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Wing Morphometric and DNA Barcoding Analysis of Two Different Public Health Important *Anopheles* Mosquito Species

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ABSTRACT

Accurate identification and bio-ecology of mosquito vectors are of paramount importance in several respects including the development of species-specific vector control strategies. In the current study, we explored two different advanced tools such as Geometric morphometric (GM) and DNA barcoding analysis, to supplement morpho-taxonomy-based identification of malaria vectors. In landmark-based GM analysis, the pattern of wing shape was examined and we noticed a clear variation in their shape among different *Anopheles* species. Similarly, in the DNA barcoding studies, the phylogenetic analysis is based upon the marker genes such as mitochondrial Cytochrome oxidase subunit- I (mt COI), Cytochrome b (mt Cytb), and nuclear D3 domain of 28s rDNA gene sequences were carried out. The Neighbor-joining tree was formed by distinctive conspecific clusters illustrating genetic variation among different groups of *Anopheles* species. Further, genetic exchange among different Anopheline populations was analyzed by considering the COI gene sequences. In the gene flow study, the frequent genetic divergence, as well as gene exchange among *Anopheles stephensi* mosquito populations, was observed. Our findings suggested that GM analysis of the wing shape along with the DNA barcoding approach can effectively be used together for accurate identification of mosquito vectors including *Anopheles stephensi* and *Anopheles barbirostris*.

Key words: Integrated Taxonomic approach, Geometric morphometric, Molecular analysis, Mosquito vector, *Anopheles stephensi, Anopheles barbirostris*

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INTRODUCTION

In India, Malaria, Dengue, Lymphatic filariasis, Japanese encephalitis, Chikungunya, Zika are major vector-borne diseases. Mosquitoes are the well-known vector-borne disease-transmitting agent. There are about 3,583 different Culicidae species reported across the globe (Harbach, 2021). The family Culicidae is a huge and most abundant group which is classified into two different medically important subfamilies such as Anophelinae and Culicinae, among those subfamilies, Anophelinae comprises about 488 valid species. In India, around 58 *Anopheles* mosquito species have been reported to date (Dev and Sharma 2013). Most *Anopheles* mosquito *species* act as a vector for malarial parasites and some other also transmits microfilaria and arboviruses to humans and other animals (Trent, 2005).

For surveillance as well as control of vectors and mosquito-borne diseases, precise identification of mosquito vector species, as well as an understanding of their biology, ecology, and geographical distribution, are crucial (Amini, Hanafi-Bojd, Aghapour, & Chavshin, 2020). The accurate identification of vector species is essential for developing efficient control strategies. The knowledge of taxonomy has evolved in many different ways, but still, morpho- taxonomy is rated high in mosquito species identification. Traditional mosquito identification has relied on dichotomous keys that explain the physical attributes of a certain life stage. Although this technique has proven to be extremely useful and is still widely used to distinguish many mosquito species, it does have some limitations. It requires taxonomic experts to perform accurate identifications, and it is prone to inappropriate identifications due to the loss of key morphological characters during specimen collection and preservation (Madeira, Duarte, Boinas, & Costa Osorio, 2021). Further, an integrated taxonomic approach is the need of the hour in order to resolve taxonomic ambiguity, systematics study and phylogenetic analysis in biological sciences (Joshi & Agarwal, 2021). Keeping in view the importance of the integrated taxonomic approach, during this study we applied two tools i.e. Geometric morphometric and DNA barcoding along with a traditional morpho-taxonomic approach for accurate identification and molecular analysis of mosquito vectors, *Anopheles stephensi* and *Anopheles barbirostris.*

The Geometric morphometric (GM) analysis has been introduced as a supplement for error-free identification of species. It has been demonstrated to be an incredible tool for evaluating the correlation between shapes of various organs and structures, including 2D, 3D points representing landmarks (LMs), curves and outlines. It also permits several statistical analyses and allows depicting the shape and size graphically (Lorenz et al, 2017). It became popular because of the current accessibility of modest computational power-specific software (Rohlf, 2003; 2004; 2006; Dujardin, 2010) and digitizing applications suitable even for less experienced users. The advantage of the use of this technique by multivariate regression investigation is that the allometric impact can effortlessly be evacuated from shape analysis, making it conceivable to compare shapes with the least interference from varying sizes (Adams, Rohlf, & Slice, 2004). Mosquito geometric morphometrics has previously been used to identify

between genera (Wilke et al, 2016), species within the same genus (Sumruayphol et al, 2016; Chaiphongpachara et al, 2019) and populations within a species (Vidal & Suesdek, 2012; Morales Vargas et al, 2013). In mosquitoes, wings are the excellent structures generally utilized for morphometric comparisons since it contains veins that encompass natural anatomical landmarks which are ideal for landmarking (Mondal, Devi & Jauhari 2015).

In recent time, the molecular taxonomic approaches have gained momentum in numerous taxonomic studies on dipterans (Stahls, Vujic, & Perez-Banon, 2009; Pramual, Wongpakam, & Adler, 2011) and also employed in identifying several mosquito species (Gonzalez et al, 2010; Laboudi et al, 2011; Ruiz-Lopez et al, 2012). The standard mitochondrial DNA, COI barcode region, has enough information and is very proficient for species identification. It is also helpful in resolving the identification related to ambiguous and cryptic species. In some cases, multiple gene markers have been used to distinguish closely related cryptic as well as sibling species (Lin & Danforth, 2004). Hence, nuclear gene along with mitochondrial DNA may be used as a supplementary marker to support any conclusions. Moreover, molecular data are broadly utilized for producing molecular phylogenies such as phylogenetic, population genetics, genetic variation and species identification studies. Geographical barriers and climatic differences are the main factors of genetic difference which prevents the geneflow within the same species which gradually leads to speciation. A study on the rate of geneflow gives an idea about the genetic exchange among the different populations of a particular species.

Malaria affects an estimated 219 million people worldwide each year, resulting in over 400,000 deaths (WHO, 2021). India contributes substantially to the global malaria burden with approximately 1,86,532 malaria cases reported in the year 2020 (NVBDCP, 2021). Odisha state of India accounts for about 22% of malaria cases and 10% mortality due to malaria alone (NVBDCP, 2021). Out of thirty districts of this state, most of the cases were reported from the Western, Northern and Southern regions of the state (Pradhan et al, 2016). Several mosquito vectors of *Anopheles, Culex* and *Aedes* are found in Odisha state and study site in particular. *An. culicifacies*, *An. stephensi, An. minimus* mainly act as malaria vectors and *Cx. vishnui, Cx. tritaeniorhynchus, Cx. gelidus, Cx. quinquefaciatus, An. barbirostris, An. peditaeniatus, An. subpictus* etc acts as vectors of JE in the studied area. Further, there is no sufficient data available on mosquito faunal diversity of southern districts of Odisha state and therefore we have considered this region as our study area.

The primary goal of this study is to identify the public health important *Anopheles* mosquito species through the geometric-morphometric examination of wing shape variation and by DNA barcoding approach using marker genes such as mitochondrial COI (mt COI), Cytochrome b and nuclear D3 domain of 28s rDNA. In addition, we also analyzed the genetic exchange between different *Anopheles* mosquitoes based on COI sequences to understand the genetic divergence among Anopheline mosquito species.

MATERIALS AND METHODS

Mosquito sampling

A survey of different mosquito breeding habitats was carried out for the updation of mosquito faunal diversity in some southern districts of Odisha state, India (Fig.1). Mosquitoes in their different developmental stages were collected throughout the year by using a variety of standard procedures from multiple locations and transported to the laboratory in preserved condition for identification. Morphological identification of all the field-collected samples was performed using the available identification keys (Christophers, 1933; Barraud, 1934; Tyagi, Munirathinam & Venkatesh, 2015). After successful identification; the mosquito samples were vouchered and stored for future study.

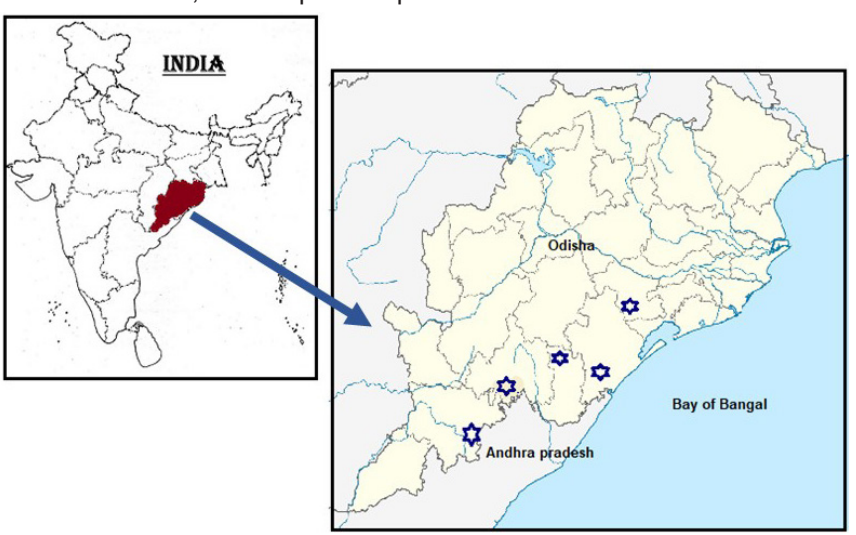

Fig.1. Map showing locations of the mosquito collection sites.

Material preparation for geometric morphometric analysis

The right-wing of individual adult female mosquito was segregated from the thorax and mounted over a microscope slide with a coverslip. Each wing was then photographed under 40x magnification using Leica DFC320 digital camera coupled with a Leica S6 microscope.

Landmarking for GM analysis

About 62 photographs of two different mosquito species (*Anopheles stephensi* (n=31), *Anopheles barbirostris* (n=31)) were chosen and saved as TPS files using the program TPSutil32. The coordinates of 19 landmarks represented through vein intersections were carried out using TPS Dig version 2.31. All the coordinates of landmarks and their descriptions are provided in (Fig.2). In this study, the software Morpho J (Klingenberg, 2011) was used for Principal Component Analysis (PCA) and Discriminate Function Analysis (DFA).

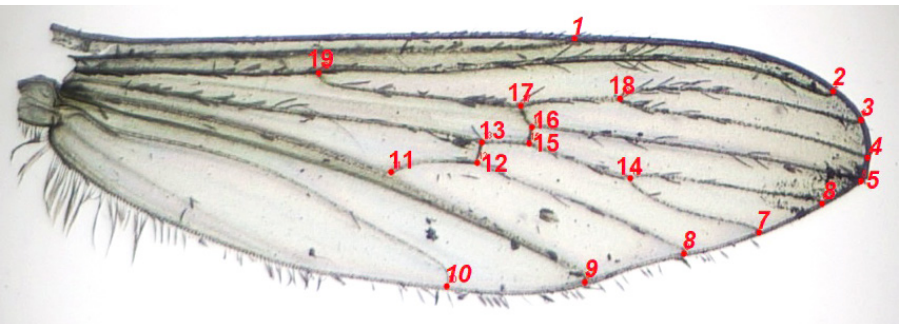

Fig. 2. Landmarks used for PCA and DFA analysis: (1) Intersection of costa(c), (2) Distal end of the radius(R), (3) Radical branch 2, (4) Radical branch 3, (5) Distal end of radius branches 4 and 5, (6) Distal end of M1 and 2, (7) Distal end of M3 and 4, (8) Distal end of cubital vein 1, (9) Distal end of cubital vein 2, (10) Anal vein, (11) Origin of cubital 1, (12) Midpoint branch of cubital 3, (13) Medio-cubital cross vein, (14) Radio-sectoral vein, (15) Midpoint branch Radio medial vein, (16) Radio medial cross vein, (17) Mid-point branch of radial vein, (18) Origin of radius branches 2 and 3, (19) Radial cross vein.

Genomic DNA isolation, PCR amplification and sequencing

DNA extraction from the whole adult mosquito was carried out as per the Bender Buffer method (Collins et al, 1987) with minor modifications. Further, the isolated DNA was used as a templet for the amplification of mitochondrial COI, Cytb and nuclear 28s rDNA gene. DNA amplification was carried out using previously described primer pairs by Folmer, Black, Hoeh, Lutz, & Vrijenhoek 1994; Lyman et al, 1999, Singh et al, 2004, respectively. The reaction mixture was the same for all the studied genes. All the gene amplification consisting of 1X PCR buffer, 0.5 U Taq DNA, 2.5mM $MgCl_2$, 200 µM dNTPs, 10pmol of each primer, 100 pmol template DNA, total dilution was made up to 25 µl. The thermal profile for the COI gene consisted of one cycle of 95℃ for 5min followed by 35 cycles of 95℃ for 30sec, 45℃-55℃ for 30sec and 72℃ for 1min with a final extension step of 7 min at 72℃. The thermal profile for Cytb gene amplification includes an initial denaturation at 94℃ for 5 min followed by 35 cycles of denaturation at 94℃ for 30 sec, annealing at 45–47℃ for 30 seconds, and extension at 72℃ for 2 min; followed by a final extension of 72℃ for 7 min. The PCR condition for the 28s rDNA gene was similar to COI amplification except for the annealing temperature, which is 48℃ for 30 sec. The amplicons were resolved in 1.5% agarose gel. Finally, the purified PCR products were outsourced for sequencing. Large no. of individual specimens were sequenced for each species but the only good quality sequence of COI, Cytb and 28S rDNA was selected and used in this study for analysis of both the species.

DNA sequence analysis

The trace files of COI, Cytb and 28s rDNA sequences were edited and assembled using Geneious version 9.0.5 (Biomatters Ltd, Auckland, NZ) (http://www.geneious.com) software and low-quality sequences were excluded at the time of data analysis. The generated nucleotide sequence from each specimen was compared with barcode sequences available on NCBI using nucleotide Basic Local Alignment Search Tool

(BLASTn), and the final obtained sequences were submitted to NCBI to get the accession number. The accession numbers of the submitted sequences are listed in Table 1. Further, to resolve the genetic relationship among different species, some mt COI gene sequences representing the same and related species of *Anopheles* taxa under study were retrieved from GenBank as replicate data for evaluating the taxonomic position of our target species.

Table 1. Sequence characteristics of COI, Cytb and 28s rDNA (D3).

Gene	*Anopheles stephensi*		*Anopheles barbirostris*	
COI	Sequence length	Accession No.	Sequence length	Accession No.
	668bp	MN329060	615bp	MN166188
Cytb	469bp	MN954649	434bp	MT036262
28s rDNA	398bp	MN319577	381bp	MN318470

Phylogenetic analysis of COI, Cytb and D3 28s rDNA sequences

Multiple sequence alignment was carried out by the CLUSTAL W algorithm embedded in software package MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) to find out the distance matrix which can be used to make a phylogenetic tree. Further, to study the evolutionary relationships among the mosquito species, the generated mitochondrial COI and Cytb gene sequences as well as the 28s rDNA gene sequences were subjected to phylogenetic analysis. The phylogenetic analysis of each gene was carried out with a published set of sequences of different mosquitoes for the mapping of the gene phylogenies by Neighbor-joining (NJ) algorithm (Saitou& Nei, 1987). The evolutionary distances were estimated using the (Kimura 2 Parameter model) K_2P-distance method in MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) with a bootstrap test of 1000 replicates (Felsenstein, 1985). $1^{st}+2^{nd}+3^{rd}$+noncoding codon positions were included in the study and for each sequence pair, all the ambiguous positions were discarded.

Gene flow estimation

Estimates of long-term inbreeding effective population sizes and rates of gene flow among regions were made under a Bayesian inference framework using the program DnaSP version 6 (Rozas et al, 2017) separately for different species. Population genetic indices were calculated separately for each mosquito species using nucleotide data of the mt COI gene. Apart from this, the haplotype diversity (Hd), nucleotide diversity (Pi), genetic distances among haplotypes (Fst), number of migration (Nm) were analyzed. Obtained datasets were compared with datasets reported from various regions of India, Pakistan, Sri Lanka, Iran, Thailand, and China.

RESULTS

Both the immature and mature stages of different types of mosquitoes were collected from different sites of the study area. The immature stages of mosquitoes were reared to the adult stage for morphological identification. The larval habitat from which specimens were obtained includes both natural and artificial container habitats

whereas adults were collected resting on the various surface from cattle sheds and other human habitats. All the adult mosquitoes were identified morphologically as *Anopheles stephensi* and *Anopheles barbirostris* using the available identification keys (Christophers, 1933; Barraud, 1934; Tyagi, Munirathinam & Venkatesh, 2015).

In the geometric morphometric analysis, the Procrustes sums of squares and the tangent sum of the square were found to be 0.1314 and 0.1310, respectively. In Principal component (PC) analysis, out of 34 principal components, PC1 and PC2 have the highest eigenvalues and % variance and thus, these two PCs were chosen for further analysis (Fig.3). The eigenvalues of PC1 and PC2 were found to be 0.0011 and 0.0002 with % variance 52.091% and 9.941% respectively, representing more than 62% of cumulative variance. The scatter plot of the first two PCs for the 62 specimens showed two distinct clusters thereby the *Anopheles barbirostris* clustered themselves in the negative axis of PC1 and *Anopheles stephensi* formed a cluster in the positive axis of PC1 (Fig.3 C). The transformation grid of PC1 showed that the lollipop structure of LM 7, 9, 10, 11, 12, 13, 14 and 18 had maximum variation (Fig.3 A). In the PC2 transformation grid, the lollipop of LM 1, 7, 8, 9, 10, 14, 18 and 19 showed maximum variation (Fig.3 B). *Anopheles stephensi* showed a significant shape difference from *Anopheles barbirostris* which is evident from the Procrustes distance, Mahalanobis distance and T- square value obtained from Discriminant Function Analysis (DFA) having values 0.0642, 15.6633, 3802.7658 respectively with p-value < 0.0001.

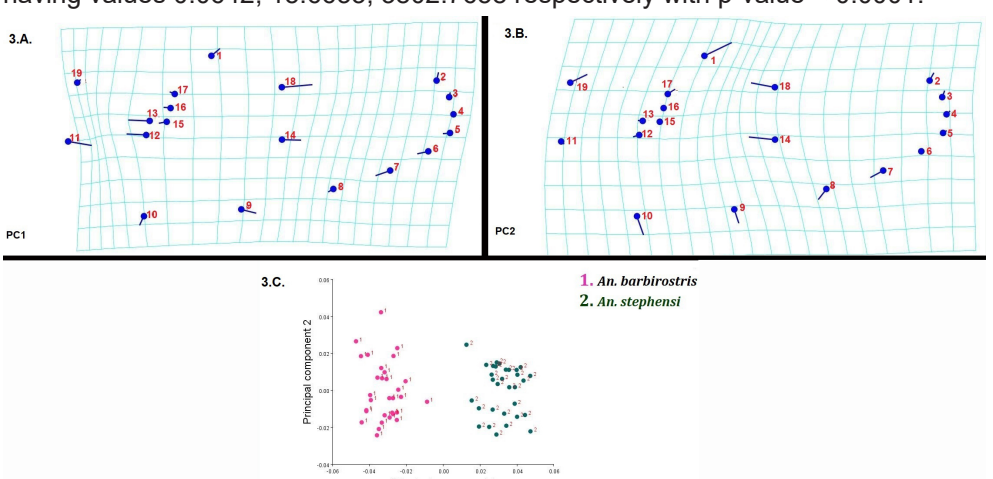

Fig.3. Scatter plot of principal component (PC1 and PC2) and transformation grid representation of variance of 62 samples. Arrows indicate the changes in the relative position of the landmarks.

The COI gene sequences for *Anopheles stephensi* (668bp) and *Anopheles barbirostris* (615bp) were obtained after the final annotation. AT- richness ranges from 67-68% in these sequences. The Neighbor-joining is conceptually related to the clustering pattern. In this study, the COI gene fragments provide phylogenetic signals and also revealed species boundaries accurately. The optimal NJ tree with the sum of branch length =0.43167063 (Fig.4). In the resulted phylogenetic tree, all

the above-studied specimens form clusters with their respective species. Likewise, the generated Cytb sequences of *Anopheles stephensi* (469 bp) and *Anopheles barbirostris* (434bp) were rich in AT content, which is about 74%. A similar NJ tree topology was observed in the case of Cytb geneset, supporting the process of molecular identification (Fig.5). Final annotation obtained from D_3 gene sequences for *Anopheles stephensi* (398 bp) and *Anopheles barbirostris* (381bp) were rich in GC content, which ranges from 55.6%-57.3%. Similar to both the mitochondrial genes, these 28s rDNA sequences of the same species cluster together in the phylogeny (Fig.6).

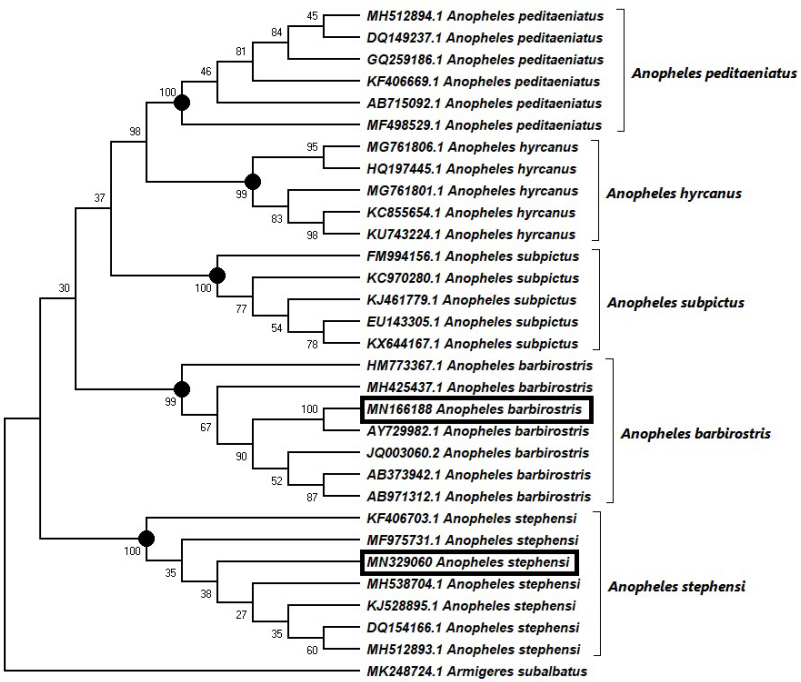

Fig.4. Phylogenetic tree of *Anopheles* mosquitoes constructed by using the Neighbor-Joining approach. The evolutionary distances have been computed utilizing the K_2P-distance comparisons among COI sequences and are in the units of the number of base substitutions per site. All the ambiguous positions were removed for each sequence pair. Next to the branches is shown the percentage of replicate trees in which the related taxa clustered together. Species within the box along with the NCBI accession no. indicates the generated sequence. A sequence of *Armigeres subalbatus* was used as the out-group.

Haplotype diversity (Hd) and nucleotide diversity (π) are two important indicators to measure the diversity of species populations among different geographical strains. Haplotype diversity (Hd) for the 34 COI sequences of *Anopheles stephensi* was calculated to be 0.631, an average number of nucleotide differences (k), and nucleotide diversity (Pi) was found to be 42.146 and 0.125, respectively. Genetic differences among the populations of *Anopheles stephensi* species are provided in Table 2. For *Anopheles barbirostris,* by considering 41 COI sequences, the haplotype diversity (Hd) was calculated to be 0.897. An average number of nucleotide differences (k) and nucleotide

diversity (π) was found to be 89.092 and 0.342, respectively. Genetic differences among the population of *An. barbirostris* are provided in Table 3. The Nm value between Indian and Pakistan *An. stephensi* population was greater than 1, which was an indication of frequent genetic exchange between these two places. However, the Nm value was less than 1 between Indian and other studied populations of *An. barbirostris* species which indicates a less or no genetic exchange observed among these mosquito populations.

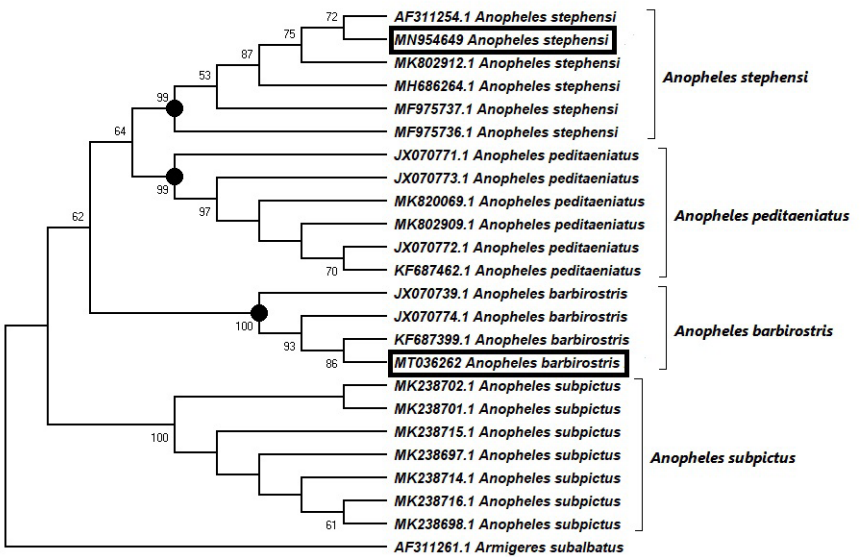

Fig. 5. Phylogenetic tree of *Anopheles* mosquitoes constructed by using the Neighbor-Joining approach. The evolutionary distances have been computed utilizing the K_2P-distance comparisons among Cytb sequences and are in the units of the number of base substitutions per site. All the ambiguous position were removed for each sequence pair. Next to the branches is shown the percentage of replicate trees in which the related taxa clustered together. Species within the box along with the NCBI accession no. indicates the generated sequence. A sequence of *Armigeres subalbatus* was used as the out-group.

Table 2. Gene flow properties between different populations of *Anopheles stephensi* calculated from nucleotide sequence of mtCOI gene. AN1- India, AN2- Pakistan, AN3- Sri Lanka, AN4- Iran. (Hd=Haplotype diversity, Pi= nucleotide diversity, Kt=average number of nucleotide difference, Kxy= inter-population nucleotide differences between the populations, Gst= Genetic differentiation index based on the frequency of haplotypes, Fst= genetic distances among haplotypes, Dxy= average number of nucleotide substitutions per site between all the populations, Da= average no. of net nucleotide substitutions per site between all the populations, Nm= number of migration)

Population 1	Population 2	Hd	Pi	Kt	Kxy	Gst	Fst	Dxy	Da	Nm (Based on Fst)
AN1	AN2	0.395	0.089	30.193	30.333	0.096	0.092	0.090	0.008	2.46
AN1	AN3	0.699	0.110	37.921	30.416	0.096	0.093	0.088	0.008	2.42
AN2	AN3	0.404	0.001	0.500	0.833	0.348	0.480	0.002	0.001	0.27
AN2	AN4	0.058	0.136	66.858	123.40	0.617	0.499	0.252	0.125	0.25
AN3	AN4	0.854	0.205	78.690	107.833	0.197	0.495	0.281	0.139	0.25
AN1	AN4	0.794	0.213	72.882	89.816	0.171	0.184	0.262	0.048	1.10

Table 3. Gene flow properties between different populations of *Anopheles barbirostris* calculated from nucleotide sequence of mtCOI gene. P1- Thailand, P2- India, P3-Srilanka, P4- China. (Hd=Haplotype diversity, Pi=nucleotide diversity, Kt=average number of nucleotide difference, Kxy= inter-population nucleotide differences between the populations, Gst= Genetic differentiation index based on the frequency of haplotypes, Fst= genetic distances among haplotypes, Dxy= average number of nucleotide substitutions per site between all the populations, Da= average no. of net nucleotide substitutions per site between all the populations, Nm= number of migration)

Population 1	Population 2	Hd	Pi	Kt	Kxy	Gst	Fst	Dxy	Da	Nm (Based on Fst)
P1	P2	0.839	0.198	65.930	93.433	0.169	0.539	0.281	0.151	0.21
P2	P3	0.757	0.234	65.990	115.175	0.281	0.849	0.409	0.348	0.04
P2	P4	0.774	0.224	77.792	125.00	0.292	0.749	0.360	0.269	0.08
P1	P4	0.942	0.368	160.100	209.66	0.065	0.499	0.481	0.240	0.25
P1	P3	0.923	0.191	70.456	74.466	0.096	0.153	0.202	0.031	1.38
P3	P4	0.830	0.276	60.566	97.944	0.273	0.746	0.447	0.334	0.08

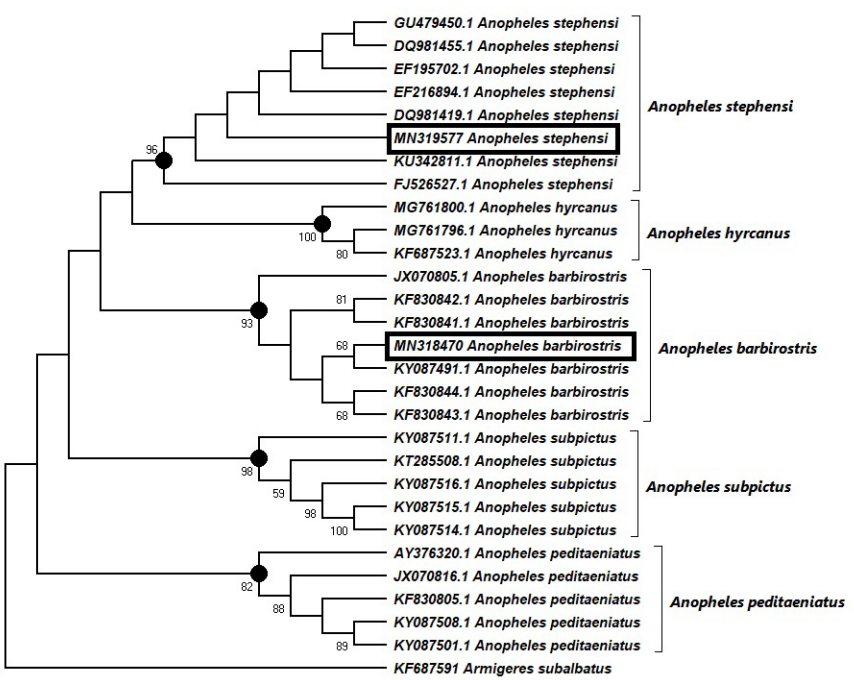

Fig. 6. Phylogenetic tree of *Anopheles* mosquitoes constructed by using the Neighbor-Joining approach. The evolutionary distances have been computed utilizing the K_2P-distance comparisons among 28s rDNA sequences and are in the units of the number of base substitutions per site. All the ambiguous position were removed for each sequence pair. Next to the branches is shown the percentage of replicate trees in which the related taxa clustered together. Species within the box along with the NCBI accession no. indicates the generated sequence. A sequence of *Armigeres subalbatus* was used as the out-group.

DISCUSSION

Vector-borne diseases account for more than 17% of all infectious diseases, causing more than 7,00,000 deaths annually (WHO, 2020). The species *Anopheles stephensi* is a dominant urban vector for both *Plasmodium falciparum* and *Plasmodium vivax,* distributed throughout the geographical range of middle-east Indian subcontinent (Nalin et al, 1985; Sharma, 1995; Oshaghi, Yaaghoobi, & Abaie, 2006). *Anopheles barbirostris* is also reported as a vector of Japanese encephalitis in India (Tyagi, Munirathinam & Venkatesh, 2015). Therefore, the unequivocal identification of mosquito specimens is one of the backbones of mosquito and vector surveillance programs and therefore major attention should be given to their genetic diversity study. The combined use of genetic markers provides an idea about the population structure, genetic differentiation and gene flow of species, which is an essential component to design strategies for the management of vector-borne disease (Weeraratne, Surendran, Walton, & Karunaratne, 2018b).

Even though morpho-taxonomy is regarded as the gold standard method for discrimination of mosquito species, it seems to be quite difficult in the identification of field-collected mosquitoes as they may lose some of their important identifying features during handling. For this reason, there is a need for an alternative technique for identification especially in the case of cryptic and ambiguous species. Various other techniques were also available for mosquito species identification such as cytotaxonomy involving karyotyping of polytene chromosome (Tyagi et al, 2015), isozyme analysis (Knight & Nayar, 2004), next-generation sequencing approach (NGS) (Muturi, Dunlap, & Tchouassi, 2021), SNP barcoding with a decision tree algorithm based on machine-learning approach (Swain, Makunin, Dora, & Barik, 2019), deep learning technique (Mulchandani, Siddiqui, & Kanani, 2019), convolutional neural networks (CNNs) using image database (Goodwin et al, 2021) etc. Herein, we examined the accuracy of the integrated taxonomic tool using the geometric-morphometrics and DNA barcoding technique.

Geometric morphometric analysis based on quantitative characterization of mosquito wing venation has proved to be a reliable technique for identifying cryptic mosquito species (Morais, Moratore, Suesdek, & Marrelli, 2010; Lorenz, Marques, Sallum, & Suesdek, 2012) and also efficiently classify up to species level (Wilke et al, 2016). This was proved to be an effective tool for the rapid, inexpensive and reliable classification of six species of the *Aedes* genus in France (Martinet et al, 2021). The use of multiple gene markers coupled with landmark-based wing morphometric analysis of insect species including Lepidoptera and Diptera was evidenced from the various studies of Laparie, et al, (2016); Sontigun, et al, (2017); Jin, Hu, Han, & Chen, (2018). Earlier studies on morphological features of mosquito wings for the identification purpose were also reported by Sumruayphol et al, (2016); Lorenz, et al, (2017); Haarlem, & Vos, (2018). In the present study, PCA analysis compares two different Anopheline mosquito groups that segregate, forming two separate clusters. LM7, LM9, LM10, LM14 and LM18 showed the highest displacement along the transformation grid of both PC1 and PC2 which specifies that both species are separated from each

other at these four landmarks. Further, in DFA analysis between two species, the P-value is < 0.0001 which indicates that each group is significantly different from each other with high variation. Thus, our finding indicates that two epidemiologically critical mosquitoes *Anopheles stephensi* and *Anopheles barbirostris* were accurately recognized by utilizing wing shape through GM analysis.

DNA-based methodologies for mosquito identification (Manonmani et al, 2001; Kang, & Sim, 2013), molecular phylogeny (Shepard, Andreadis, & Vossbrinck, 2006) and genetic diversity (Pfeiler, Lopez Flores-Lopez, Mada-Velez, Escalante-Verdugo, & Markow, 2013) have gained increasing adoption in recent years as it is faster to perform and more reliable. Phylogenetic trees are utilized for analysis of gene duplication, estimating rates of diversification, polymorphism, recombination, population dynamics and inferring organismal phylogenies by combining it with other data sources.

Earlier studies have proved the use of the mitochondrial COI marker in finding more biodiversity and increasing species richness than traditional taxonomic approaches by uncovering undescribed and cryptic species (Hebert, Ratnasingham, &de Waard, 2003; Schmidt, Schmid-Egger, Morinière, Haszprunar, & Hebert, 2015; Wilson, Sing, Floyd, & Hebert, 2017). This COI gene as a molecular marker was used to infer the phylogeny of various dipteran taxa within the genera *Aedes, Anopheles* and *Culex* mosquitoes (Ashfaq et al, 2014; Weeraratne, Surendran, & Parakrama Karunaratne, 2018a; Chan-Chable, Martínez-Arce, Mis-Avila, & Ortega-Morales, 2019). Recently, a DNA barcode reference library was developed for identifying the mosquito species from Portuguese mosquito fauna, including the most significant vector species (Madeira, Duarte, Boinas, & Costa Osorio, 2021). In previous reports, rDNA such as ITS_2, D_3, and Mt DNA COII sequences were also used for differentiation of siblings from five species of Maculatus group (Ma, Li, & Xu, 2006) collected from China. Similarly, phylogenetic relationships among the Anopheline species was inferred using COI, COII, D3, ITS genes in various other regions of Odisha state of India which proved useful in properly defining species distribution and resolving the ambiguity that normally arises due to morphological taxonomy (Mohanty, Swain, Kar & Hazra, 2009). Therefore, multiple genes may be required as a supplementary marker to draw any conclusion. Thus, in addition to geometric morphometric analysis of wing shape variation, we employed DNA barcoding of COI, Cytb and D_3 domain of 28s rDNA sequences for mosquito species identification in the present study. We observed that the composition of generated COI sequences was AT-rich which is similar to the findings of Cywinska, Hunter, & Hebert, (2006); Rivera, & Currie, (2009) on dipterans. In this study, the mt COI-based analysis strongly supports the positioning of *Anopheles stephensi* and *Anopheles barbirostris* by forming distinct clusters with their respective group. Similarly, the phylogenetic repositioning with other two markers such as Cytb and 28s rDNA also supports the cladogenesis of both species. The clustering patterns agreed with the morphological identification, enable distinguishing the individual species based on both the studied gene sequences. Further, in a study, the genetic diversity and evolutionary relationships among *An. tessellatus* in nine Asian countries at the COI gene were described by Bourke, Wilkerson and Linton in 2021 which revealed

the exceptional levels of genetic diversity in populations across its known range and identify up to six putative species in the newly determined Tessellatus Complex. The existence of such cryptic diversity has potentially important consequences for vector management and disease control.

Population genetic study illustrated the occurrence of genetic differentiation within the population and the frequent exchange of genes among the population. Fang, et al, (2018) noticed a positive correlation between differences in genetic material among *Aedes albopictus* population with respect to their geographical distances and concluded that the genetic diversity might occur due to genetic mutation or due to ecological factors which accumulate genetic differences that may lead to reproductive isolation and gradually formation of a new species. We also analyzed the rates of gene flow of two different *Anopheles* mosquito species in the present study. When the gene flow Nm value is greater than 1, it means that the gene exchange is frequent, which can prevent the inter-population differentiation caused by genetic drift (Morton, 1977). It may be inferred from this study that due to geographical barriers and differences in climatic conditions between India and other studied regions except for Pakistan, there was a slight difference in COI gene sequences of *An. stephensi*. A frequent genetic exchange between Indian and Pakistan populations might be happening due to human activity or fewer physical barriers. However, the Nm value between different studied populations of *An. barbirostris* indicates, there might be less or no genetic exchange occurs between Indian with other populations might be due to different environmental and climatic conditions.

In conclusion, although *Anopheles stephensi* and *Anopheles barbirostris* are two different species that can be distinguished easily by using identification keys based on morpho-taxonomy. Our study proved here the efficiency of the combined use of wing geometric morphometric analysis and DNA barcoding approach for mosquito identification which can be utilized in the future for the identification of species that are difficult to distinguish. No doubt, only the COI gene as a molecular marker alone can confirm the species identification along with morpho-taxonomy and geomorphometric analysis but in the present study, we used multiple marker genes to validate the data to strengthen our findings. Furthermore, the result also validated the use of COI gene sequences for genetic flow analysis that helps to understand genetic diversity among geographically distant populations of different mosquito species.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest with respect to the research, authorship and/or publication of this article.

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