Available online freely at www.isisn.org

Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2022 19(2): 1000-1006. **OPEN ACCESS**

PCR based identification of mosquitoes species in the Western belt of Khyber Pakhtunkhwa, Pakistan

Khan Niaz Khan[1], Muhammad Ali[1]and Muhammad Zahid[2]

[1]Institute of Biotechnology and Microbiology Bacha khan University Charsadda KP, **Pakistan**
[2]Department of Zoology Islamia college University Peshawar kp, **Pakistan**

***Correspondence:** knk.edwardian@gmail.com **Received 11-04-2022, Revised:18-05-2022, Accepted: 19-05-2022 e-Published: 20-05-2022**

The study was conducted in selected districts of Western belt of KP. The molecular identification of 14 species of *Culex, Anopheles* and *Aedes* was done using RAPD (Random Amplified Polymorphic DNA) PCR in order to determine their genetic variance. 52 fragments of DNA were produced with 5 bands as an average; indicate the polymorphism when bands of *An. subpictus* (U) were compared with *An. subpictus* (R). Similarly, a little variation in banding pattern of *Ae. albopictus* was seen in urban and rural species. Lower level of polymorphism was observed in many monomorphic fragments. Gene diversity range was calculated as 0.296 to 0.412 both in urban and rural species. Moreover, the genetic variation Gst for urban and rural species was calculated as 0.114 and 0.130 respectively. The value for migration rate in urban species was more than in rural species. (Nm=4.11 for U and 2.89 for R). According to dendogram, group A is the largest group which is further divided into 8 sub-groups and showing genetic distance among 10 species of *Aedes* and *Culex* both from rural and urban areas of the belt, whereas group B is the smaller one which is divided into further 3 sub-groups showing genetic distance among *Anopheles* species. The dendogram revealed a close relation among *Cx. quinquefaciatus* and both urban and rural *Culex* species. Similarly, *Cx.pseudovishnui* (R) possses genetic association with *Cx. Pseudovishnui* (U) and genetic detachment to *Aedes* species. In group B, genetic association was found to be existed among rural and urban species of *Anopheles* found in the western belt of KP. The dendogram revealed a weak association among *An. stephensi* R and U species whereas a strong association was existed among *An. subpictus* species. Moreover, *An. stephensi* (R) exhibited more genetic divergence from *Aedes* and *Culex* species with rural and urban origin. *An. stephensi* and *An. subpictus* were found to be more preserved naturally in comparison to other.

Keywords: Diversity, Mosquitoes, PCR, Molecular Identification, Western Belt

INTRODUCTION

Insects are the world's biggest and most diversified creatures. The number of species described, reaches close to one million in about 30 orders. Scientists believe that 20 million can be exceeded. These amazing animals make for roughly 75% of all animal species documented and are very abundant. Insects are present in soil, water and air in almost every environment and on every continent, even Antarctica .Flies and mosquitoes are essential elements in the food chains of ecosystems due to the diversity of their eating habits and as prey (Bouchet,20060). First, with the paradox of anophelism without malaria, the necessity to comprehend the variety of Anopheles mosquitoes became apparent, as it became obvious that there is an enormously varied number of *Anopheles* species and that not every species transmits malaria (Morgan et al.2013) In Southeast Asia, the variety of anopheline mosquito fauna is higher than in any other place on earth and, for some, the cryptic species complexes, at least 19 species are known to be contributing significantly to the transmission of malaria. Of the 24 known Anopheles complexes in Asia, 50% underline the complexity of the variety within the Asian continent, 13%, 4% and 21 % respectively, are in the Americas, Africa, Australia-Pacific and Europe (Obsomer et al.2007). In this research study different species of the mosquitoes have been document from various parts of the world. Nearly, 113 species are collected from the Asian country, Bangladesh. Around 22 Culex, Aedes and Anopheles species are taken from the neighbouring country India. On the same pattern, 25 species of mosquitoes are gathered from Southern Israel. As for the rest of the mosquitoes, they were collected from around the world, including 39 species of Egyptian mosquitoes (Hayes 2019); between 1934 and 1971, Pakistan's mosquito fauna grew by three *Culex* species and one *Anopheles* species identified 16 mosquito species in Pakistan. Pakistan has twenty-four *Anopheles* species (seventeen of the subgenus *Cellia* and seven of the subgenus *Anopheles*) (Qasim et al.2014) identified 30 mosquito species in Changa Manga Lahore, including *Cx. tritaeniorhynchus, Cx. Quinquefasciatus,* and

Aedeslineatopennis, which are responsible for West Nile virus transmission. In Lahore, Pakistan, twenty-nine mosquito species were discovered for the first time (Qasim et al.2014).

MATERIALS AND METHODS

2.1 Sampling method

A simple random sampling approach was used to identify all eligible biotopes across the research region. Adult mosquitoes were taken from human dwellings (indoor) and their surroundings (outdoor), whilst mosquito larvae were obtained from containers, stagnant pools, discarded tyres, cesspits, domestic runoffs, and tree holes.

2.2 Processing of traps for molecular characterization

For molecular characterization the trap bags were frozen at -20°C overnight and mosquitoes collected were removed from the trap bag carefully to avoid damaging the specimens. Enamel trays were used for rearing the larval forms of the mosquitoes. The reared adults were shifted and preserved in the plastic vials.

2.3Primer designing

From different databases (NCBI, EMBL, Vector base etc.) the gene sequences of CO1 were recovered. To align all the sequences, the multi-align interface page (http://multalin.toulouse.inra.fr/multalin) was used. Several of the most desirable sequences were considered. A 422bp fragment between nucleotides 211 and 623 was chosen for primer construction using vector NTI (Version advance 11) (Zahir et al.2021). The CO1 gene was retraced using CO1 forward (CO-F) and CO1 reverse (CO-R) primers.9 Chappell, 1997)

2.4 DNA extraction and DNA amplification

The DNA was obtained from the hind leg of the dried mosquitoes. Minor modifications were adopted to optimize the procedure.

To determine the mosquitoes phylogeny, we used gene-specific primers to amplify the mitochondrial gene cytochrome oxidase I. (COI). To optimize the PCR conditions, various volumes of PCR ingredients were used, including NTPs (0.4 l, 0.8 l, 1 l, and 1.5 l), DNA (0.5 l, 1, 1.5 l, and 2 l), $MgCl_2$ (1 l, 1.5 l, 2 l, and 2.5ul), DMSO (1%, 2%, 3%, and 4%), and primers (0.5 l, 0.75 l, and 1l) (Christian,2018) The following components were included in a total optimized reaction volume of 20l: 10.4 liters PCR grade water, 0.4 liters dNTPs, 2 liters 10X T*aq* buffer, 1.6 liters $MgCl_2$, 1-liter forward primer (CO1-F), 1-liter reverse primer (CO1-R) 1 liter T*aq*, 0.5 liter polymerase, and 2 liter template DNA. The optimized thermal profile consisted of an initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 10 minutes

Using a 2% agarose gel pre-stained with Ethidium bromide, a small amount of the amplified product (6-8l) was detected,. Gel documentation was performed using the Uvi-Tech Gel Documentation System, and sequencing was performed using the remaining PCR product (12-14l) of the amplified gene, By using the big dye chain termination method.

2.5 Diversity data and statistical analysis

Diversity of mosquitoes and their statistical analysis was interpreted by using Relative abundance, Species richness, Simpson diversity index and Shannon-wiener diversity index SPSS version 15 was used for statistical analysis. The value of P less than 0.05 was conceived statistically significant (Elumalai et al.2020)

RESULTS

3.1 Molecular analysis

The molecular identification of 14 species of *Culex, Anopheles* and *Aedes* was carried out using RAPD (Random Amplified Polymorphic DNA) PCR. A total of 52 fragments of DNA were produced with 5 bands as an average. The primers used in PCR are given in table 3.1

Table 3.1: Primers used in PCR

Primer	Nucleotide sequence	Size (base pair)	No of bands amplified
GL-Decamer A-01	F-5'-CAG GCC CTT C-3'	250-2100	5
GL-Decamer A-02	F-5'-TGC CGA GCT G-3'	240-1800	7
GL-Decamer A-03	F-5'-AGT CAG CCA C-3'	220-1800	8
GL-Decamer A-04	R-5'-AAT CGG GCT G-3'	200-1260	10
GL-Decamer A-05	F-5'-AGG GGT CTT G-3'	250-2160	6
GL-Decamer A-06	F-5'-GGT CCC TGA C-3'	280-1400	8
GL-Decamer A-07	R-5'-GAA ACG GGT G-3'	280-1820	4
GL-Decamer A-08	R-5'-GAA ACG GGT G-3'	230-2400	8
GL-Decamer A-09	F-5'-GAA ACG GGT G-3'	270-1480	6
GL-Decamer A-10	F-5'-GGG TAA CGC C-3'	220-1800	0

The amplification profile of all the samples is shown in the figure 3.1. It clearly indicates the polymorphism when bands of *An. subpictus* (U) were compared with *An. subpictus* (R). Similarly, a little variation in banding pattern of *Ae. albopictus* was seen in urban and rural species. Lower level of polymorphism was observed in many monomorphic fragments. The polymorphism shown by

every primer is mentioned in figure 3.2

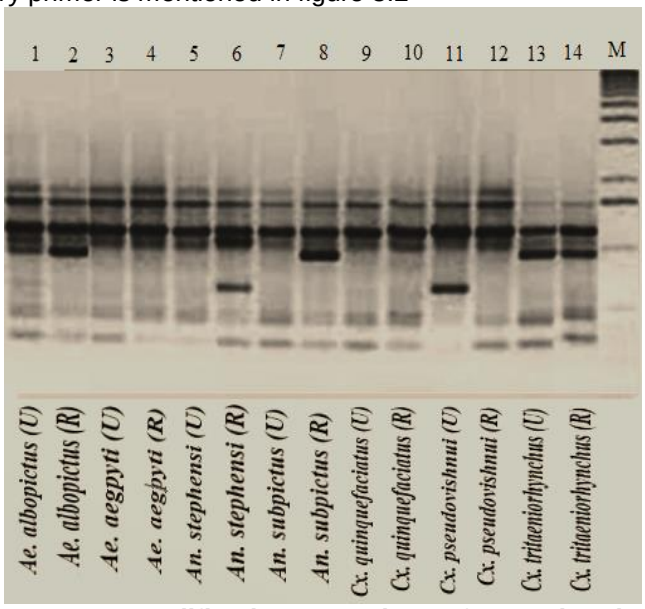

Figure 3.1. Amplification overview of samples by RAPD-PCR

From the figure 3.3 below, one can easily assess that the monomorphic bands are divergent from one another. Similarly, monomorphic bands were also seen in *Culex* species collected both from urban as well as rural areas excepting a few one that exhibited polymorphic banding fashion.

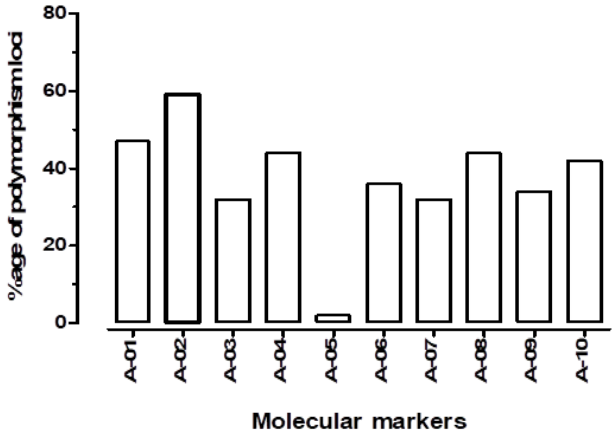

Figure 3.2: Polymorphic loci % (amplification done by each RAPD-primer)

As for as gene diversity is concerned, the range was calculated as 0.296 to 0.412 both in urban and rural species. Moreover, the genetic variation Gst for urban and rural species was calculated as 0.114 and 0.130 respectively. The value for migration rate in urban species was more than in rural species. (Nm=4.11 for U with *P*<0.001 and 2.89 for R) with *P*<0.001 (Table 3.2).

Table 3.2: Gene diversity analysis in urban and rural species

Species	Gst	Nm	Gene diversity
Urban (U)	0.114	4.11	0.296
Rural (R)	0.130	2.89	0.412
Urban Rural	0.141	3.80	0.374

3.2 Cluster study

The dendogram constructed showed two groups of species collected from both urban and rural areas of Western belt of KP. The species were classified and identified as 7 from urban and 7 from rural areas of the entire belt. According to dendogram, group A is the largest group which is further divided into 8 sub-groups and showing genetic distance among 10 species of *Aedes* and *Culex* both from rural and urban areas of the belt, whereas group B is the smaller one which is divided into further 3 sub-groups showing genetic distance among *Anopheles* species. The dendogram revealed a close relation among *Cx. quinquefaciatus* and both urban and rural *Culex* species. Similarly, *Cx.pseudovishnui* (R) possses genetic association with *Cx. Pseudovishnui* (U) and genetic detachment to *Aedes* species. In group B, genetic association was found to be existed among rural and urban species of *Anopheles* found in the western belt of KP. The dendogram revealed a weak association among *An, stephensi* R and U species whereas a strong association was existed among *An. subpictus* species. Moreover, *An. stephensi* (R) exhibited more genetic divergence from *Aedes* and *Culex* species with rural and urban origin. *An. stephensi* and *An.subpictus* were found to be more preserved naturally in comparison to other species.

3.3 Species Richness

Margelel's index was used to calculate species richness. Table 3.3 displays the abundance, Margelel's index (Dmg), Shannon index (H), and Simpson index (D) results.

The species abundance in July was 312 with 3.14 Dmg (Margelel's index), 2.22 H (Shannon index) and 0.6 D (Simpson index). In August abundance was 540 with 4.10 Dmg, 1.90 H and 0.8 D, followed by September with 460 abundance and 4.0 Dmg, 1.90H nd 0.8 D. October shows abundance as 90 with 2.22 Dmg, 0.88 H and 0.10 D and 54 abundance in November with 1.90 Dmg, 0.66 H and 0.20 D. December showed 66 adundance with 1.98 Dmg, 0.72 H and 0.25 D. January showed no abundance, Margelel's, Shannon and Simpson indexes. February showed the lowest in all abundance as 3 with 0.40 Dmg, 0.30 H and 0.42 D. March had 44 abundances with 1.78 Dmg, 0.70 H and 0.18 D followed by April with 80 abundance and 1.98 Dmg, 0.90 H, 0.10 D. May and June both had abundance of 180 with 2.56 Dmg, 1.24 H and 0.44 D

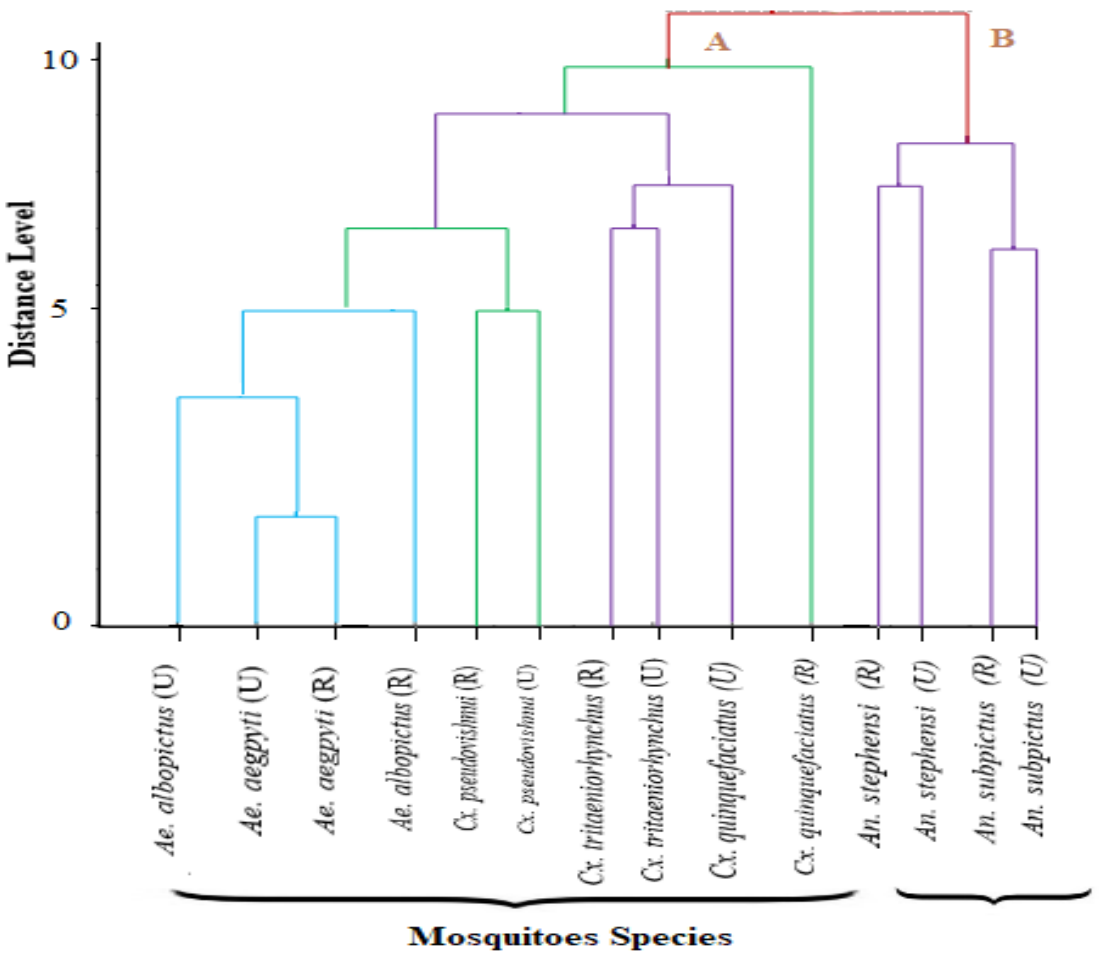

Figure 3.3: Dendogram constructed using unweight pair-group mean analysis (UPGMA) showing genetic distance analyzed through RAPD PCR.

Table 3.3: Monthly analysis of mosquito species composition collected from various towns in Western belt of KP.

Months	Abundance	Margelel's index (Dmg)	Shannon index (H)	Simpson index (D)
July	312	3.14	2.22	0.6
August	540	4.10	1.90	0.8
September	460	4.0	1.56	0.10
October	90	2.22	0.88	0.14
November	54	1.90	0.66	0.20
December	66	1.98	0.72	0.25
January	0	0	0	0
February	3	0.40	0.32	0.42
March	44	1.78	0.70	0.18
April	80	1.98	0.90	0.10
May	180	2.56	1.24	0.44
June	180	2.56	1.24	0.44

DISCUSSION

In this study, overall, 14 species belonging to 3 different genera (*Culex*, 6 species, *Aedes*, 4 and *Anopheles*, 4 species) were identified that were collected from both urban and rural areas of the study scenario. In a study that was conducted in Murree-Pakistan, 13 species were mainly identified that were collected from the various localities of the Murree. The most abundant species were *Aedesaegypti, Armigerusobturbans* and *Cx. Fuscitarsis* respectively followed by *Cx. Nilgiricus* and *Cx. vagans*. The all reported species in Murree were the same as reported by excepting one species that was *Cx. raptor.* The aforementioned study revealed all the collected species majorly in the month of July to November whereas April was the month in which, minimum number of species were collected. The months of January, February, March and December were the one in which no sampling was done because of rainfall and snowfall (Khan et al. 2022)

In a study that was conducted in Thailand, SNP (single nucleotide polymorphism) markers were incorporated in order to analyze the genetic variability and populace diversity of *Ae. aegypti* in four recognized neutral fragments i.e. *Tsf* (Transferrin), *AelMUC1*(Mucin-like protein), *ApoLp-2* (Apolipophorin II) and *CPA* (Carboxypeptidase A) (Steffler et al.2016). The location of *Tsf* gene is on chromosome I and is responsible for Fe transportation. The location of AelMUC1 gene is on chromosome II and is found to be involved in immune actions. The involvement of *ApoLp-2* gene which is located on chromosome III is found to be linked with biosynthesis of neutral fats as well as transportation of lipoprotein. Finally, the *CPA* gene which is known to be existed on chromosome II is responsible for protein degradation in the GI tract. The 4 evaluated genes were carefully selected on the basis of a previous report which demonstrated an appropriate marker for *Ae. Aegypti* genetic study. Apart from that, the PCR amplification reliability was also a criterion for the selection of markers. (Platten et al.2019)

In a study that was conducted in China, *Ae. albopictus* species displayed common gene flow with the inhabitants it was demonstrated that *Ae. albopictus* from Hainan only presented recurrent gene flow with the Yunnan inhabitants and that gene exchange between Hainan and Guangdong or Fujian populations was obstructed(Fang et al.2018)

The current study shows genetic resemblance among *Ae. aegypti* rural and urban species with having preserved pattern of genome but in contrast, the rural species of *Ae. albopictus* is divergent (Gd=0.412). The gene flow (Nm) value as well as the resemblance value for the assessment of gene flow showed that *Ae. Albopictus(*R) populations exhibited gene flow among populations (Gst: 0.114; 0.130). The data demonstrated their travel between rural and urban areas (4.22:2.89) and it is factual for other species. The overall gene diversity was found to be 0.389 observed in rural and urban areas and it was in consistence with a Brazilian study that was conducted through RAPD markers (Vianna et al.2020)

In order to differentiate isolated mosquitoes population genetically and geographically, the RAPD markers technique is a powerful and an efficient method. This method incorporates the PCR principle for amplification of DNA sequences (Aputri et al.2021)The RAPD-PCR is a leading molecular marker that cannot distinguish between homozygotes and heterozygotes .(Al-Hadeithi, and Jasim 2021) These markers help in studying a huge number of loci and facilitate a random sampling of DNA and thus, show higher level of polymorphism in comparison to protein markers and RFLP (Hasan et al.2021). Yet, these markers have remarkable limitations in comparison to micro satellites and mtDNA. (Caballero et al.2022)

The highest mosquito diversity (H= 2.24) was reported in August in a study conducted in Lahore due to the availability of high rainfall and suitable humidity. This, in turn, increases the spread of vector-borne diseases. The least number of rare species were recorded in August due to the highest diversity of mosquitoes. Similarly, September had the highest species richness (Dmg= 4.17)[19]. During these months, high water temperatures promote larval development, making mosquito survival more likely. The highest mosquito diversity (H=2.22) was recorded in our study during the month of July-16, which is consistent with the findings of the aforementioned study. Similarly, the highest species richness was found in August (Dmg=4.10), which is consistent with the above study's findings.

CONCLUSION

The outcomes of this study concluded the morphological and molecular characterization, prevalence, and incidence of numerous mosquitos' species in various districts of western belt of KP-Pakistan. In this study, overall, 14 species belonging to 3 different genera (*Culex*, 6 species, *Aedes*, 4 and *Anopheles*, 4 species) were identified on molecular basis that were collected from both urban and rural areas of the study sites. (Manzoor et al.2020)]The results of our findings revealed the presence of *Culex* genera in all the habitats and remained the dominant one among the others followed by *Anopheles, Aedes* and *Psorophora .*

The current study shows a genetic resemblance among some of the rural and urban species of mosquitoes with having a preserved pattern of genome. The molecular characterization of 14 species of *Culex, Anopheles* and *Aedes* was done using RAPD PCR in order to determine their genetic variance (Iyiola, O.A., 2020)52 fragments of DNA were produced with 5 bands as an average. During this research work, the analyzed species displayed genetic variability among both rural and urban localities. The amplified products that were obtained, possessed a range

between 200-2500bp.. It is obvious from the Dendogram that a genetic similarity among certain areas is there due to the travelling of mosquitoes from one locality to the other. A significant genetic variability among rural and urban area mosquitoes is prominent from their genetic relation and Nm (gene flow) value and the fact is supported by their unrestricted movement.

Based on our research in Western belt of KP, we recommend a more comprehensive and detailed study having larger number of mosquitoes from the untouched areas in the country to compare their molecular characterization with the one investigated during this current research work. Techniques other than RAPD should be applied if feasible and available. More areas and more samples collection will be a helping hand in exploring the actual picture of the diversity of mosquitoes throughout the country.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

ACKNOWLEDGEMENT

The research article is part of the PhD thesis of the author. The author is highly thankful to the Edward College Peshawar for providing Faculty development fund.

AUTHOR CONTRIBUTIONS

Khan K.N designed and performed the experiments. Ali M wrote the manuscript. Zahid M data analysis and reviewed the manuscript. All authors read and approved the final version.

REFERENCES

Al-Hadeithi, Z.S. and S.A. Jasim, *Study of Plant Genetic Variation through Molecular Markers: An Overview.* Journal of Pharmaceutical Research International, 2021. **33**(45B): p. 464-473.29.

Aputri, F. N., Hanum, L., Pratama, R., & Windusari, Y. Analysis of Mosquito Genetic with PCR-RAPD Approach. *IEEE International Conference on Health, Instrumentation & Measurement, and Natural Sciences,* 2021 (pp. 1-6).

Bouchet, P., *The magnitude of marine biodiversity.* The exploration of marine biodiversity: scientific and technological challenges, 2006: p. 31-62.

Caballero-Huertas, M., et al. *The current knowledge status of the genetic population structure of the European sardine (Sardina pilchardus): uncertainties to be solved for an appropriate fishery management.* Reviews in Fish Biology and Fisheries, 2022: p. 1-19.

Chappell, J. D. *Structural and functional analysis of the reovirus attachment protein, sigma-1*. 1997 Vanderbilt University.

Christian, R. N. *The effects of pyrethroid resistance on transcription of metabolic enzymes in a major african malaria vector, anopheles funestus.* 2011. Doctoral dissertation, Faculty of Health Sciences, University of the Witwatersrand.

Elumalai, K., et al. *Entomofaunal survey and larvicidal activity of greener silver nanoparticles: A perspective for novel eco-friendly mosquito control.* Saudi Journal of Biological Sciences, 2020. 27(11): p. 2917-2928.

Fang, Y., et al. *Genetic polymorphism study on Aedes albopictus of different geographical regions based on DNA barcoding.* BioMed research international, 2018. 2018.

Hasan, N., Choudhary, S., Naaz, N., Sharma, N., & Laskar, R. A. Recent advancements in molecular marker-assisted selection and applications in plant breeding programmes. 2021. *Journal of Genetic Engineering and Biotechnology*, *19*(1), 1-26.

Hayes, C.G., *West nile fever*, in *The arboviruses: epidemiology and ecology*. 2019, CRC Press. p. 59-88.

Iyiola, O.A., *Genetic Diversity and Molecular Characterization of Mosquitoes (Diptera: Culicidae) In North-Central Nigeria Using Ribosomal DNA ITS2 and Mitochondrial 16S-DNA Sequences: Oluyinka A. Iyiola1*, Rahmat D. Shaibu1, Olalere Shittu2, Olukayode J. Adelaja3, Aishat T., Kamaldeen-Ibrahim1, Temitope O., Fadipe4, Ademola E. Alaba1, 5, Seun F. Adejuwon1, Bashirudeen O. Oyinlola1.* The Iraqi Journal of Veterinary Medicine, 2020. 44(2): p. 78-91.

Khan, K.N., et al. *Diversity of Mosquitoes Collected from the Southern Areas of Khyber Pakhtunkhwa Pakistan.* Journal of Gandhara Medical and Dental Science, 2022. 9(1): p. 3-8.

Manzoor, F., et al. *Determination of Species Composition of Mosquitoes in Lahore, Pakistan.* Journal of Arthropod-Borne Diseases, 2020. 14(1): p. 106.

Morgan, K., P. Somboon, and C. Walton, *Understanding Anopheles diversity in Southeast Asia and its applications for malaria control.* Anopheles mosquitoes-new insights into malaria vectors, 2013. 327: p. 355.

Näslund, J., et al. *Emerging Mosquito-Borne Viruses Linked to Aedes aegypti and Aedes albopictus: Global Status and Preventive Strategies.* Vector-Borne and Zoonotic Diseases, 2021. 21(10): p. 731-746.

Obsomer, V., P. Defourny, and M. Coosemans, *The Anopheles dirus complex: spatial distribution and environmental drivers.* Malaria journal, 2007. 6(1): p.

1-16.

Platten, J.D., J.N. Cobb, and R.E. Zantua, *Criteria for evaluating molecular markers: Comprehensive quality metrics to improve marker-assisted selection.* PloS one, 2019. 14(1): p. e0210529.

Qasim, M., M. Naeem, and I. Bodlah, *Mosquito (Diptera: Culicidae) of Murree Hills, Punjab, Pakistan.* Pakistan Journal of Zoology, 2014. 46(2).

Steffler, L.M., et al. *Genetic variability and spatial distribution in small geographic scale of Aedes aegypti (Diptera: Culicidae) under different climatic conditions in Northeastern Brazil.* Parasites & vectors, 2016. 9(1): p. 1-9.

Vianna, R., et al. *An NGS-based HLA haplotype analysis and population comparison between two cities in Rio de Janeiro, Brazil.* HLA, 2020. 96(3): p. 268-276.

Zahir, F., et al. *Epidemiological characteristics and genetic diversity of clinically isolated dengue vector in Khyber Pakhtunkhwa, Pakistan.* Clinical Epidemiology and Global Health, 2021. 12: p. 100863.