Molecular identity and evolutionary history of the *Culex quinquefasciatus* mosquito from the Udaipur area in Rajasthan, India

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Abstract

The present study aimed to identify and confirm the *Culex quinquefasciatus* mosquitoes collected to the species level. To evaluate the molecular taxonomy and phylogeny, the mt DNA COI gene of the Culex mosquitoes that were sampled was amplified using the COI gene specific forward and reverse primers. Bioinformatics analysis was conducted using NCBI's BLAST program. Based on DNA barcoding data, it was concluded that *Culex quinquefasciatus* was the extant Culex species.

Keyword: *Culex quinquefasciatus,* COI gene, NCBI Blast, phylogeny, molecular taxonomy

Introduction

Mosquitoes are found all over the world except Antarctica. They belong to the order Diptera of the class Insecta. Members of the genera *Anopheles, Culex* and *Aedes* bite primarily humans. Mosquitoes impact human welfare both directly as a nuisance and through transmission of pathogens that cause diseases such as malaria, filariasis, chikungunya, Japanese encephalitis, dengue fever and variety of other diseases in animals. They spread diseases to more than seventy crore people each year and will be responsible for the death of 1 out of every 17 people currently alive. Therefore, to control mosquitoes, many states and agencies have established mosquito control programs (Williams, 2000; Rueda and Kline, 2007; Manguin and Boete, 2011; Naseem *et al.*, 2016).

We report for the first time the molecular identification of *Culex quinquefasciatus* and *Culex pipiens* species while the molecular identification of *Culex tritaenirhynchus* and *Culex sitiens* was reported for the first time in the entire Saudi Arabia and Jazan region. This study used PCR for the first time to identify *Culex* mosquito species in the Jazan region. PCR is a complementary and integrated taxonomy-based identification tool for mosquito species. This integrated has the potential to promote and enhance programs, as well as define the genetic diversity of species in the region (Noureldin *et al.*, 2021).

Understanding the climatic factors that affect adults and larvae is the first step to controlling the survival, production, development, abundance and distribution of the mosquito vector6. Temperature has been reported to influence mosquito populations and activity; for example, higher temperatures accelerate mosquito development from egg to adult and increase their numbers (Alahmed,2012; Bayoh and Lindsay, 2013; Hopp *et al.*, 2000). Random enhanced polymorphic DNA (RAPD) is a fast, modest polymerase chain reaction (PCR) based technique that permits the identification of genetic polymorphism utilizing inconsistent primers (Welsh and McClelland, 1990; Williams, 1990). Therefore, it is the most suitable succession for phylogenetic investigations of strongly related species (Coleman and Vacquier, 2002). Gene sequences, for example, ITS-1 and ITS-2 of r DNA, COI and COII are useful in establishing phylogenetic relationships among species (Kaura *et al*., 2010; Park *et al.,* 2008).

Materials and methods

Mosquito collection

During the pre-monsoon, monsoon, and post-monsoon seasons of April 2021–March 2023, *Culex* mosquitoes were collected in the Udaipur district. After being collected, the mosquitoes were sent to the Department of Zoology at UCOS, MLSU, Udaipur, Rajasthan, where they were identified.

Morphological identification

To identify *Culex* mosquito species, a visual identification key (Reuben *et al.,* 1994; Rattanarithikul *et al.,* 2005) was used. The body is hunched when at rest, so that the thorax and abdomen form an angle. Parallel to the surface is where *Culex* rests. It breeds in large numbers in fecal-contaminated water. There are black scales all around the proboscis. There is no yellow scale stripe in the middle. In the laboratory, a stereoscopic microscope was used to view and use the identifying feature.

Molecular phylogeny of *Culex*

For molecular phylogenetic analysis, the adult specimens were sent to Siriprogen Life Sciences Lab in Hyderabad, Telangana. The steps listed below were taken:

DNA ISOLATION:

As directed by the manufacturer, genomic DNA isolation was carried out using the QIAamp DNA Mini Kit. Using 20.0 uL of elution buffer, DNA was extracted. The quality was evaluated by PCR using 2.0 uL of DNA that had been examined on the gel.

DNA PCR:

The mtDNA COI gene was amplified using forward and reverse primers specific to the COI gene. The PCR amplification has a size of about 600 bp.

For a single DNA sample, PCR reaction mixes were made. For every sample, two primers were used in PCR reactions. Each reaction had a final volume of 25.0 uL. All samples' reaction mixes were made and put into 200 uL PCR tubes. Each tube was then filled with genomic DNA.

Agarose Gel Electrophoresis of PCR product

Using a 2% (w/v) agarose gel and standard 0.5X TBE gel electrophoresis buffer, PCR products were subjected to agarose gel electrophoresis. After running the agarose gel for 30 minutes at 5 volts per centimeter, the gel image below was captured. The DNA marker 100-1000 bp size standard is shown in the first lane of the gel image. PCR products for samples amplified using COI primers are put into the remaining wells. The gel image below further demonstrates that the amplicons produced by this primer pair are around 600 bp in size:

Results

It takes a lot of time and work to distinguish between the several taxonomic species of Culex mosquito larvae because they all seem so similar. Although DNA barcoding for COI quality helps to standardize species, it is not possible to do so for every mosquito collected due to their high prevalence and the lack of an easily accessible gene bank information base. The reaction mixture must be cleaned up by eliminating any dNTPs and primers that are not being used before PCR amplicons are sequenced. To clean up ExoSAP, we used 10 ul of PCR products. Thermo Fisher's ExoSAP-ITTM PCR product cleanup reagent is used to clean up amplified PCR products. It simultaneously breaks down additional primers and nucleotides. ExoSAP-IT-cleaned samples are now prepared for use in further processes, such as DNA sequencing. There is no need to move the ExoSAP-IT reagent to tubes, wells, or columns because it can be added straight to the PCR product. This eliminates the need for additional processing and safeguards the PCR amplicons, reducing the possibility of cross-contamination.

To create a 7 ul reaction volume, 5 ul of a post-PCR reaction product and 2 ul of ExoSAP-IT reagent were used. To break down any leftover primers, the reaction was maintained at 37°C for an hour. In one step, surplus primers and nucleotides are hydrolyzed. Samples that have been purified with ExoSAP-IT are prepared for use in subsequent processes like DNA sequencing. No additional processing is required when the ExoSAP-IT reagent is added straight to the PCR product, eliminating the requirement for transfer steps to tubes, wells, or columns and conserving PCR amplicons, which helps lower the risk of cross contamination. For a total reaction volume of 7 ul, 5 ul of a post-PCR reaction product and 2 ul of ExoSAP-IT reagent were combined. To break down the remaining primers, the reaction was incubated for 60 minutes at 37°C.

DNA sequencing of PCR products

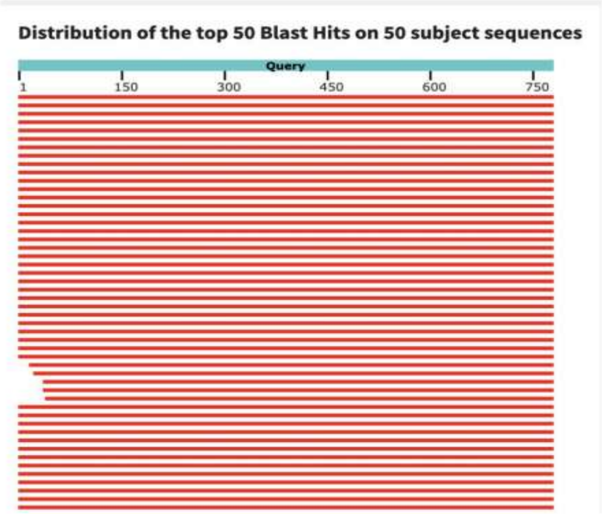
The Biosystem Big Dye Terminator V3.1 cycle sequencing kit was used to sequence the PCR product's DNA using both primers. On an Applied Bio system 3130xL genetic analyzer, a device for sequencing DNA, the sequencing items were stacked on top of one another. The sequencing machine came with software called sequencing examination 5.1, which was used to break up the sequences. Additionally, chromasPro v 1.34 was used to copy and fragment these patterns. To frame the contig with the best sequence calls, the forward and reverse sequences were altered. In this manner, two sequences were used to create one contig for every sample (Forward and Reverse). The test samples' COI region layouts were obtained using ChromasProV3.1 sequence assembly software. A different sequence study tool available on the NCBI database was utilized to examine the effects of the obtained sequences. The reference sequences of the BLAST hit data were obtained from the database. The reference sequences that were extracted from the NCBI Genebank data set were described in part by the organization of the question sequences. The sequences were identified by their end point name and promotion number, as shown by the BLAST result. Ten strains were selected from the BLAST data to examine their phylogenetic relationships. Additionally, phylogenetic relationships between the reference and question genes were examined.

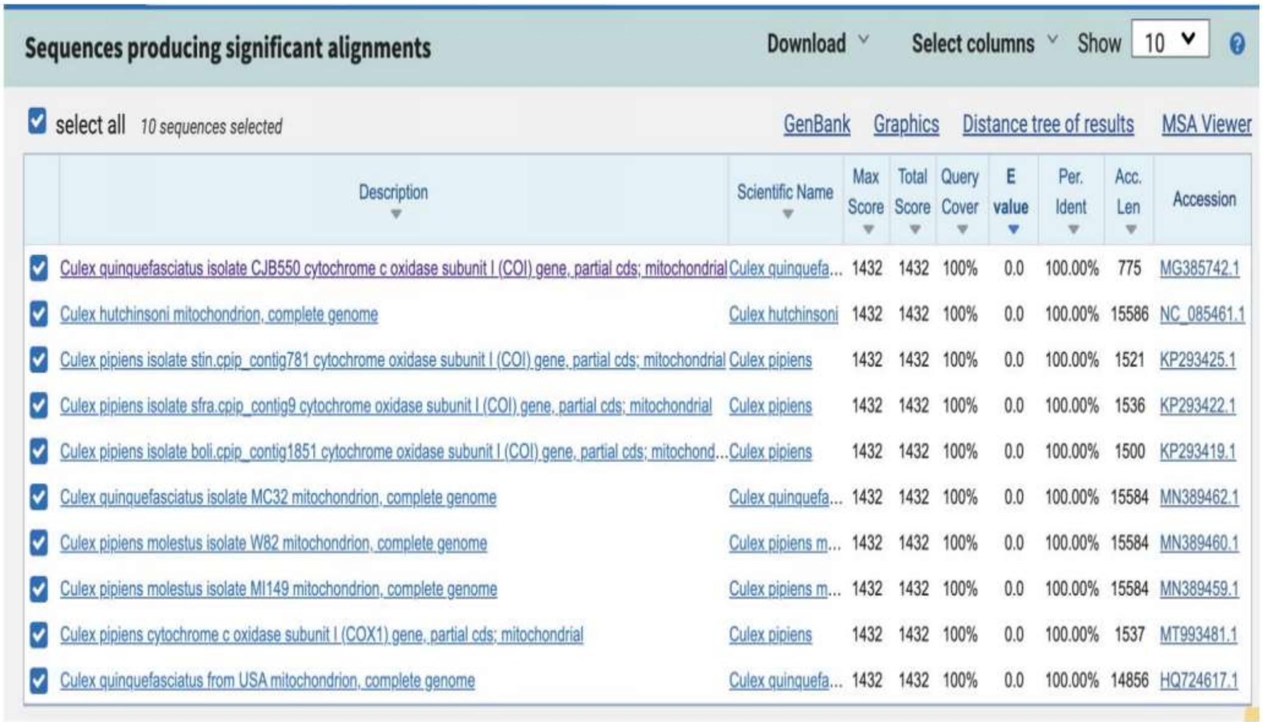
The Clustal W online tool was used to arrange the DNA sequences, and the MEGAX software was used to modify the structure in MEGA design. The neighbor-joining approach was used to create the phylogenetic tree on DNA distance networks identified by the Kimura two-boundary model. The issue of assurance numbers in branches was resolved by bootstrap research. In the lower portion of the figure, this is displayed on a scale. Branches length is measured using normal nucleotide replacements per site. Although they can also be expressed as a percentage change, which is the number of changes per 100 nucleotide locations, this is the number of modifications or replacements divided by the length of the sequence.

CONSENSUS SEQUENCE OF SAMPLE ID – S-2

GGGGCTTGAGCTGGAATAGTTGGAACTTCTTTAAGTTTACTAATTCGAG
CAGAATTAAGTCAACCAGGTGTATTTATTGGAAATGATCAAATTTATA
ATGTTATTGTAACTGCTCATGCTTTTATTATAATTTTTTTATAGTAATA
CCAATCATAATTGGAGGATTTGGAAATTGATTAGTTCCTTTAATGTTAG
GAGCTCCAGATATGGCCTTTCCTCGAATAAATAATATAAGTTTTTGAAT
ACTACCTCCTTCATTGACACTACTACTTTCAAGTAGTTTAGTAGAAAAT
GGAGCTGGGACTGGATGAACAGTGTATCCCCCTCTTTCATCTGGAACA
GCTCATGCTGGAGCTTCAGTAGACTTAGCTATTTTTTCTTTACATTTAGC
AGGAATTTCATCAATTTTAGGTGCAGTAAATTTTATTACAACAGTAATT
AATATACGATCTTCAGGAATTACTCTTGATCGAATACCTTTATTTGTTT
GATCAGTAGTAATTACTGCAGTTTTATTACTTCTTTCTTTACCTGTTTTA
GCTGGTGCTATTACTATGTTATTAACAGATCGAAATTTAAATACTTCAT
TCTTTGATCCAATTGGAGGAGGAGATCCAATTTTATATCAACATTTATT
TTGATTCTTTGGACATCCAGAAGTTTATATTTTAATTCTTCCAGGGTTTG
GAATAATTTCTCATATTATTACTCAAGAAAGAGGAAAAAAGGAAACAT
TTGGAACTTTAGGAATAATTTATGCTATATTAGCTATTG

BLAST ANALYSIS





Sequences producing significant alignments

☑ select all 10 sequences selected

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Culex quinquefasciatus isolate CJB550 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	Culex quinquefa...	1432	1432	100%	0.0	100.00%	775	MG385742.1
☑	Culex hutchinsoni mitochondrion, complete genome	Culex hutchinsoni	1432	1432	100%	0.0	100.00%	15586	NC_085461.1
☑	Culex pipiens isolate stin.cpip_contig781 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	Culex pipiens	1432	1432	100%	0.0	100.00%	1521	KP293425.1
☑	Culex pipiens isolate sfra.cpip_contig9 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	Culex pipiens	1432	1432	100%	0.0	100.00%	1536	KP293422.1
☑	Culex pipiens isolate boli.cpip_contig1851 cytochrome oxidase subunit I (COI) gene, partial cds; mitochond...	Culex pipiens	1432	1432	100%	0.0	100.00%	1500	KP293419.1
☑	Culex quinquefasciatus isolate MC32 mitochondrion, complete genome	Culex quinquefa...	1432	1432	100%	0.0	100.00%	15584	MN389462.1
☑	Culex pipiens molestus isolate W82 mitochondrion, complete genome	Culex pipiens m...	1432	1432	100%	0.0	100.00%	15584	MN389460.1
☑	Culex pipiens molestus isolate MI149 mitochondrion, complete genome	Culex pipiens m...	1432	1432	100%	0.0	100.00%	15584	MN389459.1
☑	Culex pipiens cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	Culex pipiens	1432	1432	100%	0.0	100.00%	1537	MT993481.1
☑	Culex quinquefasciatus from USA mitochondrion, complete genome	Culex quinquefa...	1432	1432	100%	0.0	100.00%	14856	HQ724617.1

Fig 1: NCBI hits obtained for *Culex quinquefasciatus*

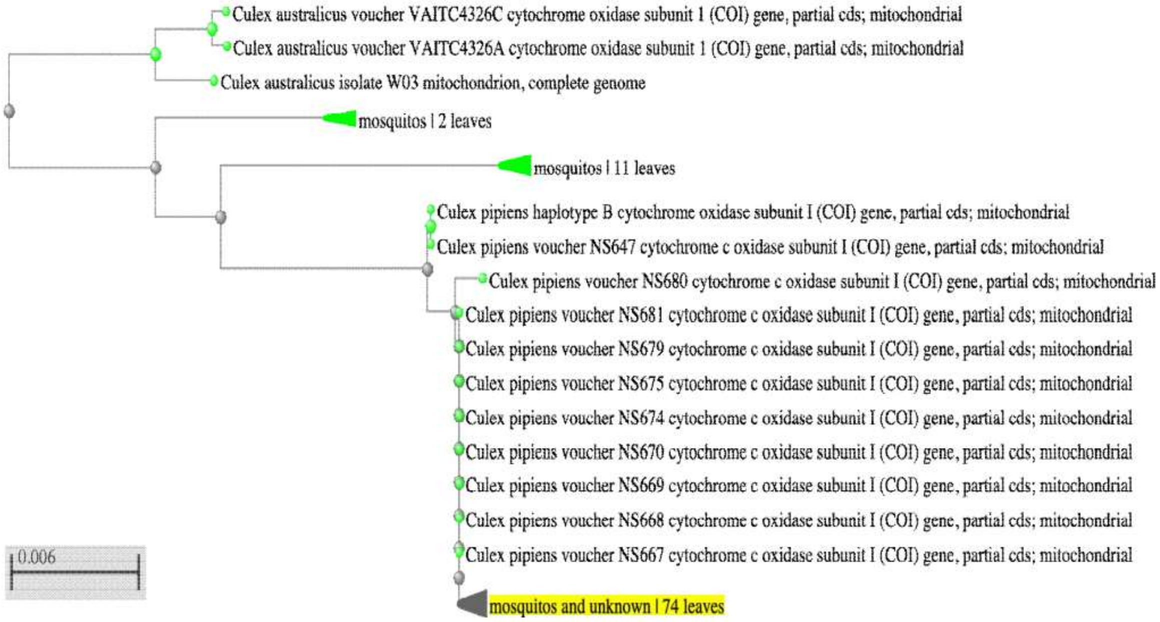


Fig 2: Phylogeny tree of *Culex quinuuefasciatus*

Conclusion

Molecular taxonomy, phylogenetic evidence, and DNA bar coding have given us complete confidence that the specimen we are examining is a *Culex quinquefasciatus*. This is the hierarchy and classification of the species.: -
Domain- Eukaryota/Eukarya; Kingdom- Animalia;
Sub kingdom- Bilateria; Infra kingdom- Protostomia;
Super Phylum - Ecdysozoa; Phylum- Arthropoda;
Sub phylum- Hexapoda; Class- Insecta; Sub class- Pterygota;
Infra class- Neoptera; Super order- Holometabola; Order- Diptera;
Suborder- Nematocera; Infra order- Culicomorpha;
Family- Culicidae; Subfamily- Culicinae; Tribe- Culicini;
Genus- *Culex;* Species- *Culex quniquefasciatus.*

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