INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
14–647/2015/17–4–809–814
DOI: 10.17957/IJAB/14.0027
http://www.fspublishers.org

Full Length Article

Genetic Analysis of Mosquitoes from Rural and Urban Areas of Sialkot, Pakistan

Maryam Bibi[1], Muhammad Kashif Zahoor[1*], Muhammad Asif Zahoor[2], Hafiz Muhammad Ashraf[1], Humara Naz Majeed[3], Shabab Nasir[1] and Bilal Rasool[1]

[1]*Department of Zoology, Wildlife and Fisheries, Government College University Faisalabad, Pakistan*
[2]*Department of Microbiology, Government College University Faisalabad, Pakistan*
[3]*Pharmaceutical and Biochemistry Department, School of Pharmacy, University College London, UK*
[*]For correspondence: kashif.zahoor@gcuf.edu.pk

Abstract

The current study was conducted to demonstrate the genetic variability, gene flow and rate of migration in mosquito populations between rural and urban areas in Sialkot, Pakistan. The adult mosquitoes were collected with the help of sweep net and battery-operated aspirator, whereas the larvae were collected using standard dippers. DNA extraction was performed through TNE salt extraction method. Fourteen samples of mosquito populations for the selected seven species of three genera were studied using RAPD *loci*. Ten oligonucleotide decamer primers produced 92 polymorphic fragments ranging from 120 to 3000 base pairs. The data generated through RAPD markers were analyzed through POPGENE software. The UPGMA dendrogram demonstrated two distinct groups comprising of seven mosquito species of three genera' *Culex*, *Anopheles* and *Aedes*. All the species from both urban and rural areas showed genetic relatedness with the corresponding species. *Aedes albopictus* from urban areas was found more closely related to *Ae*. *aegypti*. *Aedes* species group originated from *Ae*. *albopictus* of rural areas. The genetic diversity observed in population from urban areas was G_{ST}=0.113 (Nm=4.014) with heterozygosity of 0.3691; and the rural areas showed genetic variation of G_{ST}=0.134 (Nm=2.62) with a total heterozygosity of 0.4019. The overall genetic variation among fourteen populations showed G_{ST}=0.147 and rate of migration Nm=3.73. The genetic relatedness and Nm value showed low level of genetic variations in mosquito populations from rural and urban areas of Sialkot. Moreover, the genetic data show that mosquitoes are freely moving between rural and urban areas.

Keywords: Mosquitoes; Genetic variation; Gene flow; RAPD Markers

Introduction

Mosquitoes are medically important arthropods, vectoring numerous agents which adversely affect millions of people annually (WHO, 2009; Khan *et al*., 2010; Idrees and Ashfaq, 2012). Mosquitoes of the genera *Culex*, *Anopheles*, *Culiseta*, *Mansonia* and *Aedes* may act as vectors. The known vectors for human malaria belong to the genus *Anophele*, whereas the bird malaria vector is harbored by the genus *Culex*. It has been reported that *Plasmodium falciparum* results in the death of more children each year than any other single infectious agent (Murray *et al*., 2008). In addition, *Aedes aegypti* and *Ae*. *albopictus* are responsible to cause dengue fever which is a major health concern particularly in tropical and subtropical areas (Gibbons and Vaughn, 2002; Schulze *et al*., 2004). About 2.5 billion people (more than 40%) are at risk of dengue fever around the world (Itrat *et al*., 2008; Raheel *et al*., 2011; WHO, 2014).

Dengue fever has emerged as disaster and repeatedly reported in many cities of Punjab (Lahore, Faisalabad and Sialkot) and Sind province (Karachi), Pakistan. The prevalence of humid environment and the natural ponds around the city could be a reason of its existence in Lahore (Attaullah, 2013). According to information data collected from urban and rural areas, old-dumped tires play a major role for dengue mosquitoes (Hoffmann and Willi, 2008; Ashraf, 2013; Attaullah, 2013).

It has been widely accepted that genetic variability occurred in different insects both at intra and inter-species level as a result of differences in environmental processes, genetics and various demographic factors. In contrast, the genetic homogeneity in insect species occurs due to free movement, lack of barriers (Batool, 2012; Ashraf, 2013; de Lourdes *et al*. 2013), migration as well as transportation through various means (Franco *et al*., 2002; Souza *et al*., 2001; Ayres *et al*., 2003).

To cite this paper: Bibi, M., M.K. Zahoor, M.A. Zahoor, H.M. Ashraf, H.N. Majeed, S. Nasir and B. Rasool, 2015. Genetic analysis of mosquitoes from rural and urban areas of Sialkot, Pakistan. *Int. J. Agric. Biol*., 17: 809–814

The comparative analysis of DNA is a potent approach to estimate genetic variation, genetic relatedness and the genetic exchange within species. Over the last decade, DNA makers have made a significant contribution to rapid rise of molecular studies of genetic variation, phylogeny, population dynamics and genome mapping in insects (Ravel *et al*., 2002; Scarpassa *et al*., 2008; Santos *et al*., 2011). Monomorphism in amplification profile of the same species belonging to different areas shows gene flow among mosquito populations (Ayres *et al*., 2002); whereas the polymorphic pattern of DNA profile shows genetic variability from different areas (Ayres *et al*., 2003; Zahoor *et al*., 2013). Thus, it became necessary to elucidate the population structure of mosquitoes on genetic basis using molecular techniques in order to devise and launch a successful control program in specific areas.

Despite the epidemiological importance of mosquitoes, the studies on the genetic basis of mosquito species have been neglected in Pakistan. The mosquito-borne diseases have been also reported to increase in Punjab (Gilani, 2012). The outbreak of dengue fever in Lahore, Faisalabad, a few cases from Sialkot and some other cities, are the few examples. Therefore, studies are needed on molecular level to help combat mosquito borne diseases. Despite of few reported cases of dengue, district Sialkot has been neglected from studying mosquito populations. In the present study we investigated the genetic variability, gene flow, genetic exchange and migration in mosquito populations from urban and rural areas of Sialkot, Punjab, using random amplified polymorphic DNA (RAPD) technique.

Materials and Methods

Mosquito Collection

The rural and urban areas of district "Sialkot" were selected for the study due to high prevalence of mosquitoes. This district lies on south east to Gujrat district and southwest to Jammu district. It has 15,078 acres (61.02 km^2) of forest, 12.295 km of roads and 3229 industrial units where wheat and rice are frequently grown. About 28.52% of population of district is urban. It is hot and humid during summer and cold during winter. The land is generally considered as plain and very fertile.

A total of 14 populations were collected as from rural and urban area of Sialkot (Fig. 1 and Table 1). Adult mosquitoes were collected using sweep net and battery-operated aspirator (Herrel *et al*., 2001; Shortall *et al*., 2009; Qasim *et al*. 2014), while larvae and pupae of mosquitoes were collected from both natural and artificial breeding places (lawns, homes, tree holes, tires, waste material, stagnant water, sewer water, etc) using dipper method from each collection site (Nikookar *et al.,* 2010; Naeem-Ullah *et al*., 2010). The collected adult samples were preserved in 70% alcohol in vials labelled with date, collection site etc. and then stored at 4°C for DNA extraction. The larval forms of mosquitoes were reared in enamel trays in the laboratory. The emerged adults were collected and stored in plastic vials.

Identification of Mosquitoes

The collected adult samples containing 70% ethanol were identified up to the specie level on the basis of morphological characteristics using different taxonomic keys and the available literature (Harbach, 1985; Cranston *et al*., 1987; Rueda, 2004; Azari-Hamidian, 2009). A few samples were also identified by comparing against the samples present in "Entomology Lab", Department of Zoology, Wildlife and Fisheries, Faculty of Science and Technology, Government College University Faisalabad, Pakistan. Seven species were used for molecular study representing three the genera *Culex*, *Anopheles*, and *Aedes*.

DNA Extraction

Individual mosquitoes were homogenized in 400 µL of TNE buffer and then 100µL of 20µg/µL of Proteinase-K and 40 µL of 20% sodium dodecyl sulfate (SDS) were added. The homogenates were incubated at 55ºC for 1 h, 300 µL of 5 M 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 and kept at -21°C for 1 h and then centrifuged at 15,000 rpm for 10 min. DNA pellet was washed with 70% ethanol, air-dried and re-suspended in 50 µL of sterile water (d_3H_2O). Estimation of DNA concentration was made by measuring optical density (OD) at 260 nm and DNA quality was checked through 1% agarose gel electrophoresis (Batool, 2012; Ashraf, 2013; Zahoor *et al*., 2013).

RAPD-PCR

Gene Link-A series RAPD primers were used for PCR amplification (Table 2). Each PCR reaction was carried out in a final volume of 25 µL, containing 2.5 µL of genomic DNA, 3 mM of $MgCl_2$, 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 72ºC for 1.5 min, final extension at 72ºC for 10 min and then hold at 4°C until tubes were removed. The PCR products were run on 1.2–1.5% agarose gel electrophoresis at 80 voltages for 1 h (Batool, 2012; Ashraf, 2013; Zahoor *et al*., 2013).

Statistical Analysis of Data

The fingerprints were examined under UV Transilluminator and photographed by gel documentation system (SynGene).

The amplified bands (*loci*) were read against DNA marker. The fragments were scored as present (1) or absent (0) for each sample. Ambiguous bands which were not clearly distinguished were not scored. The bands were counted starting from top to the bottom in all lanes.

The RAPD markers were analyzed using the following assumptions: (1) The alleles (RAPD alleles) segregate following a Mendelian genetics fashion; (2) the bands which co-migrate are homologous; (3) different *loci* segregate independently and (4) populations are in Hardy-Weinberg equilibrium (Ayres *et al*., 2002). A dendrogram was constructed using unweighted pair-group mean analysis (UPGMA) (Nei, 1978). Effective migration rates (Nm) were estimated based on inbreeding indices (GST) where Nm= 0.5 (1-GST)/GST (McDermott and McDonald, 1993; Humeres *et al*., 1998). Calculations were performed with the help of the POPGENE (version 1.32) software.

Results

Fourteen population samples of *Anopheles*, *Culex* and *Aedes* mosquitoes were molecularly characterized to reveal their genetic variations using RAPD primers during the study. A total of 57 DNA fragments were generated with an average of about 6 bands per primer (Table 2). Fig. 2 shows the amplification profile of all samples with primer A-04. *Anopheles subpictus* from urban areas shows polymorphic band compared to *An*. *subpictus* to that from rural areas. Moreover, *Ae*. *albopictus* from urban and rural areas also showed a slight difference of banding pattern. Mostly, monomorphic fragments (48 fragments; 84%) were observed with low to moderate level of polymorphism. The percentage of polymorphism by each primer is shown in Fig. 3. A low degree of dissimilarity (monomorphic bands), indicated "low divergence". The samples of corresponding *Culex* species from urban areas compared to those from rural areas showed monomorphic banding pattern except for some samples collected from rural areas.

The gene diversity (Ht) ranged from 0.369 in Urban (U) populations to 0.401 in Rural (R) populations. The Gst value for the seven populations from urban areas was 0.113 with high rate of migration (Nm=4.014), the Gst for seven populations from rural areas of the district was 0.134 with low number of migrants (Nm=2.62). The overall genetic differentiation among 14 populations from urban and rural areas presented Gst =0.147 and Nm=3.73. An average genetic diversity in all populations of district Sialkot was 0.391 (Table 3).

Cluster Analysis

The dendrogram demonstrates two distinct groups of mosquito populations from urban and rural areas of Sialkot (Fig. 4). There are seven populations from urban areas of Sialkot, i.e. Pasrur park, Chawinda, Iqbal park Sialkot, Qila kalarwala, Kotli loharaan, Sambrial, Pindi bhago, Ban bajwa; and seven populations from rural areas i.e. Saiduwali, W. Sadhuan, Mundeke, Dheera Sadhna, Motra and Shazada (Table 1). Group 'A' showed eight subgroups (A1, A2, A3, A4, A5, A6, A7 and A8) representing the genetic distances among 10 species of *Culex* and *Aedes* from urban and rural areas, while group B consists of three subgroups (B1, B2 and B3) representing genetic relatedness among *Anopheles* spp, *Cx. quinquefaciatus* (R) is more closely related to other *Culex* species (R, U) and also genetically related to *Aedes* species. *Ae. albopictus* (U) is showing close relationship not only with *Aedes* (U), but also with *Aedes* species (U, R) and *Culex* species from both urban and rural areas, respectively. The same case is happened to be with *Cx. pseudovishnui* (R). It shows more genetic relatedness to *Cx. pseudovishnui* (U, R) and genetically distant to *Aedes* spp. In group B, genetic relationship is described among *Anopheles* species from urban and rural areas of Sialkot. *An. stephensi* (R) is closely

Table 1: Detail of Mosquito samples collected from Urban and Rural areas of District Sialkot

Area/village of district Sialkot	Type of Collection	Date of Collection	Code
Chawinda	Net/Aspirator	Aug./Sep. 2012	U1
Iqbal park Sialkot	Net/Aspirator	Aug./Sep. 2012	U2
Qila kalarwala	Net/Aspirator	Sep. 2012	U3
Kotli loharaan	Net/Aspirator	Sep. 2012	U4
Sambrial	Net/Aspirator	Sep. 2012	U5
Pindi bhago	Net/Aspirator	Aug./Sep. 2012	U6
Ban bajwa	Net/Aspirator	Sep. 2012	U7
Pasrur park	Net/Aspirator	Aug./Sep. 2012	R1
Saiduwali	Net/Aspirator	Aug./Sep. 2012	R2
W. Sadhuan	Net/Aspirator	Aug./Sep. 2012	R3
Mundeke	Net/Aspirator	Aug./Sep. 2012	R4
Dheera Sadhna	Net/Aspirator	Sep. 2012	R5
Motra	Net/Aspirator	Sep. 2012	R6
Shazada	Net/Aspirator	Aug./Sep. 2012	R7

Table 2: RAPD Primers and their sequences used for PCR

Primers	Nucleotide Sequence	Size (bp)	Number of amplified bands
GL-Decamer A-01	5'-CAG GCC CTT C-3'	250-2000	7
GL-Decamer A-02	5'-TGC CGA GCT G-3'	200-1600	8
GL-Decamer A-03	5'-AGT CAG CCA C-3'	240-1800	6
GL-Decamer A-04	5'-AAT CGG GCT G-3'	250-1200	5
GL-Decamer A-05	5'-AGG GGT CTT G-3'	250-2000	4
GL-Decamer A-06	5'-GGT CCC TGA C-3'	200-1200	9
GL-Decamer A-07	5'-GAA ACG GGT G-3'	240-1890	0
GL-Decamer A-08	5'-GAA ACG GGT G-3'	250-2500	6
GL-Decamer A-09	5'-GAA ACG GGT G-3'	210-1600	7
GL-Decamer A-10	5'-GGG TAA CGC C-3'	250-2000	5

Table 3: Nei's Analysis of Gene diversity among populations of Urban and Rural areas of Sialkot

Populations	Genetic variation Gst	Gene flow Nm	Heterozygosity Ht
Urban	0.113	4.014	0.3691
Rural	0.134	2.62	0.4019
Urban Rural	0.147	3.73	0.3914

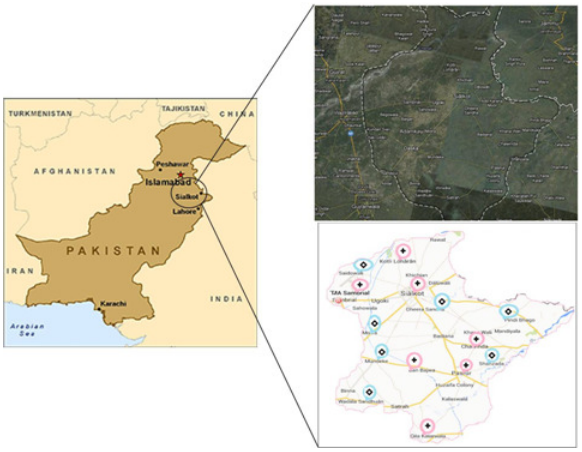

Fig. 1: The selected rural (◆) and urban (✚) areas of district Sialkot.

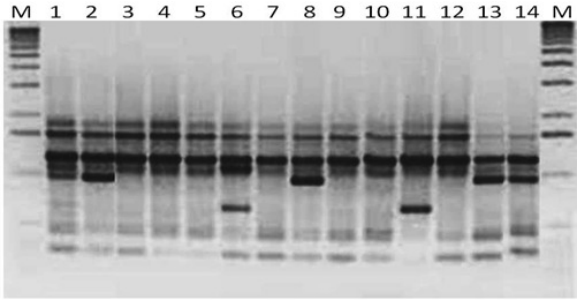

Fig. 2: Amplification profile of mosquitoes with primer A-04 by RAPD-PCR from urban and rural areas of Sialkot
1: *Ae. albopictus* (U); 2: *Ae. albopictus* (R); 3: *Ae. aegpyti* (U); 4: *Ae. aegpyti* (R); 5: *An. stephensi* (U) ; 6: *An. stephensi* (R); 7: *An. subpictus* (U); 8: *An. subpictus* (R); 9*: Cx. quinquefaciatus* (U); 10: *Cx. quinquefaciatus* (R); 11: *Cx. pseudovishnui* (U); 12: *Cx. pseudovishnui* (R); 13:*Cx. tritaeniorhynchus* (U); 14:*Cx.tritaeniorhynchus* (R); M: 1Kb Ladder

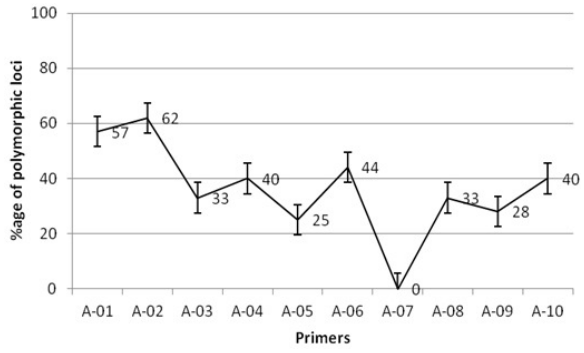

Fig. 3: Percentage of polymorphic *loci* amplified by each RAPD primer

related to *An. stephensi* (R), while there is low genetic similarity seen in *An. stephensi* from urban areas. *An. subpictus* showed more genetic similarity in this species. The maximum genetic dissimilarity of *An. stephensi* (R) is seen with *Culex* and *Aedes* species (U, R). In addition, both of the *Anopheles* spp. were genetically more conserved compared to the other species.

Discussion

Mosquito-borne diseases are rapidly spreading during the last decade, threatening thousands of people due to prevailing peculiar socio-economic conditions and epidemiological situation in Pakistan (Jahan *et al*., 2010). The humid environment, accumulation of water bodies in human dwellings, lifestyle and non-compliance of public could be the reason of mosquito prevalence in and around the city (Hoffmann and Willi, 2008; Idrees and Ashfaq, 2012; Attaullah, 2013). The dispersal due to transportation, indiscriminate use of insecticide and the elimination of natural and artificial breeding places had a significant effect on migration, genetic exchange and the genetic structure of mosquitoes (Paupy *et al*., 2000; Lerdthusnee and Chareonviriyaphap, 2002).

The genetic diversity in mosquito populations had been widely reported to exist and inferred through various molecular techniques (Franco *et al*., 2002; Paduan *et al*., 2006). Subsequent molecular studies have been conducted for mosquitoes in developed countries and being applied to control diseases spread by mosquitoes. However, in case of developing countries like Pakistan these studies are being continuously ignored and no commendable work on molecular characterization of mosquitoes has been reported yet (Hussain *et al*., 2011) except very few studies like Rasheed *et al*. (2013).

In this study, 14 mosquito populations were genetically analyzed and all the species showed genetic variability between urban and rural areas. The amplified fragments obtained were in the range of 200 bp to 2500 bp as defined by Ayres *et al*. (2002). Noteworthy, the RAPD primers clearly differentiate the populations from both urban and rural areas. Dendogram among *Aedes*, *Culex*, and *Anopheles* populations showed that *Anopheles* spp. had a distinct pattern with genetic variability. The *Culex* species from rural and urban areas are more closely related to each other while less closely related to *Aedes;* however, certain areas showed genetic similarity among populations due to transportation of mosquitoes from one area to the other (Rasheed *et al*., 2013). The genetic relatedness and Nm value shows high level of genetic variations in the population of mosquitoes from rural and urban areas of Sialkot. In addition, the diversity and the genetic data show that mosquitoes are freely moving between rural and urban areas.

The present results showed that *Ae. aegypti* from both urban and rural areas are genetically similar and have conserved pattern of genetic make-up; however, *Ae. albopictus* from rural areas is genetically diverse (Ht: 0.4019).

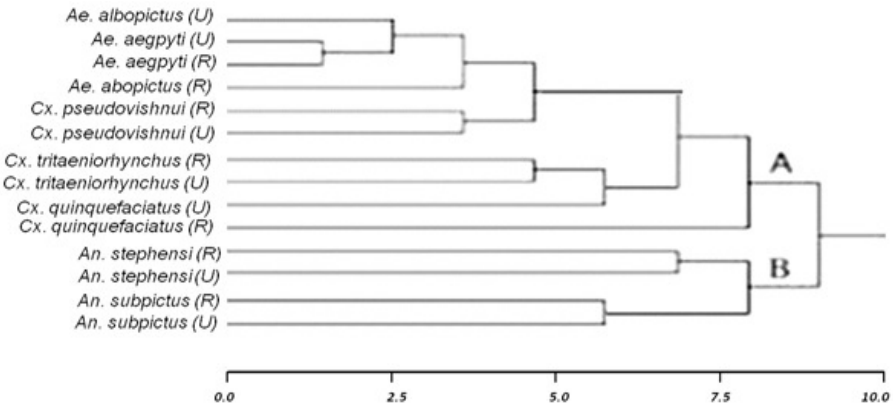

Fig. 4: Dendrogram based on Nei's genetic distances among *Aedes, Culex,*and *Anopheles* populations analyzed through RAPD-PCR

The relatedness and Nm (gene flow) value for an estimate of gene flow showed that *Ae. albopictus* (R) populations exhibit gene flow among populations (Gst: 0.113; 0.134). The data also suggested that it can be transported from rural to urban and vice versa (Nm: 4.014; 2.62) which is true for other mosquito species (Paupy *et al*., 2000; Lerdthusnee and Chareonviriyaphap, 2002; Rasheed *et al*., 2013). The overall heterozygosity (Ht = 0.391) observed in urban and rural areas of Sialkot was consistent as previously reported in Brazilian mosquito populations through RAPD markers (Apostol *et al*., 1996; Ayres *et al*., 2003).

The genetic diversity data showed that these mosquito populations were genetically less differentiated (GST = 0.147) with high gene flow (Nm=3.73). In contrast, Souza *et al*. (2001) described low migration rate in mosquitoes in Argentina (GST = 0.249; Nm = 0.75). However, Santos *et al*. (2011) studied four mosquito populations through RAPD technique and found high level of polymorphism. They distinguished 52 markers 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 a percentage of polymorphism varied from 25 to 62 (Fig. 3). Paduan *et al*. (2006) described a low genetic variation (GST = 0.208) with a higher degree of gene flow (Nm = 1.90). Similarly, the current study showed a genetic variation (GST = 0.113) with a high gene flow (Nm =4.014) in urban area; whereas population differentiation (GST = 0.134) with a moderate gene flow (Nm =2.62) found in rural area indicated low intra- and inter-population genetic variability in mosquito populations. However, further investigations are needed on large scale using gene specific markers in order to devise mosquito control program to combat mosquito-borne diseases.

Conclusion

Aedes aegypti from both urban and rural areas are genetically similar; however, *Ae. albopictus* from rural areas is genetically diverse but closely related to *Ae. aegypti*. *An. subpictus* from rural and urban areas also shows polymorphism. High gene flow (Gst) occurs due to high migration rate (Nm) and thus, low level of genetic variations exists in mosquito populations between both rural and urban areas. The findings suggest that mosquitoes are freely moving between rural and urban areas.

Acknowledgement

The facilities provided by Department of Zoology, Wildlife and Fisheries, GCUF and the financial support from HEC Project are highly acknowledged to conduct this research work. The authors declare that there is no conflict of interest.

References

Apostol, B.L., W.C. Black IV, P. Reiter and B.R. Miller, 1996. Population genetics with RAPD-PCR markers: the breeding structure of *Aedes aegypti* in Puerto Rico. *Heredity*, 76: 325–334

Ashraf, H.M., 2013. Studies on the comparative genetic analysis of *Aedes aegypti* populations from Lahore and Faisalabad using molecular DNA markers. *M.Phil. Thesis*, Department of Zoology, Wildlife and Fisheries, Government College University Faisalabad, Pakistan

Attaullah, 2013. Biodiversity, habitat preference and population dynamics of mosquitoes from rural areas of Faisalabad. *M.Phil. Thesis*, Department of Zoology, Wildlife and Fisheries, Government College University Faisalabad, Pakistan

Ayres, C.F.J., M.A.V. Melo-Santos, A.M. Sole-Cava and A.F. Furtado, 2003. Genetic differentiation of *Aedes aegypti* (Diptera: Culicidae), the major dengue vector in Brazil. *J. Med. Entomol*., 40: 430–435

Ayres, C.F.J., T.P.A. Romao, M.A.V. Melo-Santos and A.F. Furtado, 2002. Genetic diversity in Brazilian populations of *Aedes albopictus*. *Mem. Instrum. Oswaldo. Cruz*., 97: 871–875

Azari-Hamidian, S. and R.E. Harbach, 2009. Keys to the adult females and fourth-instar larvae of the mosquitoes of Iran (Diptera: Culicidae). *Zootaxa*, 2078: 1–33

Batool, F., 2012. Studies on the genetic variations in natural populations of *Drosophila melanogaster* using RAPD and Microsatellite markers. *M.Phil. Thesis*, Department of Zoology, Wildlife and Fisheries, Government College University Faisalabad, Pakistan

Cranston, P.S., C.D. Ramsdale, K.R. Snow and G.B. White, 1987. *Adults, Larvae and Pupae of British Mosquitoes (Culicidae). A Key*. Freshwater Biological Association, Cumbria

de Lourdes, M., M.R.F. Mercado-Curiel, A. Diaz-Badillo, G.P. Ramirez and W.C. Black IV, 2013. Gene Flow Pattern Among *Aedes aegypti* Populations in Mexico. *J. Amer. Mosquito Cont. Assoc*., 29: 1–18

Franco, F.G., M.D.L. Munoz, S.L. Fuentes, L.F Salas, J.G. Rejon, B.J. Beaty and W.C. Black, 2002. Large genetic distances among *Aedes aegypti* populations along the south pacific coast of Mexico. *Amer. J. Trop. Med. Hyg*., 6: 594–598

Gibbons, R.V. and D.W. Vaughn, 2002. Dengue: an escalating problem. *BMJ*, 324: 1563–1566

Gilani, I., 2012. 2,340 malaria cases reported. Dawn News, June 04, 2012. http://dawn.com/news/407357/malaria-plagues-faisalabad

Harbach, R.E., 1985. Pictorial keys to the genera of mosquitoes, subgenera of *Culex* and the species of *Culex* (*Culex*) occurring in southwestern Asia and Egypt, with a note on the subgeneric placement of *Culex deserticola* (Diptera: Culicidae). *Mosquito Sytematics*, 17: 83–107. Walter Reed Army Inst of Research Washington DC, USA

Herrel, N., F.P. Amerasinghe, J. Ensink, M. Mukhtar, W. Van Der Hoek and F. Konradsen, 2001. Breeding of *Anopheles* mosquitoes in irrigated areas of South Punjab, Pakistan. *Med. Vet. Entomol*., 15: 236–248

Hoffmann, A.A. and Y. Willi, 2008. Detecting genetic responses to environmental change. *Genetics*, 9: 421–432

Humeres, Silvia G., et al., 1998. Estimation of genetic divergence and gene flow between *Culex pipiens* and Culex quinquefasciatus (Diptera: Culicidae) in Argentina. *Memórias do Instituto Oswaldo Cruz*, 93: 57–62

Hussain, S., F. Malik, M.K. Ashfaq, G. Parveen, H.A. Abdul, S. Ahmad, H. Riaz, P. Akhtar and T. Saeed, 2011. Prevalence of self-medication and health-seeking behavior in a developing country. *Afr. J. Pharm. Pharmacol*., 5: 972–978

Idrees, S. and U.A. Ashfaq, 2012. A brief review on dengue molecular virology, diagnosis, treatment and prevalence in Pakistan. *Gen. Vacc. Ther*., 10: 6

Itrat, A., A. Khan, S. Javaid, M. Kamal, H. Khan et al., 2008. Knowledge, Awareness and Practices Regarding Dengue Fever among the Adult Population of Dengue Hit Cosmopolitan. *PLoS ONE*, 3: e2620

Jahan, N. and N. Mumtaz, 2010. Evaluation of resistance against deltmethrin in *Aedes* mosquitoes from Lahore, Pakistan. *Biol. Pak.*, 56: 9–15

Khan, E., M. Kisat, N. Khan, A. Nasir, S. Ayub et al., 2010. Demographic and clinical features of dengue fever in Pakistan from 2003–2007: A Retrospective cross-sectional study. *PLoS ONE*, 5: e12505

Lerdthusnee, K. and T. Chareonviriyaphap, 2002. Genetic differentiation of *Aedes aegypti* mainland and island populations from Southern Thailand. *J. Amer. Mosq. Contr. Assoc*., 18: 173–177

McDermott, J.M. and B.A. McDonald, 1993. Gene flow in plant pathosystems. *Annu. Rev. Phytopathol*., 31: 353–373

Murray, C.J., L.C. Rosenfeld, S.S. Lim, K.G. Andrews, K.J. Foreman, D. Haring, N. Fullman, M. Naghavi, R. Lozano and A.D. Lopez, 2008. Global malaria mortality between 1980 and 2010: a systematic analysis. *Lancet*, 379: 413–431

Naeem-Ullah, U., W. Akram, A. Suhail, S.A. Rana, 2010. Grouping of different mosquito species on the bases of larval habitats. *Pak. J. Agric. Sci*., 47: 124–131

Nei, M., 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, 89: 583–590

Nikookar, S.H., S.H. Moosa-Kazemi, M.A. Oshaghi, H. Vatandoost and A. Kianinasab, 2010. Species Composition and Diversity of Mosquitoes in Neka County, Mazandaran Province, Northern Iran. *J. Arthropod-Borne Dis*., 4: 26–34

Paduan, K.S., J.P. Araújo-Júnior and P.E.M. Ribolla, 2006. Genetic variability in geographical populations of *Aedes aegypti* (Diptera, Culicidae) in Brazil elucidated by molecular markers. *Genet. Mol. Biol*., 29: 391–395

Paupy, C., M. Vazeille-Falcoz, L. Mousson, F. Rodhain and A.B. Failloux, 2000. *Aedes aegypti* in Tahiti and Moorea (French Polynesia): isozyme differentiation in the mosquito population according to human population density. *Amer. J. Trop. Med. Hyg*., 62: 217–224

Qasim, M., M. Naeem and I. Bodla, 2014. Mosquito (Diptera: Culicidae) in Murree Hills from Punjab Province of Pakistan. *Pak. J. Zool*., V.46: 523–529

Raheel, U., M. Faheem, M.N. Riaz, N. Kanwal, F. Javed, N.S. Zaidi and I. Qadri, 2011. Dengue fever in the Indian subcontinent: an overview. *J. Infect. Dev. Countries*, 5: 239–247

Rasheed, S.B., M. Boots, A.C. Frantz and R.K. Butlin, 2013. Population structure of the mosquito *Aedes aegypti* (*Stegomyia aegypti*) in Pakistan. *Med. Vet. Entomol*., 27: 430–440

Ravel, S., J.P. Herve, S. Diarrassouba, A. Kone and G. Cuny, 2002. Microsatellite markers for population genetic studies in *Aedes aegypti* (Diptera: Culicidae) from Côte d'Ivoire: evidence for a microgeographic genetic differentiation of mosquitoes from Bouaké. *Acta Trop*., 82: 39–49

Rueda, L.M., 2004. Pictoral key for the identification of mosquito (Diptera: Culicidae) associated with dengue virus transmission. *Zootaxa*, 589: 1–60

Santos, J.M.M., E.C. Fraga, J.F. Maia and W.P. Tadei, 2011. Genetic Diversity in Dengue Mosquito, *Aedes aegypti* (Diptera: Culicidae) from Amazon Region: Comparative Analysis with Isozymes and RAPD Loci. *Open Trop. Med. J*., 4: 11–20

Scarpassa, V.M., T.B. Cardoza and R.P. Cardoso-Júnior, 2008. Population genetics and phylogeography of *Aedes aegypti* (Diptera, Culicidae) from Brazil. *Amer. J. Trop. Med. Hyg*., 78: 895–903

Schulze, C.H., M. Waltert, P.J.A. Kessler, R.P. Shahabuddin, D. Veddeler, M. Mühlenberg, S.R. Gradstein, C. Leuschner, I. Steffan-Dewenter and T. Tscharntke, 2004. Biodiversity indicator groups of tropical land-use systems: Comparing plants, birds and insects. *Ecol. Appl*., 14: 1321–1333

Shortall, C.R., A. Moore, E. Smith, M.J. Hall, I.P. Woiwod and R. Harrington, 2009. Long-term changes in the abundance of flying insects. *Insect Conserv. Divers*., 2: 251–260

Souza, G. B. de., A. Blanco and C.N. Gardenal, 2001. Genetic relationships among *Aedes aegypti* (Diptera: Culicidae) populations from Argentina using random amplified polymorphic DNA polymerase chain reaction markers. *J. Med. Entomol*., 38: 371–375

WHO, 2009. Dengue guidelines for diagnosis, treatment, prevention and control. New edition. Geneva, Switzerland

WHO, 2014. Dengue and Severe Dengue. *Fact sheet N* 117. Available at: http://www.who.int/mediacentre/factsheets/fs117/en/

Zahoor, M.K., A. Suhail, S. Zahoor, A. Iqbal and F.S. Awan, 2013. Molecular characterization of Scarab beetles (Scarabaeidae: Coleoptera) using RAPD markers. *Pak. J. Life Soc. Sci*., 11: 238–243

(Received 08 August 2014; Accepted 31 December 2014)