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Genetic diversity studies on *Culex quinquefasciatus* from diverse larval habitats using RAPD-PCR marker

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Abstract
The study focuses on the analysis of population genetic structure of *Culex quinquefasciatus* from various larval habitats using RAPD- PCR. The population differentiation parameters, genetic distances, molecular variance and cluster analysis were done using POPGENE, GenALEx and MEGA softwares. Out of 140 RAPD primers screened, four primers exhibited distinct RAPD banding patterns showing up to 100% polymorphism. Moderate variation was evident from value of G_{ST} among larval populations. Effective migration rates were observed to be depicting high gene flow. The AMOVA analysis revealed high intrapopulation genetic variation (92%) with only 8% variation among populations. The cluster analysis showed one main cluster which is divided into two subclusters, one between pond and waste water populations and another cluster of cement tanks along with plastics as a separate branch. The study revealed establishment of *Cx. quinquefasciatus* in diverse larval habitats with excellent adaptability as indicated by high gene flow and polymorphism.

Keywords: *Culex quinquefasciatus,* genetic structure, RAPD marker, larval habitats, gene flow

1. Introduction
Culex quinquefasciatus is the primary vector of lymphatic filariasis caused by the nematode *Wuchereria bancrofti* in the tropics and subtropics [1]. According to a recent WHO report [2] about 120 million people are infected with lymphatic filariasis globally whereas, 40 million are disfigured and incapacitated by this disease. This peridomestic species is seen all over India and commonly found around human dwellings. Rapid urbanization and industrialization without adequate drainage facilities are the key factors for its increased proliferation [3]. A number of other factors are responsible for the spread of these vectors however, transmission of disease is mainly influenced by the environmental factors and genetic variability [2].
The advents of PCR based molecular markers are providing new perspectives in the field of medical entomology for the identification of these potential vectors in order to plan effective control measures [5]. This technology has the potential to reveal large amounts of genetic variation at individual loci. Micro satellites [6] single strand conformation polymorphism (SSCP) analysis of individual loci [7] and random amplified polymorphic DNA (RAPD) analysis [8] are three techniques that have proven especially useful. RAPD PCR offers a number of advantages in that it does not require any prior knowledge of sequence and can be done on any life cycle stage of organism either dead or alive. RAPD is considered as valuable tool for the study of genetic differentiation of geographically and genetically isolated populations [8-11].
Overall studies on population genetic structure can provide important insights into key ecological processes and are especially valuable when direct observation or quantification of such processes is difficult. Moreover, analysis of population genetic structure in disease vectors may provide clues in apprehending their dispersal patterns, behaviour and resistance to insecticides. Relatively few initiatives have been taken in revealing the patterns of population genetic structure of *Cx. quinquefasciatus* despite the significant understanding that genetic structure could provide significant information about dispersal and population dynamics. Indeed the spatial genetic structure of the this mosquito has been investigated primarily at a larger geographic scale in India [12-13] and a recent study has been undertaken from southern

India [14]. Therefore, the present study evaluated the population genetic structure of *Cx. quinquefasciatus* to gain insight about the genetic diversity, gene flow and genetic differentiation in four different larval habitats in a microgeographic region using RAPD marker.

2. Materials and methods

2.1. Collection of immature stages

Monthly surveys of various sites in rural and urban areas were done from Agra (27°10'N and 78°05' E) during 2009-2012 for the presence of the immature stages of *Cx. quinquefasciatus* mosquito vectors from appropriate larval habitats *viz*., permanent and temporary ponds, waste water pond, cement tanks, diverse plastic pots etc. using the standard dipping procedure. Mosquito larvae were immediately brought to the laboratory and morphologically identified microscopically. They were separated, washed with milliQ water, transferred to new vials and preserved in 95% ethanol (-20 ºC) for subsequent DNA analysis.

2.2. Genomic DNA isolation

Modified SDS method for DNA extraction was used for isolation of genomic DNA from fourth instar larvae [15]. Individual larvae were ground in 100µl lysis buffer (100 mM Tris-HCl, pH 8.0; 0.5% SDS; 50mM NaCl; 100mM EDTA) and the mixture was treated with 5µl of proteinase K (20 mg/ml) for two hours at 55 °C. 5µl of RNAase was added (10 mg/ml) to this cell lysate and kept for 20 min for incubation at room temperature. The suspension was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) by heating the contents at 55°C for 10 min. In order to remove every trace of phenol, after centrifugation (10,000 rpm) the supernatant was extracted with chloroform and isoamyl alcohol (24:1). DNA was precipitated by the addition of 0.2 volumes of 5M NaCl and 2.5 volumes of ethanol at room temperature. The mixture was incubated for one hour at -20°C and spun at 12,000 rpm for 10 min. The pellet was resuspended in 100µl of T-10 buffer and stored at -20 °C until used. DNA concentration was checked by means of absorbance ratios A_{260}/A_{280} through a double beam UV-vis spectrophotometer. Agarose electrophoresis was done for qualitative check of DNA samples [16] with 1kb plus DNA ladder and the bands were visualized under UV light in Gel Doc XR system (Bio-Rad, USA).

2.3. RAPD-PCR amplification

Previously optimized protocol was used for RAPD-PCR amplification [15]. Each reaction was carried out in 25 µl volumes containing 20 µg of genomic DNA, 0.5 U of Taq DNA polymerase (Invitrogen), 0.4 mM of each dNTP (Fermentas) 0.2 mM $MgCl_2$ and 5 pmol of a decanucleotide primer. For amplifications thermal cycler (MJ-Mini, Bio Rad USA) was used which was programmed as: initial denaturation at 94 °C for 4 minutes, followed by 40 cycles of 94 °C for 1 minute, 36 °C for 1 minute and 72 °C for 2 minutes, a final extension step at 72 °C for 5 minutes and stored at 4 °C. A total of 140 primers from seven kits *viz.* OP-A, OP-B, OP-C, OP-AB, OP-M, OP-R and OP-S (Operon Technologies, Almeda, CA) were prepared by resuspending at a concentration of 100 pmol/µl in nuclease free water and screened with laboratory reared *Cx. quinquefasciatus* strain. The amplified products were resolved and visualized on 1.4% agarose gel and photographed with Gel Doc.

2.4. Population genetic analysis

For population genetic analysis of the RAPD-PCR data set, the gels were examined and all the amplified loci were treated as diploid dominant markers. It was assumed that each marker band represented a distinct locus where amplified bands were visually scored as binary variables assuming that 1 and 0 represented band present and band absent respectively. The presence of a band represents the dominant genotype (Homozygote and heterozygote) and absence of band represents the recessive homozygote genotype. The data sets were analysed using a population genetic software POPGENE (Version 1.32) [17] and Nei's genetic distances [18], Shanon index, Effective migration rates (Nm) were calculated. For the assessment of overall distribution of molecular variance among and within the groups of *Cx. quinquefasciatus* larval populations from various microhabitats, the RAPD data set were subjected to AMOVA analysis using GenALEx6.5 [19]. A dendrogram, summarizing the genetic relationships among all populations was built using the MEGA 5.2 [20] program using unweighted pair–group mean analysis (UPGMA) algorithm.

3. Results

Out of 140 primers screened, 4 revealed apparent, consistent and discrete RAPD banding patterns showing upto 100% polymorphism in *Cx. quinquefasciatus*. These primers are OPA02, OPM02, OPM04, and OPR08 which were selected by visually analyzing gel pictures on the basis of their ability to amplify DNA fragments from mosquito genomic DNA and resolution on gel with less background smear and brighter bands. A total of 77 bands with a minimum score of 2 and maximum score of 12 bands per sample were amplified with four primers (Fig. 1a-d). OPR08 produced minimum 17 bands whereas OPM02 generated highest 23 band score. OPA02 primer generated lowest molecular weight band of 346 bp whereas, a maximum of 2741 bp molecular weight band was generated by OPM02.

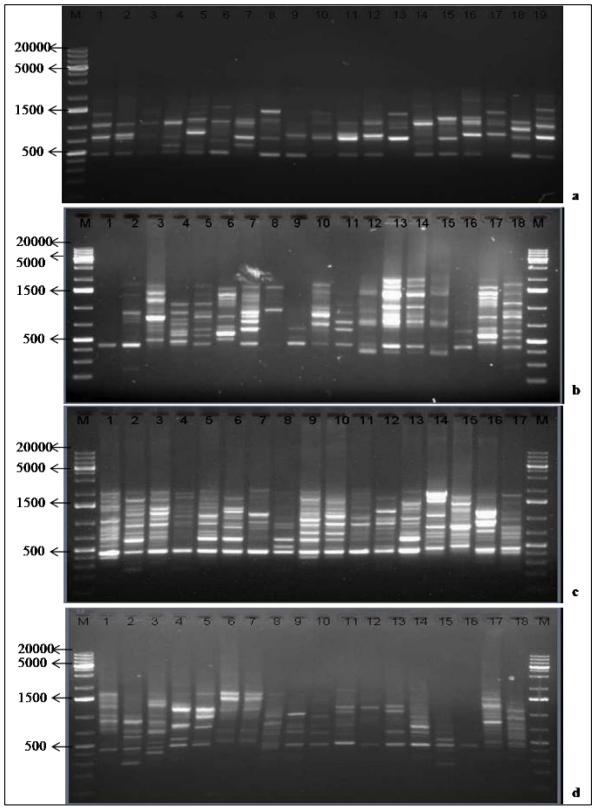

Fig 1(a-d): RAPD profile of *Cx. quinquefasciatus* larval populations from *a.* Cement tanks b. Plastics c. Pond d. Waste water microhabitats using primer OPM04. 1-19: samples; M- 1 kb Plus DNA ladder (Fermentas).

Table 1 exhibits genetic estimates of four larval populations with four different primers. The results displayed moderate genetic variation among *Cx. quinquefasciatus* populations which is evident from high value of within population heterozygosity (Hs) i.e. 0.258. Total heterozygosity was calculated as 0.276. The results displayed rich genetic variation among larval populations which is evident from high value of Gst i.e. 0.066 indicating very great genetic differentiation. Effective migration rates were also observed to be 7.1 depicting high rate of gene flow.

Table 1: Primers used in RAPD analyses showing heterozygosity, genetic differentiation, gene flow and polymorphic loci in *Cx. quinquefasciatus* population.

Primer	Primer sequence	Band size range	N	Ht	Hs	G_{ST}	Nm	NPL	(%)
OPA02	TGCCGAGCTG	346-2281	68	0.317±0.016	0.283±0.010	0.109	4.011	19	100
OPR08	CCCGTTGCCT	400-2186	64	0.353±0.022	0.333±0.019	0.061	7.079	17	100
OPM02	ACAACGCCTC	456-2741	69	0.214±0.019	0.192±0.015	0.105	4.29	23	100
OPM04	GGCGGTTGTC	358-2142	72	0.304±0.022	0.280±0.017	0.080	5.79	18	100
All over			273	0.276±0.020	0.258±0.018	0.066	7.10	77	100

Ht = expected total heterozygosity, Hs = expected within population heterozygosity, G_{ST} = genetic differentiation, Nm =migration rate, NPL= No. of Polymorphic loci, % = polymorphism

Band patterns for different populations have been depicted in Fig. 2 specifying total number of bands, unique and common bands and expected heterozygosity. Among the four populations studied, larval populations from cement tanks exhibited greatest level of variability in polymorphic loci (94.81%), followed by pond populations (92.21%), plastic pot populations (84.42%) and lowest level of variability in waste water populations (83.12%).

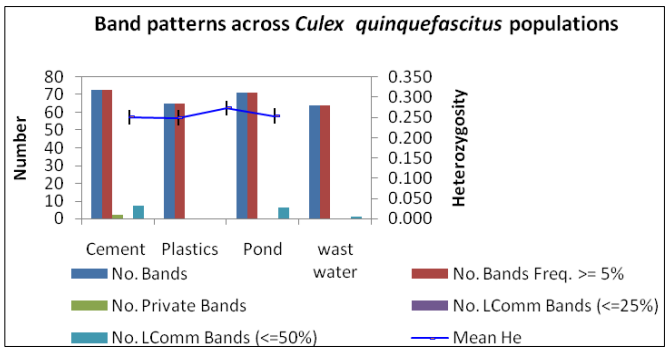

Fig 2: Total band patterns for binary (diploid) data by populations of *Cx. quinquefasciatus.*

Table 2: Details of AMOVA analysis between *Cx. quinquefasciatus* populations using GenALEx 6.5 program.

Source of variation	Df	SS	MS	Est. Var	%	Φ_{PT}	P Value
Among population	3	103.836	34.612	1.166	8%	0.080	0.001
Within population	69	920.383	13.339	13.339	92%		

Df = Degree of freedom, SS = Sum of squares, MS = Mean of squares, Est. Var. = estimated variance, % = percentage of variation.

Analysis of Molecular Variance (AMOVA) among various populations was done using GenAlEx (version 6.5) and data are shown in Table 2. It is evident from the table that very high proportion exhibited intrapopulation variation (92%) whereas only 8% variation was recorded among populations (Fig. 3). AMOVA showed that ΦPT analyzed among four larval populations was statistically significant (P<0.05).

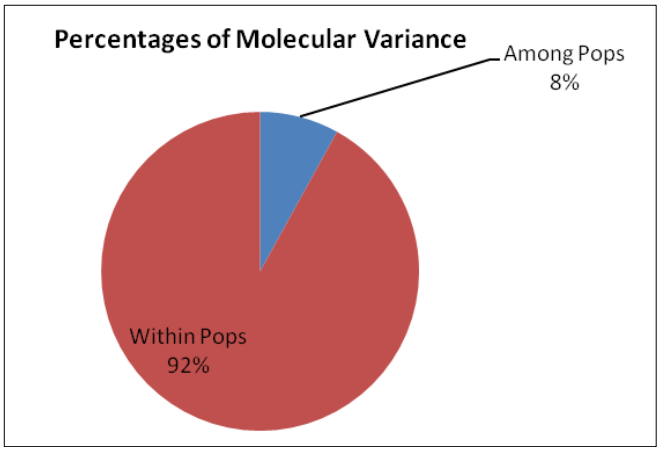

Fig 3: Percentages of molecular variance in *Cx. Quinquefasciatus* populations.

The average genetic distance between larval populations was 0.0233 showing close relation between pond water and waste water populations as indicated by genetic distance which was 0.016 (Table 3). Fig. 4 depicts dendrogram generated by MEGA 5.2 version which clearly shows one clusters with mosquito population from ponds and waste water whereas, cement tanks represented another clade for *Cx. quinquefasciatus* larval population. These three populations were distantly related with populations from plastic pots as evident from genetic matrix also. The study revealed establishment of *Cx. quinquefasciatus* in diverse larval habitats with excellent adaptability as indicated by high gene flow and polymorphism.

Table 3: Matrix of Genetic distance and similarity among the *Cx. quinquefasciatus* populations from four larval habitats based on RAPD-PCR.

Larval habitat	Cement tank	Plastic	Pond	Waste water
Cement tank	****	0.9719	0.9768	0.9826
Plastic	0.0285	****	0.9653	0.9820
Pond	0.0235	0.0353	****	0.9836
Waste water	0.0176	0.0182	0.0166	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

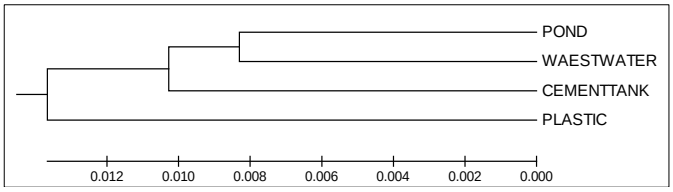

Fig 4: Phylogenetic tree generated by using MEGA 5.2 version for *Cx. quinquefasciatus* populations from different larval habitats resulting from UPGMA cluster analysis after bootstrapping.

4. Discussion

The results presented in this study are the first evaluation of the genetic analysis of *Cx. quinquefasciatus* in Agra region from India. The analysis of allele frequencies based on RAPD showed 77 markers with the percentage of polymorphic loci varying from 83.12% to 94.81% in the four different larval habitats whereas overall polymorphism was recorded as 100%. The genetic diversity was moderate as evident from heterozygosity values (Hs-0.258: Ht-0.276).

The heterozygosity and polymorphism values were similar to those described for other populations of *Cx. quinquefasciatus* from north western states of India (H_{ST}- 0.31; P-100%) [12] through RAPD markers. In our study, we have additionally determined moderate variation among larval populations which is evident from low value of G_{ST} indicating moderate genetic differentiation. Effective migration rates were observed to be depicting high gene flow.

Earlier studies on *Cx. quinquefasciatus* analyzed the variation in the ISSR marker within and among populations of *Cx. quinquefasciatus* in India [13]. The analysis of molecular variation (AMOVA) was not studied by them however, we have demonstrated that the majority of genetic variation was within population (92%) whereas, approximately 8% was among populations. This low proportion of interpopulation variance in relation to intrapopulation variance supports our data where the values of G_{ST} and Nm depict a moderate genetic differentiation indicating high rate of gene flow with stable and established populations of *Cx. quinquefasciatus* in this region.

The genetic variations may result due to various intrinsic and extrinsic factors which includes geoclimatic conditions, physiochemistry of larval habitats [21] etc. Vector- pathogen interaction plays an important role in disease transmission followed by the vector-host interactions. Different populations of *Culex* mosquitoes show varying degree of competence of pathogen acquisition and disease transmission. The studies carried out in various laboratory and different geographical strains of *Culex tritaeniorhynchus* species showed susceptibility to Japanese encephalitis virus infection in various parts of the world i.e. Japan [22], Southern Asia and Far East [23] and susceptibility of a North American *Culex quinquefasciatus* to JE virus [24]. Present research has demonstrated establishment of *Cx. quinquefasciatus* populations in diverse habitats depicting stable population with high gene flow.

The application of such genetic tools may help us as a foundation for developing biomarkers of vector competence to pathogen and novel targets for effective management of laboratory sterilized mosquito release in wild conditions and also for priorities regarding vector control [13, 25]. This investigation could be helpful in understanding the population variations with respect to preferred habitat for ovipositioning and facilitate in strategic planning for effective management of *Cx. quinquefasciatus* in their confined niches exposed to environmental stressors. Further research in utilizing RAPD marker for the development of SCAR marker for the identification of *Culex* sp. in this region is in progress.

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