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Morphological and molecular characterization of the ecological, biological and behavioural variants of the JE vector *Culex tritaeniorhynchus:* An assessment of its taxonomic status

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ABSTRACT

Background & objectives: Culex tritaeniorhynchus (Diptera: Culicidae), an important vector of Japanese encephalitis belongs to the *Culex vishnui* subgroup which includes two other vector species namely, *Cx. vishnui* and *Cx. pseudovishnui.* Many varieties and types of *Cx. tritaeniorhynchus* have been reported, besides populations that exhibit behavioural and biological differences. This study was undertaken to find out whether *Cx. tritaeniorhynchus* populations exhibiting behavioural and biological variations, and those from different geographical areas, are comprised of more than one taxon or belong to a single taxon.

Methods: Morphological characterization was done by examining 153 morphological and morphometric characters in the larval (75), pupal (60) and adult stages (18) of five geographical populations of *Cx. tritaeniorhynchus*. Molecular characterization was done by PCR amplification of mitochondrial cytochrome c oxidase (COI) gene sequences (DNA barcodes) and another hypervariable genetic marker, the ribosomal DNA (16S). One-way ANOVA, principal component analysis (PCA) and discriminant factor analysis (DFA) were done for statistical analyses using the statistical package SPSS IBM version 19.0.

Results: Morphological characterization showed that no intraspecific differentiation can be made among the five geographical populations of *Cx. tritaeniorhynchus*. Molecular characterization done by DNA barcoding also showed that the COI sequences of all the five populations of *Cx. tritaeniorhynchus* grouped into a single taxonomic clade plus the genetic differentiation among these was non-significant and the overall gene flow among the populations was very high. Analysis of the ribosomal DNA also confirmed that the *Cx. tritaeniorhynchus* populations belonged to a single taxon.

Interpretation & conclusion: Culex tritaeniorhynchus is a taxon that does not involve cryptic species.

Key words *Culex tritaeniorhynchus*; *Culex vishnui* subgroup; DNA barcode; morphology; taxonomic status

INTRODUCTION

Japanese encephalitis (JE) is a major public health problem in Southeast Asia. The mosquito vectors involved in the transmission of this viral infection mainly belong to the *Culex vishnui* subgroup which includes the three important species, namely *Cx. tritaeniorhynchus* Giles, 1901, *Cx. vishnui* Theobald, 1901 and *Cx. pseudovishnui* Colless, 1957 (Diptera: Culicidae)[1]. Larvae of these species are known to occur in fresh water habitats such as paddy fields, ground pools, ponds, wells and ditches while adults prefer to rest outdoors[2]. However, in India, *Cx. tritaeniorhynchus* was reported to rest predominantly indoors in Bellary district of Karnataka state[3]. Yet another report from Mysore indicated that larvae of *Cx. tritaeniorhynchus* inhabiting ground pools varied in some of their morphological characters from those inhabiting paddy fields[4]. Such behavioural variations have been indicative of the presence of genetically isolated populations of a taxon which have been subsequently identified as sibling species in the case of anophelines such as *Anopheles fluviatilis* and *An. subpictus* Grassi, 1899 (Diptera: Culicidae). These observations reported in the case of *Cx. tritaeniorhynchus* added a new dimension to the already complex nature of the members of the subgroup whose identification as adults also remains difficult. These circumstances warranted a morphological and molecular characterization of the geographically and ecologically variant populations of the important JE vector *Cx. tritaeniorhynchus* of this subgroup.

MATERIAL & METHODS

Study area

Geographical locations in Bellary district, Mysore and Mandya districts in Karnataka, where biological and behavioural variations have been reported in *Cx. tritaeniorhynchus* formed the primary study sites. JE prone areas such as Warangal and Karim Nagar districts

in Telangana state (erstwhile Andhra Pradesh state) and areas of repeated epidemic outbreaks of JE such as Gorakhpur district of Uttar Pradesh state were the other study sites. All these study sites are known to be erstwhile JE epidemic prone areas. In addition, samples from West Bengal, Tamil Nadu and Assam were also included for molecular characterization.

Two villages within the Bellary Developmental Block, namely Rupanagudi (15º 03.209' N, 77º 03.738' E) and Moka (15º 14.027' N, 77º 03.622' E) were selected for mosquito collections because of the reported behavioural variation of resting indoors (endophilism) in *Cx. tritaeniorhynchus*[3]. Mysore (12º 18.841' N, 76º 37.740' E) and Mandya (12º 30.359' N, 76º 50.024' E) districts are the sites where biological variation in the form of differences in the morphological structures of larvae of *Cx. tritaeniorhynchus* has been reported[4]. Chalapalli (18º 15.061' N, 79º 27.106' E)and Rajapalli (18º 13.744' N, 79º 26.073' E) villages of Chelpur Primary Health Centre (PHC), Uppalpalli village (18º 10.774' N, 79º 28.535' E) of Uppal PHC and Medipalli village (18º 03.242' N, 79º 27.135' E) of Hasanparthy PHC were the mosquito collection sites in Warangal and Karimnagar districts. Mosquito collection sites in Gorakhpur were Mahesra (26º 49.305' N, 83º 21.243' E), Rampurkhud (26º 50.350' N, 83º 26.183' E), Sekurwa (26º 49.006' N, 83º 20.834' E) (Chargawan PHC), Karimnagar (Nagar Nigam) (26º 48.364' N, 83º 23.042' E), Kusmitakia (26º 44.868' N, 83º 20.834' E), Shivpur (26º 39.918' N, 83º 29.899' E) (Pipraich PHC) and Rudrapur (26º 39.269' N, 83º 15.009' E) (Khajani PHC).

Collection of mosquitoes

Field work was carried out during February to November 2009 in study localities of Bellary, Mysore and Mandya districts for collection of mosquitoes of the *Culex vishnui* subgroup. Adult collections from both indoor and outdoor resting sites were made in the morning between 0900 and 1000 hrs and in the evening between 1700 and 1800 hrs using an oral aspirator and torch light. In Moka and Rupanagudi villages of Bellary district, apart from exclusive human dwellings and cattlesheds, most of the houses had the cattle together with the inmates sharing the dwelling, which were considered as mixed dwellings. One man hour collection of indoor resting mosquitoes was done in each of these types. Collection of outdoor resting mosquitoes was done in the paddy fields, in the vegetation along irrigation channels, hay stacks in the open, and cotton fields using an oral aspirator. Larval collection was done in the villages, from the paddy fields, irrigation channels and ditches by using a dipper.

Mosquito collection in Mysore and Mandya was mainly focused on larval collections because morphological variations have been reported in larvae of JE vector from these areas. Larval collection was done in Mysore City from Kukarhalli tank area. The vast area adjacent to the tank was a marsh grassy region with large puddles of water stagnation that formed the larval habitats. Another location of larval collection in the city was the Vidyaranyapuram farm where fodder grass is cultivated. Larval collection outside the city area was done in the paddy fields, irrigation channels, ditches and ponds in both Mandya and Mysore. Indoor and outdoor resting collection of adults was made in Chikpalya village in Mandya.

Adult and larval collections of *Cx. vishnui* subgroup mosquitoes were made in Warangal and Karimnagar districts of Andhra Pradesh during August 2010 and in Gorakhpur district of Uttar Pradesh during September 2010, since peak densities of *Cx. tritaeniorhynchus* have been reported during the respective months in these study sites. Adults were collected from both indoor and outdoor resting habitats and larval collections were done in paddy fields, ground pools and irrigation channels. Voucher specimens of mosquitoes collected from all the study areas have been deposited in the mosquito museum at the Vector Control Research Centre, Puducherry[5–6].

Morphological characterization

Specimens obtained from different study areas formed five different geographical populations, *viz.* (i) Bellary, (ii) Mysore City, (iii) Mysore outskirts and Mandya, (iv) Warangal and Karimnagar, and (v) Gorakhpur. Morphological characterization was based on the study of larvae, pupae, adult females and males of *Cx. tritaeniorhynchus* of all the five populations. Majority of the specimens examined were those obtained through associated rearing, so that the adult could be linked to its larval and pupal skins.

Larva: There are about 186 pairs of seta found in mosquito larva besides the comb scales, pecten and anal papillae, of which around 48 pairs are commonly used in different keys for identification of species. In this study, 75 characters including length, width and ratios were used, for which 197 IV instar larvae and larval skins were examined.

Pupa: About 118 pairs of seta are present in the mosquito pupa, of which around 53 pairs of seta, besides the trumpet and paddle are commonly used in keys for species identification. In this study, 60 characters which included the number, length, width and ratios were examined in 130 specimens.

Female: In the case of adult females, six characters, namely colour of erect scales on vertex, anterior 0.7 of mesonotum (dark or pale brown), mesonotum scales (fine or coarse), accessory pale scales on the ventral side of proboscis (present or absent), plume scales on wing (narrow or broad), and dark scaling in hind femur were examined in 1821 specimens. Since, the cibarial characters are generally used in differentiating closely related species of genus *Culex*, the cibarial armature was dissected and mounted for 29 specimens and five cibarial characters, namely cibarial width and length of larger dorsal papillae, number of smaller dorsal papillae, length and number of cibarial teeth were also examined.

Male: In the case of adult males, seven characters, namely length of proboscis in relation to palpal segment 5, dorsal apical pale band on palpal segment 2, dorsal median pale band on palpal segment 3, ventrolateral tufts on palpal segment 3, setae on ventral surface of proboscis, distal setae (d-f) of subapical lobe and number of finger like processes on phallosome were examined in 385 specimens.

Molecular characterization

Cytochrome c oxidase 1 gene: Altogether, 143 specimens of *Cx. tritaeniorhynchus* collected from different parts of the country (Karnataka, Kerala, Andhra Pradesh, West Bengal, Tamil Nadu, Assam and Uttar Pradesh) were subjected to PCR amplification of mitochondrial cytochrome c oxidase (COI) gene sequences (DNA barcodes), a genetic marker established to be species specific, in order to delineate whether any cryptic species existed among *Cx. triteaniorhynchus*. This included 135 specimens from the study areas (Bellary 36, Mysore City 22, Mysore outskirts 25, Warangal 29 and Gorakhpur 23), besides eight specimens of *Cx. tritaeniorhynchus* collected in Kerala, West Bengal, Tamil Nadu and Assam. Also eight specimens of *Cx. pseudovishnui* and six specimens of *Cx. vishnui* which are the other two species of the *Cx. vishnui* subgroup, collected from the study areas were included in the analysis.

About 600 bp of the mitochondrial *cytochrome* c *oxidase* gene was PCR amplified following the protocol described by Kumar *et al*[7]. Total DNA was extracted from 3 legs of the specimens using Genelute Mammalian DNA Miniprep kit (Sigma-Aldrich, U.S.A). The extracted DNA was dissolved in 30 µl of deionized water. 5' COI region of mitochondrial DNA was amplified in Eppendorff Mastercycler Gradient S (Germany) using following cycling conditions. The 50 µl reaction was set up using Qiagen Taq PCR Core kit (Qiagen, Germany). The reaction components included 1.5 U of *Thermus aquaticus* DNA Polymerase, 5 µl of 10× PCR Buffer, 2.5 mM Magnesium chloride, 10 µl Q solution, 1 µl of dNTP mixture and 1 µl of 10 pmol of forward and reverse DNA primers 5'-GGATTTGGAAATTGATTAGTTCCTT-3' and 5'-AAAAATTTTAATTCCAGTTGGAACAGC-3' along with 2 µl of template DNA[7]. The reaction parameters were as follows: Initial denaturation of 5 min (95°C) followed by 5 cycles of 94°C for 40 sec (denaturation), 45°C for 1 min (annealing), and 72°C for 1 min (extension) and 35 cycles of 94°C for 40 sec (denaturation), 51°C for 1 min (annealing), 72°C for 1 min (extension), and a final extension at 72°C for 10 min. The amplified fragments were run on a 1% agarose gel to check the integrity of the fragments and the PCR product was purified by QIAGENGmbH PCR purification kit. The purified products were eluted to 20 µl of deionized water, and a portion of it was lyophilized in a Speed Vac concentrator (Thermo Electron Corporation, Waltham, MA) followed by custom sequencing. Both reads (from forward primer as well as reverse primer) were done, and the sequences were analyzed as follows. The DNA sequences were subjected to alignment using ClustalW. Sequence divergences among individuals were quantified by using the Kimura two-parameter (K2P) distance model[8]. A neighbour joining (NJ) tree of K2P distances was constructed to provide a graphic representation of the clustering pattern among different species[9-10]. These analyses of the sequences were conducted using MEGA version 3.1 software[11].

Ribosomal DNA

Another hypervariable genetic marker, the ribosomal DNA (16S) was analysed to find out whether the populations of *Cx. tritaeniorhynchus* from the five different study areas exhibit genetic variation. Altogether, 55 specimens of *Cx. tritaeniorhynchus* collected from the five study areas were subjected to rDNA analysis. The extracted DNA from the specimens was subjected to PCR using the following conditions. The DNA forward and reverse primers used were 5'- CGCCTGTTTATCAAAAACAT-3' and 5'-CTCCGGTTTGAACTCAGATC-3' which amplify about 500 bp of hypervariable ribosomal DNA[12]. The 50 µl reactions set up included 5 µl of 10× Buffer, 10 µl of Q solution, 2 µl of $MgCl_2$, 1 µl dNTP mixture of 10 mM each, 1.5 U of Taq polymerase, 0.2 pmol of forward and reverse primers and 2 µl of template DNA (Taq PCR core kit, Qiagen, Germany). The reaction was carried out in the Eppendorff Mastercycler Gradient S and the PCR conditions were as follows. Initial denaturation of 4 min at 95°C followed by 35 cycles of 50 sec denaturation step of 94°C; annealing of 1 min at 55°C and the extension of

1 min at 72°C. The final elongation step provided was 72°C for 7 min.

Statistical analysis

Data on different morphological characters examined were subjected to one-way ANOVA for assessing the differences among the populations of *Cx. tritaeniorhynchus* and post-hoc test was used to make pair-wise comparison of each characteristic using Bonferroni method. Principal component analysis (PCA) was done to derive small number of factors, of many observed characteristics. Number of factors were considered based on eigen value of >1. Discriminant factor analysis (DFA) was done to find the variables, which were most useful for discriminating between individuals of different populations. In discriminant factor analysis, Pillai's trace statistics were performed to determine whether the clusters were significantly different from each other. Within-class covariance, matrices were assumed to be equal and prior probabilities were taken into account while performing the discriminant analysis. For all statistical tests $p < 0.05$ was considered statistical significance. All these analyses were performed using statistical package for social sciences, SPSS IBM version 19.0.

For molecular characterization, the PCR reactions were ran on a 1.5% agarose gel. The amplified fragments were purified using Qia quick PCR purification kit (Qiagen, Germany) and sent for custom sequencing. The DNA sequences were aligned in ClustalW software and were subjected to NJ phylogenetic analysis in MEGA 4.0 software[11]. The DNA sequence polymorphism was analyzed in DNASP 5.0[13].

RESULTS

In Bellary, all the three species of the *Cx. vishnui* subgroup, *Cx. tritaeniorhynchus*, *Cx. pseudovishnui* and *Cx. vishnui* were collected and *Cx. tritaeniorhynchus* was found to be the predominant species. Among the indoor resting habitats, resting density of *Cx. tritaeniorhynchus* was in the order of cattleshed > mixed dwelling > human dwelling, with per man hour densities of 315, 273.43, and 41.11, respectively. The overall indoor resting density of this species in Bellary was high (202.15 per man hour) compared to the outdoor resting density (17 per man hour). The density of *Cx. pseudovishnui* and *Cx. vishnui* was < 1 per man hour in all the resting habitats. In the larval collections done in Bellary, only *Cx. tritaeniorhynchus* was obtained. In larval collections made in Mysore and Mandya, larvae of *Cx. tritaeniorhynchus, Cx. vishnui,* and *Cx. pseudovishnui* were obtained. A total of 4048 adult specimens were obtained as study material. Of these, 387 specimens were collected as adults and 342 adults emerged from larval collections were mounted on minuten while the others were preserved in pill boxes. From the larval collections done, 928 larvae, larval skins and pupal skins were mounted on slides.

In Warangal and Gorakhpur, *Cx. tritaeniorhynchus*, *Cx. pseudovishnui* and *Cx. vishnui* were obtained in adult collections, with *Cx. tritaeniorhynchus* being the predominant species in both the areas. The per man hour density (PMD) of resting *Cx. tritaeniorhynchus* in Warangal was 30.22 in human dwellings, 88.67 in cattlesheds and 417.8 in outdoors and in Gorakhpur it was 28, 36.62 and 318.02 respectively. In both the areas, high density of the species was found in outdoor habitats, indicating the exophilic behaviour. Density of *Cx. pseudovishnui* and *Cx. vishnui* was very low in both the areas, being < 1 per man hour in human dwellings and cattlesheds. In outdoor habitats, PMD of *Cx. pseudovishnui* was 1.05 and 1.39 while that of *Cx. vishnui* was 0.52 and 2.31 in Warangal and Gorakhpur respectively. In Warangal, larvae of *Cx. tritaeniorhynchus* were obtained mostly from paddy fields while in Gorakhpur it was from the ground pools and vacant plots. Very high density (250 per dip) of larvae of *Cx. tritaeniorhynchus* was observed in a vacant plot in Karimnagar village of Gorakhpur where JE case had been recorded. A total of 830 adults of *Cx. tritaeniorhynchus* obtained from different collection sites in Warangal and 860 adults from different collection sites in Gorakhpur were mounted on minuten, while the rest were preserved in pill boxes. From larval collections made, 200 and 260 larvae, larval skins and pupal skins from Warangal and Gorakhpur were mounted on slides.

Morphological characterization

Larva: Of the 75 morphological characters examined in larvae, 66 characters, excluding those which were similar in all the populations, were considered for morphological analysis of the five populations based on one-way ANOVA. Among the 66 characters, only 35 were significantly different in the one-way ANOVA (Table 1). The pair-wise comparison of morphological characters using the Bonferroni test revealed that characters such as seta 1-A branch, 1-A length, antenna length, antenna width at middle, antennal index, head/antenna width at point of attachment of 1-A ratio, 5-C branch, 5-C length, 6-C length, 7-C length, 7-C branch, 9-C branch, 13-C branch, 6-I length, 7-I length, 1-III length, 1-IV length, 1-V length, 1-VI length, 6-IV length, 6-VI length, number of comb teeth, number of denticles in the apical pecten teeth, siphon length, siphon width at middle, siphon width at

Table 1. Morphological analysis of larvae of *Culex tritaeniorhynchus* of different geographical populations using one-way ANOVA test

Character	Bellary Mean (SD)	Mysore City Mean (SD)	Mysore outskirts Mean (SD)	Warangal Mean (SD)	Gorakhpur Mean (SD)	F	*p*-value <0.05
Seta 1-A branch	28.1 (3.16)	27.7 (2.13)	28.1 (2.58)	28.4 (1.97)	26.4 (1.94)	2.778	0.029
Seta 1-A length	0.22 (0.02)	0.22 (0.02)	0.25 (0.08)	0.23 (0.02)	0.21 (0.02)	4.947	0.001
Antenna length	0.56 (0.03)	0.56 (0.03)	0.56 (0.03)	0.54 (0.02)	0.53 (0.02)	8.887	0.0
Antenna width	0.07 (0.01)	0.07 (0.01)	0.08 (0.01)	0.08 (0.00)	0.08 (0.01)	4.469	0.002
Antenna index	7.87 (1.46)	7.83 (0.87)	7.56 (0.88)	6.82 (0.38)	6.99 (0.77)	8.436	0.0
Seta 1-A width	0.05 (0.0)	0.05 (0.0)	0.05 (0.0)	0.06 (0.0)	0.05 (0.0)	10.417	0.0
Head ratio	14.7 (1.12)	14.4 (1.28)	14.2 (1.20)	12.6 (0.94)	13.7 (1.24)	9.736	0.0
Seta 5-C branch	3.24 (0.48)	3.40 (0.45)	3.20 (0.42)	3.34 (0.54)	3.08 (0.37)	2.924	0.022
Seta 5-C length	0.49 (0.04)	0.51 (0.04)	0.52 (0.05)	0.46 (0.03)	0.47 (0.03)	13.219	0.0
Seta 6-C length	0.56 (0.04)	0.59 (0.04)	0.59 (0.05)	0.52 (0.03)	0.53 (0.03)	19.459	0.0
Seta 7-C branch	7.83 (0.98)	8.20 (1.08)	7.62 (1.05)	8.52 (1.01)	8.20 (0.97)	3.771	0.006
Seta 7-C length	0.51 (0.03)	0.53 (0.03)	0.53 (0.04)	0.50 (0.03)	0.49 (0.02)	10.205	0.0
Seta 13-C branch	2.97 (0.67)	3.03 (0.30)	2.99 (0.31)	3.04 (0.27)	3.31 (0.51)	2.929	0.022
Seta 13-T branch	8.12 (1.24)	8.32 (1.63)	7.91 (1.41)	8.98 (1.40)	8.83 (1.71)	2.922	0.022
Seta 6-I length	0.76 (0.06)	0.78 (0.05)	0.76 (0.06)	0.74 (0.04)	0.73 (0.05)	4.289	0.002
Seta 7-I length	0.57 (0.04)	0.59 (0.04)	0.56 (0.04)	0.53 (0.04)	0.55 (0.04)	13.191	0.0
Seta 1-III length	0.24 (0.03)	0.23 (0.02)	0.23 (0.04)	0.24 (0.02)	0.21 (0.03)	4.296	0.004
Seta 1-IV length	0.30 (0.03)	0.31 (0.02)	0.30 (0.03)	0.30 (0.03)	0.26 (0.03)	11.458	0.0
Seta 1-V length	0.35 (0.04)	0.38 (0.02)	0.34 (0.04)	0.35 (0.03)	0.33 (0.03)	4.473	0.003
Seta 1-VI length	0.41 (0.04)	0.44 (0.03)	0.39 (0.04)	0.40 (0.03)	0.37 (0.05)	6.137	0.0
Seta 6-IV length	0.48 (0.04)	0.49 (0.06)	0.49 (0.06)	0.46 (0.03)	0.46 (0.04)	3.021	0.019
Seta 6-V length	0.60 (0.04)	0.59 (0.06)	0.58 (0.06)	0.56 (0.05)	0.56 (0.04)	3.649	0.007
Seta 6-VI length	0.67 (0.05)	0.68 (0.07)	0.67 (0.06)	0.64 (0.05)	0.65 (0.04)	2.585	0.039
Comb scales	35.7 (3.29)	36.6 (4.34)	33.7 (4.20)	34.8 (3.27)	34.0 (2.12)	4.043	0.004
No. of denticles in the distal pecten teeth	7.88 (1.09)	7.70 (1.45)	7.94 (1.31)	6.98 (1.0)	6.73 (0.99)	8.58	0.0
Siphon length	1.79 (0.19)	1.81 (0.19)	1.81 (0.17)	1.71 (0.14)	1.66 (0.13)	5.724	0.0
Siphon width at base	0.29 (0.02)	0.28 (0.02)	0.29 (0.02)	0.29 (0.01)	0.29 (0.02)	3.341	0.011
Siphon width at middle	0.19 (0.03)	0.18 (0.02)	0.19 (0.02)	0.19 (0.01)	0.20 (0.02)	3.394	0.010
Siphon index at base	6.28 (0.82)	6.57 (0.61)	6.37 (0.67)	5.97 (0.59)	5.83 (0.75)	7.512	0.0
Siphon index at middle	9.53 (1.97)	9.94 (1.51)	9.39 (1.38)	8.84 (1.03)	8.41 (1.33)	6.232	0.0
Saddle length	0.33 (0.02)	0.32 (0.03)	0.33 (0.02)	0.32 (0.01)	0.33 (0.01)	5.431	0.0
Saddle width	0.38 (0.02)	0.35 (0.03)	0.36 (0.07)	0.36 (0.07)	0.37 (0.02)	2.510	0.043
Siphon/Saddle ratio	5.38 (0.48)	5.73 (0.46)	5.44 (0.49)	5.39 (0.43)	5.04 (0.39)	12.196	0.0
Anal papillae length	0.26 (0.06)	0.26 (0.05)	0.33 (0.08)	0.31 (0.04)	0.36 (0.05)	10.135	0.0
Anal papillae width	0.06 (0.02)	0.06 (0.01)	0.06 (0.02)	0.07 (0.01)	0.07 (0.02)	3.356	0.015

F—Fishers's test statistics based on one-way ANOVA.

base, siphon index at base, siphon index at middle, saddle length, saddle width, siphon/saddle ratio and anal papillae index were shorter in length and number of branches were lesser in Gorakhpur and Warangal populations than in Mysore City, Mysore outskirts and Bellary populations. However, between Gorakhpur and Warangal populations, the Warangal population had longer/higher number of branches.

Multivariate analysis of the larval populations

Principal component analysis (PCA) of the morphological analysis extracted five factors with eigen-values of more than one. The Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy was 0.566. Cumulatively, these factors explained 73.03% of the total variability in the data. The first factor (F1) explained 19.57% of the total variability, while the second factor (F2) explained 11.28%. Similarly, the third factor (F3) and fourth factor (F4) explained 6.05% and 5.13% of the total variability, respectively. Altogether, the first four factors explained 42.02% of the total variability. Characters such as seta 6-V length, 6-IV length, 6-III length; 6-VI branch, 6-I length, 5-C length, antennal length, 6-C length, 7-I length and saddle width showed high magnitude on F1. Siphon index at base, siphon/saddle ratio, siphon index at middle, siphon length, siphon width at base, and siphon width at middle showed high magnitude on F2. Seta 13-T branch, number of denticles on apical pecten teeth, number of pecten teeth, seta

9-C branch, and seta 7-C branch showed high magnitude on F3. Antenna width and antennal index showed high magnitude on F4. DFA could distinguish five significant clusters for Mysore City, Mysore outskirts, Bellary, Warangal and Gorakhpur populations (Pillai's trace = 0.923, F = 1.57, p <0.012). Characters that showed high factor loading on F1 included length of setae 6-I, 6-III, 6-IV, 6-V, 5-C, 6-C, 7-I, antenna width and saddle width, while characters that showed high factor loading on F2 included only siphon length, siphon index at middle, siphon index at base, and siphon/saddle ratio.

Analysis based on variables related to length and indices

Principal component analysis was also done by considering only the variables related to length and indices. The KMO measure of sampling adequacy was 0.672. Cumulatively, these factors explained 76.16% of the total variability in the data. The first factor (F1) explained 30.31% of the total variability, while the second factor (F2) explained 16.96% of the total variability; together the first two factors explained 47.27% of the total variability. Characters such as length of seta 6-III, 6-IV, 6-V, 6-I, 7-I, antenna length, saddle width, and siphon width at base showed high magnitude on F1. Siphon index at base, siphon/saddle ratio, siphon index at middle, and siphon length showed high magnitude on F2. The analysis showed that increase in length is not specific to siphonal characters alone but also for other body setae.

Analysis based on variables related to number

Principal component analysis by considering only the variables related to number of branches of setae was also performed. The KMO measure of sampling adequacy was 0.526. Cumulatively, these factors explained 68.21% of the total variability in the data. The first factor (F1) explained 13.71% of the total variability, while the second factor (F2) explained 8.81% of the total variability, the third factor (F3) explained 7.67%, the fourth factor (F4) explained 6.91% and fifth factor (F5) explained 6.17%; together the first five factors explained 43.27% of the total variability. Characters such as number of branches of seta 13-T, seta 8-C, 9-C, number of pecten teeth and number of denticles on apical pecten teeth showed high magnitude on F1. Branch number on seta 1-IV, III, and V showed high magnitude on F2. Branch number of seta 13-C and 10-C showed high magnitude on F3. Branch number of seta 6-I, and 7-I showed high magnitude on F4. Branch number of seta 1-VI and 7-C showed high magnitude on F5. It showed there was no significant difference among the five populations in relation to the number of branches of the various setae.

Larval habitat-wise analysis

Besides, analysis of the morphological characters of different geographical populations, analysis was done based on larvae obtained from different larval habitats. The mean values of the characters considered for morphological analysis showed significant difference among the seven habitats, namely ground pools, irrigation canals, paddy fields, rock pools, seepages, sewage and swamps, based on one-way ANOVA. Among the 66 characters, only 18 were found to be significantly different (Table 2). The pair-wise comparison of morphological characters using Bonferroni test revealed that characters such as antenna length, siphon length, siphon index at base, siphon index at middle, and siphon/saddle ratio were shorter in ground pool than irrigation canals, paddy fields, rock pools, seepages, sewage and swamps; length of seta 5-C and 7-C shorter in ground pools, irrigation canals and paddy fields than rock pools, seepages, swamps and sewages; length of seta 6-C shorter in ground pools and paddy fields than irrigation canals, seepages, sewage and swamps; number of comb scales higher in sewage and swamps than ground pools, irrigation canals and paddy fields.

Pupa

Of the 60 morphological characters examined in pupae, 42 characters, excluding those which were similar in all the populations, were considered for morphological analysis of the five populations based on one-way ANOVA. Among the 42 characters only 25 were significantly different in the one-way ANOVA (Table 3). The pair-wise comparison of morphological characters using Bonferroni test showed that trumpet length of Gorakhpur and Mysore City populations was similar and Mysore outskirts and Warangal populations was similar while Bellary population had trumpet length greater than all other populations. Trumpet width of Bellary and Warangal populations was similar while other populations significantly differed from each other. Seta 5-CT branch number of Gorakhpur and Mysore outskirts populations was similar while other populations significantly differed from each other. Paddle width of Bellary and Mysore outskirts populations was similar while other populations significantly differed from each other. Seta 9-VIII branch number of Mysore City and Mysore outskirts populations was similar while other populations significantly differed from each other. Seta 5-VII branch number of Gorakhpur and Bellary populations was similar while other populations significantly differed from each other.

Mysore City population differed significantly from Warangal population with respect to branch number of

Table 2. Morphological analysis of larvae of *Culex tritaeniorhynchus* from different larval habitats using one-way ANOVA test

Character	Ground pool Mean (SD)	Irrigation canal Mean (SD)	Paddy field Mean (SD)	Rock pool Mean (SD)	Seepage Mean (SD)	Sewage Mean (SD)	Swamp Mean (SD)	F	*p*-value <0.05
Antenna length	0.53 (0.02)	0.55 (0.03)	0.55 (0.02)	0.58 (0.02)	0.57 (0.02)	0.54 (0.01)	0.56 (0.03)	7.68	0.0
Antenna width	0.08 (0.007)	0.07 (0.008)	0.08 (0.008)	0.08 (0.004)	0.07 (0.007)	0.08 (0.006)	0.07 (0.008)	3.16	0.01
Antenna index	6.79 (0.63)	7.58 (0.80)	7.36 (1.26)	7.44 (0.34)	7.94 (0.81)	6.76 (0.43)	7.92 (0.83)	5.15	0.0
Seta 5-C length	0.47 (0.03)	0.49 (0.05)	0.49 (0.04)	0.53 (0.03)	0.52 (0.04)	0.50 (0.03)	0.52 (0.04)	5.73	0.0
Seta 6-C length	0.53 (0.03)	0.56 (0.05)	0.54 (0.04)	0.56 (0.03)	0.58 (0.04)	0.58 (0.04)	0.59 (0.04)	10.10	0.0
Seta 7-C length	0.49 (0.02)	0.50 (0.04)	0.51 (0.03)	0.54 (0.03)	0.53 (0.04)	0.52 (0.04)	0.53 (0.03)	7.45	0.0
Seta 13-C branch	3.36 (0.53)	3.1 (0.92)	2.95 (0.34)	3.08 (0.58)	3.03 (0.21)	3.20 (0.44)	3.02 (0.29)	2.69	0.02
Seta 6-I length	0.74 (0.04)	0.77 (0.07)	0.75 (0.05)	0.75 (0.05)	0.76 (0.06)	0.73 (0.05)	0.78 (0.05)	2.95	0.01
Seta 7-I branch	1.92 (0.23)	2	2	2	2	2.10 (0.22)	2.01 (0.07)	3.52	0.0
Seta 7-I length	0.54 (0.04)	0.57 (0.05)	0.55 (0.04)	0.57 (0.04)	0.57 (0.03)	0.56 (0.02)	0.59 (0.04)	7.28	0.0
Comb teeth	33.48 (2.03)	34.72 (3.16)	34.71 (3.72)	35.07 (2.30)	35.54 (3.61)	38.80 (3.33)	36.47 (4.43)	2.76	0.01
Siphon length	1.62 (0.14)	1.82 (0.22)	1.77 (0.15)	1.77 (0.2)	1.78 (0.17)	1.88 (0.18)	1.81 (0.19)	4.34	0.0
Siphon width at base	0.29 (0.02)	0.30 (0.02)	0.28 (0.02)	0.27 (0.01)	0.28 (0.01)	0.28 (0.02)	0.28 (0.02)	4.69	0.0
Siphon width at middle	0.21 (0.02)	0.20 (0.02)	0.19 (0.02)	0.19 (0.02)	0.19 (0.02)	0.21 (0.008)	0.18 (0.02)	5.64	0.0
Siphon index at base	5.57 (0.68)	6.17 (0.86)	6.23 (0.67)	6.55 (0.92)	6.43 (0.62)	6.75 (0.34)	6.55 (0.63)	7.06	0.0
Siphon index at middle	7.96 (0.99)	9.04 (1.88)	9.39 (1.60)	9.58 (1.71)	9.55 (1.08)	9.04 (0.75)	9.99 (1.54)	5.65	0.0
Saddle length	0.33 (0.01)	0.34 (0.03)	0.33 (0.02)	0.34 (0.03)	0.33 (0.02)	0.31 (0.03)	0.32 (0.03)	2.76	0.0
Siphon/Saddle ratio	4.91 (0.35	5.43 (0.52)	5.43 (0.43)	5.15 (0.32)	5.45 (0.48)	6.10 (0.24)	5.69 (0.46)	11.28	0.0

F—Fishers's test statistics based on one-way ANOVA.

Table 3. Morphological analysis of pupae of *Culex tritaeniorhynchus* of different geographical populations using one-way ANOVA test

Character	Bellary Mean (SD)	Gorakhpur Mean (SD)	Mysore City Mean (SD)	Mysore outskirts Mean (SD)	Warangal Mean (SD)	F	*p*-value <0.05
Trumpet length	0.60 (0.047)	0.56 (0.03)	0.56 (0.04)	0.59 (0.07)	0.59 (0.05)	4.76	0.001
Trumpet width	0.12 (0.01)	0.13 (0.01)	0.10 (0.01)	0.11 (0.01)	0.12 (0.01)	12.12	0.0
Trumpet ratio	5.14 (0.41)	4.63 (0.46)	5.41 (0.59)	5.44 (0.78)	4.86 (0.39)	7.46	0.0
Seta 5-CT branch	4.86 (0.82)	4.45 (0.57)	4.57 (0.72)	4.43 (0.49)	5.25 (0.81)	4.84	0.001
Sect 6-CT branch	3.43 (0.71)	3.77 (0.79)	2.98 (0.61)	3.17 (0.68)	3.35 (0.72)	4.04	0.004
Seta 10-CT branch	7.21 (2.20)	7.38 (1.30)	6.13 (1.40)	6.42 (1.73)	8.09 (2.09)	4.89	0.001
Seta 12-CT branch	3.79 (0.56)	3.45 (0.50)	3.47 (0.53)	3.67 (0.73)	3.94 (0.58)	2.67	0.035
Seta 1-II branch	21.05 (4.96)	19.55 (6.97)	17.64 (4.48)	19.39 (3.95)	20.11 (3.44)	2.47	0.048
Seta 1-III branch	10.67 (1.96)	10.42 (2.49)	9.49 (1.85)	10.57 (1.77)	11.44 (2.08)	3.88	0.005
Seta 1-IV branch	8.69 (1.48)	9.45 (2.23)	8.07 (1.46)	8.52 (1.58)	9.32 (1.80)	2.94	0.023
Seta 1-V branch	7.75 (0.87)	8.00 (1.58)	7.22 (1.25)	7.31 (1.04)	8.69 (1.06)	6.13	0.0
Seta 1-VI branch	6.70 (0.67)	6.59 (1.30)	6.16 (1.12)	6.19 (0.97)	7.06 (0.95)	3.37	0.012
Seta 1-VII branch	4.65 (0.61)	4.67 (0.72)	4.38 (0.59)	4.25 (0.49)	5.03 (0.66)	5.75	0.0
Seta 5-IV branch	5.61 (0.94)	5.35 (0.58)	4.96 (0.84)	4.82 (0.52)	5.37 (0.91)	3.84	0.006
Seta 5-V branch	2.10 (0.41)	2 (0.0)	1.95 (0.18)	2 (0.15)	2.22 (0.43)	3.75	0.007
Seta 6-V branch	5.34 (0.53)	5.83 (0.81)	4.97 (0.67)	5.32 (0.78)	6.08 (0.73)	10.21	0.0
Seta 6-VI branch	5.45 (0.79)	5.88 (0.64)	5.15 (0.66)	5.26 (0.85)	6.03 (0.81)	5.76	0.0
Seta 7-III branch	5.72 (0.81)	6.33 (0.29)	5.05 (1.07)	5.07 (0.90)	5.53 (0.90)	3.12	0.018
Seta 9-VIII branch	6.95 (0.76)	6.64 (0.80)	6.44 (0.99)	6.45 (1.32)	7.60 (1.50)	3.55	0.01
Seta 4-VIII branch	2.61 (0.46)	2.63 (0.61)	2.33 (0.46)	2.52 (0.54)	2.71 (0.45)	2.89	0.025
Paddle width	0.49 (0.05)	0.51 (0.04)	0.45 (0.04)	0.49 (0.05)	0.50 (0.07)	5.7	0.0
Paddle ratio	1.46 (0.16)	1.49 (0.12)	1.60 (0.13)	1.48 (0.12)	1.45 (0.11)	7.73	0.0
Seta 5-VII branch	1.95 (0.21)	1.95 (0.15)	1.73 (0.42)	1.81 (0.36)	2.03 (0.26)	3.54	0.009
Seta 8-VI branch	3.82 (0.87)	4.25 (0.58)	3.40 (0.92)	3.83 (0.80)	4.66 (0.69)	8.61	0.0
Seta 8-VII branch	4.02 (0.75)	4.71 (1.25)	3.52 (0.69)	3.83 (0.70)	4.44 (0.66)	8.5	0.0

F—Fishers's test statistics based on one-way ANOVA.

seta 12-CT, seta 1-III, seta 1-IV, seta1-VI, seta 5-CT, seta 4-VIII, and seta 5-VII. In characters such as trumpet width, branch number of seta 5-CT, seta 10-CT, seta 1-V, seta 1-VI, seta 6-V, seta 6-VI, seta 9-VIII, and seta 8-VI, both Mysore City and Mysore outskirts populations were significantly different from Warangal population. With respect to trumpet width, branch number of seta 6-CT, seta 6-V, seta 8-VI, seta 8-VII, and paddle width, Mysore City population was significantly different from Gorakhpur population. Paddle ratio of Mysore City population was significantly different from all other populations. With respect to 22 characters related to number of branches,

Mysore City, Mysore outskirts and Bellary populations were similar having lesser number than the Gorakhpur and Warangal populations, but between the Gorakhpur and Warangal populations, the Gorakhpur population had lesser number.

Multivariate analysis of the pupal populations

Principal component analysis of the morphological analysis extracted five factors with eigen-values of more than one. The KMO measure of Sampling Adequacy was 0.662. Cumulatively, these factors explained 67.18% of the total variability in the data. The first factor (F1) explained 31.64% of the total variability, while the second factor (F2) explained 11.74% of the total variability; together the first two factors explained 43.38% of the total variability. Characters such as branch number of seta 1-V, seta 1-VI, seta 1-IV, and seta 1-III showed high magnitude on F1. Trumpet ratio and trumpet width showed high magnitude on F2. F1 was significantly different in all five populations, whereas F2 was different in all five populations except the trumpet width, which was similar in Bellary and Warangal populations. DFA could distinguish five significant clusters for Mysore City, Mysore outskirts, Bellary, Warangal and Gorakhpur populations (Pillai's trace = 0.447, F = 2.37, p <0.001). Characters that showed high factor loading on F1 included branch number of seta 1-V, 1-VI, 1-IV, 1-VII, and 1-III, while characters that showed high factor loading on F2 included trumpet width and trumpet ratio.

Female

Morphological characters examined in adult females did not show any variation between the different populations. For most of these characters including that of the accessory pale scales on the ventral side of proboscis, over 90% of the specimens of Mysore City, Mysore outskirts, Bellary, Warangal and Gorakhpur populations were similar. For characters related to the cibarial armature, one-way ANOVA showed that only the cibarial width significantly (p <0.05) differed among the populations.

Male

All the morphological characters examined in adult males including the length of proboscis in relation to palpal segment 5, and genital characters of distal setae (d-f) of subapical lobe, and number of finger like process on phallosome, were similar in the Mysore City, Mysore outskirts, Bellary, Warangal and Gorakhpur populations.

Molecular characterization

Cytochrome c oxidase 1 gene: The phylogenetic tree generated along with an outgroup is provided (Fig. 1). In the analysis it was found that all the COI sequences (143) of *Cx. tritaeniorhynchus* grouped into a single taxonomic clade. Also, the other two species included in the study branched as separate two taxonomic clusters. The statistical significance of this branching pattern was found to be highly significant (the interior Branch test with 1000 replications showed the statistical significance of the clades to be 99% for *Cx. tritaeniorhynchus*; *Cx. pseudovishnui* and *Cx. vishnui*) indicating these to be three distinct species. The K2P genetic distance (d) recorded for these three species were found to be negligible (0.0073; 0.0080 and 0.0088 respectively) indicating the specimens included under each category to be of single taxonomic units. The interspecific 'd' was found to be 0.0768 between *Cx. tritaeniorhynchus* and *Cx. pseudovishnui*; 0.0586 between *Cx. tritaeniorhynchus* and *Cx. vishnui*. The interspecific genetic distance between *Cx. pseudovishnui* and *Cx. vishnui* was recorded to be 0.0591. These interspecific values also denote these three taxonomic clades to be three distinct species categories. Besides, the study clearly indicated the utility of DNA barcode approach to distinguish these three morphologically similar species, which are important vectors of Japanese encephalitis.

Also, the five different populations of *Cx. tritaeniorhynchus* from Bellary, Mysore City, Mysore outskirts, Warangal and Gorakhpur were subjected to phylogenetic analysis to understand the genetic variability in geographically isolated populations. The K2P genetic distance (d) values between the populations were found to be negligible. The genetic differentiation estimated among these five populations (Fst = 0.00500) was found to be non-significant (χ^2 = 347.92; p = 0.6103) and the overall gene flow (Nm) among the populations computed was found to be very high (99.51). The maximum genetic differentiation was recorded among Mysore outskirts and Gorakhpur populations (Fst = 0.03996), which also recorded the least gene flow (Nm = 12.01). However, this highest genetic differentiation value recorded among these populations was also not significant genetically (χ^2 = 34.643; p = 0.5331). The above observations indicate negligible genetic variability among the five populations of *Cx. tritaeniorhynchus*.

Ribosomal DNA (16S) analysis

The K2P genetic distance and mean nucleotide diversity (pi) recorded for 55 specimens analyzed were found to be very negligible (0.0007 and 0.00067 respectively). The phylogenetic tree using NJ analysis of K2P genetic distances is given (Fig. 2). Thus, this hypervariable genetic marker also confirmed that *Cx. tritaeniorhynchus*

Fig. 1: Phylogenetic (Neighbour-joining) analysis of mitochondrial cytochrome C oxidase sequences of *Culex vishnui* subgroup from different study areas; BLR—Bellary; GKP—Gorakhpur; MND—Mysore outskirts; MYS—Mysore City; WGL—Warangal.

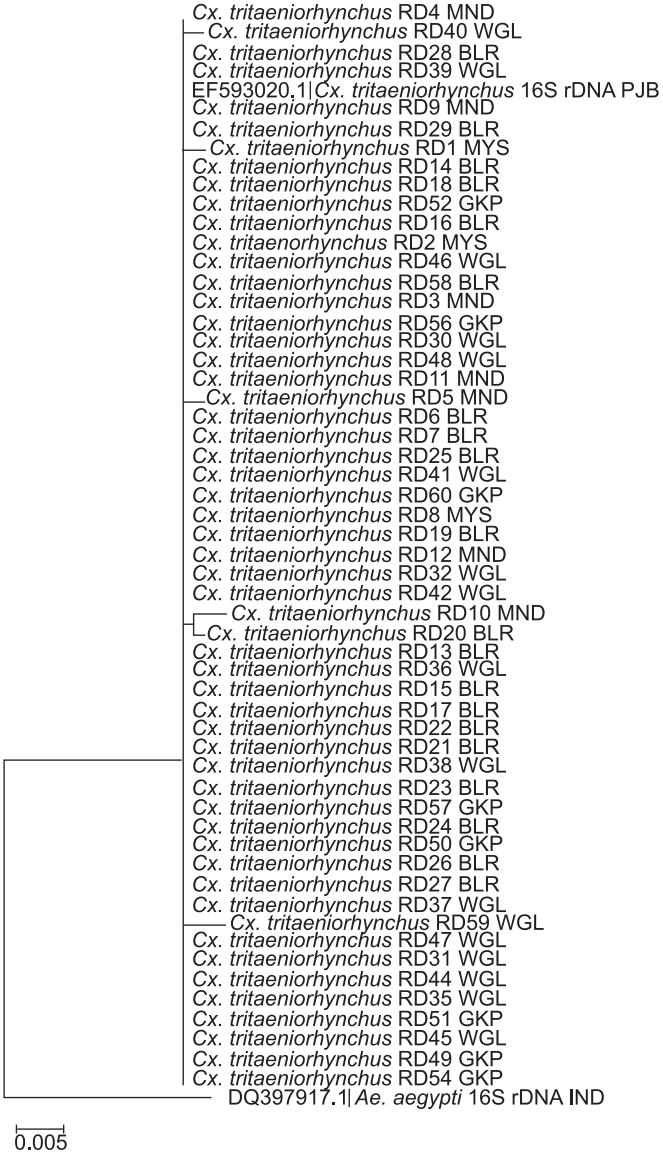

Fig. 2: Phylogenetic (Neighbour-joining) analysis of the hyper variable ribosomal DNA sequences of *Culex tritaeniorhynchus* from different study areas; BLR—Bellary; GKP—Gorakhpur; MND—Mysore outskirts; MYS—Mysore City; WGL—Warangal.

specimens collected and analyzed from different geographical localities in the study belonged to a single taxonomic unit.

DISCUSSION

A total of 153 morphological and morphometric characters in the larval (75), pupal (60) and adult stages (18) of *Cx. tritaeniorhynchus,* were examined in this study to find out whether *Cx. tritaeniorhynchus* populations exhibit behavioural and biological variations, and those from different geographical areas, are comprised of more than one taxon or belong to a single taxon.

ANOVA results indicated that several morphological characters differ significantly among the five populations of *Cx. tritaeniorhynchus* studied. Larvae from Bellary, Mysore City, and Mysore outskirts had setae with lesser number of branches/shorter in length compared to those from Gorakhpur and Warangal. But no relationship could be established in terms of geographical distribution from the southern to northern region because Gorakhpur population had the lesser number/shorter length compared to Warangal population. Principal component analysis (PCA) revealed that among the larval characters, variables related to the body hair accounted significantly for variations among the populations rather than the variables related to the siphon and the saddle. PCA done independently for variables related to number and variables related to length and indices showed that there was no significant difference among the five populations in relation to the number of branches of the various setae, but it differed significantly in relation to length and indices. In larval chaetotaxy, based on which larval description of a species is done, emphasis is given to the branch number of various body setae rather than on the length of the setae, for the reason that the former is considered as fixed characters, while the latter is subject to variability. In the discriminant factor analysis also, the characters that were responsible for differentiating the five populations as five different clusters were those related to length and indices of the setae and not the number of branches. It is, therefore, evident that there is no basis to consider that any of the five populations of *Cx. tritaeniorhynchus* is different from the others.

Earlier reports on morphological variations in *Cx. tritaeniorhynchus* were all based on very few characters examined in the larvae. Significant morphological differentiation in four populations collected from Bellary, Cuddalore, Pune and the Microbial Containment Complex laboratory culture in India, based on siphon, saddle and pecten teeth related variables has been reported[14]. However, all the 11 characters considered by them for analysis were only of length and indices related to siphon, saddle and anal gills. The present study has shown that these variables are not reliable for differentiating the populations. This is also substantiated by the values obtained for the siphon index in the two studies, while the earlier study found the siphon index to be highest in Cuddalore population ranging from 9.42–16 compared to other populations including that of Bellary, this study showed that the siphon index of Bellary population ranged from 6.45–16 indicating that Cuddalore population cannot be differentiated from Bellary population based on the siphon index.

In another study, *Cx. tritaeniorhynchus* populations from Mysore City (ground pools) and outside Mysore (paddy fields) were differentiated as two varieties, based on morphological variations in comb scales, pecten teeth, denticles on the apical pecten tooth, siphon index, anal gills index and the length of basal hair tufts on siphon[4]. Mysore City (ground pools) population was named as variety one having shorter siphon index, shorter anal gills index, less comb scales and longer basal hair tufts on siphon, while outside Mysore (paddy fields) population was named as variety two having longer siphon index, longer anal gills index, more comb scales and shorter basal hair tufts on siphon. Analysis of the same two populations collected by us also showed that ground pool larvae had shorter siphon index, length of siphon shorter, less comb scales and width of siphon at middle greater, and compared to larvae of paddy fields. But larvae collected in swamp (Mysore City) showed longer siphon index, length of siphon longer, and more comb scales compared to larvae in paddy fields indicating that characters related to length can be influenced by the habitats. Further, habitat-wise analysis of the larvae from all the five study areas showed that, of the 18 variables found to be significantly different between the various larval habitats, 16 variables are related to length and indices, which again substantiates that these are not reliable for differentiating the *Cx. tritaeniorhynchus* populations into varieties.

The range values of the different morphological characters of larvae recorded in our study, as well as the earlier studies[4, 14], were compared with that of taxonomic descriptions and larval chaetotaxy tables available for *Cx. tritaeniorhynchus* of Thailand, Oriental region, Japan and Korea and South-western Asia[2, 15-17]. It was found that values obtained in our study to that of the other two earlier studies[4, 14] were all within the range provided by these authors, showing that any conclusion based on variations in the larval characters in considering *Cx. tritaeniorhynchus* as variant populations is not reasonable.

In systematic studies it is important to include all life stages because evidence of relationships cannot always be found readily in the same stage[18–19]. While earlier studies on morphological variations in *Cx. tritaeniorhynchus* have not considered the pupal stage, the present study is the only one which fulfils this requirement, as the pupal stage of *Cx. tritaeniorhynchus* of different geographical populations has been extensively examined for morphological characters. Discriminant function analysis of the characters distinguished five significant clusters for Mysore City, Mysore outskirts, Bellary, Warangal and Gorakhpur populations, the characters contributing to the

variability being the branch number of seta 1-V, 1-VI, 1-IV, 1-VII, and 1-III. But for these characters also, Mysore City, Mysore outskirts and Bellary populations were similar having lesser number than the Gorakhpur and Warangal populations, while between the Gorakhpur and Warangal populations, the Gorakhpur population had lesser number. Further, it was seen that the values obtained for different characters in our study were all within the range provided for pupal chaetotaxy of *Cx. tritaeniorhynchus*[2, 17, 20]. Thus, none of the five populations can be treated as specifically different from the others.

In the adult stage, the females of the different populations of *Cx. tritaeniorhynchus* did not show any variation in the morphological characters studied. More than 90% of the specimens examined were similar for these characters. Among the cibarial armature characters also, many were similar, and though the cibarial width differed significantly among the populations, it was found that Bellary and Warangal populations were similar for this character. Further, it was found that our results of the cibarial characters were comparable to that of the baseline data of the same characters provided for *Cx. tritaeniorhynchus* in Taiwan and the Oriental region[2, 21].

In Thailand, *Cx. tritaeniorhynchus* of the different provinces were categorized into three types based on the number of cibarial teeth, siphon index ratio and mating behaviour[22]. But their results for the three types show overlapping values for these characters. With regard to the cibarial teeth, both type A and type C have < 16 teeth, while the siphon index ratio is shorter, moderate and long and the mating behaviour is moderate stenogamous, non-stenogamous and moderate stenogamous respectively in type A, type B and type C. Their observation that besides the morphotaxonomic studies, other studies such as cytogenetic and biochemical studies and cross mating were also carried out but were not found significantly useful as markers to separate strains, shows that there is no basis to consider different populations of *Cx. tritaeniorhynchus* as types A, B and C.

In the case of males also, none of the characters studied were found to vary between the different populations of *Cx. tritaeniorhynchus*. The male genitalia characters considered to be of importance in taxonomic differentiation also showed no evidence to consider that any of the five populations is different from the others. The intraspecific variations reported in *Culex vishnui* subgroup species included one variant in *Cx. tritaeniorhynchus* which was based on the length of the finger-like processes on the lateral plate of phallosome[23]. Measurement of this character is highly subjective as it could vary with the condition of the mounting of the specimen. It should also be noted that *summorosus*, which was described as a subspecies of *Cx. tritaeniorhynchus* mainly based on this character has been synonymized with *Cx. tritaeniorhynchus* and is now not a valid taxon[24].

The results of the morphological characterization done in the present study by examining a total of 153 characters in the larva, pupa, female and male of *Cx. tritaeniorhynchus* of the Bellary, Mysore City, Mysore outskirts, Warangal and Gorakhpur populations showed that all populations is of the same species and no intra-specific differentiation can be made. This is conclusively proved by the molecular characterization done by DNA barcoding in which it was found that the COI sequences of all the five populations of *Cx. tritaeniorhynchus* grouped into a single taxonomic clade and the genetic differentiation among these five populations was found to be non-significant, and the overall gene flow among the populations was found to be very high. Further, the ribosomal DNA, which is a hypervariable genetic marker, also confirmed that *Cx. tritaeniorhynchus* specimens collected and analyzed from different geographical localities in the study belonged to a single taxonomic unit.

Extensive study of the morphological characters and their analysis done in the present study has made it clear that for a given species one population can differ from another population in few morphological characters, but unless this variation is consistent and is significantly outside the known chaetotaxic range of that character for that species, there is no rationale to categorize the populations into Variety or Type.

The present study has also shown that the endophilic resting behaviour of *Cx. tritaeniorhynchus* in Bellary reported earlier is not a function of genetic variability in the population[3]. Besides, the arid conditions of Bellary to which these authors have attributed the indoor resting, our observations indicated that cohabitation of cattle along with humans in the same dwelling (mixed dwelling) attracts this zoophilic species to the cattle and also provides a suitable humid condition for resting inside the mixed dwellings that constitute the majority of dwellings in the villages of Bellary. This is substantiated by the results of our resting densities which showed that resting density in the mixed dwellings was 6.65 times higher than that in exclusive human dwellings.

The phylogenetic analysis of the three species of the *vishnui* subgroup namely, *Cx. tritaeniorhynchus*, *Cx. pseudovishnui* and *Cx. vishnui* showed that *Cx. pseudovishnui* and *Cx. vishnui* are more closely related. Very low densities of these two species in almost all the study areas may be a reflection of this close

relationship between these species compared to the *Cx. tritaeniorhynchus* which is found to be predominant.

The COI sequences (DNA barcodes) of the three species, *Cx. tritaeniorhynchus*, *Cx. pseudovishnui* and *Cx. vishnui* deposited in the Gene Bank is an additional molecular tool which is now available for identification of these different taxa. While morphological identification of these species can be done with keys already available, the problem faced in identification of rubbed specimens that have lost the scales, can now be resolved with this new molecular tool.

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