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Morphological and Molecular Identification of *Aedes Aegypti* through Genetic Marker Mitochondrial Encoded Cytochrome C oxidase I (MT-COI) Gene in Pakistan

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Abstract

Aedes aegypti are slender, fragile, long-legged mosquito, which are distributed worldwide. In general, it is a terrestrial and nocturnal species that act as pollinator for few plant species as well as it is a hematophagous insect and act as main vector for many arboviruses (dengue, yellow fever, etc.). In many cities of Pakistan, dengue fever is becoming more prevalent as an infectious disease. as there is currently no vaccine for the dengue virus and control of the disease depends on vector control. The present study aims to investigate morphological and molecular identification of *Aedes aegypti* in district Buner. A total of 365 samples of mosquitoes were randomly collected from six tehsils of the district Buner. Before examination, all the collected samples were preserved in labelled Eppendorf tubes containing silica gel and transported to Veterinary Research & Disease Investigation Center (VR&DIC), Balogram Swat, Khyber Pakhtunkhwa, Pakistan for further laboratory analysis. Morphological identification was performed with the help of stereomicroscope using taxonomical keys. To increase identification accuracy, each sample was reconfirmed by PCR using mitochondrial Cytochrome oxidase first gene (COI). The amplified PCR product was sequenced, and the obtained sequences were trimmed by Bio Edit and BLAST in NCBI. After BLAST, phylogenetic tree was constructed by using MEGA-X. In phylogenetic tree, the sequence (COI) genes of *Aedes aegypti* species as previously reported from Sri Lanka and India. Furthermore, the results of this study will generate basic information about mosquitos spp; and

it will help to understand the epidemiology of *Aedes aegypti* disease in Khyber Pakhtunkhwa, especially in district Buner, Khyber Pakhtunkhwa, Pakistan.

Keywords: Aedes aegypti, Buner, cytochrome oxidase-I, MT-COI, mosquitos, genetic marker

1. Introduction

The word mosquito is come from Spanish language, which means little fly. It has slender, fragile, long-legged insects, with usually size of 3-6 mm in length. (CDC, 2020). it can be differentiating from other flies through its body part including its proboscis and hair like scale on the thorax and wings (Gullan *et al.*, 2014). It enjoys cosmopolitan distribution, Although the only area form which they are absent are some islands like Antarctica and few islands (Dhakane *et al.*, 2021). Mostly mosquitoes are terrestrial as well as nocturnal species and act as a vector (Aziz, 2023). In all over the world, there are approximately 3,581 species of mosquitoes has recorded (mosquito-taxonomic-inventory.myspecies.info, n.d.).

Like other insects, mosquitoes have small body and consists of 3 regions i.e., head, thorax and abdomen. The head region consists of paired compound eyes, mouth parts and a pair of antennae. Antennae in male are plumose (feather-like) while in female are pilose (hairy-like). Thorax are cover dorsally and laterally with scales of different colors, and containing three pair on appendages and two pair of wings. Only the forewings are functional used in flight while the hindwings are in form of small knob like halters which help in flight as a balancing organ. The abdomen is typically consisting of 10 abdominal segments, out of which first seven or eight are visible. The last abdominal segment of the female mosquito terminates in a small finger like cerci where as in male a pair of prominent claspers (Gullan *et al.*, 2014).

Mosquitoes act as a pollinator for few plant species and plays a vital role in food web and act as primary food source for many birds, bats and aquatic larva (Mizejewski, 2020). Similarly, mosquitoes also act as an ectoparasites, Although *Aedes aegypti* is hematophagous insect and main vector for many arboviruses (dengue, yellow fever, etc.) (Escobar *et al.*, 2022). According to the World Health Organization, between 50 and 100 million dengue cases are reported worldwide each year. (Ali *et al.*, 2016).

Dengue virus is causative agent of dengue fever Which belong to family Flaviviridae and genus Flavivirus, however five different species of genetically distinct serotypes e.g., DENV 1, DENV 2, DENV 3, DENV 4 and DENV 5 has been recognized (Khan *et al.*, 2017). A complicated disease, dengue fever can range in severity from minor symptoms like dengue fever and flu-like discomfort to more serious illnesses like hemorrhagic fever. A high fever of 40 °C, a severe headache, muscular and joint discomfort, swollen glands, nausea, pain in the eyes, and vomiting are possible symptom (World Health Organization, 2019).

In Pakistan, first outbreak of Dengue was reported from Karachi in 1994, after that its outbreak was become prominent in all over Pakistan (Khan *et al.*, 2017). Outbreak of dengue fever required three main factors e.g., *A. aegypti* (its vector), the dengue virus and huge number of susceptible human hosts. In recently decade, entomologist develops a number of taxonomical keys which are based on morphology of *A. aegypti*. However, to increase identification accuracy, molecular biology develops numerous tools which perform accurate identification as well as its helps in studies of genetic variation due to continuous used of insecticides and ecological changes (Chan *et al.*, 2014).

Several mitochondrial genes, notably the main mitochondrial cytochrome c oxidase subunit 1 (COI) gene, the mitochondrial NADH dehydrogenase subunit 4 (ND4) gene, and the internal transcribed spacer (ITS2) gene, are used for molecular identification (Galtier *et al.*, 2009; Wan *et al.*, 2004). Each species has a particular genetic profile that can be used to identify it and to investigate its evolutionary relationships (Knowlton *et al.*, 1998). Mt-DNA testing is used for determining ancestry because mt-DNA does not change as rapidly (Ali *et al.*, 2016). In this study, barcoding of DNA based on COI was found to be an effective genetic marker for taxonomically classifying mosquitoes.

2. Material and Methods

2.1. Approval of Ethical Committee

This research project was duly approved by the ethical review committee of the department of Zoology, Abdul Wali Khan University, Mardan Khyber Pakhtunkhwa Pakistan.

2.2. *Study design, area and sampling*

This research study was conducted in district Buner, Khyber Pakhtunkhwa, Pakistan. A total of 365 *Aedes aegypti* samples were randomly collected from 6 different tehsils in district Buner during March-August 2023 (**Figure 1**).

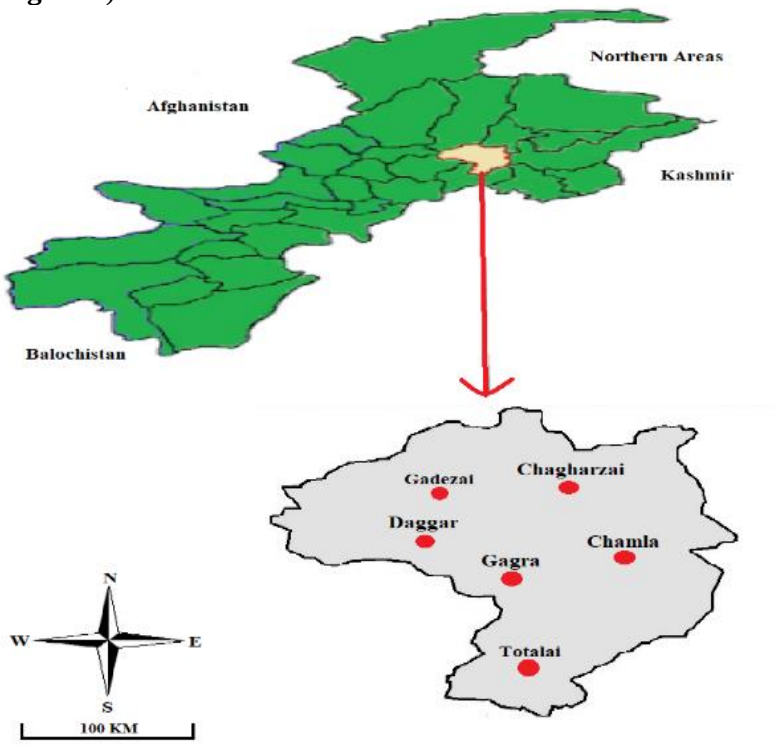

Figure 1: Map of six different tehsil of district Buner, Khyber Pakhtunkhwa Pakistan

The samples were collected from various locations including private residences, construction sites, junkyards, marshes, ponds and forested areas through the help of mosquito nets, light-traps, and spray procedures. All the collected samples were preserved in properly labelled eppendorf tubes containing silica gel and were transported to Veterinary Research & Disease Investigation Center (VR&DIC), Balogram Swat 19210, Khyber Pakhtunkhwa, Pakistan for further laboratory investigation.

2.3. *Morphological Identification*

Morphological Identification of *Aedes aegypti* was performed with the help of stereomicroscope using taxonomical keys available in literature (https://www.cdc.gov/nceh/ehs/docs/pictorial_keys/mosquitoes.pdf).

2.4. *Molecular identification*

2.4.i. *DNA Isolation and PCR*

DNA isolation was performed by a method describe by (Bibi *et al.*, 2015). Amplification of COI gene (having 735bp size) were as performed by followed the protocol with slight modification that describe by (Chan *et al.*, 2014) using Primer pair:

5'- GGATTTGGAAATTGATTAGTTCCTT – 3' (Forward) and
3' – AAAAATTTTAATTCCAGTTGGAACAGC – 5' (Reverse).

The total volume PCR reaction mixture is 25 µl that composed of 4 µl of extracted DNA, 5 µl master mix and 0.5 µl of each primer and 15 µl of dH_2O. The PCR process followed the following guidelines: it started with initial denaturation step at 95°C for 5 minutes, followed by via 5 cycles of denaturation for 40 seconds at 94°C, annealing for 1 minute at 46°C, and extension for 1 minute at 72°C. The next 35 cycles of the amplification cycle consisted of denaturation at 94°C for 40 seconds, annealing at 53.3°C for 1 minute, and extension at 72°C for 1 minute, with the final extension step take place at 72°C for 10 minutes. The amplified DNA fragments were visualizing in 1.5% agarose gels through UV-trans illuminator.

2.4.ii. *Sequencing*

The amplified products of the COI was sequenced by Sanger Sequencing (Macrogen, Inc., Seoul, South Korea). The obtained sequence undergoes trimming using the BioEdit program to remove any segments with contamination or poor reading quality. The resulting consensus sequence, produced by BioEdit, was then

submitted to the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) for verification of its morphological identity. On the bases of high percentage of identity, the FASTA format form NCBI were downloaded. Using Molecular Evolutionary Genetics Analysis (MEGA-X version 11) software the download FASTA sequences were align via Clustal-W. MEGA software was used to help generate a phylogenetic tree. The COI gene sequences from *Aedes aegypti* were used in the phylogenetic study, and an evolutionary tree was constructed using the Maximum Likelihood approach, bootstrapping with 1000 replications. A Microsoft Excel 2010 spreadsheet was used for the analysis of the data collected during field work.

3. Results

3.1. Mosquito identification

A total 365 mosquito's specimen were identified by using morphological keys that comprise to 2 species e.g., *Aedes aegypti* and *Culex quinquefasciatus* belonging to two genera. Percent Prevalence of of identified species of *Aedes aegypti* and *Culex quinquefasciatus* species are shown in **Table 1.**

Table 1: Percent Prevalence of of identified species of *Aedes aegypti* and *Culex quinquefasciatus* species are shown.

Species Identified	**Selected Tehsil of District Buner**						% Prevalence(n/N X 100)
	Gadezai	Gagra	Daggar	Chamla	Chagharzai	Totalai	
Aedes aegypti	12.33	13.70	12.02	12.33	13.42	14.25	78.08
Culex quinquefasciatus	3.84	2.47	3.84	3.56	3.56	4.66	21.92
Total	16.16	16.16	15.89	15.89	16.99	18.90	100.00

3.2. DNA Extraction

DNA extraction was successfully performed from 50 individual's mosquito specimens followed the protocol proposed by (Bibi *et al.,* 2015) as mention in the previous chapter. The DNA extracted on 0.8% agarose gel results were visualized as shown in **Figure 2.**

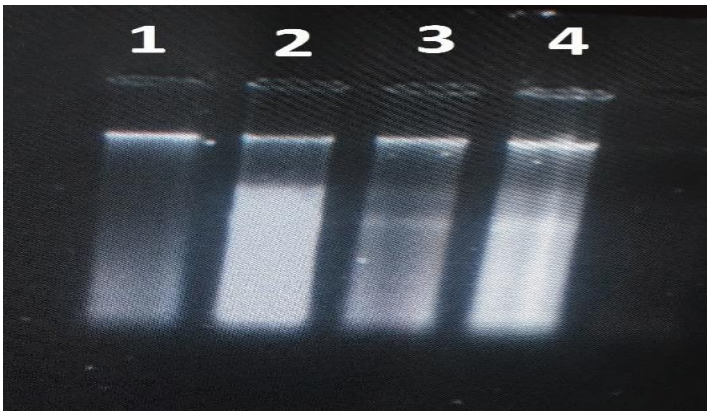

Figure 2: Results of DNA extracted from four specimen of mosquitos on 0.8% Gel

3.3. PCR Reaction

The extracted DNA were successfully amplified using COI gene having size of 735 bp by using forward and reverse primers. The PCR results were visualized on 1.5% agarose gel as shows **Figure 3**.

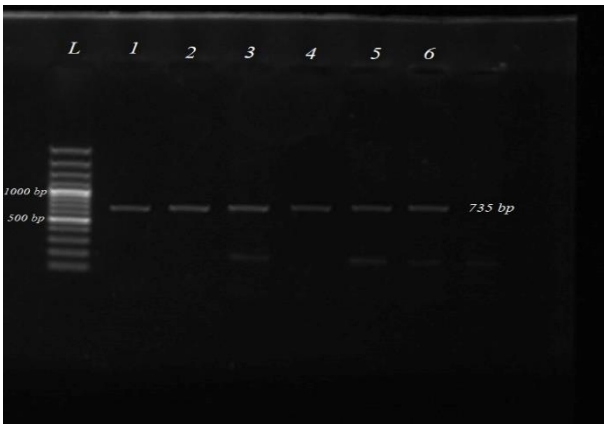

Figure 3: Samples 1-6= fragment of MT-COI gene having size of *735bp* on 1.5 % agarose gel; L= DNA leader *100bp*

3.4. Sequences phylogenetic analysis of Aedes aegypti

Sequencing was performed on the PCR-amplified *Aedes aegypti* COI gene products. The COI gene sequences that generated after that were then used in a BLAST search on the NCBI database. The BLAST results of *Aedes aegypti* showed 97.83-100% identity that represent almost similar with previously *Aedes aegypti* reported in GenBank from Sri Lanka (MG004703, MG004708, MGH004693, MGH004696, MGH004699, MG384711 and MF993580), India (MK265724, MK265725, MK265726, MK359842, MG770600, MK805532, MK805535 and MK265727), Brazil (MN019000, MN019002, MN019006, MN018999 and MN018985), Sudan (MN893711 and MN893739), West Africa (MK359830) and Tokyo (LC482630 and LC482626) which are shown in phylogenetic tree (**Figure 4**).

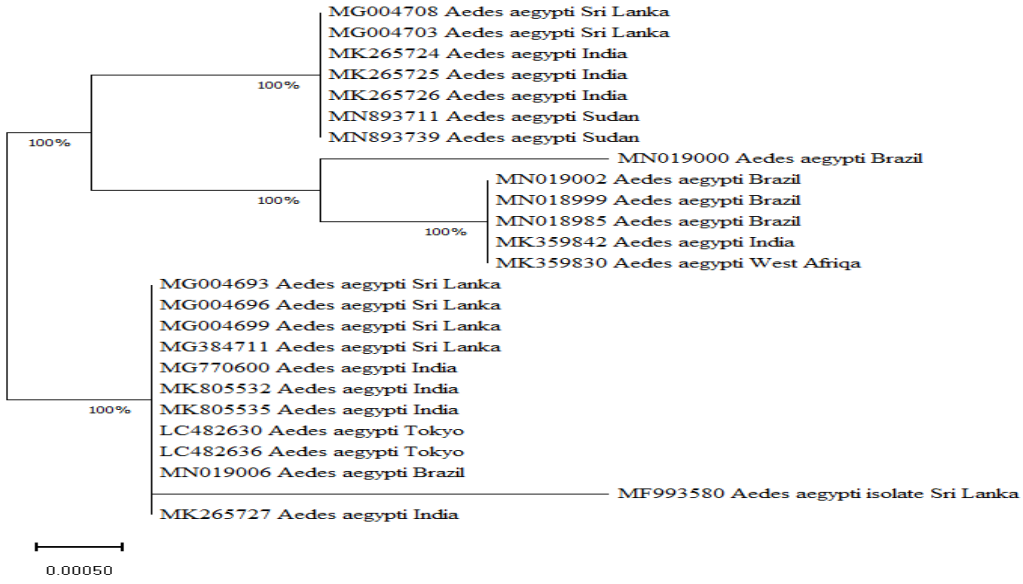

Figure 4: The *Aedes aegypti* phylogenetic tree can be seen with a scale bar representing a substitution rate of 0.0005, and was created using the Maximum Likelihood approach with 1000 bootstrap replicates. *Aedes aegypti* represent the root of tree. Evolutionary analysis were conducted in MEGA11.

4. Discussion

Mosquitoes is very important agent and play a key in ecosystem. Form different region of the different species were reported. In 2006, a study investigated a 617-bp fragment from the 5' end of the CO1 gene (cytochrome c oxidase subunit 1) in mitochondrial DNA in order to identify the mosquito species prevalent throughout Canada using DNA barcodes. A total of 37 mosquito species were collected during the study from the Canadian provinces of Ontario and New Brunswick. These species were initially classified using standard taxonomic keys. Using the Gene Elute-TM-Mammalian Genomic DNA Miniprep Kit, their DNA was extracted after identification, and the CO1 gene was amplified and sequenced. The results of the study showed that the CO1

gene among these mosquito species had an average genetic divergence of 10.4%, ranging from 0.2% to 17.2%. The quantity of genetic difference was significantly higher than the 0.5% to 3.9% range of genetic divergence generally found in closely related species (Cywinska *et al.*, 2006).

In 2012, a Chinese study studied the use of DNA barcoding to differentiate between several mosquito species. 404 mosquito specimens from the study were examined and classified into 122 distinct species by 15 taxa based on their physical characteristics. The identification procedure included examining the mitochondrial COI gene. These samples were gathered from eight different Chinese provinces, and physical characteristics were used to categorize them into species and subspecies. Based to the study's results, there is around 30 times more genetic variety in members of a single genus than there is in members of the same species (Wang *et al.*, 2012).

Another study was conducted in 2013 to evaluate the phylogenetic relationships and population genetics of Culex mosquitoes in the Sonoran Desert area of North America. The COI gene as well as microsatellite molecular markers were analyzed in the study. *Culex quinquefasciatus*, *Culex tarsalis*, and two unnamed species were among the species that were being examined. The most frequent species in each of these locations was *Culex quinquefasciatus*, while *Culex tarsalis* was only seen in Tucson, Arizona, and Guaymas, Sonora. Navojoa, in southern Sonora, also had notable populations of two undetermined species The COI gene sequences of *Culex quinquefasciatus* were specifically compared to those of the other three species in the study. The main conclusion of the study was that the increase of the *Culex tarsalis* population began during the Pleistocene era, perhaps as a result of significant genetic diversity differences between *Culex tarsalis* and *Culex quinquefasciatus*. (Pfeiler *et al.*, 2013).

Using DNA barcoding, another investigation carried out in Singapore in 2014 tries to identify the species of mosquitoes. The study using the mitochondrial COI gene to identify 128 adult mosquitoes that were collected. These specimens contained 45 species, which were divided among 13 different taxa. Note that the sequencing database does not contain the COI gene sequences for 16 species. The main conclusion of the study was that DNA barcoding was more accurate than traditional taxonomical procedures in identifying mosquito species. (Chan *et al.*, 2014).

In 2016, a study investigated in southeastern Australia for molecular identification of mosquito species, using Cytochrome Oxidase I (COI) sequences. Total 113 specimens were collected from different sites that were morphological identified which belong to 12 genera and 29 species. Previously Three out of 29 species were miss identified e.g., Culex palpalis, Macleaya macmillani and Tripteroides atripes. This study showed that taxonomical key identification is more time consumable as compare with Barcoding (Batovska *et al.*, 2016).

In 2016, a study was carried out to examine the cytochrome c oxidase I gene in order to investigate the genetic diversity of Anopheles mosquitoes. A total of 44 specimens were collected, of which 7 were from Pakistan and the remaining 37 were from different areas of the globe. The results showed that within the Anopheles species in Pakistan, intraspecific divergence varied from 0.0% to 2.5% (with an average of 0.49%), while interspecific divergence ranged from 8% to 22.3% (with an average of 12.77%). Similarly, the intraspecific divergence ranged from 0.0% to 11.2% (with an average of 0.65%) and the interspecific divergence ranged from 3.4% to 35% (with an average of 11.75%) for Anopheles species in mosquitoes collected worldwide. The main finding of the study highlighted the value of DNA barcoding as a useful tool for species identification (Tahir *et al.*, 2015).

In 2018, a study was done in Sri Lanka to use DNA barcoding to identify the mosquito fauna. In this method, the internal transcribed spacer 2 region of the nuclear ribosomal DNA and the Cytochrome c oxidase subunit 1 gene from the mitochondrial genome were used to identify the species. A total of nine administrative areas provided samples, which were then identified according to their characteristics. Among them, the Culicinae subfamily, which includes 17 genera, contained 118 species, or 84% of the nation's total mosquito species. Additionally, Anopheles mosquitoes were not mentioned among them. This study concluded that the result of DNA barcoding is more accurate than morphological identification because some characters of the specimen are damaged which affect the result (Weeraratne *et al.*, 2018).

In 2019, a study was conducted in west Africa to study Aedes aegypti and its Phytogeography and invasion history. Aedes aegypti is main vector for many arbovirus diseases. During this stud COI gene were analysis for Molecular characterization. This study shows that A. aegypti ancestor were present in Holocene period of the geographical times however A. aegypti also show similarities to the A. aegypti species reported from America (Salgueiro et al., 2019).

In 2021, research was done in in Jeddah City of Saudi Arabia for study surveillance of Dengue fever. During this study a total of 950 specimens were collected using light trapes. This study showed that aedes aegypti are founded throughout the year however two peaks of abundances were reported form December to march because of optimum temperature and humidity (Al-Azab *et al*., 2021).

A study was initiated in 2021 to apply DNA barcoding to make a phylogenetic analysis of mosquitoes in Kocaeli, Turkey. In the Kocaeli province, samples were taken at four different sites between June 2017 and September 2017, a time period. After molecular characterization using PCR and sequences all the mosquitoes were identified successful belonging to 4 genera e.g., Aedes, Anopheles, Culiseta and Culex. This study provides data to GenBank for the first time and is the first molecular analysis of the distribution of mosquito species in Kocaeli (Polat and Serkan, 2021).
In 2022, a study was conducted using the cytochrome oxidase subunit 1 (coxI) and internal transcribed spacer 2 (ITS2) genes to investigate the physical characteristics and genetic make-up of Aedes aegypti mosquitoes from India. The investigation included collecting samples from a total of eight study locations. Morphological study of all Aedes aegypti specimens was conducted, with a particular focus on changes in the dark scaling of the fifth segment of the insects' hind tarsi. The molecular analysis showed that all of the variations detected in the sample had extremely similar genetic profiles. (Kumar *et al.*, 2022).
In 2022, a study was performed to study Genetic Diversity of *Aedes aegypti* in central America through DNA barcoding by using Cytochrome c oxidase subunit 1 gene from the mitochondrial genome and the internal transcribed spacer 2 (ITS2). *Aedes aegypti* collected form four different cities. The molecular characterization of this study the showed that the *Ae. aegypti* in Honduras shows similarities with the Central American isthmus (Escobar *et al.*, 2022).
In Sri Lanka, a study try was carried out in 2018 to use DNA barcoding to determine the species of mosquitoes found present. This involves the use of the internal transcribed spacer 2 region from the nuclear ribosomal DNA and the cytochrome c oxidase subunit 1 gene from the mitochondrial genome for identification. Samples from nine administrative districts were submitted, and they were morphologically classified. These comprised 118 species, or 84% of all the mosquito species in the nation, belonging to the subfamily Culicinae. Among the 17 different genera that these species belonged to, there were no reports of Anopheles mosquitoes. This study concluded that the result of DNA barcoding is more accurate than morphological identification because some characters of the specimen are damaged which affect the result (Weeraratne *et al.*, 2018). In this study, *Aedes aegypti* of the district Buner was detected through molecular bases that is so similar to study of Weeraratne, Surendran and Parakrama Karunaratne studies. This is because molecular studies will identify every species well accurate and provides much more information about its phylogeny.

5. Conclusion

Aedes aegypti is hematophagous insect and act as main vector for many arboviruses (dengue, yellow fever, etc.). In many cities of Pakistan, dengue fever is becoming more prevalent as an infectious disease. as there is currently no vaccine for the dengue virus and control of the disease depends on vector control. Furthermore, the results of this study will generate basic information about mosquitos spp; and it will help to understand the epidemiology of *Aedes aegypti* disease in Khyber Pakhtunkhwa, especially in Buner district. A total of 365 samples of mosquitoes were randomly collected from six tehsil of district Buner and were transported to Veterinary Research & Disease Investigation Center (VR&DIC), Balogram Swat 19210, Khyber Pakhtunkhwa, Pakistan for molecular identification using genetic marker of mitochondrial encoded COI gene. In phylogenetic tree, the sequence (COI) genes of *Aedes aegypti* species as previously reported from Sri Lanka and India.

6. Recommendations

From all over the world, every year a wide range of diseases is reporting that causes by various species of mosquitoes. Although to minimized mosquitoes borne diseases, we should create focus on its taxonomic convergence using DNA barcodes. Although the DNA barcoding is so efficient but sometimes not occurs so accurately due to the lacking and unavailability's of sufficient reference sequence (mitochondrial genes) in databases such as BOLD and NCBI. Many studies that do thorough sampling and analysis may significantly address and fix these mistakes.
In addition to DNA barcoding, the control of diseases spread by mosquitoes can require using a variety of strategies, including biological, physical, chemical, and preventative measures like immunization, especially in areas where these diseases are more prevalent. Effective implementation of widespread mosquito control activities may depend significantly on the use of COI gene-based identification. It is recommended that countries worldwide use this strategy to protect the public's health and reduce the spread and invasion of dangerous mosquito species. Furthermore, the results of this study will generate basic information about

Mosquitos spp. And it will help to understand the epidemiology of *Aedes aegypti* disease in Khyber Pakhtunkhwa, especially in Buner district.

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Novelty Statement

The research and experimental work on "Morphological and Molecular Identification of *Aedes Aegypti* through genetic marker Mitochondrial Encoded Cytochrome c oxidase I (MT-COI) gene in Pakistan" is original and new in the field of zoology in Khyber Pakhtunkhwa, Pakistan.

Author's Contribution

Fida Muhammad: Investigation, Writing-original draft preparation; Asad Ullah: Supervision; Tayyaba Ilyas: Formal analysis; Tahira Tayyeb: Methodology; Mansoor Ahmad & Zulfiqar Ali: Validation; Rafiq Ullah: Data Curation; Muhammad Owais Khan & Maiz ur Rahman: Writing-review and editing; Imad Khan: Project administration; Raheela Taj: Software; Shumaila Gul: Resources; Shakirullah Khan: Conceptualization; Bushra Bibi: Visualization.

Statement of conflict of interest

The author(s) declared no potential conflicts of interest with respect to research, authorship, and/or publication with the work submitted.

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