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Studies on some species of Indian *Anopheles* based on mitochondrial gene COII (Diptera: Culicidae: Anophelinae)

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Abstract
Mosquitoes are the important members of biting Diptera and are widely distributed in almost all the regions of the world. *Anopheles* is the most studied genus of mosquitoes due to their great impact on human health. The mitochondrial COII gene has been widely used to identify and address phylogenetic uncertainties in insects at diverse taxonomic levels: among orders, within an order, within genus or species groups. The use of genetic markers has managed to recognise of a number of cryptic species and divergent evolutionary ancestries. In the current study, we analysed a partial sequence of 183 bp (approx.) of COII gene for four species of subfamily Anophelinae. The interspecific distance between these *Anopheles* species was calculated to be 9.88% while intraspecific distance between populations of same species was 0.65%.

Keywords: Mitochondrial gene, COII, Indian Anophelinae

1. Introduction
Insects alone, with approximately 970,000 known species, comprise over one-half of all kinds of life known to occur on the planet [6]. Mosquitoes are one of the most important insect vectors that affect the health and wellbeing of humans and domestic animals worldwide. Over 3,500 species of mosquitoes have already been described from various parts of the world [1]. As many as 61 species belonging to subfamily Anophelinae have been reported from India [3]. Mosquitoes are the vectors of many deadly diseases that humanity has faced. Hence they are considered as a threat to mankind in terms of global infectious diseases. Alpha and beta levels of taxonomical study on mosquitoes based on morphological characters have already been carried out extensively, worldwide. In addition to morphological identification, DNA sequences are indispensable tools for delineating and identifying species. Recent studies on DNA-based approaches show a promising trend in the rapid description of biodiversity [11; 10]. In addition to that they are quite useful in determining cryptic as well as sibling relationship between the species. The mitochondrial DNA (mtDNA) markers are often used for phylogenetic and population genetics analyses due to high information content at different evolutionary levels, including variation within and between populations [8]. The present work was carried out to generate molecular database of various collected species of genus *Anopheles* using mitochondrial gene COII and studying intra and interspecific relationship between the collected species.

2. Materials and methods
Collection of mosquitoes from different localities of Punjab (Fig 1; Table 1) was done during the year 2014-16 which was followed by killing, pinning, preservation, sorting and identification of the collected specimens. The mosquitoes were identified morphologically using identification keys given by Christophers [5] and Tyagi *et al.* [21]. The labels provided information like, location and timing records as well as other valuable data about the specimens. Legs were carefully dissected out from these specimens and preserved in absolute alcohol. The preserved legs were used for the DNA extraction process. A set protocol was followed along with different extraction kits to ensure accuracy of the procedure. The DNA was quantified using Nano drop spectrophotometer. The DNA purity was determined by analysing the ratio of the optical densities at 260 nm and 280 nm. The quality of DNA was assessed by running the extracted DNA sample on Agarose gel.

Extracted DNA was stored at -20 ºC for future use. In order to get rid of certain miscoding regions and enhancing the quality of DNA sequence, the unfinished/crude DNA was first subjected to trimming as miscoding regions could interfere with the quality of desired DNA sequences. Alignment of the sequences was done by clustalW alignment (Fig 2). Basic steps followed for analysis are: Editing and trimming of the crude DNA sequence, Translation of DNA sequences into proteins, BLAST Analysis and Evaluation of genetic distances.

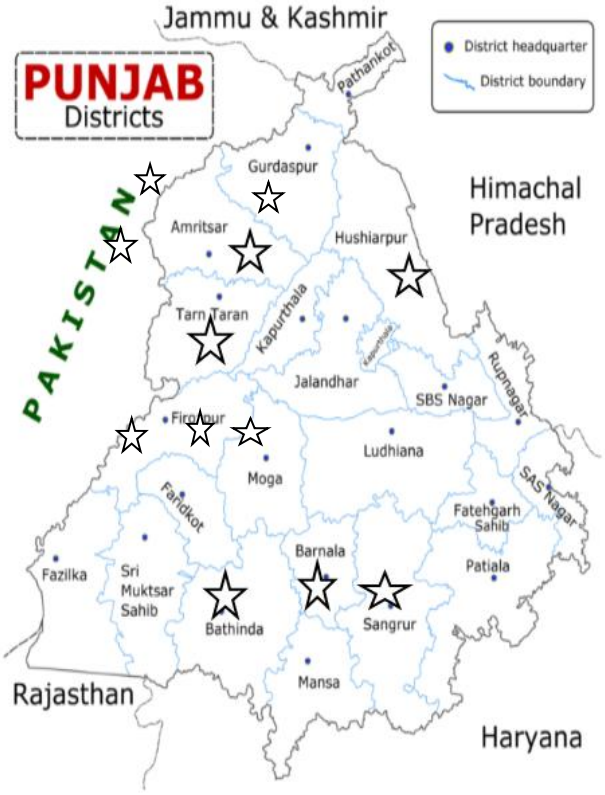


Fig 1: Map showing different collection sites from Punjab state.

Table 1: District wise list of collection areas.

S. No	District	Coordinates
1.	Amritsar	31.6340°N, 74.8723°E
2.	Tarntaran	31.4617°N, 74.9267°E
3.	Hoshiarpur	31.5143°N, 75.9115°E
4.	Bathinda	30.2110°N, 74.9455°E
5.	Barnala	30.3819°N, 75.5468°E
6.	Sangrur	30.2458°N, 75.8421°E

Amplification of the extracted DNA was the most crucial part as optimum annealing temperature was necessary to be fixed for every sample. The thermal cycler conditions were the following: initial denaturation at 98°C for 2 minutes followed by 38 cycles at 98 °C for 30 seconds, annealing at 49°C for 40 seconds, elongation at 72°C for 1 minute and final elongation at 72°C for 7 minutes. 10µL PCR cocktail constituted of Phusion DNA polymerase enzyme 0.1U/10µL, reaction, 5X Buffer 1.2µL, 1.2µLdNTP, 50Mm $MgCl_2$ 0.2µL, 0.25µL primer and MQ water (Thermo Fisher Scientific, India). The mitochondrial COII gene fragment was amplified using primers C2-J-3138 (5' AGAGCTTCTCCTTTAATGGAACA 3') and C2-N-3686
(3' CAATTGGTATAAAACTATGATTTG 5') [20]. Successful completion of PCR was followed by checking of products on 1% Agarose Gel Electrophoresis. Size of the amplicon was compared using reference gene ruler 100 bp ladder. After getting the results from the gel images, the amplified samples were sent to SciGenom Labs, Cochin (Kerala) for DNA sequencing. The sequences were then submitted in the GenBank and corresponding accession numbers were obtained for all these sequences. The accession numbers assigned to different samples are enlisted in Table 2.

Table 2: List of accession numbers obtained from GenBank for various samples.

Species	Sex	Sampling Sites	Accession Number
Anopheles stephensi			
As1	F	Tarntaran, Punjab	KU878973
As2	M	Hoshiarpur, Punjab	KX148466
As3	F	Barnala, Punjab	KX148467
Anopheles peditaeniatus			
Ap1	M	Amritsar, Punjab	KU884334
Ap2	F	Tarntaran, Punjab	KU884333
Ap3	F	Tarntaran, Punjab	KU884335
Anopheles annularis			
Aa1	F	Tarntaran, Punjab	KX015825
Aa2	F	Amritsar, Punjab	KX148465
Anopheles pulcherrimus			
Apu1	F	Sangrur, Punjab	KX298156
Apu2	F	Sangrur, Punjab	KX298155
Apu3	M	Bathinda,Punjab	KX298154

3. Results and discussion

In the present study both nucleotide sequence and the percentage of particular nucleotide have been evaluated as these parameters are important for studying the variation among different species. The average percentage of each nucleotide for the studied fragment of COII gene has been observed to be T= 41.5%, C= 13.8%, A= 35.4% and G= 9.3%. This clearly shows that it is A+T rich site. Also the results indicate that T is greater than A and C is greater in proportion to G. Domain wise nucleotide composition was also calculated (Fig 3). The current study is in total agreement with the previous studies which reported that higher A+T frequency is a characteristic of insect mtDNA [17; 2; 12; 15; 8]. The conserved, variable and parsimoniously informative sites have also been analysed. There were a total of 183 positions in the final dataset. Analyses were conducted in the software MEGA6.0. The overall numbers of conserved sites were 154 and variable sites were only 29. This clearly indicates that COII gene is highly conserved. Out of the 29 variable sites, 28 sites were found to be parsimoniously informative. Analysis of Nucleotide Substitution Pattern Estimation using Maximum Composite Likelihood (MCL) was performed using Tamura-Nei (1993) model as shown in Table 3. Analysis of Nucleotide Substitution Matrix using Maximum Likelihood was also performed using Tamura-Nei (1993) model (Table 4). Each entry in the table shows the probability of substitution (r) from one base (row) to another base (column). For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in *italics*. The estimated Transition/Transversion bias (*R*) is 1.23. Substitution rates and patterns were estimated under the Kimura (1980) 2 parameter model.

The difference between intra and interspecific percent DNA

variation has been used as a "genetic yardstick" to recognize new species for some taxa [14]. In the present study, distance analyses were conducted using Kimura 2-parameter model. The interspecific distance between *An. stephensi*, *An. peditaeniatus*, *An. annularis* and *An. pulcherrimus* ranged from 9.02% to 13.07% with an average of 9.88%. Pairwise genetic distances (in percent) of *Anopheles* species are given in Table 5.

Table 3: Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution.

	A	T	C	G
A	-	9.81	3.26	4.43
T	8.36	-	7.87	2.21
C	8.36	23.65	-	2.21
G	16.77	9.81	3.26	-

Table 4: Maximum Likelihood Estimate of Substitution Matrix.

	A	T/U	C	G
A	-	7.65	2.55	5.43
T/U	6.55	-	9.40	1.80
C	6.55	28.24	-	1.80
G	19.83	7.65	2.55	-

Table 5: Pairwise genetic distance (in percent) of *Anopheles* species.

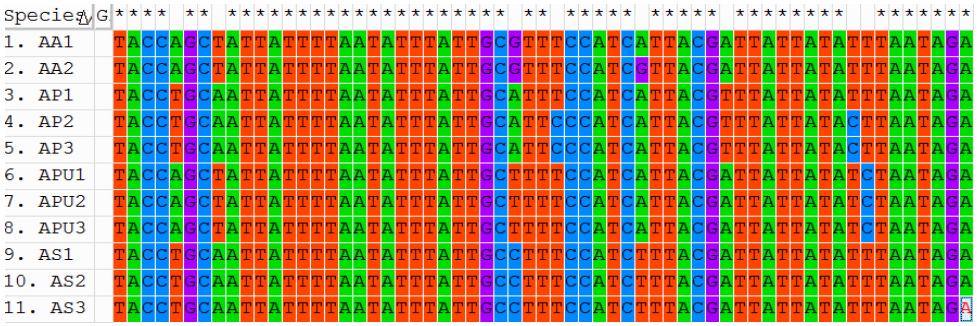
S. No	SPECIES	*An. peditaeniatus*	*An. stephensi*	*An. pulcherrimus*	*An. annularis*
1.	*An. peditaeniatus*	0.0	9.02	9.24	11.74
2.	*An. stephensi*	9.02	0.0	9.89	10.49
3.	*An. pulcherrimus*	9.24	9.89	0.0	9.89
4.	*An. annularis*	11.74	10.49	9.89	0.0

Intraspecific distance among the species was observed between the range 0-1.7% (Table 6). The intraspecific distance values found among the four species are in the range proposed for Anophelines by Bullini and Clouzzi [4]. Hebert [13] cited that intraspecific divergence would likely range between 1.0-2.0%. Wang *et al.* [22] studied the intraspecific divergence range and calculated it to be between 0-1.67% among 122 species of mosquitoes belonging to 15 genera.

Table 6: Pairwise intraspecific distance range among *Anopheles* species.

S.no.	Species	Species	Distance (%)
1.	*An. annularis* 1	*An. annularis* 2	1.1
2.	*An. pulcherrimus*1	*An. pulcherrimus* 2	0
3.	*An. pulcherrimus* 1	*An. pulcherrimus* 3	0.5
4.	*An. pulcherrimus* 2	*An. pulcherrimus* 3	0.5
5.	*An. peditaeniatus* 1	*An. peditaeniatus* 2	1.7
6.	*An. peditaeniatus* 1	*An. peditaeniatus* 3	1.1
7.	*An. peditaeniatus* 2	*An. peditaeniatus* 3	0.5
8.	*An. stephensi* 1	*An. stephensi* 2	0.5
9.	*An. stephensi* 1	*An. stephensi* 3	0.5
10.	*An. stephensi* 2	*An. stephensi* 3	1.1

The present research dealt with the generation of molecular database of collected species of genus *Anopheles* using mitochondrial DNA and decrypting intra and interspecific relationship between the collected species. It was a small effort to bring forward a few among the vast applications of mitochondrial DNA. The species under study showed inter and intraspecific divergence within the limits as proposed by numerous workers round the globe. So this can be further accentuated that COII is worthy for intra and interspecific studies. The variation observed might have been higher if sampling had been directed over greater geographical area. Many extensive studies based on species complex, sibling species and genetic population structure using COII has been carried out [9; 7; 19]. Similar studies have been carried out using COI barcode gene to study intergeneric divergence among different genera viz., *Anopheles*, *Culex* and *Aedes* as well as interspecific divergence [16]. Multiple specimens study on construction of DNA barcodes belonging to as many as 15 genera has also been done [18]. The study can be continued and many more objectives covering mitochondrial DNA studies of more species pertaining to their molecular genetics and traits can be achieved successfully. This work will act as a basis for undertaking such studies at a larger level involving more species, generating more molecular database using other mitochondrial markers as well.



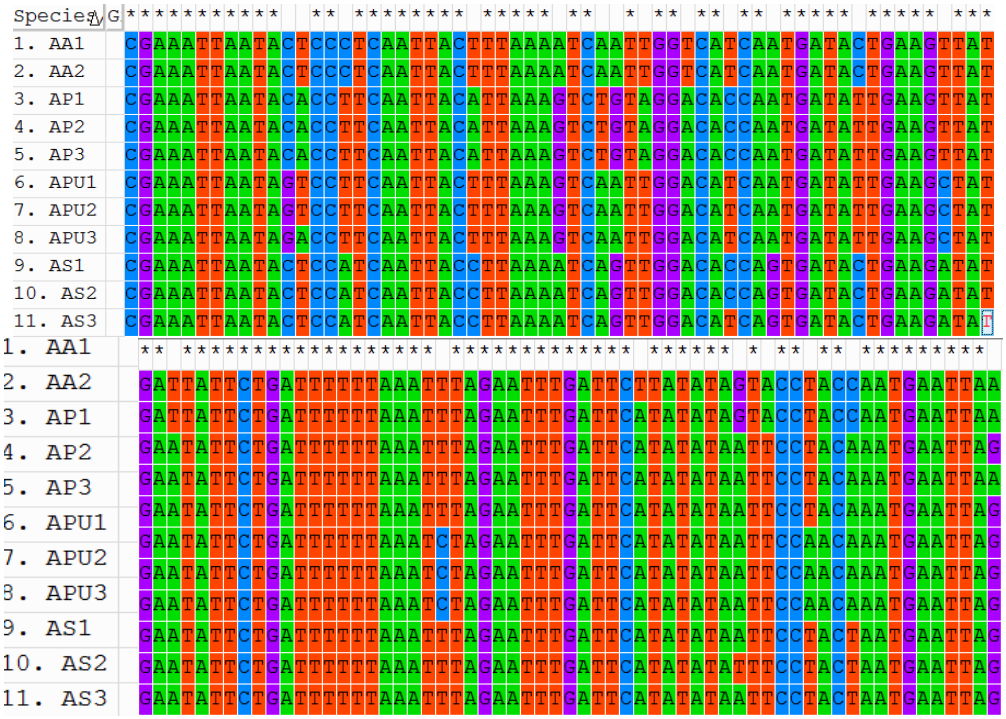


Fig 1: Aligned sequences using clustalW

Domain: Data																				
	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
AA1	43.2	13.7	33.9	9.3	183.0	43	11.5	27.9	18.0	61.0	39	23.0	29.5	8.2	61.0	48	6.6	44.3	1.6	61.0
AA2	42.6	13.7	33.9	9.8	183.0	43	11.5	27.9	18.0	61.0	39	23.0	29.5	8.2	61.0	46	6.6	44.3	3.3	61.0
APU1	41.0	13.7	36.1	9.3	183.0	38	16.4	29.5	16.4	61.0	39	21.3	29.5	9.8	61.0	46	3.3	49.2	1.6	61.0
APU2	41.0	13.7	36.1	9.3	183.0	38	16.4	29.5	16.4	61.0	39	21.3	29.5	9.8	61.0	46	3.3	49.2	1.6	61.0
APU3	40.4	13.7	36.6	9.3	183.0	38	16.4	29.5	16.4	61.0	39	21.3	29.5	9.8	61.0	44	3.3	50.8	1.6	61.0
AP1	42.1	12.6	36.1	9.3	183.0	43	11.5	27.9	18.0	61.0	39	23.0	29.5	8.2	61.0	44	3.3	50.8	1.6	61.0
AP2	41.0	13.7	36.1	9.3	183.0	43	11.5	27.9	18.0	61.0	39	23.0	29.5	8.2	61.0	41	6.6	50.8	1.6	61.0
AP3	41.0	13.7	36.1	9.3	183.0	43	11.5	27.9	18.0	61.0	39	23.0	29.5	8.2	61.0	41	6.6	50.8	1.6	61.0
AS1	41.0	14.8	35.0	9.3	183.0	41	13.1	27.9	18.0	61.0	39	23.0	29.5	8.2	61.0	43	8.2	47.5	1.6	61.0
AS2	41.5	14.8	34.4	9.3	183.0	43	13.1	26.2	18.0	61.0	39	23.0	29.5	8.2	61.0	43	8.2	47.5	1.6	61.0
AS3	41.5	14.2	35.0	9.3	183.0	41	13.1	27.9	18.0	61.0	39	23.0	29.5	8.2	61.0	44	6.6	47.5	1.6	61.0
Avg.	41.5	13.8	35.4	9.3	183.0	41	13.3	28.2	17.6	61.0	39	22.5	29.5	8.6	61.0	44	5.7	48.4	1.8	61.0

Fig 2: Domain-wise average frequency (in percent) of nucleotide bases

4. Conclusion

In the present study, identity of four *Anopheles* species was confirmed by morphological identification as well as by using mitochondrial COII gene. Interspecific variation in DNA sequences of COII gene was relatively higher than intraspecific variation. Identification based on mitochondrial DNA has been proved to be a successful tool time and again and it holds true in the present study as well. The COII gene has been found to be quite useful for identifying and distinguishing the species belonging to the genus *Anopheles.*

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