

**CULEX PIPIENS COMPLEX WITH THREE NEW RECORDS IN BANGLADESH:
A NEW CHALLENGE TO THE ELIMINATION OF LYMPHATIC FILARIASIS**

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ABSTRACT: The current study in Gulshan thana area of Dhaka North City Corporation documented new records of the members of *Culex pipiens* group. Until now, *Culex quinquefasciatus* was recorded as the sole known species of *pipiens* group in Bangladesh. Through morphological, wing morphometry and genetic analysis, some members of the *Culex pipiens* complex were identified, such as, *Culex pipiens*, *Culex pipiens pallens*, and *Culex pipiens f pipiens*. These species were observed co-existing with *Cx. quinquefasciatus*. Molecular identification was conducted by employing cytochrome c oxidase subunit 1 (COI) and 16S rRNA gene barcoding including sequence percentage identity using BLAST and performing tree-based identification through Neighbor-Joining (NJ) model using the MEGA 11 software package. The 16S sequences of *Cx. pipiens pallens* and *Cx. pipiens f pipiens* were submitted for the first time into the GenBank. This study also represents the first record of *Cx. pipiens pallens* in the Indian region, a species previously documented primarily in Japan, China and South Korea as a prevalent house mosquito. Moreover, the fact that all the newly recorded species are well known vectors of filariasis poses a substantial challenge to Bangladesh, which has recently been declared as a filaria-free country by World Health Organization.

Key words: *Culex pipiens* complex, molecular analysis, wing morphometrics, morphological identification.

INTRODUCTION

The century long perceived knowledge of bio-diversity among taxa as a result of natural selection based on eco-geographic diversification and the climate within had changed drastically after the introduction of advanced genome sequencing technology along with the new analytical software. In that context a lot of mosquito species, across the world had generated a series of novel form of sub-groups or species complex that was not recorded in that specific geographic location. *Cx. quinquefasciatus* is one of the many species, belong to the subgenus *Culex* and *pipiens* group that had a very complex species pattern throughout different geographic location.

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The current subgeneric classification system of *Culex* is primarily based on morphological characteristics, particularly those of the male genitalia and thus divided into Series, Groups, Subgroups and Complexes (Harbach 2011). With a total of 198 species, the subgenus *Culex* is currently recognized as the largest subgenus within the genus *Culex* (Harbach 2012). The current taxonomic classification by Knight & Stone (1977, 1978) in the Catalogue of the Mosquitoes of the World, members of *Cx. pipiens* group are described as *Cx. pipiens* complex that includes *Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. australicus*, and *Cx. globocoxitus*. This classification, however, remains a subject of controversy due to the reliance on morphological differences in traditional taxonomy, the absence of significant morphological distinctions among many complex members, and the occurrence of hybridization (among all the members of the complex but more between *Cx. pipiens* and *Cx. quinquefasciatus*), which further complicates the classification (Harbach et al. 1985, Mattingly 1965, Vinogradova 2000). According to Collins and Paskewitz (1996) a species complex is a group of evolutionary closely related species that often pose challenges in morphological differentiation. Thus, the two nominal forms of *Cx. pipiens*, which are morphologically indistinguishable, have been added to the complex, namely *Cx. pipiens* form *pipiens*, written as *Cx. pipiens f pipiens* and *Cx. pipiens f molestus* (Farajollahi et al. 2011, Fonseca et al. 2009). Furthermore, *Cx. pipiens pallens*, described by Tanaka et al. (1979) was added to the complex as a sub species. With the distribution across the world, *Cx. pipiens* and *Cx. quinquefasciatus* are the two most common species in North and South America, Europe, Asia, Australia and Africa (Kasai et al. 2008, Cui et al. 2007, Mogi 2012). On the other hand, some members of the group can be region specific, such as *Cx. pipiens pallens* is widely distributed in some of the temperate zone of Asia, e.g. Japan, South Korea and China (Fonseca et al. 2009, Liu et al. 2020). Australia has two indigenous species of the complex, *Cx. australicus* (Dobrotworsky and Drummond 1953) and *Cx. globocoxitus* (Dobrotworsky 1953) (Russell 2012). The *Cx. pipiens* complex (*Cx. Pipiens* s.l.) in Europe is comprised of *Cx. pipiens pipiens* Linnaeus, *Cx. pipiens f molestus* Forskal, *Cx. pipiens quinquefasciatus* Say, and *Cx. torrentium* Martini (Becker et al. 2010). In addition to *Cx. pipiens* and *quinquefasciatus*, a widespread cryptic species within the complex has been identified in South Africa. The designated name '*Culex juppi* nov. sp.' is proposed for this mosquito species (Dumas et al. 2016).

Apart from the geographical isolation, some members of the complex are somewhat difficult to distinguish solely based on morphological characters due to frequent hybridization. In addition to that, the morphological differentiation is primarily based on male genitalia (Barr 1957, Dobrotworsky 1967), which cannot differentiate the females. Hence, their stance in taxon formation, changes

in type-locality over time and the maintenance of reproductive isolation within and between species are poorly understood.

Therefore, genomic studies (Kent *et al.* 2007) would shed some lights on the mechanism of hindering taxonomic radiation and speciation. In addition to that, this study would focus on the differences in female morphology of *Cx. pipiens*, *Cx. quinquefasciatus* and *Cx. pipiens pallens* in details.

In the present era of globalization, where geographic boundaries are increasingly interconnected it is impossible for a tiny insect like mosquito to remain region specific. The widespread movement of people and goods has introduced mosquito species to a new region more frequently than ever, causing emergence of new vectors, pathogens and diseases to those regions. Thus, the possibility of the members of *Cx. pipiens* complex, acting as primary vectors, secondary or incidental vectors, or simply carriers of a pathogen during outbreak situation should be taken into consideration. Therefore, the objective of this study is to investigate the presence and abundance of the members of the *Cx. pipiens* complex within the study area, aiming to comprehend their impact on disease dynamics to mitigate the risk effectively.

MATERIAL AND METHODS

Study area: Gulshan thana area of Dhaka City Corporation is 53.59 square kilometer (23.7917°N 90.4167°E), having four wards, 19, 20, 21 and 22. This area is a mix of outstanding residential places, business hubs, schools, universities, slums, and all of the embassies of the country. The area is well divided by blocks in most places except Karail slum and Mahakhali bustand. Besides the commercial high-rise buildings and markets Gulshan thana area has a lot of greeneries, which includes parks, lakeside vegetations, buildings with large compounds as well as rooftop gardens and front or backyard gardens, and abandoned plots with vegetation, which gives plenty of suitable mosquito larval habitats.

Larval collection, rearing and dissection: A sampling strategy was developed using high resolution satellite imagery by using Arc GIS Pro (version 2015) platform by constructing 6 figures grid system (100×100 meters) of the area. The cells were numbered in a way that the randomly selected cells from each locality should be proportional to the total number of cells (Troyo *et al.* 2008). A final grid was created using the multispectral Quickbird imagery. Larval collection was conducted over a period of sixteen months, spanning from December 2022 to December 2023. Grids for collection were chosen in a random manner, and the GPS coordinates of each surveyed area were meticulously recorded.

Mosquito larvae were collected from various breeding sites, such as, drains, ponds, artificial containers, small pools, tubs, drums, cemented tanks, stored

rain waters in different objects, stagnant rain water, slowly flowing drains and a many more stagnant water bodies. It is important to note that the surveyed breeding grounds were not limited to specific species. Water temperature, pH level and TDS (Total dissolved solid) of the habitats were recorded (Table 1).

The collected larvae were brought to the laboratory, which maintained a controlled environment with a 10:14 hours of light-dark cycle, a temperature of $22\pm 2^{\circ}\text{C}$ and a humidity of $75\pm 2\%$ RH. The collected larvae were placed in 6"X6" tubs labeled with dates and allowed them to emerge without providing any larval food. The mosquito cages were 12"X12" aluminium frame cages covered with mosquito net with an opening at one side. Upon emergence, the adults were individually captured in falcon tubes and then stored in a refrigerator for further use. A total of 350 adults both males and females were dissected to get the tissue of midgut for pcr analysis. The dissection process followed the methodology outlined by Coleman *et al.* (2007). Subsequently, the guts were cleaned with 70% alcohol, repeated three times. The males and females were kept separated in distinct eppendorf tubes, each containing 35-40 cleared guts for pcr.

Morphological identification: Emerged adults were identified morphologically with taxonomic keys (Tanaka *et al.* 1979, Bram 1967). The distinguishing characteristics included the examination of proboscis, lateral view of thorax, scaling on abdomens, and wings. These morphological characteristics were visible through AmScope T390 with 4x magnification. However, differences between *Cx. pipiens* and its biotype form *Cx. pipiens f pipiens* were not identified here.

Morphometric analyses: Wings were photographed using AmScope T390B. TpsDig V1.40 software was used to create files with 18 landmarks (Fig 4) of the wings (Wilke *et al.* 2016). The landmark data was organized using Sublime text. MorphoJ1.08.01 software was used to do a series of analyses. For example, A multivariate regression of Procrustes coordinates against centroid size was done using a permutation test with 1000 randomizations were done to assess the allometric influence of wing size on wing shape. Discriminant analysis was also conducted to investigate the level of dissimilarity in wing shape among species within a morphospace generated by Canonical Variate Analysis (CVA) and to calculate the Mahalanobis distances. Thin plate splines were created by regression analysis of CVA scores against the variations of wing shape variation to visualize the shape disparity among the species compared. The analyses were done and graphs were plotted with TpsUtil 1.29 (Rohlf 2008) and MorphoJ 1.08.01 (Klingenberg 2011).

DNA extraction, PCR amplification and DNA sequencing: DNA was obtained from a 5 mg tissue sample of each specimen using the

Table 1. Mosquito larval collection sites with GPS coordinates and habitat types with pH level, temperature and TDS

location with positive sites	latitude	longitude	positive	habitats	pH	TDS	H ₂ O temp °c
	(N)	(E)	Cont				
Niketon Gulshan 1	23°77.07'	90°41.15'	4	pond & containers	6.9	360	26
Gulshan link road Gulshan 1	23°78.06'	90°41.90'	6	containers	7.2	270	27
Gulshan 2 lake	23°78.77'	90°42.16'	12	containers	6.8	290	30.5
Gulshan 2 park	23°80.20'	90°40.60'	3	tires, containers	7	285	28
Boubazar, Gulshan 1	23°78.42'	90°40.82'	25	stored water, drains	6.5	300	29.5
Korail Mandir	23°78.12'	90°40.11'	3	containers	6.8	270	28
Gulshan shooting complex	23°77.40'	90°41.72'	5	containers	7-7.2	240	28
Road 103, Gulshan 1	23°79.29'	90°42.10'	1	drains	6.9	380	30
Govt Titumir college area	23°78.33'	90°39.95'	5	drains, stored water	6.8-7	210-340	29
Road 22, Banani	23°78.07'	90°40.13'	2	drains	6.9	390	31
Banani lake rd 19	23°47.06'	90°23.01'	1	lake water	7	300	27
Six seasons hotel area	23°80.27'	90°41.59'	1	lake water	7	290	30.5
United hospital area	23°79.81'	90°41.64'	1	stored water	6.9	235	27
Park road Baridhara	23°80.46'	90°42.16'	1	containers	7.1	200	27
Beltola Adorsho nagar	23°78.42'	90°40.82'	35	stored pond water, containers, drains	6.7-7.1	285-295	30.5-31
T&T colony Banani	23°78.41'	90°40.81'	5	stored pond water	6.7-7	295	30.5
Chairman bari pond	23°78.42'	90°40.06'	1	pond water	7	275	30
Banani lake	23°47.06'	90°23.01'	1	jheel water	7	300	27
WASA water pump	23°80.20'	90°40.60'	2	containers	7.1	240	30
Justice Shahabuddin park	23°80.24'	90°41.52'	2	containers	7.2	310	28
Korail basti	23°46.98'	90°24.42'	89	drains, pots, tanks, stored water	6.3-7.2	390	27

Monarch® Genomic DNA Purification Kit according to the manufacturer's instructions. The quality and quantity of the extracted DNA were measured using a Nano Drop spectrophotometer. COI and 16S rRNA gene sequences were amplified by polymerase chain reaction with the primer LCO-1490 (forward) and HCO-2198 (reverse) (Folmer *et al.* 1994); 16Sar (forward) and 16Sbr (reverse) (Palumbi *et al.* 1991), respectively. The PCR was carried out in 25 µl volumes comprising 23 µl of PCR Master Mix and 2 µl of DNA sample, which were combined and spun for 30 seconds to homogenize the mixture. 12.5 µl Taq Polymerase, 8.5 µl Nano Pure water, 1 µl forward primer, and 1 µl reverse primer are included in PCR Master. The amplification conditions included initial

denaturation at 95°C for 5 min followed by 35 cycles of 94°C for 45 s, 52°C (COI) and 48°C (16S rRNA) for 30 s, 72°C for 45 s, and a final extension at 72°C for 7 min. Amplified gene bands were visualized on a 1% agarose gel. PCR purification and sequencing were performed by an outsourcing company (Celemics Inc., Korea). Sequencing was performed on high-quality purified PCR products with DNA concentrations greater than 10 ng/μl.

Bioinformatics study: The quality of the generated sequences was viewed using CHROMAS software. Each sequence was confirmed by a BLASTn search against the best-matching sequences in the nucleotide database and deposited in NCBI GenBank and BOLD. All COI and 16S rRNA sequences were automatically aligned using MUSCLE (Edgar, 2004). Pairwise genetic divergence was determined by calculating the Kimura-2-parameter (K2P) (Kimura, 1980) distance using MEGA 11 (Tamura et al. 2021). Phylogenetic trees were constructed for both the COI and 16S rRNA sequences based on the neighbor-joining (NJ) statistical method with gamma distribution rates by bootstrap analysis with 1000 replicates in MEGA 11.

Data analyses: Relative abundance (RA) and distribution (C) of different members of the species complex were determined according to the equations described by Rydzanicz and Lonc (2003).

$RA = I/L \times 100$ Here, “I” represents the number of specimens of a species and “L” is the total number of species collected.

With the RA value mosquito species were classified into specific categories based on Trojan’s (1992) criteria: Satellite (RA < 1%), Sub-dominant (RA < 5%) and Dominant species (RA > 5%). Distribution (C) is $C = n/N \times 100$

Where “n” is the number of sites where mosquito larvae were found and “N” is the total number of sites analyzed. Again using the value of “C”, the mosquito species were categorized according to Dzieczkowski (1972) into the following classes: C = 0-20% (Sporadic); C = 20.1- 40% (Infrequent); C = 40.1- 60% (Moderate); C = 60.1- 80% (Frequent) and C = 80.1- 100% (Constant)

RESULTS AND DISCUSSION

Among the total collection of *Cx. pipiens* group (3180 samples out of 4163), *Cx. quinquefasciatus* emerged as the dominant species, constituting 76%. Meanwhile, *Cx. pipiens* and *Cx. pipiens pallens* comprised 19% and 5% respectively. The Relative Abundance (RA) and distribution (C) value were presented in Table 2. However, it is worth mentioning that these findings may lack accuracy due to the limitations of morphological identification, which only distinguished between *Cx. pipiens* and *Cx. pipiens pallens* in addition to *Cx. quinquefasciatus*. Conversely, genetic analysis unveiled the presence of another member within the complex, *Cx. pipiens f pipiens*.

Culex (Culex) quinquefasciatus: Female abdomen: The basal bands on terga III- VII medially broadened. Tergum I with median spot of dark scales. Basal band on tergum II is short, more triangular shape. Basal bands usually not connected with laterobasal patches on anterior segments (Fig 1). Wing: Veins dark scaled. Subcosta intersects costa before the level of furcation of vein r_{-2+3} . Cell R_2 is 2.74-3.56 ($x = 3.13$) length of vein r_{-2+3} (Fig.2) (Dehghan *et al.* 2016).

Culex (Culex) pipiens pallens: Female abdomen: Tergum I has two median black spot with black and white patches anteriorly (Fig 1). The basal bands on terga III-VII often even in width, occasionally medially broadened.

Basal bands on terga V-VII usually connected with laterobasal patches of anterior segments (Fig 1). Wing: Subcosta intersects costa at or beyond level of furcation of vein r_{-2+3} , a common feature for *Cx. pipiens* group members (Fig 3). Cell R_2 is 3.68-5.57 ($x = 4.26$) length of vein r_{-2+3}

Wing Morphometric analyses: The allometric effect was significant (P-value $<.0001$), which showed the differences in variation among the species (Table 4). Canonical Variate Analysis (CVA) (repeated three times) showed variation among groups scaled by the inverse of the within group variation (Table 4), which were all significant with about 76% variance. Scatter plot from CVA of wing shape showed clusters in morphospace, which revealed three different species, however, there are overlapping observations (Fig 7). Mahalanobis and Procrustes distances among groups are given in Table 3. Subsequent pair-wise comparison of wire frame graphs among species showed that quantitative landmarks are associated with differentiation between each group (Fig 5). Principal component analysis showed the differences of wing shape variation among the sibling species (Fig 6). The higher Eigen value (15352.70170189) of the first principal component explains almost 50-76% of wing shape variations among all of the observations. These phenotypic variation among observations also showed in the scatter plot, however, the phenotypic plasticity of each species may also occur due to different ecological and seasonal conditions.

Table 2: Relative abundance (RA) and distribution (C) of the members of *Cx. pipiens* complex in Gushan Thana area

Species	%of total collection	Relative abundance(RA)	RA status	Distribution (C)	Distribution status
<i>Culex quinquefasciatus</i>	76	58.03%	Dominant	66%	Frequent
<i>Culex pipiens</i>	19	14.50%	Dominant	40%	Moderate
<i>Culex pipiens pallens</i>	5	0.12%	Satellite	11%	Sporadic

Molecular analyses: A total of 10 barcode sequences (6 COI and 4 16SrRNA) were obtained from the collected samples. Sequences were submitted to

GenBank with GB Accession numbers and BOLD (Table 4). Four sequences with GenBank accession number no. OM630674, OQ603495, OM748750 and OM749567 were downloaded from NCBI to compare with our generated sequences.

Table 3: Mahalanobis and Procrustes distances among *Cx. quinquefasciatus*, *Cx. pipiens* and *Cx. pipiens pallens*. Canonical Variate Analysis (CVA) showing variation among groups scaled by the inverse of the within group variation. Global test against the null hypothesis of no differences among group means (Permutation tests= 10000 permutation iterations)

Species	Mahalanobis distances		Procrustes distances		CVA(P-value <.0001)	
	<i>Cx. pipiens pallens</i>	<i>Cx. pipiens</i>	<i>Cx. pipiens pallens</i>	<i>Cx. pipiens</i>		
Species					Goodall's	7.4938
<i>Cx. pipiens</i>	246.6838		0.0978		Pillai's trace	1.9997
<i>Cx. quinquefasciatus</i>	187.3024	328.2048	0.1158	0.1144	Eigenvalues	15352.70170189

Table 4. K2P distances (%) among different members of *Culex pipiens* complex, genetic divergence (K2P Distance %) within and between species for COI gene with GB Accession number

	<i>Culex quinquefasciatus</i>	<i>Culex pipiens pallens</i>	<i>Culex pipiens f pipiens</i>	K2P Distance (%)	GB number	Accession number 16S rRNA	GB Accession number COI
<i>Culex quinquefasciatus</i>		0.002	0.002	Comparison	Mean	OQ780798 OQ780801	OQ780807 OQ780803
<i>Culex pipiens pallens</i>	0.002		0.001	Intraspecies	0.06± 0.01	OQ780799	OQ780803
<i>Culex pipiens f pipiens</i>	0.003	0.001		Interspecies	0.32± 0.24	OQ780800	OQ780804 OQ780805 OQ780806

The lengths of all the Cytochrome c oxidase I (COI) gene barcode sequences ranged from 501 to 609 bp, with an average of 534 bp. No stop codons, insertions, or deletions were observed in any sequence. The nucleotide analysis showed the average nucleotide frequencies to be A: 28.86%±0.19%, T: 39.88%±0.08%, G: 15.89%± 0.15%, and C: 15.37%±0.09%. The AT content (68.74%) was higher than that of GC (31.26%). The average percentages of GC contents at the first, second, and third codon positions were 44.43%±0.30%, 43.30%±0.17%, and 6.01%±0.16%, respectively. The average genetic distances within and between species, were 0.06 ± 0.01, and 0.32± 0.24, (Table 4) respectively. The phylogenetic topography based on the neighbor-joining (NJ) method was constructed for the seven COI sequences (Figure 8). The barcode gap was analyzed based on a comparison between the mean and max-intra-specific variation.

The lengths of the generated 16S rRNA sequences ranged from 483 bp to 546 bp, with an average of 510 bp. The 16S sequences of *Cx. pipiens pallens* and *Cx. pipiens f pipiens* were submitted for the first time into the GenBank. Average nucleotide composition was found to be 38.75%±0.24% (T), 9.03%±0.35% (C), 36.65%±0.19% (A), and 15.57%±0.28% (G). The percentage of GC (24.60%±0.25%) was lower than the AT (75.40%). A Neighbor-Joining (NJ) tree was also constructed (Figure 8). No other sequences were used downloading from NCBI to compare. Only our generated sequences were used for analysis because of unavailability of 16S sequences for *Cx. pipiens pallens* and *Cx. pipiens f pipiens* in GenBank.

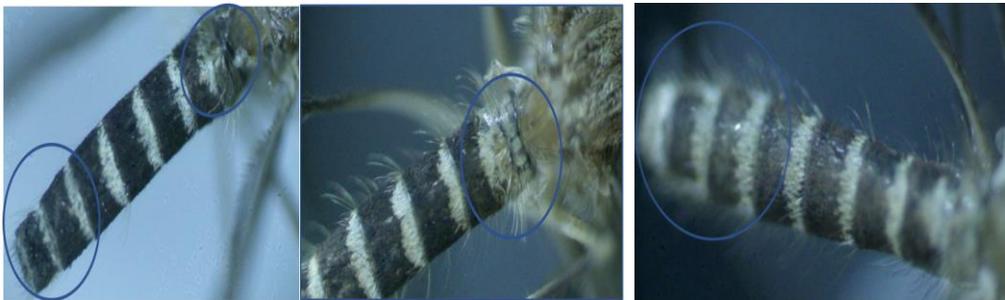


Fig 1. *Cx. quinquefasciatus* *Culex pipiens* *Culex pipiens pallens*

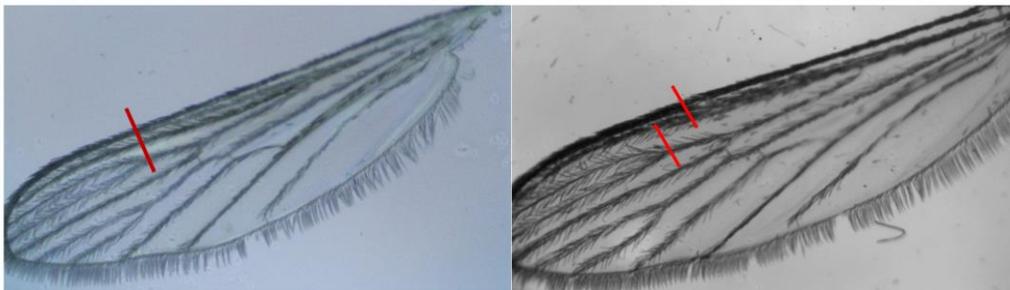


Fig 2. *Cx. pipiens* and *pipiens pallens*: subcosta intersects costa at or beyond level of furcation of R2+3

Fig 3. *Cx. quinquefasciatus*: subcosta intersects costa before level of furcation of R2+3

An intraspecific distance value of 0 indicates a complete genetic similarity between two populations, indicating minimal or no genetic diversity. Conversely, a value of 1 indicates significant genetic differentiation between the populations, reflecting maximum genetic diversity (assuming no mutations) and thus called separate species. In this study, mean intra-specific variation (K2P) was 0.06 (range 0.00-0.18), whereas mean inter-specific divergence were several-fold (5.33) higher at 0.32 (0.28-0.38) (Table 4), indicates a supporting barcoding gap.

These results show full support for separate species status of *Cx. quinquefasciatus* and *Cx. pipiens* and different stance of *Cx. pipiens pallens*. According to Ruiz-Lopez *et al.* (2012) the average genetic differences within intraspecific mosquito species measured by Kimura two-parameter (K2P) distance varying from 0.2-1.4% and a mean interspecific variation range from 2-5.6%. However, Laurito *et al.* (2013) observed that the K2P divergence within the *pipiens* lineage varied from 0-3%, which matches the findings of this study (Table 4). According to him, both *Cx. quinquefasciatus* sequences were misidentified as *Cx. pipiens*, and the *Cx. pipiens* sequences were misidentified as *Cx. quinquefasciatus* when applying the best close match (BCM) criterion.

Members of Culex pipiens complex: a recently introduced or a pre-existing species?: Since its initial recording by Barraud in 1923, *Cx. quinquefasciatus* has remained the sole species documented in this country (Ahmed 1987, Irish *et al.* 2016). This fact gave rise to numerous hypothetical scenarios that could have

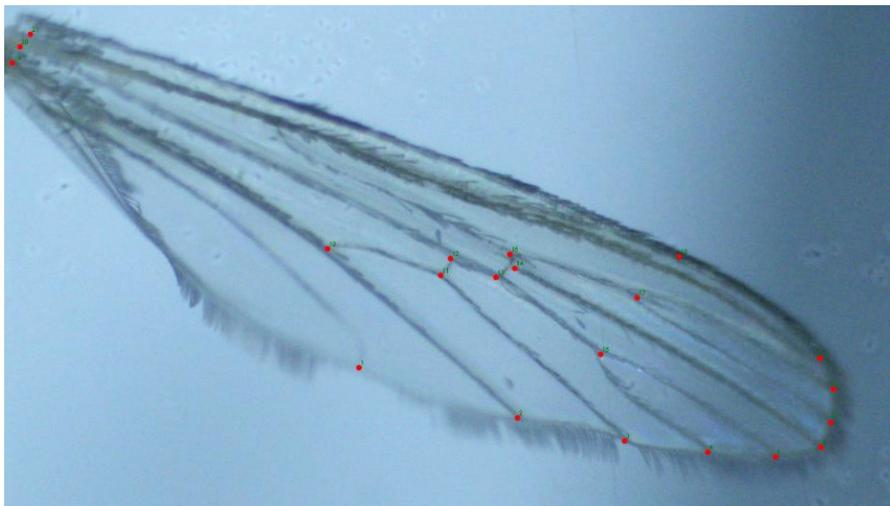


Fig 4. Eighteen landmarks on the wings created in TpsDig

unfolded over time. For instance, it is uncertain whether other members, specifically *Cx. pipiens*, have been present in the country all along. This raises questions about the accuracy of the identification of *Cx. quinquefasciatus*. Additionally, if *Cx. pipiens pallens* is indeed a hybrid of *Cx. quinquefasciatus* and *Cx. pipiens* (Harbach 2012) rather than a subspecies, its presence in this study suggests the existence of *Cx. pipiens* within the country, hence the species was not identified morphologically prior to this study. In this context, the vector status of *Cx. quinquefasciatus* also becomes questionable. This is because all the newly identified members are capable of carrying the same disease pathogens as *Cx. quinquefasciatus*, regardless of their geographical location. Therefore, further investigations are required.

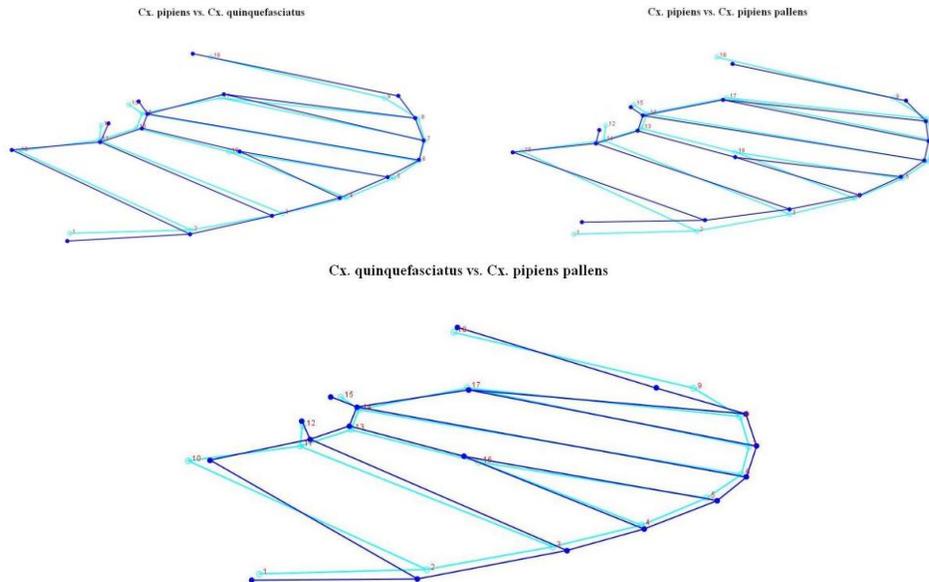


Fig 5. Thin-plate splines wireframe graphs showing the shape variation among the members of the *Cx. pipiens* complex.

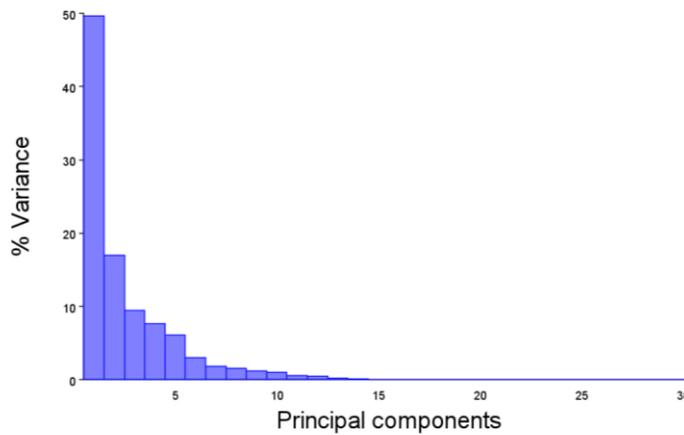


Fig 6. Eigen factor: how much of the overall phenotypic variation in the data set is explained by the each direction. First principal component explains almost 50% of all of the variations among all of the observations. The second axes is almost 20% and so on. All the variations are statistically independent to one another or orthogonal.

Filaria free Bangladesh: how sustainable it is?: In a recent news released by the WHO in May 2023, Bangladesh was declared as the fourth country in South-East Asia after Maldives, Sri Lanka and Thailand to successfully eliminate lymphatic filariasis. Lymphatic filariasis, a neglected tropical disease also known as elephantiasis, causes significant morbidity and has a major economic impact

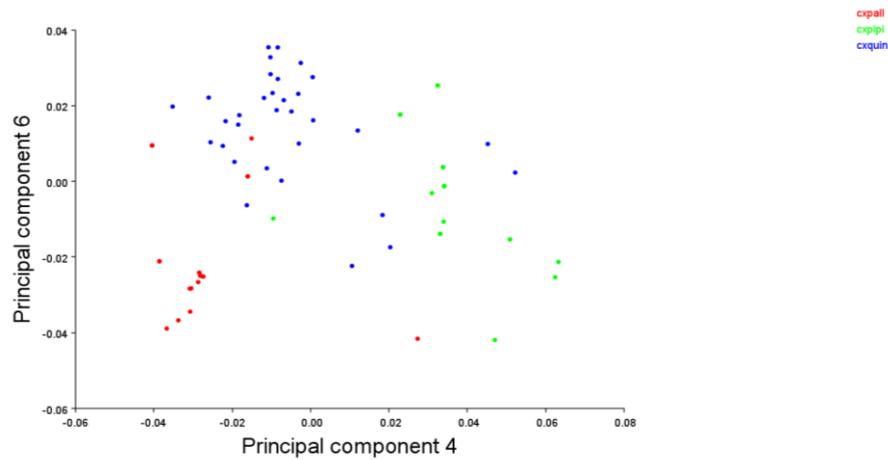


Fig 7. Morpho space between the members of *Cx. pipiens* complex produced by the second and third canonical variates based on 18 wing landmarks.

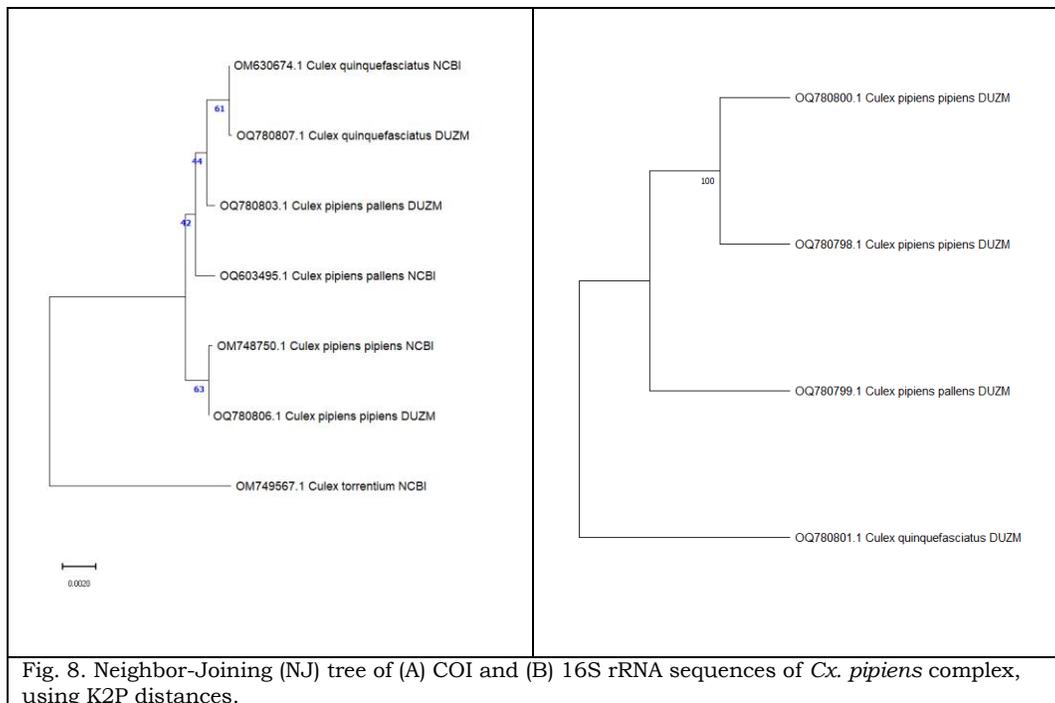


Fig. 8. Neighbor-Joining (NJ) tree of (A) COI and (B) 16S rRNA sequences of *Cx. pipiens* complex, using K2P distances.

on affected communities. In Bangladesh, 19 out of 64 districts were endemic for the filarial parasite, with reported cases from Mirpur area, Dhaka as well (Ahmed *et al.* 1986). *Cx. quinquefasciatus*, the only recorded species in Bangladesh from the *Cx. pipiens* species complex, serves as the primary vector for lymphatic filariasis (*Wuchereria bancrofti*) in the country to date (Ahmed *et al.*

2004) except for a few cases of *Brugia malayi* (Wolfe & AslamKhan 1971). However, the newly found *Cx. pipiens* and *Cx. pipiens pallens* in this study are also the principal vectors of lymphatic filariasis globally. Therefore, their presence poses a new threat to the elimination and post-validation surveillance strategy considering the slow and insidious nature of the disease.

CONCLUSION

This study documented three new country records for Bangladesh, including a species, a sub species and a biotype: *Cx. pipiens*, *Cx. pipiens pallens* and *Cx. pipiens f pipiens* respectively. However, it is important to note that further genetic investigations are necessary to validate the inclusion of these species within the *pipiens* complex and to ascertain the presence of any additional members in the species complex. Furthermore, it should be acknowledged that the study area, limited to a small section of the capital, is not representative of the entire country or a specific geographic region. Since all the members of the *Cx. pipiens* complex are vectors of filariasis, the identification of three new vectors in this study poses new challenges for the filaria free Bangladesh. Therefore, reconsidering the continuous entomological and epidemiological research on *Filaria* is crucial. With numerous factors acting as impediments to the elimination process, such as, the involvement of multiple parasitic vectors with their intricate life cycles and the longest incubation period compared to other parasites, emerging drug resistance in the parasites, the presence of asymptomatic carriers, reliance on mosquitoes as intermediate hosts, the influence of socio-economic factors on patient treatment, and the impact of global travel and tourism, achieving the “Filaria free Bangladesh” title may be a temporary accomplishment.

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