscanus (Wiedemann, 1820)
Fig 1.15 Culex bitaeniorhynchus Giles, 1901
Fig 1.16 Culex gelidus Theobald, 1901
Fig 1.17 Culex tritaeniorhynchus Giles, 1901
Fig 1.18 Culex pipiens Linnaeus,1758
Fig 1.19 Culex quinquefasciatus Say, 1823
Fig 1.20 Mansonia uniformis (Theobald, 1901)
Fig 1.21 Mansonia indiana Edwards, 1930
Fig 1.22 Mansonia bonneae Edwards, 1930

Plate 2: Breeding Habitats of Collected mosquito species from Kole wetlands of Thrissur

Burrow pit

Plastic container

Ditch

Canal basin

Banana flower bract

Leaf axil

Coconut spathe

Pit

Irrigation canal

Leaf litter

Tree hole

Stagnant water body

Marsh

Pond

Rice field

Rocky pool

Tyre

Wetland with vegetation

Temporary pool

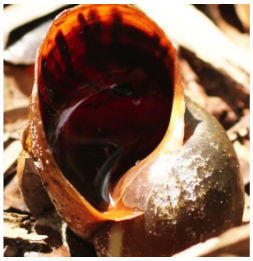
Flood plain

Leaf internode

Blocked drainage

Cemented tank

Coconut shell



Exoskeleton of snail

Broken mud pot

Plate 3: Significant Anthropological Activities seen in Kole wetlands of Thrissur

Rice cultivation

Clay mining

Construction

Fishing

Duck farming

Plate 4: Different Seasons

Premonsoon

Monsoon

Postmonsoon

Plate 5: Evidence of Insecticide Application as a part of agricultural practices in Kole wetlands of Thrissur

Spraying of Insecticides

Used bottles of Insecticides

Plate 6: Sampling and Laboratory Works