VOLUME 10 ISSUE 2 2024

ISSN 2454 – 3055

INTERNATIONAL JOURNAL OF ZOOLOGICAL INVESTIGATIONS

Forum for Biological and Environmental Sciences

Published by Saran Publications, India

International Journal of Zoological Investigations Vol. 10, No. 2, 1071-1079 (2024)

International Journal of Zoological Investigations
Contents available at Journals Home Page: www.ijzi.net
Editor-in-Chief: Prof. Ajai Kumar Srivastav
Published by: Saran Publications, Gorakhpur, India

ISSN: 2454-3055

Cytochrome Oxidase Subunit I Based Molecular Surveillance of Mosquitoes from Neyyattinkara Muncipality for Targeted Vector Control

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Received: 5th April, 2024; **Accepted:** 22nd June, 2024; **Published online:** 31st October, 2024

https://doi.org/10.33745/ijzi.2024.v10i02.105

Abstract: Mosquitoes are important vectors of several infectious diseases, posing a significant threat to public health. Accurate identification of mosquito species is crucial for understanding their ecological role and designing effective control strategies. In this study, we aimed to investigate the molecular taxonomy of mosquitoes in the Neyyattinkara municipality, Thiruvananthapuram, Kerala, India. About 103 mosquitoes were collected from the Neyyattinkara municipality area and were included in the present study. In this study DNA barcoding method was attempted to employ a complementary method alongside classical microscopy to identify species of mosquitoes collected. Samples collected were morphologically identified as *Aedes, Armigeres* and *Culex* genus. To confirm the species identity, molecular identification was exploited using mitochondrial CO1 gene specific PCR followed by sequencing of PCR amplicons. Initially, good quality total genomic DNA was extracted from mosquitoes using standard SDS-based extraction method and Cytochrome oxidase (CO1) conserved gene specific primer were used for the identification of species. Sequencing results revealed that obtained mosquito samples belong to *Aedes albopictus, Armigeres subalbatus* and *Culex pipiens* species. The findings indicated the dominance of *Armigeres subalbatus* in Neyyattinkara municipality, providing important insight into the mosquito species composition in this region. Therefore, by understanding the prevalence and distribution of *Armigeres* specific mosquito species, it is easy to formulate effective targeted vector control strategies thereby helpful in eliminating mosquito borne diseases to a greater extent.

Keywords: DNA barcoding, Cytochrome oxidase, *Aedes albopictus*, *Armigeres subalbatus*, *Culex pipiens*

Citation: Nair Aneesh, Pooja Krishna L., Jayakrishnan J.T. and Bindu O.: Cytochrome oxidase subunit I based molecular surveillance of mosquitoes from Neyyattinkara muncipality for targeted vector control. Intern. J. Zool. Invest. 10(2): 1071-1079, 2024.
https://doi.org/10.33745/ijzi.2024.v10i02.105

Introduction

Mosquito-borne diseases represent a substantial public health risk on a global scale, and India, given its distinct economic, social, demographic and climatic features, faces heightened

vulnerability to these ailments. Nevertheless, these conditions also foster an environment conducive to the spread of disease-carrying vectors, such as various species of the *Culex, Anopheles,* and *Aedes*. Over 415 mosquito species have been identified in India (Tyagi, 2015). Mosquitoes are the main carriers of vector-borne illnesses that cause human death and morbidity, including Zika fever, Lymphatic filariasis, Dengue, Chikungunya, Kala-azar, Yellow fever and West Nile fever. Recent emerging vector-borne diseases like Chikungunya and Zika fever have spread globally, necessitating increased surveillance, which calls for knowledge of the species composition and distribution of potentially dangerous mosquito species. It is important to correctly identify and understand the species because it allows the investigation of other biological aspects that differ between species such as larva ecology, resting behavior, and insecticide resistance that are crucial to implement effective vector control. This has been the driving force behind the development in mosquito identi-fication. Mosquitoes of a species complex are genetically distinct from each other although they appear morphologically indistinguishable. Therefore, these traditional techniques are challenging since characteristics such as presence of broken scales and bristles make the identification biased. For a quicker and more accurate method of identifying a vector species, molecular markers are a better choice. Present study used Cytochrome oxidase subunit 1 (CO1) gene specific PCR to identify and characterize mosquito species in addition to the traditional morphological investigations that are used as tools for taxonomic keys to identify individual species (Ratnasingham and Hebert, 2007).

Materials and Methods

Mosquito sampling

Mosquito specimens used in the study were collected from different sites of the study area (Neyyattinkara municipality) (Fig. 1) using light traps, human baited net traps and mosquito aspirators.The collecting sites majorly included semi-permanent groundwater pits in the open areas, fields and water storage containers filled with stagnant rainwater.

Preliminary identification was done using authentic taxonomic keys with the help of experts from ICMR-Vector Control Research Centre (VCRC), Kottayam and the assorted specimens were subjected to molecular taxonomic studies. The morphological features of the mosquitoes were observed and recorded under the stereo binocular microscope in the laboratory of VCRC Kottayam. Identified mosquitoes were assigned with an identification number. Females and males were separated. Initially, a total of 3 sampling sites were selected for sample collection with a considerable distance (at least 12 km) between the two sites and identified mosquitoes were classified into 3 morphological groups based on their external features and were stored at -20 ^{0}C for further investigations.

DNA extraction from collected samples

Genomic DNA was extracted from collected mosquito samples using standard isolation protocol. Precisely, samples were ground in 100 µl of Lysis buffer (100 mM Tris HCl, pH 8.0; 10% Sodium dodecyl sulphate, 50 mM NaCl, 100 mM EDTA). An entire adult mosquito from each species was taken and ground properly in lysis buffer and the mixture was treated with 5 µl of proteinase K (20 mg/ml) and kept for incubation at 55^{0}C for 2 h and to this cell lysate 5 µl of RNAase (10 mg/ml) was added and kept for 20 min for incubation at room temperature. The suspension was extracted by adding 100 µl of phenol-chloroform isoamyl alcohol (25:24:1) and centrifugation was done at 10,000 rpm for 10 min. The upper aqueous layer was extracted without disturbing the organic solvent layer and was transferred to a fresh micro centrifuge tube and DNA was precipitated by adding 0.2 V of 5M NaCl and 2.5V of absolute ethanol (100%) and mixed properly by inverted mixing and the mixture was kept overnight at -20 ^{0}C. The tubes were centrifuged at 12,000 rpm for 10 min and the supernatant was removed and to the pellet, 500 µl

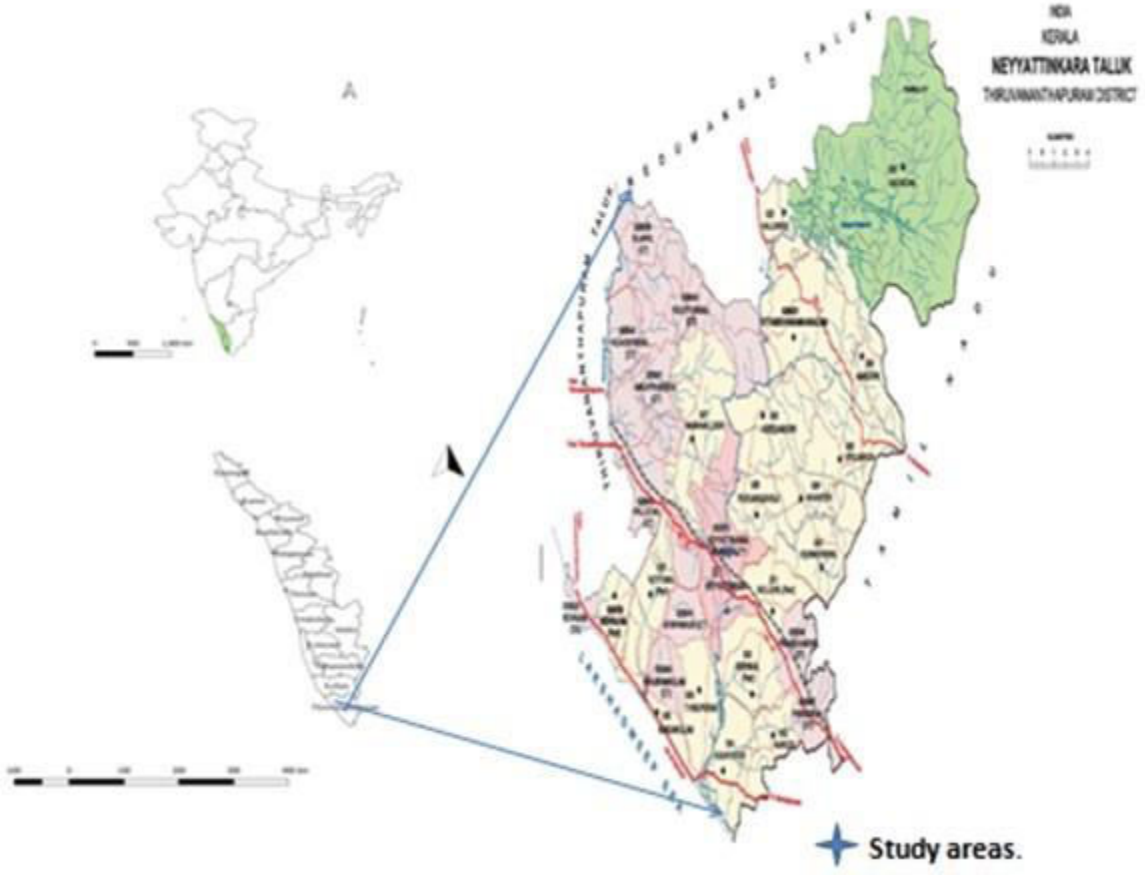

Fig. 1: Geographic distribution of Neyyattinkara municipality (Area of present study).

of 70% of ethanol was added and centrifuged for 5 min. The supernatant was removed, pellet was air dried and resuspended in 30 µl TE buffer by keeping at 50 ^{0}C incubation for 10 min. Resuspended DNA were stored at -20 ^{0}C until use. The concentration of DNA samples was determined by a Qubit 5-fluorometer. Further, samples were run on a 0.8% agarose gel and visualized under UV light using a Gel documentation system, Applied Biosystems, India.

PCR amplification

Gradient PCR

Mitochondrial Cytochrome Oxidase I (mtCOI) gene sequences from different world isolates, available at NCBI database, were aligned to retrieve the common conserved region and designing the primers. Forward primer COI-F 5'-GGTCAACAAATCATAAAGATATTGG-3' and the Reverse primer COI-R 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' were used in the study and the mtCOI region was amplified through PCR reactions in a total volume of 15 µl per reaction. Each reaction tube contained 50 ng of genomic DNA, 1 U/µl of Taq DNA polymerase, 1 µl of 10 mM dNTP mix, 1.5 µl of 10 x PCR buffer having 2.5 mM $MgCl_2$ and 1 µl of each 20 pmol of forward and reverse primers. PCR was carried out in SimplyAmp Thermal Cycler (Applied Biosystems). The annealing temperatures set for gradient PCR were 50^{0}C, 50.7^{0}C, 52^{0}C, 53.9^{0}C, 56.3^{0}C, 58.3^{0}C, 59.4^{0}C and 60 ^{0}C. PCR program was set with initial denaturation at 95^{o}C for 2 min followed by 35 cycles of denaturation at 94^{o}C for 30 sec, annealing at gradient temperatures for 30 sec and extension at 72^{o}C for 1 min. Final extension was done at 72 °C for 7 min. The

Table 1: Details of mosquito samples collected for the present study

Mosquito genus (Identified from VCRC Kottayam)	Total number of samples collected
Armigeres	85
Aedes	17
Culex	28

Fig. 2: Zoom stereomicroscopic view of (A) *Aedes*, (B) *Armigeres* and (C) *Culex* species.

amplified products (900 bp) along with PCR Marker (1 kb plus) were loaded on agarose gel (1%). The gel was run at 80 V for 45 min and viewed under UV transilluminator for analyzing the results.

COI gene specific PCR Analysis

PCR conditions (including annealing temperature) were optimized and the program was set with a single cycle of pre-denaturation at 95°C for 2 min followed by 35 cycles of 94°C for 30 sec, 52°C for 30 sec, and 72°C for 1 min and final extension 72 °C for 7 min. Amplification products (900 bp) were purified using chloroform extraction and used for direct sequencing using Sanger sequencing method.

Sequence analysis

The quality of sequencing data obtained were analyzed using freely available Chromas Lite software and confirmed through the comparison of its release species using NCBI BLAST tool.

Results

Microscopic studies

Based on the morphological characters, collected mosquitoes were taxonomically grouped into three distinct genera namely *Armigeres, Aedes* and *Culex* (Table 1, Fig. 2) and according to the collected population, it was identified that in Neyyattinkara municipality, the female mosquito population exceeded that of males, with 85 females and 45 males collected from various locations (Table 2, Fig. 3).

Morphological Characterization of mosquito species in Neyyattinkara municipality

The characteristics of wings, head, scales, thorax legs, palpi and proboscis were considered for the identification of mosquitoes and the observed results are illustrated in Table 3.

DNA isolation and PCR analysis

Good quality DNA was extracted from individual adult mosquitoes using standard SDS method of extraction and mtCOI regions were amplified using COI specific primers. The expected 900 bp PCR product of COI gene with optimum intensity without any primer dimers were obtained at 52 °C annealing temperature (Fig. 5). Optimized PCR conditions were used for the amplification of COI gene (Fig. 6) and the amplicons were purified and sent for sequencing.

Discussion

Mosquitoes are one of the important disease

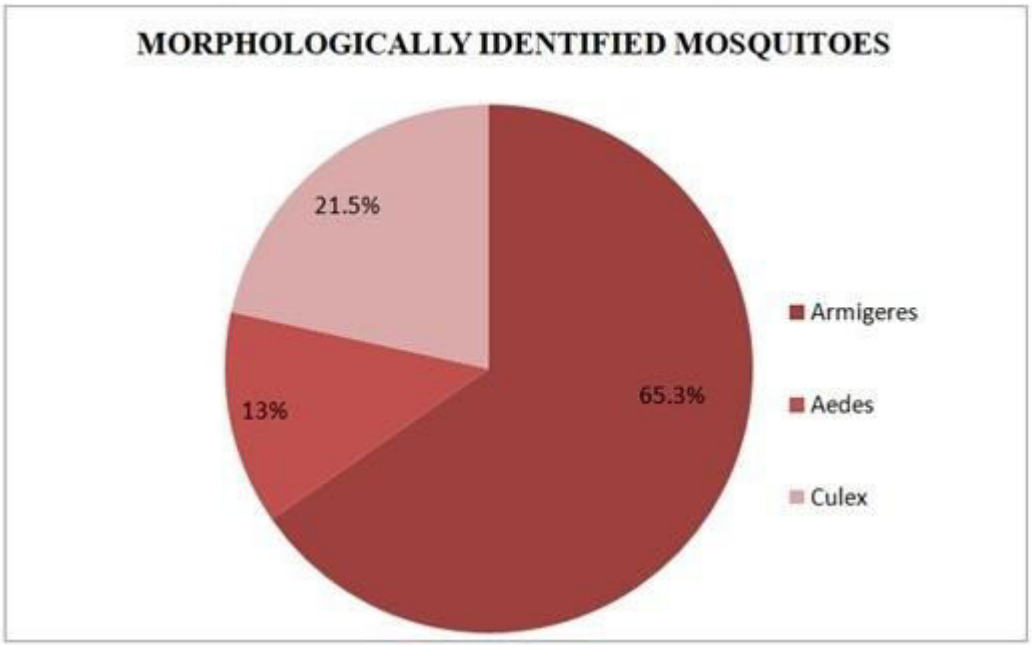

Fig. 3: Fig. 3:Morphological Characterization of mosquito species in Neyyattinkara municipality.

Table 2: Distribution of male and female mosquito samples collected for the present study (Identified in the lab of VCRC, Kottayam)

Species	Males	Females
Armigeres	40	45
Aedes	03	14
Culex	19	09

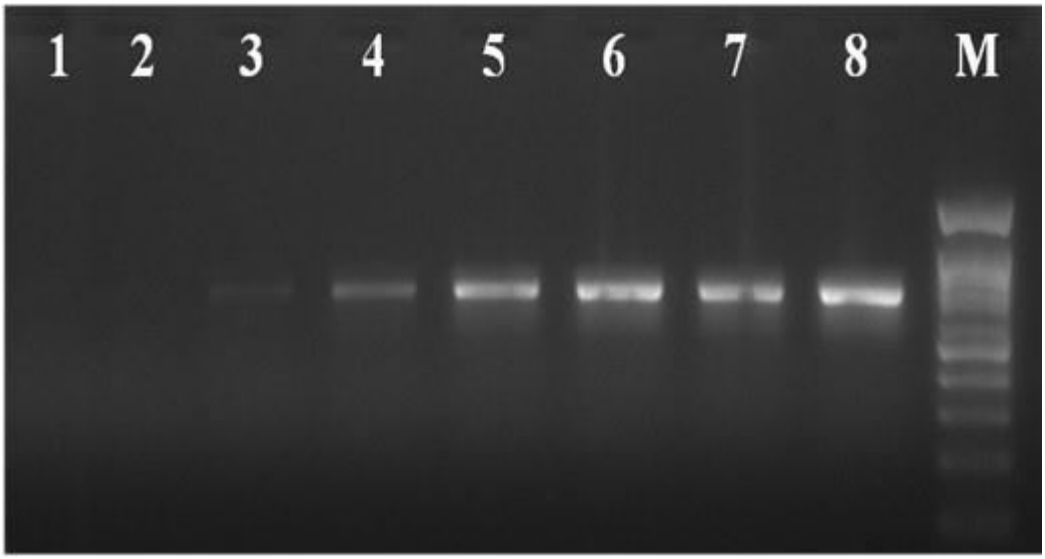

Fig. 4: Agarose Electrophoretic gel image showing the DNA bands of mosquito specimens [(Lane 1-8) and Lane M: molecular weight marker (ladder)].

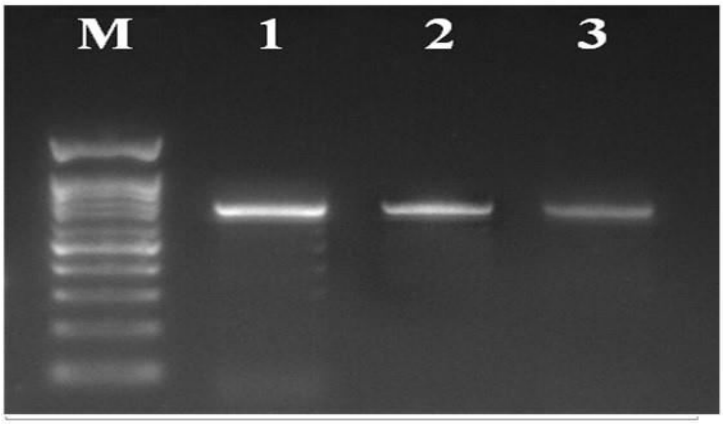

Fig. 5: Electrophoretic gel image showing the PCR products (900 bp) of genomic DNA isolated from individual adult mosquitoes using the mtCOI primer set. Lane M represents the 1 kb plus DNA marker used as a reference for estimating the size of the PCR products.

Table 3: The major characteristics considered for morphological identification of mosquitoes collected for the study

Mosquito genus	**Distinct morphological characteristics**
Armigeres	Male palpus about as long as proboscis; female proboscis laterally compressed and slightly down-turned at tip; palpus long, slender and upturned, three-segmented with the last two segments nearly bare. Eyes separated ventrally by two long rows of scales. Head scales primarily broad and flat dorsally. Wing: Alula and upper calypter with marginal hair-like scales.
Aedes	Proboscis entirely dark-scaled; palpus with white scales at apex; pedicel with scales on lateral surfaces.Thorax: Scutum with median longitudinal stripe band; antealar area with patch of broad pale scales. Legs: Silvery or white scale patches on legs;. Abdomen: Tergal scales basal, often not connected with lateral pale scales
Culex	Brown and grey scales on thorax and abdomen with no distinctive marking, wings are uniformly brown with dark veins. Head: Proboscis without distinct median pale band. Thorax: scutal integument yellowish or pale brown. Wings: Wing entirely darkscaled. Abdomen: Terga bands with pale yellowish basal bands

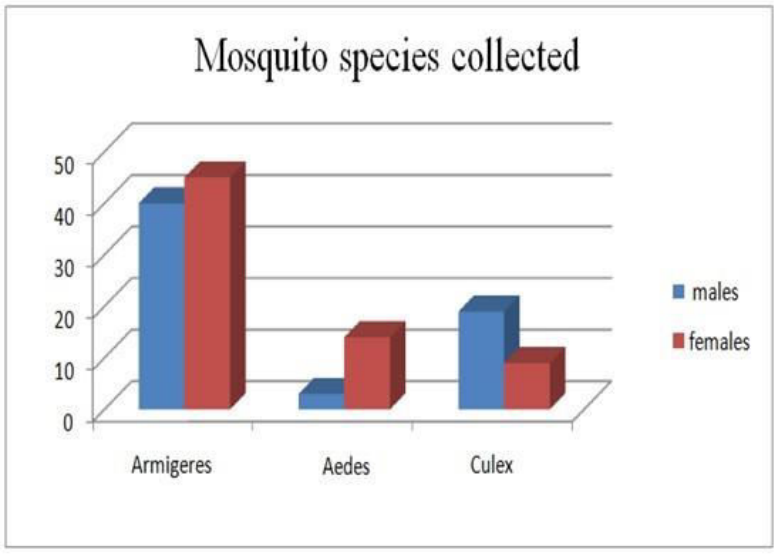

Fig. 6: Gender-wise distribution of 3 species of adult mosquito samples collected in the study.

vectors causing large number of deaths every year, and therefore, justifies the attention that has been given to its genetic diversity. Several combinations of genetic markers are used to examine population structure, genetic differentiation, and gene flow of these species. Precise identification of these species is an essential component of vector-borne diseases management strategies including vector surveillance and control activities, as the geographic origins of mosquito populations have epidemiological significance.

Neyyattinkara, is a small region situated in the southern part of Kerala, has not been extensively studied in terms of mosquito diversity. For risk assessment and disease prevention in Neyyattinkara, accurate mosquito identification and monitoring are crucial because just a small number of mosquito species play a significant role in disease transmission. The research of arboviral illnesses depends critically on the proper identification of vector species, which enables the local health authorities to focus resources on vector control measures.

The present study achieved the identification of three mosquito species that are mainly associated with rural environments. Conventional microscopy revealed the morphological characteristics of the mosquitoes based on their shape, size, and pattern such as, head, proboscis, maxillary palp, antenna, thorax, wings, legs and abdomen etc (Haarlem and Vos, 2018). Out of total 130 mosquito specimens collected, 86 (66%) were collected from outdoor resting habitat like banana plantation, stagnant water, hospital areas, railway station etc. and the remaining mosquitoes 44 (33%) were collected from indoor resting habitat. Specimens were collected using light traps and mosquito aspirators from the hospital area during the evening time. This sampling strategy allows as analyzing the spatial distribution of mosquito in Neyyattinkara municipality. The data indicates whether certain areas like hospital areas act as hotspots for mosquito activity.

Using the traditional morphology-based taxonomy, 3 mosquito species belonging to the genera *Aedes, Armigeres*, and *Culex* were identified which were consistent with previous studies (Eleni Patsoula *et al.*, 2006). The majority of mosquitoes collected belonged to *Armigeres* species followed by *Culex* and *Aedes*. Both male and female mosquitoes were identified within the majority of *Armigeres* species. Greater number of *Armigeres* suggests the dominance in which predominance of female *Armigeres* mosquitoes are noteworthy, as this species is known to be a nuisance biter and a potential vector of arboviruses such as Japanese encephalitis.

The presence of *Aedes* population in the study area is significant because this species are very important vectors of dengue, chinkungunya and Zika viruses. A recent study by Taissa Pereira-dos-Santos (2020) showed that *Aedes* mosquitoes can also transmit Arumowot (AMTV), Bujaru (BUJV), Bussuquara (BSQV), Cache Valley (CVV), Chandipura (CHPV) and Chilibre (CHIV). *Aedes* species, especially *albopictus* species has a remarkable ability to adapt and thrive in adverse environments.

The combined use of morphological and molecular techniques like PCR and sanger sequencing offers more accuracy in identifying the mosquitoes thereby making the identification process faster and reliable.

The present study used a modified SDS method for the extraction of good quality mosquito DNA. Increasing the volume of extraction buffer (from 100 µl to 200 µl) and increasing the DNA precipitation time from 1 h to overnight gave drastic increase in the quantity of extracted DNA. Extended incubation time promotes the formation of larger DNA clumps and improves the efficiency of DNA recovery during subsequent centrifugation steps.

Optimization of annealing temperature using gradient PCR gave good results (900 bp band without primer dimers) and the amplicons were sequenced to confirm the identity of species.

It was found that specimens belong to *Aedes albopictus* (98.7% identity and 97 % query cover),

Armigeres subalbatus (100% identity and 100% query cover) and *Culex pipiens* species (100% identity and 100% query cover). The results of the BLAST search revealed that the COX-I region of these mosquitoes had intra-species variation in the sequences. All three were found to be 100% identical to earlier reported studies. The homology-based BLAST results effectively identified all three species that were morphologically identified. Further genetic analyses using next generation sequencing tools will enable better understanding of these important vectors and their comprehensive molecular characterization (Chan *et al*., 2014).

Conclusion

The present study concluded that in Neyyattinkara municipality, three major vector mosquito species namely *Aedes albopictus, Armigeres subalbatus* and *Culex pipiens* are seen abundant and proper vector control measures need to be taken immediately to control the vector population thereby reducing transmission of deadly infectious diseases.

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