https://doi.org/10.33307/entomon.v46i1.589
Entomon 46(1): 73-80 (2021)
Short Communication No. ent. 46109

Genetic diversity of *Aedes aegypti* (Diptera: Culicidae) in rural and urban settings in Tamil Nadu, India

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ABSTRACT: *Aedes aegypti* populations collected from four different localities in Tamil Nadu, India were analysed using Random Amplified Polymorphic DNA (RAPD) markers to assess the level of genetic variations within and between populations. RAPDs were found to be polymorphic enough to detect genetic polymorphisms at both micro (within the city, <10 km) and macro spatial scales (between three districts, ~500 Km apart). Hetrozygosity within populations varied from 0.1150 ± 0.2140 to 0.3715± 0.1545, but pattern of genetic diversity was not found to be associated with the geographical distance between the populations and the prevalence of dengue infections.

KEY WORDS: Dengue vector, Nei's genetic distance, Shannon's index, polymorphic loci, dendrogram

Dengue is the most widespread and rapidly growing mosquito-borne disease in India, which frequently attains epidemic proportion in several parts of the country (Banik *et al*., 1994, Agarwal *et al*., 1999, Dar *et al*., 1999, Chaturvedi and Nagar, 2008). Dengue is primarily transmitted by *Aedes aegypti*, and it's anthropophilic, endophagic (indoor resting) nature, and the potential to breed in and around thickly populated urban areas enhances its epidemiological and social impact. Understanding biology, population dynamics and population genetics of *Ae. aegypti* are the major areas of research interests for developing effective and novel vector control strategies. The geographical distribution and prevalence of *Ae. aegypti* (Rao, 1967, Reuben, 1970, Das *et al*., 2014, Dev *et al*., 2014, Shriram *et al*., 2018), its potential to carry dengue virus (Kumar *et al*., 2015a, Mukherjee *et al*., 2017), and susceptibility to insecticides (Madhukar and Pillai, 1970, Biswas *et al*., 1988, Montada Dorta *et al*., 1993, Muthusamy and Shivakumar, 2015, Yadav *et al*., 2015) are well studied in India. However, the knowledge on genetic structure and the gene flow across *Ae. aegypti* populations are scanty. Previous genetic diversity studies on *Ae. aegypti* populations in India have revealed high level of genetic differentiation at both micro (Tyagi *et al*., 2017) as well as macro levels (Gokhale *et al*., 2015; Kumar *et al*., 2015b). A preliminary survey of *Ae. aegypti* populations from 31 districts of Tamil Nadu using

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Cytochrome oxidase I (Vadivalagan *et al*., 2016) (750 bp, potentially conserved genetic marker) reported different haplotypes circulating in each district, indicating high genetic diversity in the mosquito populations that needs to be investigated at the finer geographical scales. In this view, a pilot study was conducted to analyze the genetic diversity of *Ae. aegypti* at different spatial scales in Tamil Nadu, India.

A total of 39 *Ae. aegypti* adults were collected during 2008 from four different localities of Tamil Nadu, India; ten samples each from two municipal corporation zones of Chennai city namely; Chennai A (Kodambakkam) and Chennai B (Kolathur), eight from Madurai city and 11 from Thiruppuvanam village, Sivaganga. Since population genetic structure and the gene flow of mosquitoes are influenced by the geographical distance and the other ecological features (anthropogenic and environmental), the study sites were selected based on the prevalence of dengue and their spatial positioning (geographical distance). Chennai is a metropolitan city and hyper endemic for dengue, however, the land use pattern of the two sites within the city is different. While Chennai A is a typical urban area with automobile industries located in the middle of the city with high density of humans) and Chennai B is a peri-domestic area with humans having more open space with gardens around the house and less density in population. Madurai city (urban setting) has sporadic cases of dengue every year and Thiruppuvanam (rural area) is non-endemic for dengue. Spatial distance between the study sites vary from <10 km (Two Chennai city zones), ~50 km (Madurai and Thiruppuvanam) and ~500 km (Chennai and Madurai/ Thiruppuvanam). Sample collection was performed by collecting eggs of *Aedes* mosquitoes by Ovitrap method as described by Silver (2008). The Ovitraps were kept in the residential area for 24 hours and paddle of the ovitraps were air-dried, wrapped in separate polyethylene bags and safely transported to the laboratory. The paddles were checked for the presence of eggs with the dissection microscope. Collected eggs were allowed to emerge in to adult and identified as per the standard keys (Christophers, 1933; Pocock, 1933). Single female mosquito from each egg collection was used for this study for further analysis.

Whole adult mosquito was used for DNA isolation. Individual mosquitoes in the micro-centrifuge tubes were homogenized with lysis buffer (20 mM tris-HCl, pH 8.25, 25 mM EDTA, 25 mM NaCl, 1% SDS) in an indigenously designed homogenizer with the autoclaved glass rod. The suspension was incubated with 100 mg/ml of proteinase K (Boehringer Mannheim, Germany) for one hour at 56 ºC. The DNA was extracted with 100 µl of potassium acetate 3 M, ice incubation for one hour and centrifugation at 8000 xg for 10 min at 4 ºC. The DNA was precipitated by adding two volumes of absolute ethanol containing 0.3 M sodium acetate and placed at - 20 ºC for 30 minutes. The precipitated DNA was centrifuged at 10000 xg for 20 min. and the pellet washed in 70% ethanol. After air drying, the DNA was dissolved in 50 µL tris-EDTA buffer (TE) (1 mM tris-HCl pH 8.0, 1 mM EDTA pH 8.0). Any remaining RNA was eliminated with RNaseH (Boehringer Mannheim, Germany) and the suspension was incubated for one hour at 37 ºC. After extraction with equal volume of chloroform-isoamyl alcohol (24: 1), the aqueous phase was conserved at - 20 ºC. The DNA concentration was estimated spectrophotometrically by reading absorbance at 260 nm and the purity of the sample was examined by electrophoresis with a 0.8% agarose gel in TBE buffer (TBE 0.5x) (0.045 M tris-borate, 0,001 M EDTA) containing ethidium bromide (0.5 mg/ml) with visualization using a UV transilluminator (Vilber lourmet, France).

RAPD markers were used to reveal genetic diversity among *Ae. aegypti* populations. RAPD amplification was performed in a final volume of 25 µl using PCR master mix supplied by Bangalore Genei, India. Negative controls for each assay were run without DNA template to rule out contamination. The PCR amplification was carried out in a Thermal Cycler (MJ Research PTC 100, CA, USA). The following temperature profile was used: denaturation at 94^0 C for 4.00 minutes followed by 45 cycles of 94^0 C for 1.00 minute, 36^0 C for 1.00 minute for primer annealing and 72^0 C

for 2.00 minutes for strand extension. Final extension was allowed for 5.00 minutes at 72^0 C. The amplified products were visualized in 1.2% agarose gel in TBE buffer containing Ethidium Bromide (0.5 mg/ml). The RAPD profile was recorded with a gel documentation system. The presence or absence of each band was scored visually. One Kb DNA ladder (Fermentas Inc., www.fermentas.com) was used as a marker. The molecular weight of each band was estimated by comparing it with the co-migrating 1Kb DNA ladder. Unique fragments were identified and used as diagnostic profile.

Individual bands were scored for presence or absence using binary code (1 or 0, respectively) for each sample to count the level of genetic polymorphisms within and between mosquito populations. For genetic diversity analysis, it is assumed that *Ae. aegypti* populations are in Hardy-Weinberg equilibrium and therefore no selection processes favoring any particular genotype, RAPD markers used in this study segregate in a Mendelian fashion with constant evolution or substitution rate and recessive (band absent) and dominant (band that is present) alleles are identical in state among and between individuals. Genetic polymorphisms were analyzed based on the heterogeneities in the RAPD banding pattern. The extent of genetic similarity within each population was estimated using Nei's gene diversity (Nei, 1978) and Shanon's index. To understand the level of differentiation between populations, Nei's genetic distance was estimated. All the genetic analyses discussed above were performed using PopGene 1.31 software (Yeh and Boylet, 1997). To reveal genetic relationship between and within populations, dendrogram was constructed using Nei's genetic distance using unweighted pair group method with arithmetic averages (UPGMA) clustering strategy. Dendrograms were generated using RAPDPLOT computer programme.

Genetic diversity of four *Ae. aegypti* populations was estimated using RAPD markers. Total three RAPD markers A-3 (AGTCAGCCAC), A-6 (GGTCCCTGAC) and A-12 (TCGGCGATAG) were tested for this study, however only one marker (A-12) was found to be informative with scorable bands amplified in all the mosquito samples. The results of A-12 marker were also found to be reproducible on multiple testing. Therefore, only A-12 was used for further analysis in this study. Total 17 bands were scored from A-12 marker among 39 mosquito samples. The molecular size of the amplified bands varied from 250bp to 6 Kb. A band of ~400 base pair was found common among all the mosquito samples genotyped in the study (Fig. 1). The visual comparison of RAPD profiles of each population revealed variation in the level of genetic diversity among the four *Ae. aegypti* populations. The individuals from Chennai B population showed maximum diversity in banding pattern, whereas majority of the individuals from Chennai A seemed to share the common banding patterns. Chennai B banding pattern looked different from all the other three populations on visual examination of RAPD profiles. Maximum banding patterns were observed in Chennai B (n=8), followed by Madurai A (n=6), Chennai A (n=5) and minimum patterns were observed in Thiruppuvanam (n=3). This observation was also supported by the number of polymorphic bands observed in each population. The maximum number of polymorphic bands (85%) was observed in Chennai B and the lowest was found in Thiruppuvanam (25%).

The genetic diversity of each population was estimated based on the heterogeneity in RAPD banding patterns. The expected heterogeneity estimated as Nei's gene diversity, varied from 0.1150 ± 0.2140 in Thiruppuvanam population to 0.3715 ±0.1545 in Chennai B population. Similar pattern was observed for the Shannon's diversity index, which varied from 0.1630 ± 0.3026 in Thiruppuvanam population to 0.5383 ± 0.2210 in Chennai B. The values of both Nei's Genetic distance and Shannon's index were almost similar for Madurai and Chennai A populations. The total diversity of all the populations was 0.3428 ± 0.1623 with Shannon's index 0.5051 ± 0.2244 (Table 1).

The genetic differentiation between populations was estimated using Nei's genetic distance. Noticeably, maximum genetic distance was observed between Chennai B and Thirupuvanam, while the minimum distance was observed between

Table 1. Genetic analysis of four populations of *Aedes aegypti* from four geographical areas

Areas	No. of samples	Nei's gene diversity	Shannon's index	Polymorphic loci (%)
Chennai- A	10	0.2396 -± 0.2569	0.3360± 0.3598	50.00
Chennai- B	10	0.3715 ± 0.1545	0.5383 ± 0.2210	87.50
Madurai	8	0.2286 ± 0.2443	0.3248 ± 0.3472	50.00
Thiruppuvanam	11	0.1150 ± 0.2140	0.1630 ± 0.3026	25.00
Overall	39	0.3428 ± 0.1623	0.5051 ± 0.2244	87.50

Thiruppuvanam and Chennai A. Chennai B was found to be highly distant from all the populations with Nei's Genetic distance varying from 0.2656 to 0.4146. Other three populations were found genetically closer with genetic distance varying from 0.05444 (Chennai A and Thiruppuvanam) to 0.1024 (Chennai A and Madurai) (Table 2). Since the spatial positioning of each population is different based on geographic distance between them, we estimated the correlation between genetic distance and the geographical distance between populations. There was a negative correlation between population genetic diversity and geographical distance between populations (r = -0.0838, P = 0.8745), however the relation between two parameters was found to be non-significant. The similar pattern of genetic relatedness between populations was also observed based on the dendrogram constructed using Nei's genetic distances (Fig. 2). The dendrogram revealed two clearly distinguishable clusters: (1) Chennai A, Madurai and Thiruppuvanam and (2) Chennai B, showing no effect of geographic origin or dengue endemicity on clustering pattern.

Even a single RAPD marker detected higher level of genetic differentiation within and between populations. The genetic distance between four populations varied from 0.05 to 0.4616. Notably, Chennai B (one of the sampling site within Chennai city) was found highly diverse showing maximum genetic distance from other three populations. The pattern of genetic diversity was found almost similar in Chennai A and Madurai. This might be due to the small sample size and the single genetic marker used for genotyping. The reason for the increased genetic differentiation in the metropolitan city, (Chennai, a hyper-endemic area for dengue) might be due to the selective pressure exerted by the periodic application of insecticides (Larvicide and adulticides) and the availability of a variety of breeding habitats created by the water storing practices of the community. Moreover, the man made changes in the metropolitan area has also been expected to provide breeding habitats for the profuse breeding of *Ae. aegypti*. Similarly, *Ae. aegypti* population collected from Madurai, showed high genetic diversity. Hemme *et al.* (2010) reported the influence of urban landscape in the population dynamics of *Ae. aegypti* in Trinidad. Similar observations were reported in many countries (Mousson *et al*., 2002; Ocampo and Wesson, 2004). Thiruppuvanam on the other hand showed minimum genetic diversity with only three banding patterns that too identical to the ones

Table. 2. Nei's genetic distance (below diagonal) between four *Aedes aegypti* populations

Populations	Chennai- A	Chennai- B	Madurai	Thiruppuvanam
Chennai- A	-	-	-	-
Chennai- B	0.3493	-	-	-
Madurai	0.1024	0.2656	-	-
Thiruppuvanam	0.0544	0.4146	0.1010	-

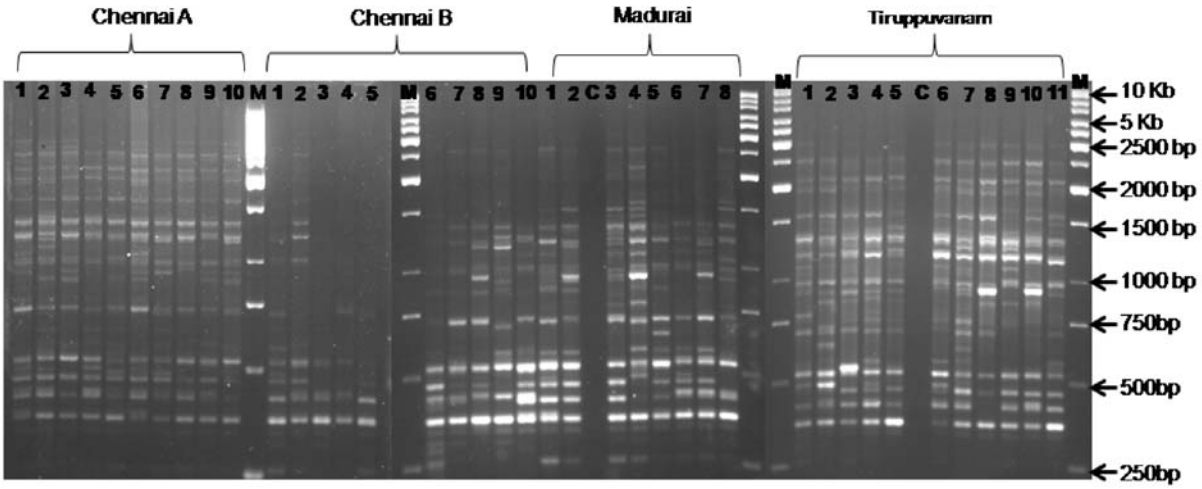

Fig. 1 RAPD banding pattern obtained from four *Aedes aegypti* mosquito populations using A-12 RAPD primer. Lane M: Molecular weight 1 Kb marker (size of the bands are shown with arrow marks), Lane C: negative control without DNA, Lanes covered by bracket above are showing the RAPD banding pattern obtained from individual mosquitoes collected from each population.

observed in Madurai and Chennai A. The low level of genetic diversity in Thiruppuvanam (rural area) might be the consequence of lower prevalence of *Ae. aegypti* in rural areas. Moreover, passive dispersal of the mosquitoes between populations can-not be under-estimated; however, confirmation needs a more detailed sampling to prove the hypothesis.

The dramatic pattern of genetic diversity was observed between two sites of Chennai city. Although the geographical distance between two sites is only ~7Km, the man made changes and land use pattern might have an influence on the mosquito populations. Chennai B has peri-domestic area with humans having more open space with gardens around the house and less density in population. Such landscapes can provide breeding spaces for the mosquitoes in the form of small flower pots, tree bases, tree holes etc and thus could lead to more genetic diversity.

Though the study included limited area for analysis, the results have demonstrated a spatial variation in genetic structure and a high level of genetic differentiation among the *Ae. aegypti* populations. It is worthwhile to correlate these findings with the transmission dynamics of dengue and the differential endemicity of the regions as the higher

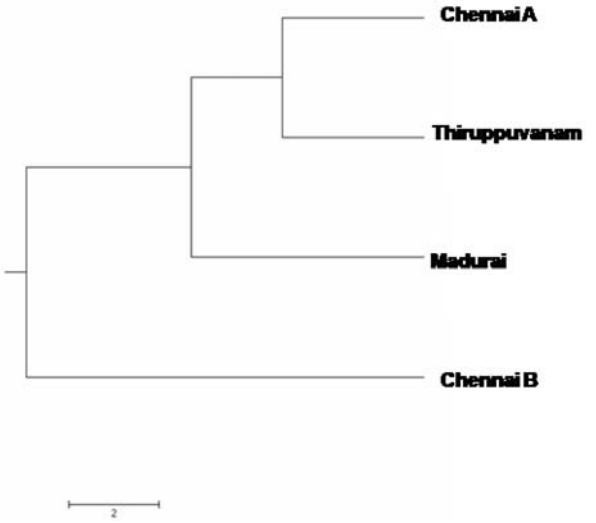

Fig. 2 Dendrogram constructed using Nei's genetic distance between populations using unweighted pair group method with arithmetic averages (UPGMA) clustering strategy.

genetic differentiation might favour the virus transmission as it has been experienced in city of Tartagal, North Argentina (Rotela *et al*., 2007). Further in-depth studies are highly warranted by improving the sample size, covering more areas would help in better understanding the genetic structure of the vector in southern India where dengue is a serious public health problem.

ACKNOWLEDGEMENTS

Authors would like to thank Dr. P. Jambulingam, for valuable suggestions for improving the manuscript. Authors also want to thank ICMR, New Delhi for financial support. BG would like to thank funding agency Science and Engineering Research Board (SERB), Government of India for Early Career Research Award "ECR/2018/001473".

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(Received November 26, 2020; revised ms accepted February 18, 2021; printed March 31, 2021)