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Genetic diversity based on 28S rDNA sequences among populations of *Culex quinquefasciatus* collected at different locations in Tamil Nadu, India

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Abstract. The basis of the present study was to distinguish the existence of any genetic variability among populations of *Culex quinquefasciatus* which would be a valuable tool in the management of mosquito control programmes. In the present study, population of *Cx. quinquefasciatus* collected at different locations in Tamil Nadu were analyzed for their genetic variation based on 28S rDNA D2 region nucleotide sequences. A high degree of genetic polymorphism was detected in the sequences of D2 region of 28S rDNA on the predicted secondary structures in spite of high nucleotide sequence similarity. The findings based on secondary structure using rDNA sequences suggested the existence of a complex genotypic diversity of *Cx. quinquefasciatus* population collected at different locations of Tamil Nadu, India. This complexity in genetic diversity in a single mosquito population collected at different locations is considered an important issue towards their influence and nature of vector potential of these mosquitoes.

INTRODUCTION

Mosquitoes are medically important insects which are known to play a significant role in the transmission of several infectious diseases (Nauen, 2007). Of the 3200 species of mosquitoes described, only few hundred species are known to be vectors that transmit several human and animal diseases (Zavortink, 1990; Harbach & Kitching, 1998). The mosquito *Culex quinquefasciatus* Say (Diptera: Culicidae) is an abundant species in tropical as well as temperate countries. Traditional mosquito taxonomy is based largely on comparisons of morphological features and life histories (Edwards, 1932). The easily recognized morphological characters have greatly facilitated identification at the genus and species level in mosquitoes. These morphological or physical characters may not be informative enough to resolve evolutionary relationships (Shepard *et al.*, 2006). This is also relevant to individual populations of a particular species at different geography or location. Environmental factors and identification of variations at the genetic level are also the most important factors that may influence especially, the propagation of pathogen and transmission of disease (Hill & Crampton, 1994). The application of genetic analyses using molecular biological techniques on mosquitoes thus provides opportunities for the development of new disease control strategies (Hill *et al.*, 2005).

During the last twenty years, DNA analysis has contributed greatly to the knowledge of evolutionary relationship among organisms. The field of molecular systematics which uses various molecular data to infer the phylogenetic relationships among taxa has been rapidly developed and

improved with progress of molecular biological tools (O'Brien *et al.*, 1989; Bernatchez & Danzmann, 1993). Intra-specific population genetic variation in insect vectors assumes great significance in disease transmission. Generally, mosquito vectors have been known to demonstrate genetic variations leading to different strain and sibling species that have been demonstrated in some of the *Culex* species (Cornel *et al.*, 2003; Kent *et al.*, 2007). Biologists such as parasitologists, geneticists and taxonomists have also preferred the use of sequence based molecular markers as tools to resolve phylogenetic problems, because DNA sequencing is the best way to directly estimate genetic variations of specific genes among taxa examined (Behura, 2006; Vijayan *et al.*, 2006; Nespolo *et al.*, 2007). Eukaryotic ribosomal DNA (rDNA) has several properties and was found useful for studying genetic variability and divergence within and between species (Fritz *et al.*, 1994).

In the present investigation, populations of *Cx. quinquefasciatus* that are primarily responsible for elephantiasis was collected from various locations in Tamil Nadu and were analyzed for the first time for their genetic variation based on nucleotide sequence analysis and sequence based secondary structure of 28S rDNA-D2 region.

MATERIALS AND METHODS

The larvae of *Cx. quinquefasciatus* were collected from various locations of Tamil Nadu, South India (Table 1; Figure 1). The larvae of collected mosquitoes from each of the locations were pooled together and reared until adult stage under standard rearing conditions (temperature 27±2°C, relative humidity 80–90% and 12-h light/dark cycle). Pupae were isolated and emerging virgin adults were morphologically identified and used for the isolation of genomic DNA (Sharma *et al.*, 2009).

The genomic DNA was isolated from unfed adult mosquitoes from each location using the method of Coen *et al.* (1982). Qualitative analysis of DNA was carried out on 1% agarose gel for 1h using 1X TAE buffer (40mM Tris–acetate, pH 7.6, 1mM EDTA). Gels were stained with ethidium bromide (Sambrook *et al.*, 1989).

The D2 region of 28S rDNA was amplified from the total genomic DNA by polymerase chain reaction (Schmidt *et al.*, 2006). The forward and reverse primer sequences used were 5'-CGTGTTGCTTGATAGTGCAGC-3' and 5'-TTGGTCCGTGTTTCAAGACGGG-3'. PCR was conducted with a total reaction volume of 25µl consisting of 200nM of each primer, 100 µM of each dNTP mix, PCR buffer

Table 1. Locations, coordinates and codes for *Culex quinquefasciatus* mosquitoes collected at various locations inTamil Nadu, India

Location	Latitude/longitude	Sample code
Kanyakumari	8°04′41″N 77°32′28″E / 8.078°N 77.541°E	KAN
Tirunelveli	8°44′N 77°42′E / 8.73°N 77.7°E	TNV
Tiruchirapalli	10.81°N 78.69°E	TRI
Madurai	9°48′N 78°06′E / 9.8°N 78.10°E	MDU
Villupuram	11°56′31″N 79°29′56″E / 11.942°N 79.499°E	VIL
Chennai	13°5′2″N 80°16′12″E	CHE
Kanchipuram	11°39′N 78°10′E / 11.65°N 78.16°E	KAC
Virudhunagar	9.583°N 77.958°E	VNR
Vellore	12°52′07″N 79°07′08″E / 12.868719°N 79.119000°E	VEL
Salem	11.65°N 78.16°E	SAL

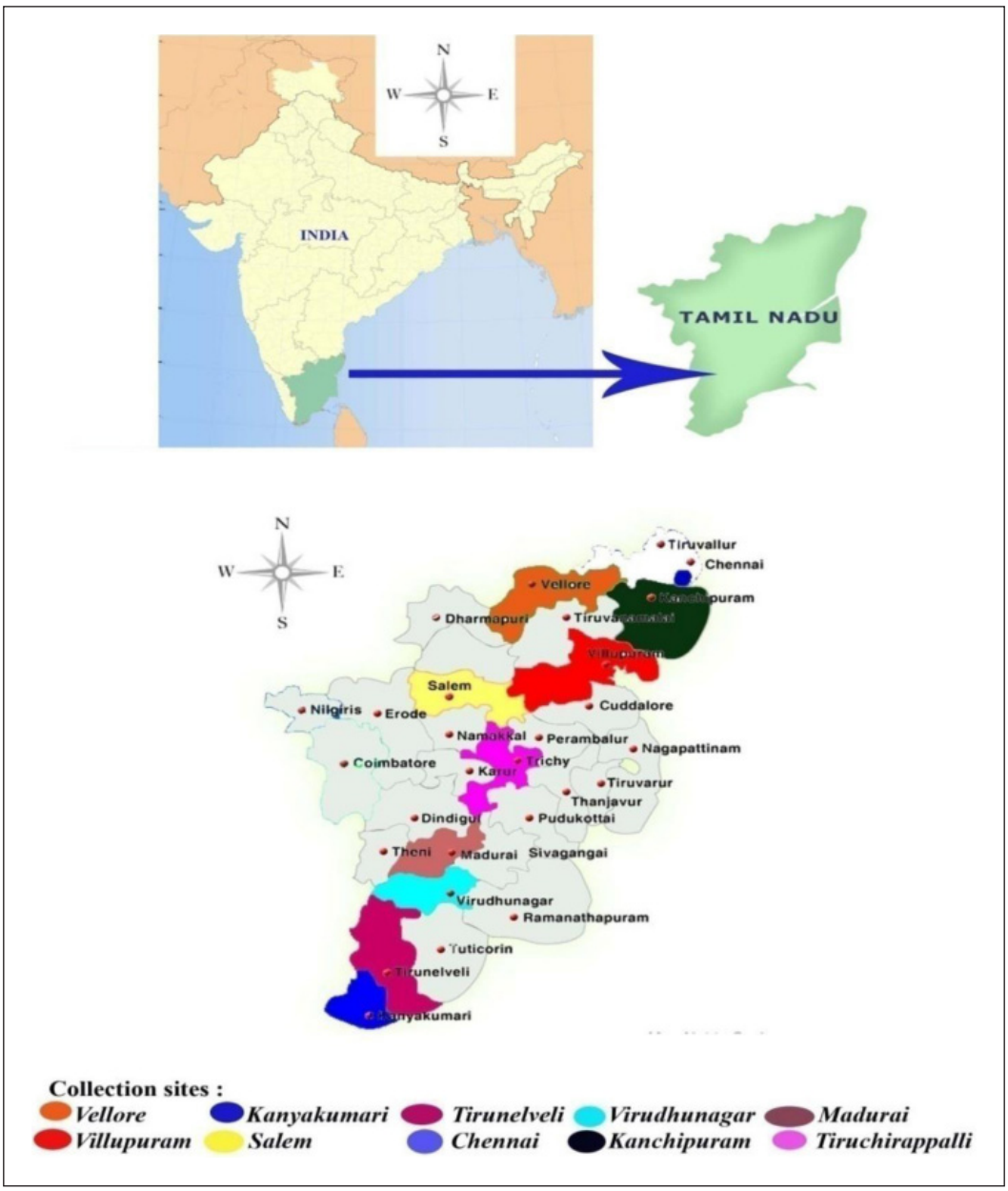

Figure 1. Map showing the locations of collection of *Culex quinquefasciatus* mosquitoes in Tamil Nadu

with 15mM $MgCl_2$ and 0.5 U of *Taq* DNA polymerase with 0.1µg of template DNA. It was mixed well and centrifuged briefly. A control reaction was prepared without template DNA (negative control). The tubes were then placed in a thermal cycler using an amplification profile consisted of first cycle at 94°C for 5 min.; 35 cycles, each cycle with 94°C for 1 min., 56°C for 1 min. 30 sec, and 72°C for 1 min.; and a final extension cycle of 72°C for 7 min. It was then stored at 4°C. To 5µl of the amplified products, 1µl of sample buffer was mixed and the product was checked using 1.2% agarose gel followed by electrophoresis at 50V for 3 h using 1X TAE buffer (Tris–acetate 40mM pH 8.0, EDTA 1mM, pH 8.0).

The amplified fragment was then gel purified and sequenced using the service of Chromous Biotech Private Ltd, Bangalore, India. Percentage of GC content, similarity index and distance matrix of all nucleotide sequences of 28S rRNA-D2 region in the mosquito populations collected at various locations were determined using BioEdit programme (Hall, 1999). The phylgram was constructed based on the multiple sequence alignment carried out using ClustalW (Thompson *et al.*, 1994; Sullivan *et al.*, 2004). The 28S rRNA-D_2 region sequences were manually aligned in the FASTA format. The sequences were then subjected individually to RNAfold core programs and the secondary structure for the individual sequences was predicted (Zuker & Stiegler, 1981).

RESULTS

The amplified 28S rDNA-D2 region from the total genomic DNA of the mosquito species *Cx. quinquefasciatus* collected at various locations in the agarose gel was resolved at 0.5kb region. The size of the nucleotide sequence or the total number of base pairs for the amplified fragment in the mosquito population collected at different places varied between 438-492 bp. The nucleotide sequence of the amplified region for the mosquito species collected at various locations were submitted to NCBI GenBank database and GenBank accession numbers were obtained (Kanyakumari - HM807287; Tirunelveli - HM807288; Tiruchirappalli - HM802160; Madurai - HM802155; Villupuram - HM802156; Chennai - HM802158; Kanchipuram - HM802159; Virudhunagar - HM802154; Vellore - HM802153; Salem - HM802157). The nucleotide frequency distribution on the gene sequences revealed that the distribution of nucleotide adenine (A) varies from 20.20 to 22.97% and that for thymine (T) from 19.03 to 19.73% whereas, for nucleotides guanine and cytosine (G and C), the distributions observed were 27.44 to 29.45% and 30.08 to 30.59% respectively (Table 2). The results clearly deduced the occurrence of higher percentage of guanine and cytosine (GC) content in all the populations of mosquitoes collected at various locations when compared to the content of adenine and thymine (AT).

The 28S rDNA-D2 region specific nucleotide sequences obtained for various populations collected at different locations were subjected to NCBI-BLAST and these sequences were found to match with the available database sequences of *Cx. quinquefasciatus*. The D2 region sequences were analyzed under multiple sequence alignment programme to observe the sequence homology among various populations. The phylogram obtained out of multiple sequence alignment was used to reveal similarities and differences among

Table 2. 28S rDNA sequence information of *Culex quinquefasciatus* mosquitoes collected at various locations in Tamil Nadu, India

Sample code	GenBank accession numbers	A (%)	C (%)	G (%)	T (%)	G+C (%)	A+T (%)	Size of 28S rDNA-D_2 region (bp)
KAN	HM807287	89(20.32)	134(30.59)	129(29.45)	86(19.63)	60.05	39.95	438
TNV	HM807288	105(22.20)	143(30.23)	135(28.54)	90(19.03)	58.77	41.23	473
TRI	HM802160	89(20.32)	134(30.59)	129(29.45)	86(19.63)	60.05	39.95	438
MDU	HM802155	113(22.97)	148(30.08)	135(27.44)	96(19.51)	57.52	42.48	492
VIL	HM802156	109(22.20)	148(30.14)	137(27.90)	97(19.47)	58.04	41.96	491
CHE	HM802158	105(21.78)	146(30.29)	136(28.22)	95(19.71)	58.51	41.49	482
KAC	HM802159	105(21.74)	147(30.43)	136(28.16)	95(19.67)	58.59	41.41	483
VNR	HM802154	105(21.78)	146(30.29)	136(28.22)	95(19.71)	58.51	41.49	482
VEL	HM802153	93(20.85)	135(30.27)	130(29.15)	88(19.73)	59.42	40.58	446
SAL	HM802157	102(22.57)	137(30.31)	127(28.10)	86(19.03)	58.41	41.59	452

Table 3. 28S rDNA sequence homology of *Culex quinquefasciatus* mosquitoes collected at various locations in Tamil Nadu, India

Sample code & accession No.	KAN	TNV	TRI	MDU	VIL	CHE	KAC	VNR	VEL	SAL
KAN (HM807287)	100									
TNV (HM807288)	99.77	100								
TRI (HM802160)	100	99.77	100							
MDU (HM802155)	99.32	99.15	99.32	100						
VIL (HM802156)	100	99.79	100	99.19	100					
CHE (HM802158)	100	99.79	100	99.38	100	100				
KAC (HM802159)	100	99.79	100	99.38	100	100	100			
VNR (HM802154)	100	99.58	100	99.38	100	99.79	99.79	100		
VEL (HM802153)	100	97.98	100	98.65	99.78	98.21	98.21	98.21	100	
SAL (HM802157)	89.73	94.47	89.73	97.12	96.9	96.68	96.9	96.46	88.12	100

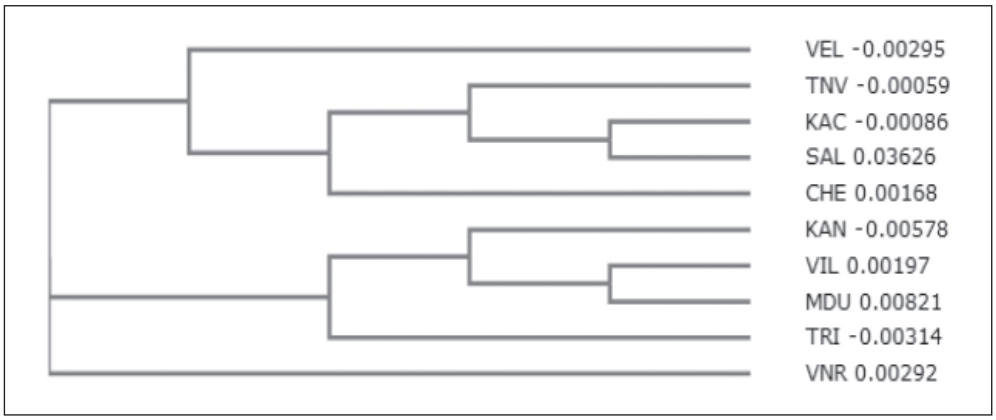

Figure 2. The phylogram showing the genetic relationship among different population of *Culex quinquefasciatus* collected from various locations of Tamil Nadu, India
VEL - Vellore (HM802156), KAN - Kanyakumari (HM807287), TNV - Tirunelveli (HM807288), VNR - Virudhunagar (HM802154), MDU - Madurai (HM802155), VIL - Villupuram (HM802156), SAL - Salem (HM802157), CHE – Chennai (HM802158), KAC - Kanchipuram (HM802159) (KAC) and TRI - Tiruchirappalli (HM802160)

rDNA sequences of mosquitoes collected at different locations. The 28S rDNA-D2 region specific nucleotide sequences of the populations collected at different locations showed 88.12 to 100% in their similarity. Absolute homology was found between mosquito populations of Kanyakumari and Tiruchirappalli and Chennai and Kanchipuram (Table 3). Mosquito populations collected at Salem were found to be very distinct and showed a minimum homology of 88.12% with Vellore population mosquitoes and a maximum homology of 97.12% with Madurai population mosquitoes. The phylogram generated based on the nucleotide sequence data revealed two major clusters of various populations of *Cx. quinquefasciatus* collected at different locations (Figure 2). One major cluster consisted of populations of mosquitoes

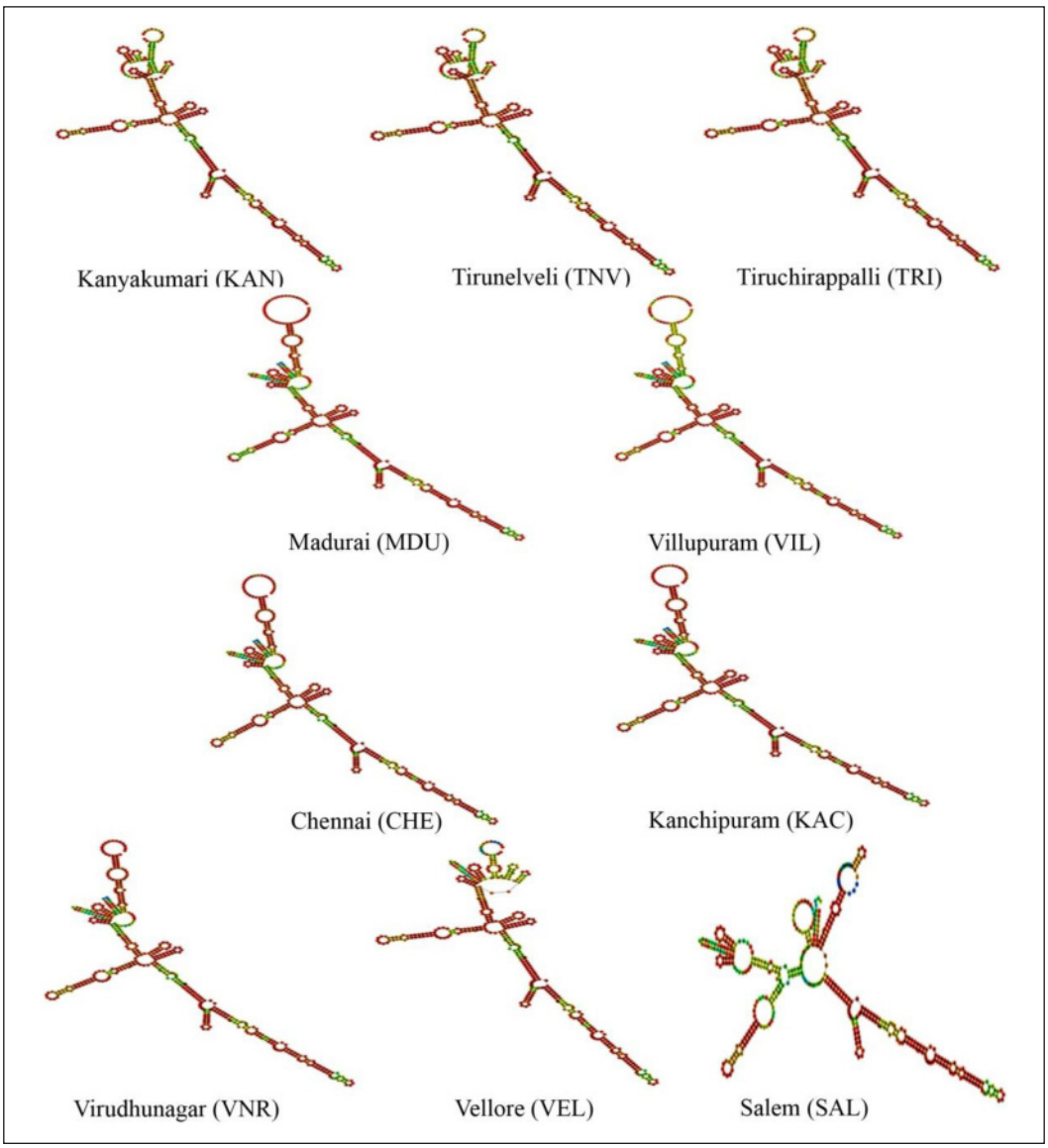

Figure 3. The secondary structure for the D2 region of 28S rDNA among different population of *Culex quinquefasciatus* collected from various locations of Tamil Nadu, India

collected from Vellore, Tirunelveli, Kanchipuram, Salem and Chennai and the other major cluster with remaining mosquito populations collected at Kanyakumari, Villupuram, Madurai, Tiruchirappalli and Virudhunagar.

The secondary structure predicted for the expansion segment D2 of 28S rDNA revealed three major arms for all the mosquito populations collected at different locations in Tamil Nadu. Each arm was characterized by various numbers of stems and loops (Figure 3). An overall structural similarity was found among mosquito populations of Kanyakumari, Tirunelveli and Tiruchirappalli as population-set A; mosquito populations of Madurai and Villupuram as population set-B; and Chennai and Kanchipuram mosquito populations as population set-C for the D2 expansion segment of rDNA. The other populations such as mosquitoes collected at Virudhunagar, Vellore and Salem were found to be dissimilar in the structures of D2 expansion segment (Figure 3).

DISCUSSION

Accurate identification and classification of mosquitoes is necessary for planning effective vector control strategies and for the better understanding of their potential role in disease transmission. A number of reports are available for both morphological and molecular phylogenetic studies with reference to various insect species but such studies are limited with members of mosquitoes (Paduan & Ribolla, 2008). Molecular data was used for the first time to examine relationships among *Culex* mosquitoes (Miller *et al.*, 1996). These authors used sequence divergence in the ITS1 and ITS2 regions of rDNA to infer relationships between 14 species representing four subgenera of *Culex*. It was explored that the generic and subgeneric relationships within Culicini using morphological characters of larvae, pupae and adults (St John, 2007). The studies related to molecular phylogeographies were carried out with reference to *Cx. quinquefasciatus* mosquitoes in the central Bangladesh using mitochondrial COII gene, nuclear elongation factor-1 alpha, acetylcholinesterase-2 and ribosomal internal transcribed spacer-2 (Hasan *et al.*, 2009). Molecular phylogenetic studies using 16S rRNA in *Cx. quinquefasciatus* from different geographical regions based on sequence data was also carried out (Sharma *et al.*, 2010). In the present study 28S rDNA-D2 region specific sequences were used for the molecular phylogenetic study for different populations of *Culex* mosquitoes collected at various locations in Tamil Nadu, South India.

Our present study reported a significantly higher percentage of GC ratios in all the sequences of various populations of mosquitoes when compared to ratios of AT. Accordingly, earlier reports revealed the relationship of GC richness with insertions or deletions of repetitive motifs, probably generated by DNA slippage mechanism (Hancock & Dover, 1988). There was substantial genetic homogeneity as well as heterogeneity among various populations of mosquitoes collected at different locations in Tamil Nadu. Our results on the phylograms based on the 28S rDNA sequence data also supported such kind of heterogeneity among various populations of *Cx. quinquefasciatus* collected at different locations. A number of other such reports towards the study of populations have also shown considerable homogeneity as well as heterogeneity among natural populations of mosquitoes (Komalamisra, 1989; Farid *et al.*, 1991; Sharma *et al.*, 2010).

The consideration of rDNA secondary structures has been advocated in recent times to increase the reliability of rDNA sequence alignment (Buckley *et al.*, 2000; Misof *et al.*, 2006). Our results on the rDNA secondary structural model predicted for the D2 expansion segment from various populations of *Cx. quinquefasciatus* revealed the distinctive populations of Salem, Vellore and Virudhunagar without any structural similarities either in stem or loop structures. Secondary structure prediction in our study was executed in order to make reliable comparison among various populations of mosquitoes collected at different localities for the 28S rDNA-D2 gene region. But, on the contrary, existence of genetic variation was observed among mosquito populations collected at different locations due to dissimilar rDNA secondary structures. The rationale behind this approach is the fact that evolutionary structure conservation surpasses primary sequence conservation. The physiological function of ribosomal RNA in protein biosynthesis is mostly maintained by the molecule's secondary and tertiary structures. Hence, these structural features are the targets of natural selection (evolutionary) whereas the primary sequence may vary, as long as the functional domains are structurally retained. Consequently, structure variation should harbor phylogenetic signal beyond time scales of primary sequence variation. Structure variation in RNA sequences can be an interesting complement to the phylogenetic tool box (Kjer, 1995). In the present study, in some distinct populations of mosquitoes, variable regions were noticed on the stems and loops in the secondary structures of the 28S rDNA-D2 expansion segment using a global structural model alignment tool. It revealed

the structural features as targets of natural selection as reported earlier (Waegele, 2001) that harbor the phylogenetic signal for divergence of the organisms or formation of subspecies.

The results based on rDNA sequences and their secondary structures suggested the existence of a complex genotypic diversity among various populations of *Cx. quinquefasciatus* obtained at different locations in Tamil Nadu, India. The complex genetic nature in a single mosquito population is an important issue in terms of their influence and nature of vector potential.

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