Original Article

Genetic Analysis of *Aedes aegypti* Using Random Amplified Polymorphic DNA (RAPD) Markers from Dengue Outbreaks in Pakistan

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Abstract

Background: Keeping in view the havoc situation of dengue fever in Pakistan, the current study was designed to demonstrate the genetic variations, gene flow and rate of migration from Lahore and Faisalabad.
Methods: The larvae were collected from both natural and artificial breeding places from each collection site. The adult mosquitoes were collected by means of sweep net and battery-operated aspirator. DNA extraction was performed using TNE buffer method. Ten GeneLink-A series RAPD primers were used for PCR amplification and the data was analyzed through POPGENE.
Results: The number of amplification products produced per primer varied from 8-12, ranging from 200 to 2000 bp with an average of 10.0 bands per primer. The percentage of polymorphic loci amplified by each primer varied from 22.5 to 51%. The UPGMA dendrogram demonstrates two distinct groups from Faisalabad and Lahore populations. The genetic diversity ranged from 0.260 in Faisalabad to 0.294 in Lahore with a total heterozygosity of 0.379. The G_{ST} value for nine populations within Lahore was 0.131 (Nm= 3.317), whereas for nine populations in Faisalabad G_{ST} value was 0.117 (Nm= 3.773). The overall genetic variation among eighteen populations showed G_{ST}= 0.341 and Nm= 1.966.
Conclusion: The genetic relatedness and Nm value show that *Ae. aegypti* populations exhibit intra-population gene flow both in Faisalabad and Lahore. Although, both cities show a distinct pattern of genetic structure; however, few areas from both the cities show genetic similarity. The gene flow and the genetic relatedness in few populations of Lahore and Faisalabad cities need further investigation.

Keywords: *Aedes aegypti*, Dengue, Genetic diversity, RAPD markers

Introduction

Aedes aegypti and *Ae. albopictus*, mosquitoes are responsible to transmit dengue fever virus which is a major health concern in tropical and subtropical areas (Pinheiro and Corber 1997, Fradin and Day 2002, Gibbons and Vaughn 2002). World Health Organization (WHO) reported that about 2.5 billion people (more than 40%) are at risk of dengue fever around the world and about 50 million new cases of dengue would be estimated mated to occur every year (WHO 2009, 2014). Dengue disease is considered endemic in more than 100 countries, with South-East Asia as a major affected region (Itrat et al. 2008, Khan et al. 2010, Raheel et al. 2011, Fatima et al. 2011, Idrees and Ashfaq 2012).

Aedes aegypti originated from Africa and became spread to the Southeast Asia. With the passage of time, it established here and now it has become a major vector of dengue

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in the region (WHO 1997, Fraga at al. 2003). Better road infrastructure and good transportation facilities caused dispersal of *Ae. aegypti*. It can easily find breeding places in human dwelling areas; a calamity of living style of people in the sub-continent. In addition, it can withstand desiccation, which also serves as a factor for its success (Raheel et al. 2011, Sajid et al. 2011). Thus, the spread mosquito and thereby the virus, has led to a resurgence of dengue (Cologna et al. 2005).

The climatic change and use of insecticides cause appreciable effect on the genetic population structure of *Ae. aegypti* (Huber et al. 2002, Paupy et al. 2005, Scarpassa et al. 2008). This vector has been considered as homogeneous species, however, it shows both morphological and genetic variations (Morlais et al. 2003). Due to epidemiological importance, various molecular studies have been conducted on *Ae. aegypti* using different DNA markers (Ayres et al. 2002, Gorrochotegui et al. 2002, Ravel et al. 2002, Ayres et al. 2003, Fraga et al. 2003, Beebe et al. 2005, Herrera et al. 2006, Bracco et al. 2007, Rasheed et al. 2013). Random amplified polymorphic DNA (RAPD) and RFLP markers are most commonly used molecular markers to elucidate the genetic variations in *Ae. aegypti* (Severson et al. 1999, 2002, Fulton et al. 2001, Ayres et al. 2003, Julio et al. 2009). Unfortunately, the genetic analysis of *Ae aegypti* has been neglected in Southeast Asia despite of its serious damaging aspect of dengue outbreaks.

In Pakistan, dengue fever has emerged as havoc for the last few years and has become one of the key public health priorities. From the first diagnosed case in 1994, now the virus has rapidly spread across the country. The dengue fever has shaped a cyclic pattern with high infection rate in the monsoon season and low in winter season. More than 2,000 people were reported with dengue infection in 2010. Over 14,000 cases were reported with more than 300 deaths from Lahore alone in November 2011, likewise, 11,500

people were affected until September 2012 (Gilani 2012, Sajid et al. 2012). The latest outbreak resulted in 18,000 cases across the country including significant number of confirmed cases reported from Lahore and Faisalabad (Siddiqui et al. 2009, Gilani 2012, Sajid et al. 2012). According to WHO reports, majority of infected people belonged to Lahore area in Punjab, Pakistan (WHO 2013).

To counter this threat and to devise effective control measures, there is essentially a dire need of time to know maximum about dengue and its vector. Despite the fact of not significant ecological differences in both cities, more reported cases of dengue reflect the severity of disease in Lahore. Therefore, it was hypothesized genetic variation might exist in *Ae. aegypti* populations in Lahore. Thus, the current study was designed to analyze genetically the *Ae. aegypti* populations to demonstrate the genetic variations, gene flow and rate of migration from Lahore and Faisalabad, Punjab using random amplified polymorphic DNA (RAPD) markers.

Materials and Methods

Mosquito collection
Eighteen populations were collected from Faisalabad and Lahore (Fig. 1). The adult mosquitoes were collected with the help of sweep net and battery-operated aspirator (Herrel et al. 2001, Shortall et al. 2009, Florencio et al. 2014). The larvae and pupae of mosquitoes were collected from both natural and artificial breeding places (tree holes, stagnant water, tires, waste material, lawns, homes) by using dipper from each collection site (Naeem-Ullah et al. 2010, Nikookar et al. 2010). The collected samples were preserved in 70% alcohol in labelled vials and stored at 4 °C for DNA extraction (Zahoor et al. 2013).

DNA extraction
DNA was extracted from individual mos-

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quitoes from each sample using salt extraction method. The mosquitoes were homogenized in 400 µl of TNE buffer (Tris-NaCl-EDTA), and then 100 µl of 20 µg/µl of Proteinase-K and 40 µl of 20% Sodium Dodecyl Sulphate (SDS) were added. The homogenates were incubated at 55 °C for one hour, 300 µl of 5M NaCl was added and vortexed. The mixture was centrifuged at 15,000 rpm for 10 min and the supernatant was shifted to separate eppendorf tube. DNA was precipitated by adding of 300-400 µl isopropanol or ice-cold 100% ethanol, kept at -21 °C for 1 hour, and then centrifuged at 15,000 rpm for 10 min. DNA pellet was washed with chilled 70% ethanol, air-dried and re-suspended in 50 µl of sterile water (d_3H_2O). The DNA concentration was estimated by measuring optical density (OD) at 260nm and its quality was checked on 1% agarose gel electrophoresis (Ashraf 2013, Zahoor et al. 2013, Bibi 2015).

RAPD-PCR analysis

Ten Gene Link-A series RAPD primers were used for PCR amplification (Table 4). Each PCR reaction was carried out in a final volume 25 µl, approximately 100 mg of genomic DNA, 3 mM of MgCl2, 20 pmol of primer, 2.5 µl buffer, 1.0 units of Taq DNA polymerase and 0.3 mM of each dNTPs. PCR program comprised of 35 cycles with initial denaturing of DNA at 94 °C for 5 min, denaturation at 94 °C for 1 min, primer annealing at 36 °C for 1.5 min, extension at 72 °C for 1.5 min, final extension at 72 °C for 10 min and then hold at 4 °C until the tubes were removed. The PCR products were run on 1.6% agarose gel electrophoresis at 80 voltages for one hour (Ashraf 2013, Zahoor et al. 2013).

Statistical Analysis of Data

The fingerprints examined under ultra violet Transilluminator and photographed using (SynGene) Gel documentation system. The size of amplified bands or loci was compared against the DNA marker. All the fragments scored as present (1) or absent (0) for each sample. Ambiguous bands were not scored. The bands counted by starting from top to the bottom in all lanes.

The RAPD markers were analyzed using the following assumptions: (1) The RAPD alleles follow the Mendelian pattern of segregation, (2) monomorphic fragments are homologous (co-migrate), (3) loci are independent from each other and (4) and mosquito populations are in Hardy-Weinberg equilibrium as described by Ayres et al. 2003. A dendrogram was constructed using UPGMA method (unweight pair-group mean analysis) (Nei 1978). Effective migration rates (Nm) were estimated based on inbreeding indices (G_{ST}), where Nm= 0.5(1- G_{ST})/ G_{ST} (Ayres et al. 2002). The genetic analysis was performed through POPGENE (version 1.32) software.

Results

Aedes aegypti populations were analyzed by RAPD-PCR using 10 oligonucleotide primers (Genelink, Table 4). Four primers were further selected which produced distinct and easily detectable bands (Table 1). The reproducible bands over repeated PCR reactions were used for analysis. The number of fragments per primer varied from 8–12, ranging from 200 to 2000 bp size, with an average of 10.0 bands per primer (Table 1 and Fig. 1). Various amplified fragments differentiated the populations of *Ae. aegypti* from Lahore and Faisalabad (Fig. 2). The percentage of polymorphic loci amplified by each primer varied from 22.5 to 51% (Fig. 3). Total 55 polymorphic loci were scored in all samples, with an average 5.5 polymorphic loci per primer (data not shown). Four primers were selected for further due to their reproducible and distinct banding pattern (Table 2). The heterozygosity, gene flow and the variation

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among eighteen populations were estimated using Nei's analysis. The genetic diversity ranged from 0.260 in Faisalabad to 0.294 in Lahore with a total heterozygosity of 0.379. The G_{ST} value for nine populations within Lahore was 0.131 (Nm= 3.317), whereas for nine populations in Faisalabad G_{ST} value was 0.117 (Nm= 3.773), respectively. The overall genetic variation among eighteen populations showed G_{ST}= 0.341 and no. of migrants Nm= 1.966 (Table 3).

Cluster Analysis

The UPGMA dendrogram based on similarity matrix of Nei's genetic distances demonstrates two distinct groups from Faisalabad and Lahore populations (Fig. 4). One group (A) shows seven populations from main Faisalabad City, ie. Nazim Abad, Gulfishan Colony, University of Agriculture Faisalabad (UAF) Jail road, Government College University Faisalabad (GCUF), Awami Colony, Narwala road, National Institute for Agriculture and Biology (NIAB). The second group (B) represents four populations from Lahore, i.e. Govt. Muhammadan Anglo Oriental College (MAO College), Qila Gujersing, UC69 and UC 81(A). There are three subgroups in group C, comprising of two populations from Faisalabad (C2, Rescue office Jhang road and AARI Jhang road) and four populations from Lahore (C3, UC 73(A) and Ghawal mandi, and and C4, Mazang chungi and UC73 (B), respectively). The BISE Lawrence road shows that both the groups A and B were originated from Lahore.

The subgroup A5 and A4 comprising of UAF Jail road, Gulfishan Colony and GCUF found to be closely related population groups in Faisalabad The subgroup A3 consists of populations from Narwala road and NIAB.

The genetic similarity could be speculated due to the transportation of mosquitoes in these areas. The subgroup B2 consists of closely related populations from Gujersing area and MAO College. This subgroup shows more genetic similarity to the mosquito population from UC69 site. The subgroup B1 represents relatedness to the subgroup B2, thereby shows more genetic similarity to Gujersing area and MAO College mosquito populations.

Within the main group C, there are three subgroups converged at C1 (C2, C3 and C4). The subgroup C3 shows that UC 73(A) is closely related to the mosquitoes from Gawal mandi area. Similarly, the subgroup C4 represents that Mazang chungi and UC 73(B) are more closely related to each other. Interestingly, the main group C also contains populations from Faisalabad. The subgroup C1 consists of areas from the main high way of Jhang road, Rescue office and AARI. The collected mosquito populations show a genetic similarity to Lahore populations.

The RAPD primer (A-04) produced fragments, which showed genetic variation in *Ae. aegypti* populations from both Lahore and Faisalabad. The genetic relatedness in Faisalabad populations and the Nm value for an estimate of gene flow shows that the dengue vector *Ae. aegypti* populations exhibit gene flow among different populations. Similarly, *Ae. aegypti* populations from Lahore also show intra-population gene flow (Table 3). Although, both cities present a distinct pattern of genetic structure, however, few areas from both the cities show that *Ae. aegypti* populations are genetically related to each other (Fig. 4).

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Table 1. Localities from Lahore and Faisalabad selected for sampling of *Ae. aegypti* populations

Sr. No.	Samples from Faisalabad District		Sr. No.	Samples from Lahore District	
	Sampling areas	Code		Sampling areas	Code
1	Rescue office Jhang road	F_1	10	UC 73 (A)	L_1
2	Awami colony Tariq road	F_2	11	UC 81 (A)	L_2
3	AARI Jhang road	F_3	12	Ghawal mandi	L_3
4	Gulfishan colony	F_4	13	Mazang chungi area	L_4
5	Nazimabad	F_5	14	Qila Gujersing	L_5
6	Narwala road	F_6	15	BISE lawerence road	L_6
7	GCUF	F_7	16	UC69	L_7
8	UAF Jail road	F_8	17	UC 73 (B)	L_8
9	NIAB	F_9	18	MAO college	L_9

Table 2. Primers used in RAPD analysis showing size and number of amplified fragments obtained (No.) from *Ae. aegypti* populations from Lahore and Faisalabad

Sr. No.	Primers	Nucleotide sequence	Size (bp)	No.
1	A-03	5'- AGTCAGCCAC - 3'	200–1500	12
2	A-04	5'- AATCGGGCTG - 3'	350–1850	11
3	A-06	5'- GGTCCCTGAC - 3'	375–1200	9
4	A-18	5'- AGGTGACCGT - 3'	210–1300	8

Table 3. Summary of Nei's Analysis of Gene diversity among between *Aedes aegypti* populations from Lahore and Faisalabad through RAPD markers

Populations	Genetic variation G_{ST}	Gene flow Nm	Heterozygosity Ht
Lahore	0.131	3.317	0.294
Faisalabad	0.117	3.773	0.260
Lahore, Faisalabad	0.341	1.966	0.379

Table 4. RAPD Primer Sequence (Genelink) and their codes

Sr. No.	Primer name (code)	Primer sequence	GC contents
1	GL Decamer A-03 (A-03)	AGTCAGCCAC	60%
2	GL Decamer A-04 (A-04)	AATCGGGCTG	60%
3	GL Decamer A-05 (A-05)	AGGGGTCTTG	60%
4	GL Decamer A-06 (A-06)	GGTCCCTGAC	70%
5	GL Decamer A-08 (A-08)	GTGACGTAGG	60%
6	GL Decamer A-10 (A-10)	GTGATCGCAG	60%
7	GL Decamer A-11 (A-11)	CAATCGCCGT	60%
8	GL Decamer A-12 (A-12)	TCGGCGATAG	60%
9	GL Decamer A-18 (A-18)	AGGTGACCGT	60%
10	GL Decamer A-19 (A-19)	CAAACGTCGG	60%

Fig. 1. Map showing different collection sites from Lahore and Faisalabad

(**Faisalabad**: 73°74 E and 30°31.5 N, **Lahore**: 74°01-74°39 E and 31°15 -31°45 N)
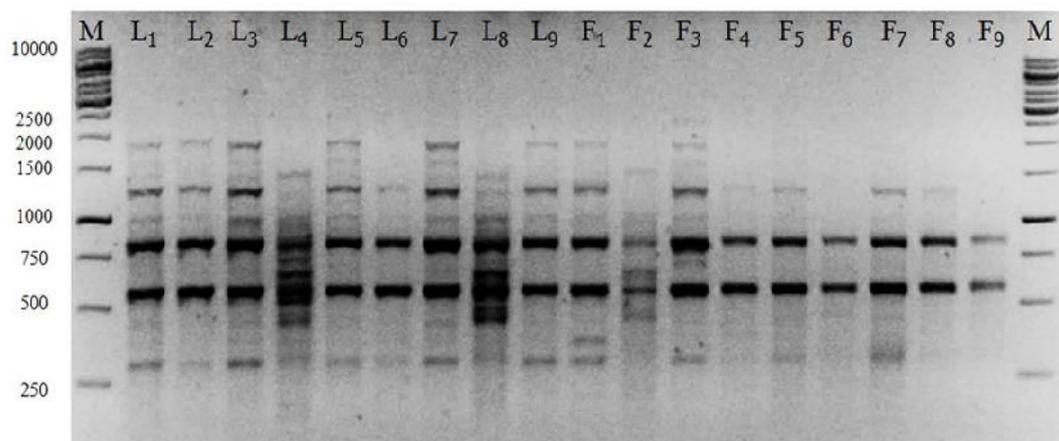
Fig. 2. Amplification profile of *Aedes aegypti* populations with primers A-04 from Lahore and Faisalabad. (L$_{1-9}$: Lahore samples, F$_{1-9}$: Faisalabad samples, Lane M= DNA Marker (size 1kb)
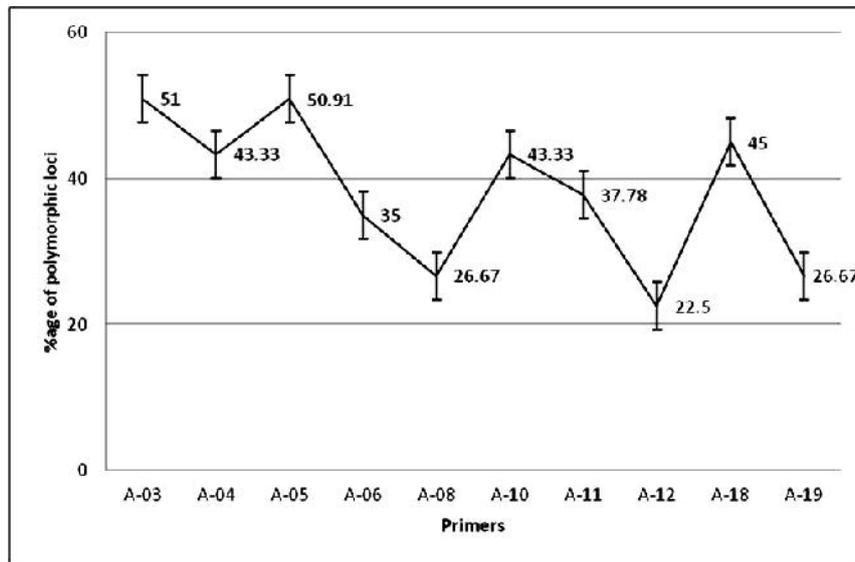
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Fig. 3. Percentage of polymorphic loci amplified by each RAPD primer
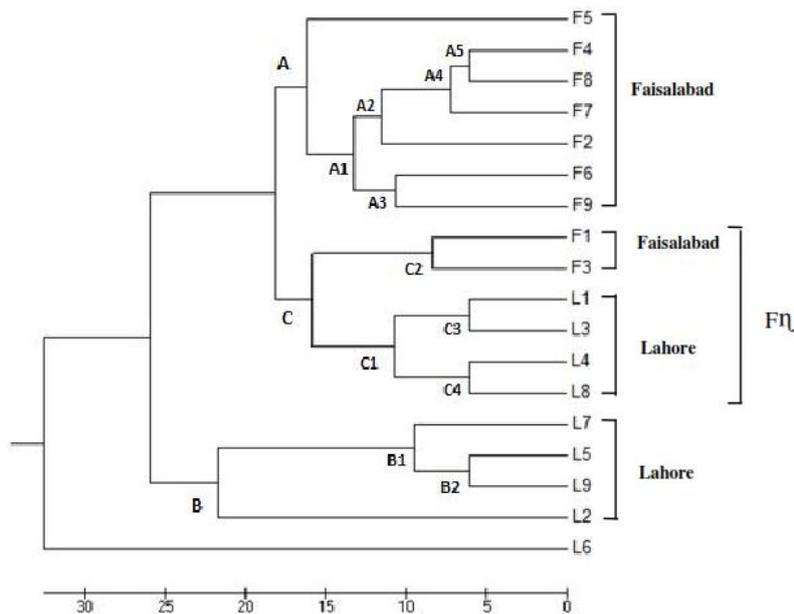
Fig. 4. Dendrogram based on Nei's genetic distances among *Aedes aegypti* populations from Lahore and Faisalabad. (Scale Bar: branch length, *F/L: Faisalabad/Lahore)

F1: Rescue office Jhang road	L1: UC 73 (A)
F2: Awami colony Tariq road	L2: UC 81 (A)
F3: AARI Jhang road	L3: Ghawal mandi
F4: Gulfishan colony	L4: Mazang chungi area
F5: Nazimabad	L5: Qila Gujersing
F6: Narwala road	L6: BISE lawerence road
F7: GCUF	L7: UC69
F8: UAF Jail road	L8: UC 73 (B)
F9: NIAB	L9: MAO College

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Discussion

Aedes aegypti is one of the most important mosquito vectors of viral agents in world, due to its role in transmitting chikungunya virus (CHIKV) and dengue virus (DENV) (Thavara et al. 2001, Bosio et al. 2005). The genetic diversity has been reported in *Ae. aegypti* populations (Gorrochotegui-Escalante et al. 2000, Souza et al. 2001, Ayres et al. 2002, Franco et al. 2002). Many DNA techniques such as RAPD, microsatellites and mitochondrial DNA markers have been widely used to detect variation in geographically and genetically isolated populations (Ballinger-Crabtree et al. 1992, Apostol et al. 1994, Ayres et al. 2003, Paduan et al. 2006). In Pakistan, few studies have been conducted on barcoding of mosquitoes through mitochondrial COI gene and genetic structure of *Ae. aegypti* populations using microsatellite markers (Ashfaq et al. 2013, Rasheed et al. 2013). However, still genetic analysis of *Ae. aegypti* populations has not been performed yet, particularly in the dengue outbreaks. Thus, the present study reinforced the significance of genetic analysis of *Ae. aegypti* populations in dengue affected areas in Pakistan.

The level of genetic diversity was revealed among various mosquito species and multiple haplotypes were reported by Ashfaq et al. (2013) in Pakistan. However, they mainly focused on biodiversity and distribution of mosquitoes in KPK and Punjab provinces. Furthermore, Rasheed et al. 2013 used 13 microsatellite markers and found low genetic diversity in mosquitoes collected from 13 different cities. Low level of genetic diversity with less differentiation was revealed and considered due to better road infrastructure. Although present results are consistent to their results however, based on the amplified fragments in our findings, it is suggested that *Ae. aegypti* populations are genetically more diverse as previously reported in Pakistan. In addition, the genetic similarity in populations at the border area of Faisalabad to few of Lahore populations supports the idea of transportations (Rasheed et al. 2013).

The heterozygosity in *Ae. aegypti* populations from Faisalabad and Lahore (Ht. 0.311) found similar to the results obtained from Brazil using 27 RAPD loci (Ht= 0.390) (Ayres et al. 2003) and to the average heterozygosity of 21 loci (Ht= 0.388) in Brazil, 57 loci in Puerto Rico (Ht= 0.354) (Apostol et al. 1996) and in 131 loci of 20 *Ae. aegypti* populations from Mexico through RAPD markers (Franco et al. 2002). Gorrochotegui-Escalante et al. (2000) also distinguished populations of *Ae. aegypti* from ten cities of the Mexican northeast coast, using 60 RAPD polymorphic loci. The gene flow and genetic variations were observed within populations separated within a range of 90 to 250 km. While, Lahore-Faisalabad *Ae. aegypti* populations correspond to ~100 km distance. Thus, the polymorphic pattern in our findings showed genetic variations by distance (55 out of 70 loci, data not shown).

The genetic variation ($G_{ST}= 0.341$, Nm= 0.966) and polymorphism (51%) found in the present study were consistent to those reported in Argentina ($G_{ST} = 0.249$, Nm= 0.75) (Souza et al. 2001) and Brazil ($G_{ST} = 0.317$, Nm= 0.54, HS= 0.274) (Ayres et al. 2003). Santos et al. (2011) found high level of polymorphism and distinguished 52 markers in the four populations through RAPD analysis, ranging in size from 300 to 2072 bp. Their percentage of polymorphic loci varied from 82.69 to 94.23. In contrast, we amplified loci ranged from 200 to 2000 bp with 22.5 to 51 percentage of polymorphism, which is closer to the genetic variability and 50% similarity in *Ae. aegypti* populations as demonstrated by Hiragi et al. (2009) using RAPD markers. However, current findings showed maximum similarity (0.8846) and genetic distance (0.045) among *Ae. aegypti*

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populations (data not shown). A population differentiation from Faisalabad ($G_{ST} = 0.131$) and Lahore ($G_{ST} = 0.117$) with higher degree of gene flow (Nm= 3.317 Faisalabad, Nm= 3.773 Lahore) showed similarity to a low population differentiation ($G_{ST} = 0.208$) with higher degree of gene flow (Nm= 1.90) as described by Paduan et al. (2006) in Brazil indicating both intra- and inter-population genetic variability.

Current findings agree to the study of De Sousa et al. (2001). They successfully used RAPD markers to estimate the level of polymorphism and genetic relatedness in five populations of *Ae. aegypti* from Argentina and Puerto Rico. However, contrary to the present study, heterozygosity and genetic distance values were found significant at all loci suggesting that the population from Puerto Rico was different from Argentina populations. Subsequently, de Patarro et al. (2013) assessed the genetic variability through RAPD markers in five *Ae. aegypti* populations from Brazil and USA. A total of 165 polymorphic DNA loci were generated with six primers compared to 55 loci using ten primers in the present study. The mean value of inter-population genetic diversity (G_{ST}) ranged from 0.044 to 0.289 suggesting that genetic variation existed in *Ae. aegypti* populations. Similarly, we found genetic diversity (G_{ST}) ranged from 0.260 in Faisalabad to 0.294 in Lahore. Ocampo and Wesson (2004) found genetic variation in *Ae. aegypti* populations in Colombia. The genetic heterogeneity ranged from 19% to 60% revealed through RAPD markers closer to our findings. In addition, the genetic distance and migration rate (Nm) demonstrated a moderate genetic differentiation among *Ae. aegypti* populations which is consistent to the current findings of genetic differentiation in *Ae. aegypti* populations existed in Faisalabad and Lahore.

Despite of RAPD, other DNA markers are also being used to analyse genetically mosquitoes. Paupy et al. (2012) examined genetic structure and geographical origin of *Ae. aegypti* in Bolivia and found polymorphism at nine microsatellite loci and in two mitochondrial DNA regions (COI and ND4). Similar to the present work, two genetic lineages were obtained after genetic analysis of mtDNA sequences. One lineage was restricted to rural localities and the other was found closely related to African *Ae. aegypti* populations. Similarly, Caldera et al. (2013) genetically analyzed two populations of *Ae. aegypti* from dengue outbreak areas in Colombia using mitochondrial gene ND4. The genetic differentiation with a limited gene flow (Nm= 1.40) indicated that both the populations of *Ae. aegypti* are genetically distinct. Hlaing et al. (2010) also found genetic heterogeneity in *Ae. aegypti* populations using microsatellite markers in Myanmar, Cambodia, Thailand, Sri Lanka and Nigeria. The genetic variation even in a distance of 500 m and genetic similarity in distant locations due to dispersal were observed. In parallel, Costa-da-Silva (2005) also found a distinct genetic pattern in *Ae. aegypti* populations in Peru using mitochondrial DNA. Furthermore, Yáñez et al. 2013 tested 51 samples of *Ae. aegypti* populations from Peru using mitochondrial ND4 gene and identified five haplotypes of *Ae. aegypti* grouped in two lineages. This variability occurred mainly due to migration of this vector and partly through human activity. Thus, transportation has been considered as a main source of spread of mosquito borne diseases, however, other factors like use of insecticides and elimination of larval habitats in and around dwellings had a demonstrable impact on gene flow and genetic structure of *Ae. aegypti* populations (Wallis et al. 1984, Failloux et al. 1995, Paupy et al. 2000, Lerdthusnee and Chareonviriyaphap 2002) which needs to be further investigated on larger scale using gene specific molecular markers.

Conclusion

The genetic relatedness and Nm (number of migrants) value show that *Ae. aegypti* populations exhibit intra-population gene flow between Faisalabad and Lahore. Although, both cities show a distinct pattern of genetic structure, however, few areas from both the cities show genetic similarity. It is also concluded that *Ae. aegypti* populations are genetically more diverse as previously reported in Pakistan.

Acknowledgements

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