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Gene Sequencing Study of Mitochondrial Cytochrome Oxidase Subunit I (COI) in *Aedes aegypti* Mosquito

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Aedes aegypti is an important vector from the public health point of view. It transmits pathogens of Dengue and various other diseases. Controlling vector populations is key to reducing disease transmission and it is possible by understanding the taxonomic characteristics of the vectors and their genetic analysis helps in the identification of the genetic variations in species of different geographical areas. The main aim of the present study is the screening of the Aedes mosquito genome of local species as genetic studies give some insights into the vaccine for the treatment of the diseases caused by mosquito species. In the present study, the adult *Ae. aegypti* collected from

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Suryapet town where Dengue fever is endemic, used for the mitochondrial *Cytochrome Oxidase subunit I* (COI) gene sequencing using the techniques of polymerase chain reaction, Electrophoretic separation, spectrophotometric analysis, and BLAST. The mitochondrial COI sequence of 583 bps is published in the National Centre for Biotechnology Information (NCBI) with accession number ON413769. The sequence data was matched with the database available in the NCBI to identify variation in the gene as this gene keeps changing in the organisms and may contribute to the species establishing itself in a particular region due to congenial environmental conditions. One match with the protein called haem copper oxidase subunit I. The transmission of different pathogens in different areas can be ascertained by comparing similar sequence studies documented. Genomic studies involving genetic markers are of immense help in developing the vaccine. The mitochondrial COI gene is the marker often used for evolutionary research. The single nucleotide polymorphisms (SNP) or Restricted fragment length polymorphisms (RFLPs) that occur in DNA also contribute to the vector's adaptability to the different geographical areas and also the survival benefit which are the basis for understanding the vectors thoroughly and their role in disease transmission.

Keywords: Aedes aegypti; gene sequencing; mitochondrial COI gene; polymerase chain reaction; BLAST.

1. INTRODUCTION

Animal kingdom with millions of species and their transformation is a big challenge for taxonomists to identify those species depending on their morphological characteristics and it is cumbersome as there is a great diversity of organisms. Additionally, studying improperly handled specimens is challenging, especially when similar species share external characteristics. In addition to this, the similar external characteristics shared by the members of species make identification a difficult task based on taxonomic keys alone Therefore, an alternative identification method is widely in use to identify the individuals at molecular level based on sequencing particular genes which can be used as markers. One such marker gene is the mitochondrial *Cytochrome Oxidase subunit I* (COI) gene (Fernando et al. 2020). The method is based on the concept that every species has a unique genetic identity that can be used for species identification and is also used to compare the species of geographical diversity (Sana et al. 2021). The mitochondrial genes are often sequences of choice for phylogenetic studies because they are highly conserved among phyla, maternally inherited, present in the highest copy numbers, and evolve faster than the nuclear DNA (Elias et al. 2020 and Jaimes-Duenezet al. 2015). The mitochondrial COI gene is the marker often used for evolutionary study because it is the largest of the three mitochondrial encoding Cytochrome Oxidase subunits (Gloria-Soria et al. 2016) and the protein sequence contains highly conserved functional domains and variable regions. COI is the terminal catalyst in the mitochondrial respiratory chain involved in electron transport and proton translocation across the membrane (Mikko et al. 2016; Mouna et al. 2021; Paul). The *Aedes aegypti* mosquito plays a significant role in Dengue disease transmission. They can be easily identified with their appendages having white markings on appendages and Thorax. These mosquitoes are more active at dawn and dusk usually biting on ankles and elbows of yellow fever along with Dengue fever. *Ae. aegypti* mosquitoes' distribution has increased in the past two to three decades worldwide and it is the most widespread mosquito species by adapting itself to warm temperate climates (Susanta et al. 2011). They also transmit different viruses causing diseases like Zika, and Chikungunya. Therefore, the present study of the mitochondrial sequence of the COI gene was undertaken to understand the intraspecific variability of the individual in terms of phylogeny as well as for species identification (Sousa et al. 2023 and Thabiani,2023). The studies on Aedes aegypti about the causes of disease are many including external morphology, anatomy, and physiology in India and abroad but the studies related to the sequencing in the state are lacking. The disease is widespread in several districts including the city during the rainy season with cases reported every year. Therefore, there is a dire need for the screening of the *Aedes* mosquito genome of local species as genetic studies give some insights into developing the vaccine for the treatment of the diseases caused by mosquito species.

2. METHODOLOGY

Mosquito collection: The larvae of *Ae. aegypti* were collected from Suryapet town during breeding seasons from stagnant water of the dumped containers like coconut shells, tires disposed of plastic bottles, etc which are the breeding grounds and maintained under specific conditions with temperature and humidity (27°C to 29°C and 65% humidity according to the experimental techniques published by America mosquito control Association (AMCA, 1979) and the adult mosquitoes were used for genomic DNA extraction (Padmavati, 2020).

2.1 DNA Extraction

The mosquito was homogenized with a lysis buffer and incubated at 55°C for 1- 2 hours. The clear lysate was transferred to another fresh tube to which low potassium chloride was added and incubated on ice for 5 minutes. Now the content was centrifuged for 10-15 mins at maximum speed and transferred the supernatant to another tube and an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifuged again for 10ming at 10000 rpm. The upper aqueous phase was transferred to a new centrifuge tube, and an equal volume of absolute ice-cold isopropanol was added and incubated for 5-10 min at room temperature and centrifuged at 10000 rpm for 10 mins. The Supernatant was discarded and the pellet was washed with 20%. Ethanol. The Pellet was air dried at room temperature and then dissolved in 50 ul Tris EDTA buffer The DNA sample was stored at -20°C until further use.

2.2 Agarose Gel Electrophoresis

Principle: For the majority of DNA samples, the DNA molecules and their fragments are considerably longer than protein under an electric field any given fragment should toward the anode with the same mobility. Separation on Agarose gel is achieved due to the resistance to their movement caused by the gel matrix. Agarose was weighed and transferred to a conical and 25 ml of IX Tris-acetate EDTA (TAE) was added and Agarose was melted to a clear solution by heating. It was allowed to cool until it reached a comfortable temperature. 5 ul of ethidium bromide Stock solution was added. The casted gel was placed in an electrophoretic tank and an IxTAE buffer was added until the gel was completely submerged. The DNA sample was mixed with the gel and loaded into the well. The samples were then electrophoresed at 50V until the gel loading dupe reached 2/3rd of the gel. This gel was then viewed under the UV trans-illuminator.

2.3 Polymerase Chain Reaction (PCR)

2.3.1 Principle

PCR is an in vitro method of enzymatic synthesis of specific DNA sequence characterizing, analysing, synthesizing, specific DNA or RNA from any living organism. It makes use of the natural polymerase function present in all living organisms to copy genetic material. It consists of three steps involving denaturation; during which the two strands melt to form single-stranded DNA with all enzymatic reaction stoops and is generally carried out at 92°C-96°C; annealing; annealing is carried out to join each separated original strand for the new strand synthesis at 40°C–60°C and finally extension; in which the polymerase adds dNTPs complementary to the template strand at the 3'end of the primers. Both the strands are copied in the PCR resulting in an exponential increase in the number of copies of the required gene. The above procedure is repeated about 30-40 times in an automated thermal cycler, which heats and cools the reaction mixture in the tube in a very short time.

2.4 Gene amplification

The isolated DNA is used for molecular-based detection of mosquito samples. The mitochondrial col gene of 686 bps was amplified by primers, the forward primer: COI-5'F: GGTCAACAAATCATAAAGATATTGG3' and reverse primer COI-5'R: TAAACTTCAGG GTGACCAAAAAATCA3'. The band length was between 600 and 700 bps. The reaction mixture of 25ul containing 50 ng of DNA template, 2mM of dNTP mix, 1 ul of DNA polymerase,1X buffer, and 3 ul of each primer was used. PCR reaction conditions: denaturation for 3 mins at 94°C followed by 35 cycles for 45 secs at 94°C, followed by annealing for 1min at 48°C and extension for I min at 72°C. The PCR products were validated by agarose gel electrophoresis followed by analyzing the gel under UV light. The amplicon was sequenced at Bizone company using an Applied Biosystems 3730xl sequencer.

2.5 Qualitative and Quantitative Determination of DNA by Spectrophotometric Method

Nucleic acid solutions exhibit maximum absorbance at 260 nm and absorb at 280 nm as well. This absorbance is directly proportional to

the concentration of nucleic acids (DNA and RNA) and can also indicate the level of contamination in the solution. The strong absorption is mainly due to the aromatic rings present in the nitrogenous bases, such as purines and pyrimidines. Nucleoproteins, often the primary contaminants in nucleic acid extracts, show a maximum absorbance at 280 nm. The ratio of absorbance at 260 nm to 280 nm indicates the degree of contamination. A ratio between 1.7 and 1.9 typically suggests pure DNA extraction, with a pure DNA ratio being approximately 1.8. Values higher than 1.8 indicate the presence of organic solvents in the DNA extract, while values lower than 1.8 suggest protein contamination. The absorbance of the solution with the sample was read at the wavelength of 260 nm and 280 nm and the concentration of DNA in the sample was calculated as 1.8 considered pure DNA which was acceptable to PCR.

2.6 Sequence Analysis

The amplified sequence underwent Basic Local Alignment Search Tool (BLAST) to determine its similarity to other sequences and to elucidate its genetic relationship with previously reported sequences in the gene bank. (ON413769https://blast.ncbi.nlm.nih.gov/Blast.cgi#)

3. RESULTS AND DISCUSSION

PCR The DNA was isolated from mosquito tissue and carried out electrophoresis with agarose gel and the gel was then viewed under a UV Transilluminator (Fig.1)

Fig. 1. The PCR product gel picture of the COI gene

PCR successfully amplified the desired mitochondrial DNA sequence. The sequence was analysed using a Bioanalyzer.

1 tatgatccgg aatagtcgga acttctctaa gaattttaat tcgtgctgaa cttagccacc
61 ctggtatatt tattgggaat gaccaaattt ataatgtaat tgtaacagct catgcattta
121 ttataatttt ctttatagta ataccaatta taattggagg atttggaaat tgattagttc
181 ctttaatatt aggagcccct gatatagcct ttcctcgaat aaataatata agtttttgaa
241 tactacctcc ttcattgact cttctattat caagctcaat agtagaaaat ggggcaggaa
301 ctgggtgaac agtttatcct cctctctctt caggaacagc tcatgctgga gcttctgttg
361 atttagctat tttttctctt catttagctg gaatttcctc aattttaggg gcagtaaatt
421 ttattacaac tgtaattaat atacgatcgt caggaattac tttagatcga ctacctttat
481 ttgtttgatc tgtagttatt acagctatct tattacttct ttctcttcct gttttagctg
541 gagctattac tatgttatta acagaccgaa acttaaatac atc

3.1 Electrophoretic Separation of DNA

The electropherogram of the amplified mitochondrial DNA sequence showed a significant peak in the middle at 600-800. Maximum peak noted at above 1000 by the nucleotide G at 210 and 577 positions. Fig.2 (a,b,c,d,e)

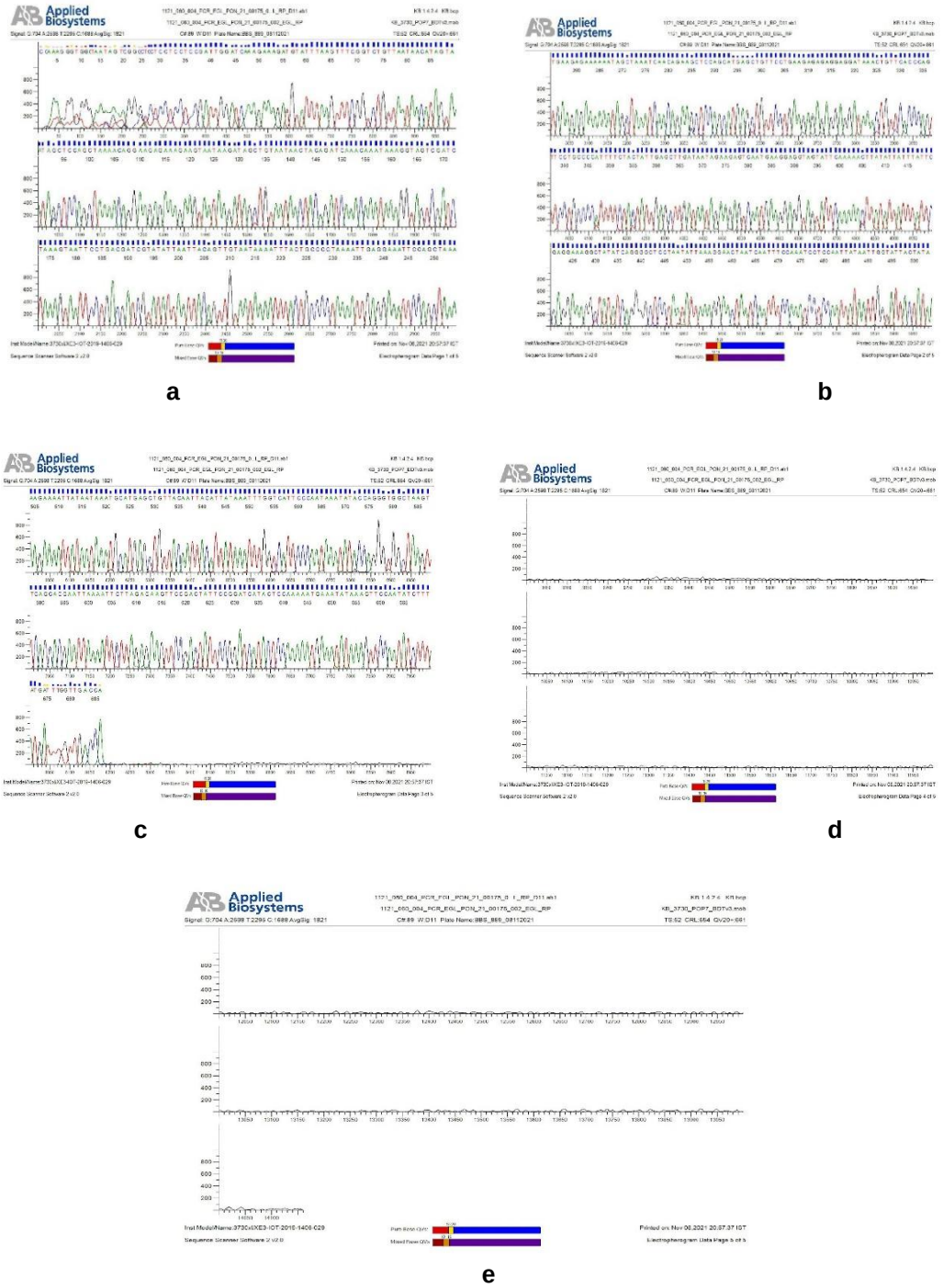

Fig. 2. Electropherogram shows, the Y-axis representing the intensity of the signal and The X-axis represents the time expressed in scan numbers

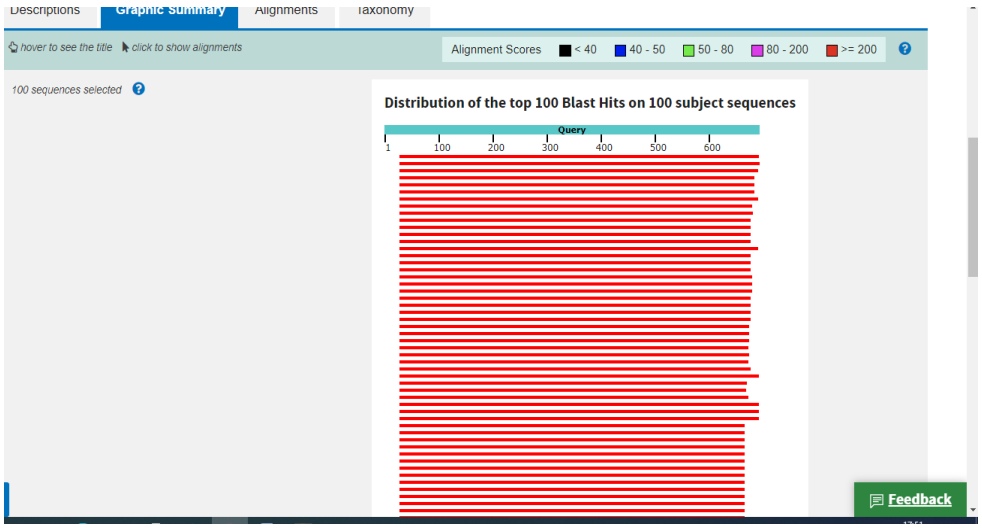

Fig. 3. BLAST

3.2 Spectrophotometric Determination

The quality and quantity of DNA were determined by the spectrophotometric method and calculated the purity of DNA by the ratio of absorbance at 260nm and 280nm. The absorbance determined is the purity of DNA i.e. 1.8.

Purity of the DNA
A260: A280ratio = A260/ A280 = 1.8: pure DNA

= 1.7 – 1.9; fairly pure DNA (acceptable ratio for PCR)
= less than 1.8; presence of proteins.
= greater than 1.8; presence of organic solvent

3.3 BLAST

When the query sequence was matched with BLAST search, it showed a 99% with other similar species of *Ae. aegypti* (Fig.3).

4. DISCUSSION

The Aedes aegypti as a vector plays a major role in transmitting different disease-causing viruses in different parts of the world The diseases caused are Dengue, Chikungunya, Zika, and yellow fever. Aedes aegypti mosquitoes can be identified with the white markings on their appendages and thorax, but morphology alone cannot define the species and their adaptations at the molecular level to different areas in the world indicating their widespread distribution across the globe. The combination of molecular identification and morphological characteristics can be effective in understanding the variations between populations of the same species that are geographically diversified[13]. The complete mitochondrial DNA barcoding of Culicidae mosquito species was also suited in Thailand[14] The molecular study suggests that Aedes aegypti from different regions show similarity in certain genes like COI, for example, mosquito species from South Iran are 100% identical and are undergoing similar evolution elsewhere also as observed by Dorzaban et al, 2020. The phylogenetic analysis of minimal nucleotide differences and low haplotype diversity in the southern region of Iran indicated an association between Aedes aegypti populations and mosquitoes from Saudi Arabia and Pakistan (Paksa et al. 2024 & Fida et al. 2024). Another study from the Pilani region of Rajasthan, India showed that *Ae. aegypti* has high similarity with an Indian isolate from Thirumala, Andhra Pradesh (Ramanadeep and Devinder 2021). The mitochondrial COI gene sequence when matched with the available databases in NCBI (Kenya, QPC41196),(Iran, WLF41753), (Mexico, WBW88600), MN299016, MT434121, MK635053, LC 485553, KX051585, HQ688294, AF425846 and (China, AWD75254) (Huiying et al.) showed 100%similarity and 99.83% similarity with, Spain(OQ144315), Iran(OR398783), Thailand(OP47750)and South Korea(OM883904) (Hajin et al. 2020) and shows 99.83% similarity with Tamilnadu(OQ630000) and with Odisha(PP892775) from India, about 99% similarity with other population with MW664686

and MW664682 also in Canada and France. In the present study, an identical protein was found with the accession number UPW57840 region 1>194 regions namely "heme copper oxidase subunit I" (BLAST)a transmembrane protein complexes in the respiratory chain that catalyzes the reduction of O2 and simultaneously pumps protons across the membrane. The study of both morphological identification and gene sequencing expands our basic knowledge about evolution in the gene flow of geographically diversified species of mosquitoes and is helpful in further investigation using the latest biotechnological applications to assist in the control of the vectors leading to a decrease in the disease burden and also surveillance in a particular area.

5. CONCLUSION

Ae. aegypti is a prominent vector for various pathogens transmitting diseases all over the world. The study provides the gene sequence of Aedes aegypti in Suryapet town which causes the high risk of local transmission of dengue is showing 99.83% in BLAST search with genotypes available in NCBI from other states like Tamilnadu and Odisha. Using the latest techniques like during the present study it may be established if there are any factors contributing to the disease transmission like geographic distribution, polymorphisms, and variations that are caused in different regions. It gives some clue about the cause of the disease outbreak due to selection pressures operating on the organism which differs in changes in different environments. Therefore, more investigation and analysis in DNA barcoding of mosquito species aid in the study of the molecular evolution of mosquito species to control mosquito-borne diseases in a better ecological and sustainable way. The transmission of different pathogens in different areas can be ascertained by comparing the similar sequence studies documented. Genomic studies involving genetic markers are of immense help in developing the vaccines. The mitochondrial COI gene is the marker often used for evolutionary study. The SNPs or RFLPs that occur in DNA also contribute to the vectors' adaptability to the different geographical areas and also the survival benefit. These polymorphisms are the basis for understanding the vectors thoroughly and their role in disease transmission. Further studies are needed to determine the significance of these polymorphisms, which is a critical gap to be addressed. Therefore, additional investigation and analysis of DNA barcoding in mosquito species will contribute to understanding their molecular evolution, ultimately supporting more effective and sustainable ecological strategies for controlling mosquito-borne diseases. Certain genetic markers like the mitochondrial *COI* gene may be looked for in further investigations regarding endemicity, disease occurrence, frequency, etc. Therefore, there is a dire need for the screening of the COI genes for the genetic studies of the *Aedes* mosquito genome of local species.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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