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SERVICES FOR SCIENCE AND EDUCATION

Water Strider Fauna and its Phylogenetic Relationship in Tehsil Wazirabad

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ABSTRACT

This examination is intended to investigate the water strider fauna and their phylogenetic relationship in Tehsil Wazirabad, Punjab, Pakistan. Being a decent predator of mosquitoes and successful bioindicator of substantial metal contamination it assumes the imperative job to keep up the manageability of the biological system. The examples of water strider bug were gathered from different oceanic locales during summer and spring from 2018-2019 in Wazirabad City. The type of water strider that was distinguished during this exploration is *Aquarius adelaidis* (Zettel) having a place with distinct genera Aquarius of the family Gerridae. It likewise uncovered that Aquarius adelaidis disengaged from Wazirabad area has homology a with arrangement of water strider detailed from Cyprus island situated in the north-eastern piece of the Eastern Mediterranean. The arrangement examination found that cytochrome c oxidase subunit I is profoundly monitored locale inside all Aquarius species around the world, and is less inclined to varieties and transformations. Aquarius adelaidis is increasingly bountiful with 58.2% of complete assortment.

INTRODUCTION

Water Striders for the most part related with request Hemiptera (Stys and Kerzhner, 1975), that includes around 2000 species from all the tropical, subtropical and calm areas of world (Polhemus and Polhemus, 2008). They are genuine bugs and can skate on water surface and belimp on still and streaming water bodies (Andersen, 1982). Various types of water strider have different territories like lakes, lakes, streams, tree trunks and bamboo internodes (Polhemus and Polhemus, 1991). A lot of water strider species happens in marine environment like mangroves and estuaries (Andersen, 1991) and some of them are living in open seas to turn out to be full pelagic (Damgaard et al., 2000a; Andersen and Weir, 2004a).

Acknowledgment of eigth groups of Gerromorpha happened for example Mesoveliidae (water treaders), Hebridae (velvet water bugs), Hydrometridae (swamp treaders or water measures), Hermatobatidae (coral treaders), Veliidae (expansive bore water striders, riffle bugs, water crickets), Gerridae (water striders, lake skaters) and Paraphrynoveliidae and Macroveliidae with no common names of last two families (Polhemus and Polhemus, 1991). There is distinctive measure of species that lie in these families like there are just two species in Paraphrynoveliidae, three species in Macrovelidae, nine in Hermatobatidae, one-thirty in Hydrometridae, two-twenty in Hebridae, 700 ten in Gerridae and 900 in Veliidae that both are assorted families (Polhemus and Polhemus, 2008).

Cytochrome Oxidase C subunit I (COI) that encoded mitochondrial quality is the most bountiful utilized hereditary marker that is utilized to distinguish the interspecific and intraspecific phylogenetic connections in water strider (Caterino et al., 2000). COI is fundamentally used to recognize the connections in the taxa of water strider, for example, Halobates (Damgaard et al., 2000a; Andersen et al., 2000) and Potamobates (Galacatos et al., 2000). These genera identified with gathering of monophyletic species and they are tried with DNA arrangement information consistently (Damgaard, 2005; 2008).

MATERIALS AND METHODS

The aim of this study is to explore the diversity and phylogenetic association of water striders in various aqauatic habitats in Wazirabad City. This study was conducted in 2018-2019 from Tehsil Wazirabad, District Gujranwala in the Department of Zoology, University of Gujrat.

Sampling Sites

Sampling was done at various water bodies in Tehsil Wazirabad, District Gujranwala. The water bodies that are pointed out for sampling was Nala Aik, Nala Palkho, Gammon Lake, Chenab River and Head khanki. Sampling was done on weekly basis and fortnightly. The most suitable and favourable time for sampling was spring and summer season. A map showing all the sampling sites in Wazirabad City, Punjab, Pakistan in order to collect the insects from these pointed aquatic areas.



Figure - 3.1: Selected Localities of Wazirabad

Sampling Method

The sampling sites were visited at regular intervals on weekly basis and fortnightly. The water striders were collected with the aid of sweep nets and aquatic nets. The humidity and temperature of the air will be observed will a hygrometer along with the temperature of water with a thermometer.



Figure - 3.2: Collection of Specimens at Different Sites

Preservation Method

The collected water striders were first separate from other insects, dried and then transferred into the small bottles or vials containing 75% ethanol. I labelled the vials with site area name and date of sampling. Then I placed the vials containing specimens in freezer at 20^{o} C for preservation.

Morphological Identification of Specimens

The identification was done in the lab of Department of Zoology, University of Gujrat. All the preserved specimens were morphological identified from family to specie level by using various taxonomic keys and dissecting microscope

Techniques Used

Following techniques are used to identify the phylogenetic relationship of water strider based on molecular identification:

DNA Extraction:

COI-1(Cytochrome C Oxidase) is a gene that is often used as a DNA barcode to identify different species. COI is present in mitochondria and it is used to extract because its sequence is conserved among conspecifics.

DNA extraction was done by the help of QIAamp DNA Minikit. The QIAamp DNA Minikit extraction is used for the purification of complete genomic and mitochondrial DNA from the tissues. The protocol of DNA extraction through the QIAamp DNA Mini kit consist of following steps:

The tissue from the storage sample of water strider was removed. Then the amount of tissue is determined to 25mg. To determine the amount of tissue of insect sample, weighing is the most accurate way. The amount and type of tissue are the factors on which yield of DNA is depend. 0.2-1.2 µg of DNA was yield from the 1mg of tissue. First, excised the tissue sample of water strider upto 25 mg into small pieces. Then placed this sample in the microcentrifuge tube of 1.5 ml and 180 µl of ATL buffer is added in it. To decrease the lysis time, it is important to cut the tissue into small pieces and microcentrifuge tubes of 2 ml are better for lysis of tissue of insect. The sample tissue of 25 mg was placed in liquid nitrogen and then grind it with the help of micropipette and poured the tissue sample along with liquid nitrogen into the microcentrifuge tube of 1.5 ml. The the liquid nitrogen was allowed to evaporate but tissue should not be dissolved in it and the 180 µl of ATL buffer is added in it.



Figure-3.4: Showing the steps of Cutting, Crushing and Lysis of Tissue of Water Strider

The tissue of 25 mg was added in the microcentrifuge tube of 1.5 ml having approximately 80 µl PBS. Equivalent rotor-stator homogenizer is used to homogenize the sample then I added ATL buffer of 100 µl. In ATL buffer the samples cannot be directly homogenized but crushing in liquid nitrogen is suitable. Then 20 µl of Pk was added, mixed it by using vortex and incubated it at 56^0 C till the complete lysis of tissue is occurred. Occasionally vortex the microcentrifuge tubes during incubation period for proper mixing and lysis of tissue.

The microcentrifuge tubes of 1.5 ml are centrifuged to take off the drops from inside of the lid. Then 4 µl of RNase A was added (100 mg/ml), vortex it for 15 seconds and incubated it for 2 min at room temperature. After that centrifuged the 1.5 ml microcentrifuge tubes to take off the drops from inside of the lid. Again, vortex it for 15 seconds and incubated it at 70^0 C for 10 minutes. Then centrifuged it again.

The buffer AL of 200 µl was added into the sample, vortex it for 15 sec for proper mixing and incubated it for 10 min at 70^0 C and centrifuged it again. After that 200 µl of ethanol was added into the sample tubes, vortex it and centrifuged the tubes to remove the drops from lid. To yield a homogenous solution, it is essential that the sample, buffer AL and ethanol was mixed thoroughly. By applying the ethanol, the white precipitates may form and then applied all the precipitates to the spin column of QIAamp Mini Kit. Without wetting the rim applied the mixture to the QIAamp Mini kit spin column. Then centrifuged it at 8000 rpm for 1 min after closing the caps of spin columns. The QIAamp Mini spin column was placed in a clean 2 ml collection tube and discard the filtrate-containing tube. To avoid aerosol formation, it is essential to close each spin column during centrifugation. Centrifuged it again until the solution sample became transparent. Added 500 µl AW1 buffer after opening the QIAamp mini spin columns. Then centrifuged it again at 8000 rpm for 1 min after closing the caps. The mini spin columns were placed in a clean 2 ml collection tube and discard the filtrate containing tube. Added 500 µl AW2 buffer in mini spin column without wetting the rim, centrifuged it at 14000 rpm for 3 min.

The mini spin columns were placed in a clean microcentrifuge tube of 1.5 ml and dispose the filtrate containing tube. Then added 200 µl of buffer AE or distilled water in the spin columns, incubated it at room temperature for min and then centrifuged the columns at 8000 rpm for 1

min. This step was repeated again. The DNA yield increases due to the 5 min incubation of mini spin columns loaded with buffer AE or distilled water. The yield of DNA was increased upto 15% by further 200 µl of AE buffer in elution step. The microcentrifuge tube of 1.5 ml are not suitable for the elution of volume more than 200 µl because it leads to the aerosol formation during centrifugation due to the contact of spin columns with eluate.

The final DNA concentration increases due to elution with less than 200 µl volume. The DNA was eluted with buffer AE and placed it at -20^0 C for long-term storage.

Primer Set:
Universal Primers were used for the characterization of water strider species on molecular basis.

Table-3.2: Primers Used for PCR Amplification

Sr #	Primer ID	Primer Sequence	# of Nucleotides
1	LCO 1490	5'ggtcaacaaatcataaagatattgg-3'	25.2
2	HCO2198	5'-taaacttcagggtgaccaaaaaatca-3	26

PCR Technique:
After the gene extraction PCR was done to amplify the genes. In PCR technique various steps and primers was used to separate the DNA strands and identical copies of DNA are made.

Amplification of 710 bp Segment of C0*1* Gene:
PCR reaction mixture was prepared as following 12.5 µl of dream Taq Green mstermix (Fischer-Thermo-scientific), 7.5 µl of water, 1.0 µl each forward and reverse primers and 3 µl of template DNA was used in each reaction. PCR profile was set with following parameters, initial denaturation at 95 ^{0}C for 2 minutes then 35 cycles of 94 ^{0}C for 45 seconds, 40 ^{0}C for 35 Seconds and 72 ^{0}C for 60 seconds and final extensions of 72 ^{0}C for 05 minutes was applied.

Sequencing of 710 bp Segment of C0*1* Gene:
Amplified segments were purified using Gene. All DNA purification kit according to manufacturer instructions. PCR product were shipped to 1st Base Lab 41 Science Park Rd, Singapore 117610 for sequencing, which applied Sanger Di deoxy method to sequenced the genes. All nucleotides' sequences were submitted to NCBI GenBank with the accession numbers.

Agarose Gel Electrophoresis:
One percent agarose gel electrophoresis was performed for gradient PCR and conventional gene amplification PCR reactions with following recipes. 60 mg of agarose was added in 60 ml of 1X TAE (Tri's acetate EDTA, pH 8.5) buffer in a flask. 600-watt current was used in microwave oven to dissolve and homogenize the agarose in buffer, 2µL of ethidium Bromide (0.5 µg/mL) was added and gel was poured in gel tank with comb setting. 70 volts current at 140 milli-amperes for 40 minutes was applied to separate the PCR products on agarose gel.

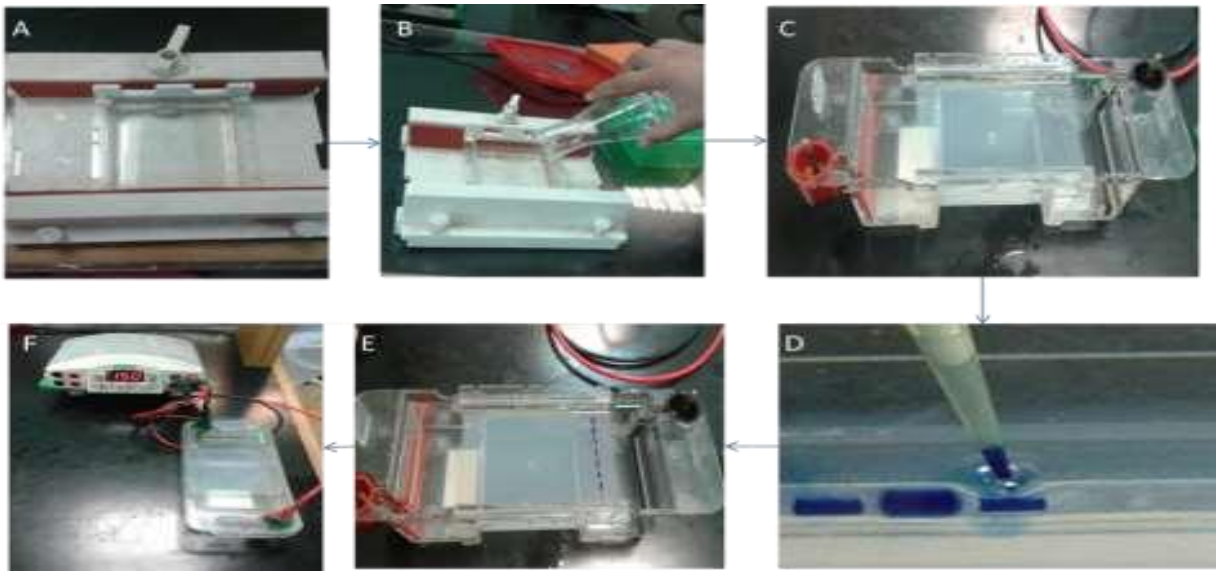


Figure-3.7: Preparation of Agarose Gel

Phylogenetic Analysis:

All nucleotide data was analysed and multiple sequence aligned using BioEdit version 7.2.6 and MEGA X software package (version 10.0.5) was used to develop NeighborJoining phylogenetic tree with Tamura-Nei model. Online server ExPASy was applied to translate the sequence into amino acids to see any possible conservations or mutations in all isolates.

Statistical Analysis:

Various indices like Shanon index and Simpson index were applied on the collected data to check the diversity of water strider and find their diversity along with phylogenetic association (Mehmood et al., 2015). The data was calculated and analysed by the help of Microsoft Excel.

RESULTS AND DISCUSSION

Diagnostic Features for Genus *Aquarius*

Eyes are prominent on a short head and pronotum is moderately prolonged. Claws are present on the fore leg and wings give black appearance which covers the whole body.

Diagnostic Features for Specie *Aquarius adelaidis*

They are macropterous having long slender body. The forewings having light brownish color along with the dark brown veins. They have two prominent spines present on the posterior region of the body.



Figure 4.1 Aquarius adelaidis (Dorsal view)

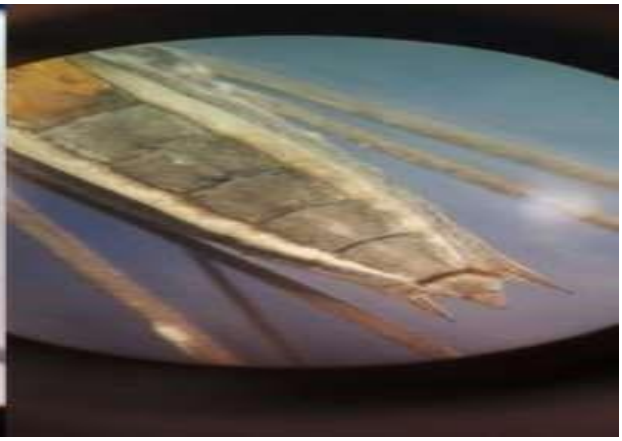


Figure 4.1 Aquarius adelaidis (Ventral view)

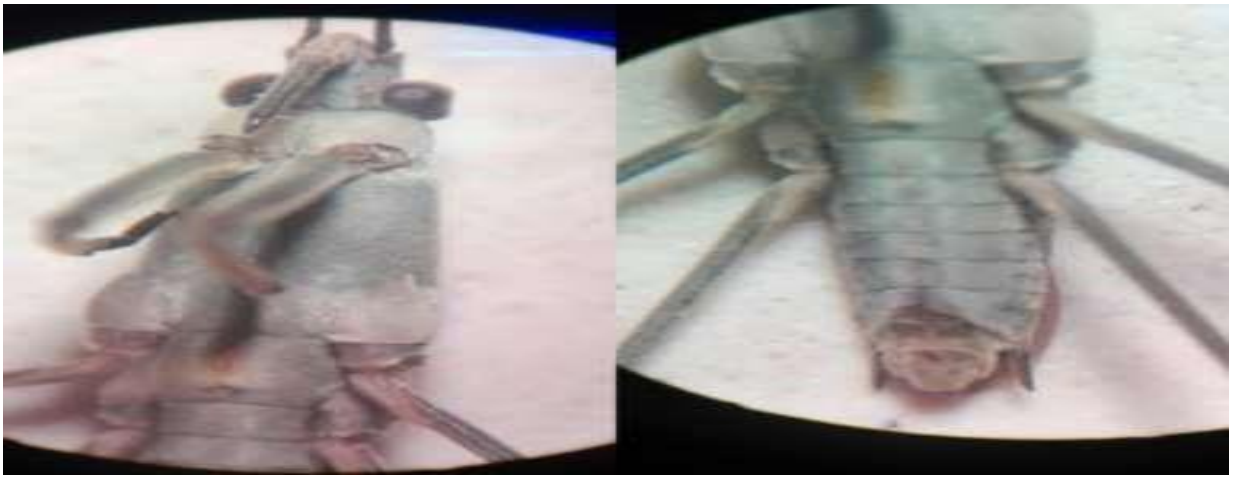


Figure 4.2 Aquarius adelaidis (Ventral view)

Table-4.2: Concentration of Extracted Genomic DNA Measured by Nano-Drop

Sample	Quantity µg/100 µL	Sample	Quantity µg/100 µL	Sample	Quantity µg/100 µL	Sample	Quantity µg/100 µL
1	22.1	**6**	29.1	**11**	21.9	**16**	20.9
2	24.4	**7**	28.6	**12**	27.8	**17**	30.1
3	27.5	**8**	23.6	**13**	26.0	**18**	27.6
4	21.9	**9**	25.3	**14**	25.0	**19**	23.8
5	22.9	**10**	21.6	**15**	24.8	**20**	23.4

Extracted DNA from insects' samples was also run on an agarose gel to ensure the quality of DNA yields.

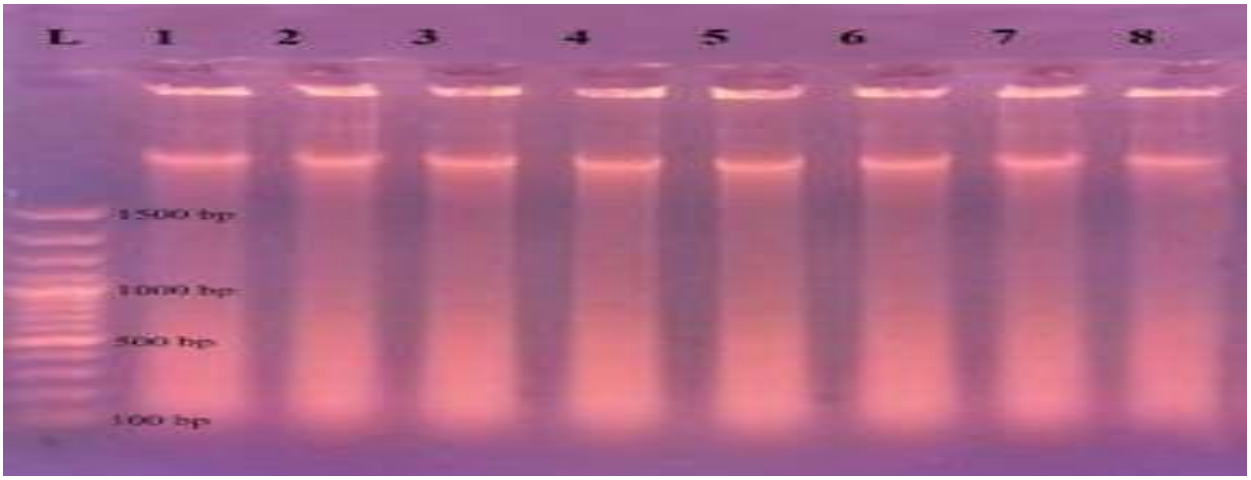


Figure-4.5: 1% Agarose gel electrophoresis. First Lane Represents 100 bp Pair DNA Ladder. Lane from 1 to 8 are DNA from (*Aquarius adelaidis*)

Gradient PCR

First of all, a gradient PCR was applied to determine the optimum annealing temperature conditions for the primer's pairs against cytochrome oxidase gene. Bio-Rad thermocycler named I-Cycler was applied with the following parameters as shown in the fig. a range of annealing temperature between 40 to 50 ^{o}C was applied. It was found that 40 ^{o}C is the best optimum annealing temperature for primes binding to target template, whereas temperature above 48 ^{o}C did not amplified any target gene.

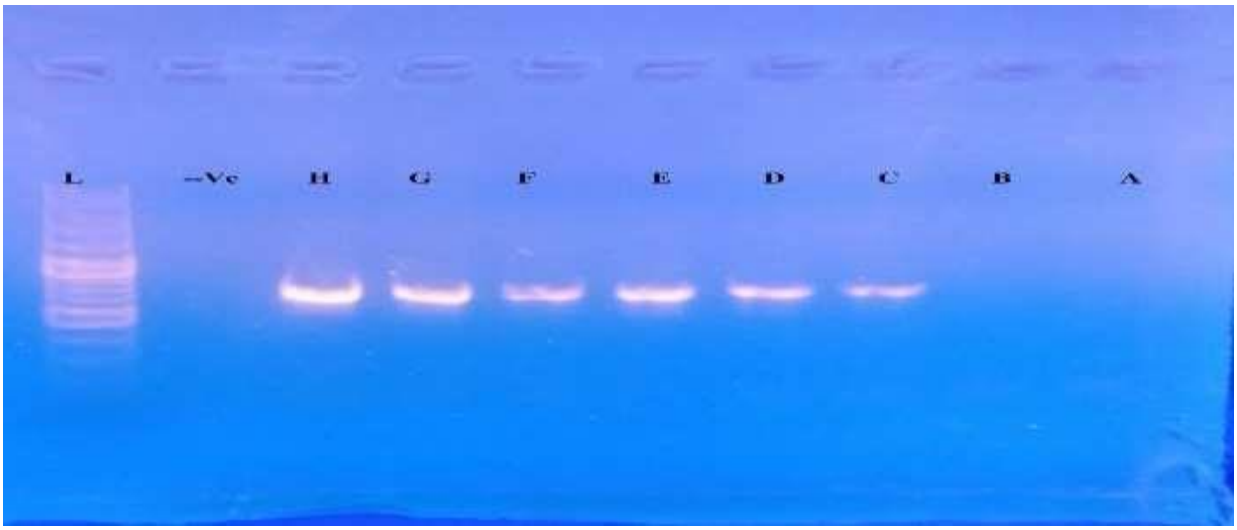


Figure-4.6: Gradient PCR 1 % Agarose Gel Run. First Lane contains 100 bp Ladder Followed by H to A PCR Reaction. Brightest Band at H indicate Best Annealing Conditions

Amplification of 710 bp segment of Cytochrome Oxidase Gene 1 (CO*1*)

AB applCO*1*gene of invertebrates with the parameters as discussed in the methodology.

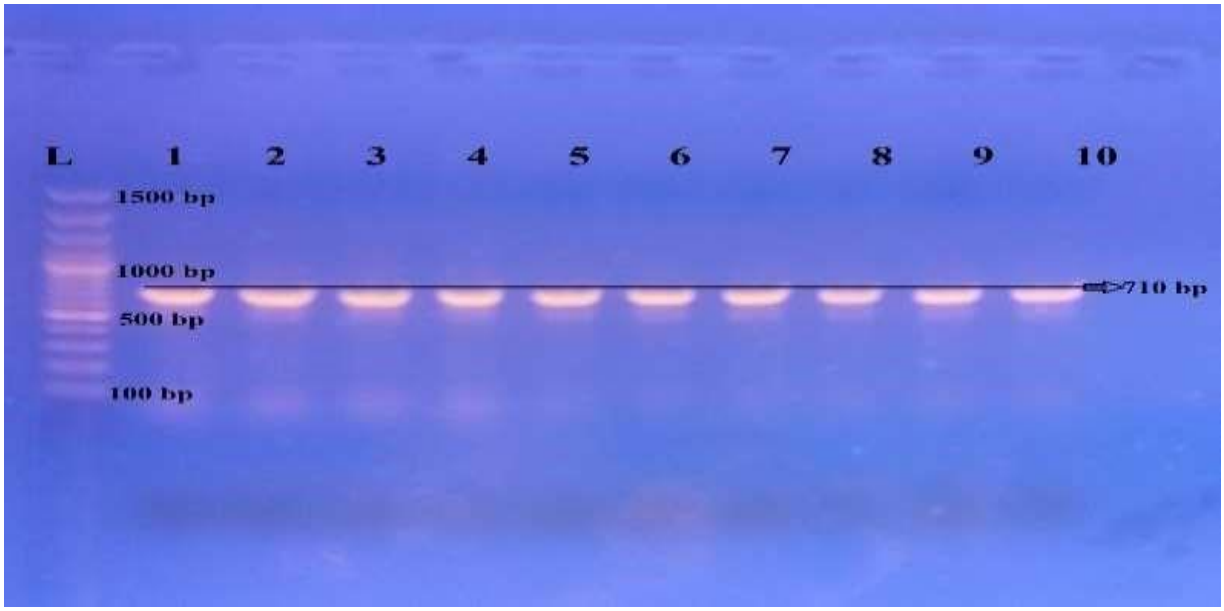


Figure-4.7: 1% Agarose Gel Electrophoresis of Amplified C0*1* of (*Aquarius adelaidis*). First lane is 100 bp Ladder Followed by PCR Products of 10 Samples (*Aquarius adelaidis*)

Phylogenetic Analysis of *Aquarius adelaidis*

Aquarius adelaidis Wazirabad isolate 01 cytochrome oxidase subunit I (COI) mRNA partial cds mitochondrial

CCCAAATTTTAATTAGGAATGTGATCAGGAATAATTGGTATATCAATAAGTTG
AATTATTCGAATTGAATTGGGACAACCAGGATCATTTATTGGAAATGATCAA
ATTTATAACGTAATTGTTACAGCACATGCATTCATTATAATTTTCTTTATAGTA
ATACCAATTATAATTGGGGGTTTTGGAAATTGATTGGTGCCCTTAATAATTGG
TGCACCAGATATAGCATTCCCCCGAATAAATAATATAAGATTCTGATTATTGC
CCCCATCGTTAACTTTACTTCTAGTAGGAAGTCTTGTAGATGCGGGGGCAGGA
ACAGGATGAACTGTTTACCCACCGCTATCTAGAAATATTGCCCATAACGGAG
CATCTGTAGATTTAACCATCTTCTCATTACACCTGGCAGGTGTTTCATCAATTC
TTGGAGCAGTAAACTTTATCTCAACTATTATTAATATACGAACCAGGGGAATA
ACAAGAGAAAAAATTCCACTATTTGTATGATCTGTAGGAATTACTGCATTACT
ACTTTTATTATCACTACCTGTACTGGCAGGAGCAATCACAATATTATTAACTG
ATCGTAATCTTAACACATCATTCTTTGACCCGGCAGGAGGAGGGGATCCAGT
ACTTTATCAACACTTGTTCTGATTTTTTGGTCACCTTGGGAAAGTTTAAA

This nucleotide sequence was aligned and compared with sequences of cytochrome oxidase subunit I of Aquarius species isolates available at NCBI GenBank, the accession number of nucleotide sequences is provided in the following table.

Table-4.4: Accession Numbers (NCBI) of Aquarius Species Isolates, Cytochrome Oxidase Subunit I Gene, Partial Codons, Mitochondrial

DQ683265.1	AY425245.2	DQ231218.1	AF200256.2	AF200259.2
KX821855.1	AB679145.2	DQ231219.1	AF200737.2	AF200257.1
AF251106.2	DQ231226.1	DQ231235.1	AY425249.2	AF200258.2
AF251101.2	DQ231236.1	AF200254.1	DQ231238.2	AY336948.2

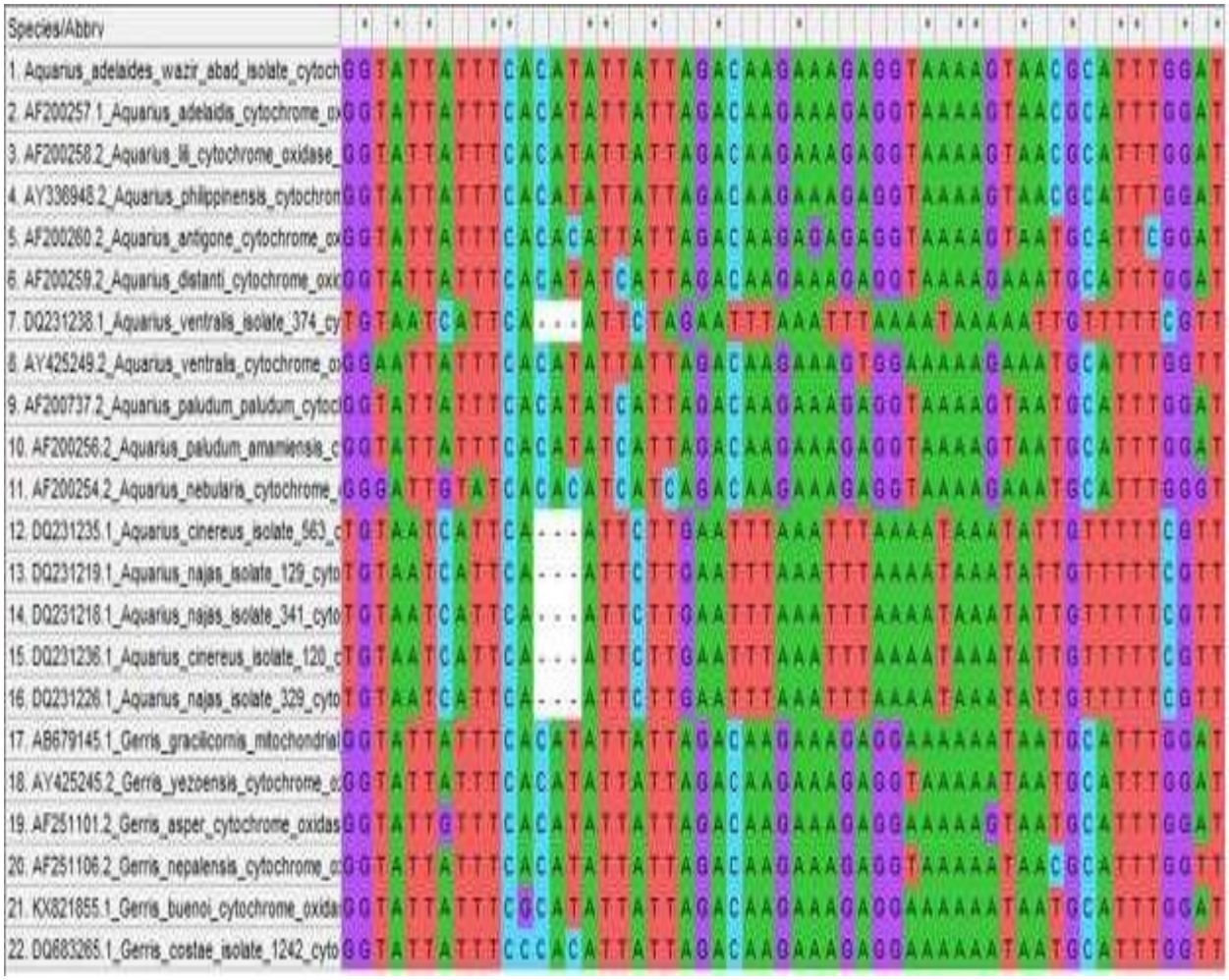


Figure-4.11: Multiple Sequence Alignment of Cytochrome Oxidase Subunit I Nucleotide Sequences of Aquarius Species

Multiple sequence alignment was performed with Clustal w setting using MEXA X software of cytochrome oxidase subunit I nucleotide sequence of *Aquarius adelaidis* along with nucleotide sequences of other Aquarius species obtained from NCBI.

Phylogenetic Tree and Evolutionary Relationships of Taxa

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.86859177 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tajima-Nei method and are in the units of the number of base substitutions per site. The analysis involved 32 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 2235 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

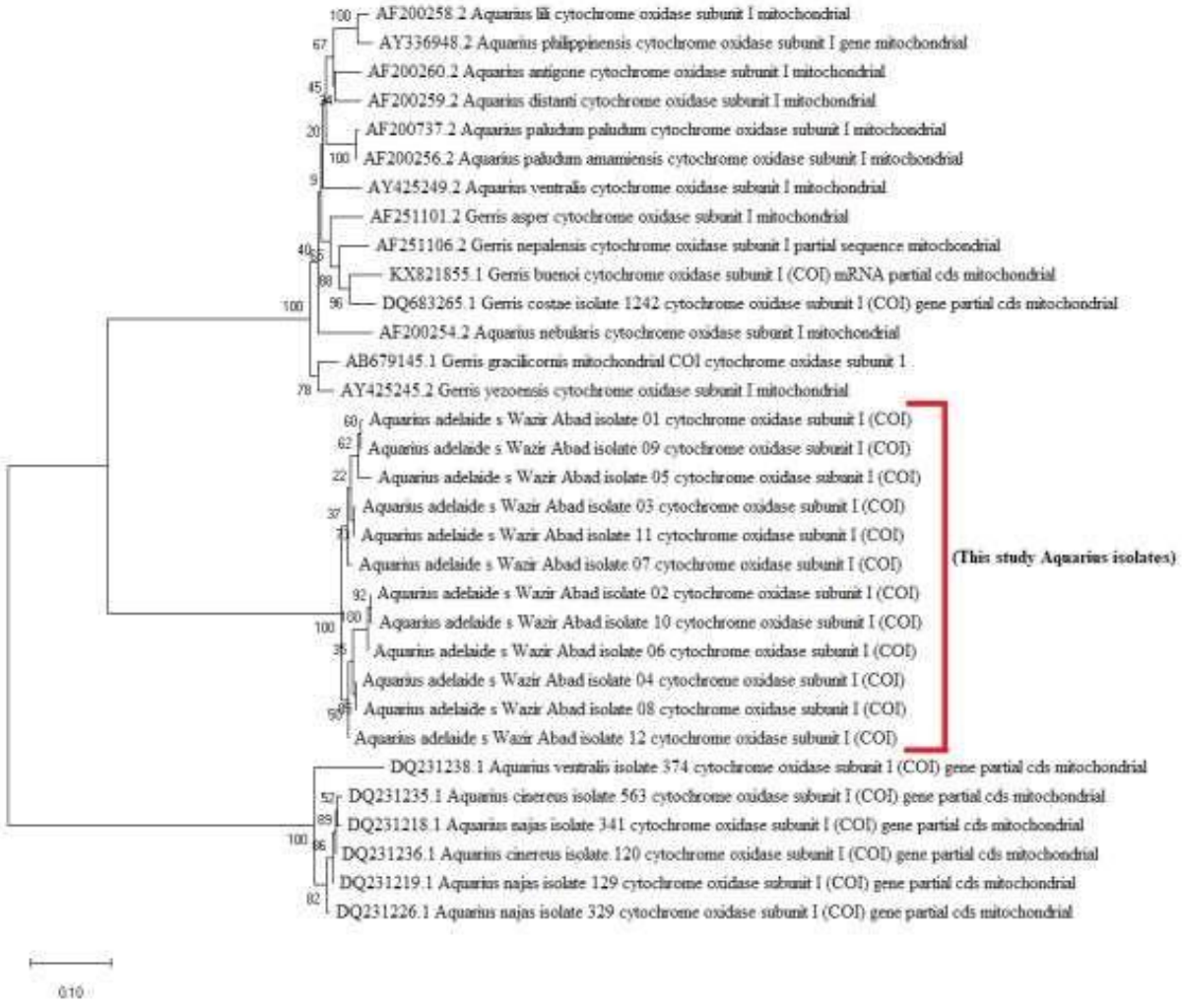


Figure-4.12: Neighbor-Joining Phylogenetic Tree with Tamura-Nei Method and 1000 Boot Strap Replicate Values. Nucleotide Sequence of Cytochrome Oxidase Subunit I Gene, Partial Codons, of *Aquarius adelaidis*, Wazirabad Isolate is Indicated with Red Triangle

Phylogenetic analysis revealed that *Aquarius adelaidis* isolated from Wazirabad region has homology with sequence of water strider reported from Cyprus island located in the northeastern part of the Eastern Mediterranean. Water strider Aquarius *Garris yezoensis* isolated from Denmark also seems to be close relative of our isolates. Other species like *Aquarius lili*, *philippinesis*, *distanti, antigone*, and *paludum* has close phylogenetic relations with *Aquarius adelaidis*. Whereas *Aquarius nebularis*, *ventralis, najas, cinereus*, *Gerris costae*, *Gerris buenoi* and *Gerris asper* have distant phylogeny relations with *Aquarius adelaidis*.

CONCLUSION AND RECOMMENDATIONS

Wazirabad has an extremely industrialized irrigation system to enhance vegetation and it has various freshwater bodies like Chenab River, Head Khanki and seasonal streams like Nala Aik and Nala Palkho along with an enormous number of Ponds like Fish Ponds which inhabit the various communities, particularly insects. Aquatic species of insects have breeding sites on these water bodies for reproduction. Water Striders are present in abundance due to the huge number of aquatic ecosystems according to their seasonal fluctuation pattern. This research explored 1 species of Water Strider belonging to Tehsil Wazirbad, Punjab, Pakistan. There are *A. adelaidis* (58.2%) was reported. This specie was collected from various water bodies during spring-summer crops in Wazirabad.

Recommendation

The presence of the reported species after the conduction of this research should be confirmed in future research. As water striders are the biological predators of mosquitoes and bio-indicators of heavy metal pollution so it is essential to keep a check and balance to maintain the diversity of water striders. The efficient role of water striders in the sustainability of the ecosystem should be studied. Being predators, their effective role as biological regulators should be comprehensively studied in Integrated Pest Management (IPM).

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