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Full Length Article

Morphological and Phylogenetic Evaluation of Libellulidae Dragonflies from District Attock, Punjab, Pakistan

Malika Mehreen Nisar[1], Khushi Muhammad[1*], Sardar Azhar Mehmood[2], Shabir Ahmed[2], Bibi Nazia Murtaza[3] and Muhammad Shahid Nadeem[4]

[1]*Department of Biotechnology and Genetic Engineering, Hazara University Mansehra 21300 Khyber Pakhtunkhwa, Pakistan*
[2]*Department of Zoology, Hazara University Mansehra 21300 Khyber Pakhtunkhwa, Pakistan*
[3]*Department of Zoology, Abbottabad University of Science and Technology (AUST) Abbottabad Pakistan*
[4]*Department of Biochemistry, King Abdulaziz University Jeddah, 21589, Saudi Arabia*
[*]For correspondence: Khushisbs@yahoo.com; ORCID: 0000-0003-4425-4742
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Abstract

Dragonflies under the family Libellulidae and order Odonata have included among the ecosystem friendly insects. In the present study, we aimed to evaluate the phylogenetics and evolutionary history of dragonflies at the cross junction of Punjab and Khyber Pakhtunkhwa provinces of Pakistan. The studies were principally based on the morphological characters of head and wing venation and phylogenetic analysis based on the nucleotide sequence of 12S rRNA gene. DNA was extracted using phenol-chloroform method and the DNA fragment was amplified through Polymerase Chain Reaction using 12S rRNA primers. A total of 233 collected specimens were identified into ten species from four genera (*Crocothemis, Orthetrum, Sympetrum* and *Zygonyx*) according to their morphological and morphometric characterization. The nucleotide sequence analysis of 12S rRNA gene had shown genetic affinities among the subject genera. The phylogenetic tree constructed by morphological data and 12S rRNA revolved two clades and supported the grouping of collected specimens. Further phylogenetic analysis based on nucleotide sequences of 12S rRNA from GenBank generated the phylogenetic tree with four clades of related species. On the basis of our findings, *Crocothemis erythraea* (Bulle 1832) were placed phylogenetically adjacent to *Orthetrum cancellatum* (Linnaeus 1758), *O. sabina* (Drury 1770) to *Libellula nodistica* (Hagen 1861) (EF640400.1), *O. glaucum* (Brauer 1865) to *Libellula saturate* (Uhler 1857) (EU054935.1), *O. brunneum* (Fonscolombe 1837) to *O. brunneum* (Fonscolombe 1837) (DQ021416.1), *Sympetrum fonscolombii* (Selys 1840) to *Orthemis ferruginea* (Fabricius 1775) (EF640402.1), and *Zygonyx torridus* (Kirby 1889) to *O. pruinosum* (Burmeister 1839) (EF640403.1) with minute differences in bootstrap values. The present report describes an aspect to record and catalogue the ecosystem friendly insects mostly being threatened. © 2021 Friends Science Publishers

Keywords: Dragonflies; Ecosystem friendly; Threatened; Phylogenetics; 12S rRNA

Introduction

The dragonflies are the most abundant and globally distributed insects except Antarctica. A total of 6500 species globally and 124 species of dragonflies have been reported from Pakistan reported (Mehmood 2016; Mehmood *et al*. 2020a). Members of the family Libellulidae, dragonflies come under sub order Anisoptera and order Odonata. Generally, these dragonflies are known as Skimmers and possess medium to large body size. Their fossil history showed the origin from Triassic period (250–200 million years ago) (Zia 2010). The existence of these dragonflies around Indus River shows that the area is clean and showing the goodness of environment health as these dragonflies are flying green flags of environment health. The dragonflies contain 3 phases of life (egg, naiad and adult). Naiad of these dragonflies are aquatic while adult is terrestrial but at both stages of life they depend on aquatic environment as they need clean water to survive so their presence shows the health of aquatic system as well (Zada *et al*. 2016; Mehmood *et al*. 2020b). Economically, the dragonflies can be used as biological control as there adults are important predators of serious insect pests of crops like rice and cotton. The larvae of dragonflies also feed on mosquito larvae and therefore, these can be used indirectly to control the dengue and malaria diseases (Zia *et al*. 2008).

The Lebellulid dragonflies are differentiated from other flies as they contain variable body colors and colored patterned wings with a soaked shaped vein in a hind wing called as anal loop (Samsudin 2013). The veins of wings are very important tool of Libellulidae family for the study of phylogenetic based on morphological characters. Even

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though these characters show similarity with other species of Odonata, but many characters are still show association when used to segregate other species. The Odonata species highly depend on characters of wing venation to separate Libellulidae into different families (Pfau 2005).

For molecular study, 28S and 16S ribosomal RNA are significant part to check the association between Libellulidae families (Ware *et al.* 2007). Furthermore, the nucleotide sequence of EF-1a of nuclear and mitochondria were also used for phylogenetic study (Ware *et al*. 2017). To investigate the evolutionary history of Libellulids dragonflies, the 12S rRNA of mitochondrial DNA analysis revealed monophyletic relationship of Anisoptera (Saux *et al*. 2003; Mehmood *et al*. 2020a). The present research work was based on morphological and molecular characters. Previously, the molecular characterization of Libellulids dragonflies was performed by Mehmood (2016) and Mehmood *et al.* (2020a) in Pakistan. In recent years, several other workers have performed molecular analysis on Odonata taxa (Kim *et al.* 2007; Bybee *et al.* 2008; Fleck *et al*. 2008; Dumont *et al.* 2010; Ballare and Ware 2011; Davis *et al.* 2011; Kohli *et al.* 2013; Carle *et al*. 2015; Bybee *et al.* 2016, Phan 2019; Saetung and Boonsoong 2019). Keeping in view the importance of Libellulidae family, the morphological and molecular analysis was done to confirm phylogenetic association of the related species.

Materials and Methods

Study Area

Libellulids were collected from District Attock which is in Pothohar Plateau, Punjab (Pakistan). The area is positioned in the Northern borderline of Punjab (side of river Indus) and covers an area of 6857 square kilometer at geographical coordinates 33° 46' 20" North latitude and 72° 22' 6" East longitude. The average rainfall is 783 mm in this district and the weather is cold in winters and in warm summers, while Northern part is moist due to high altitude (Chaudhry 2010).

Collection and Preservation

The specimens were collected during the months of April to November 2018 from District Attock with the help of hand net and then they were carefully transferred into entomological bottles (pour bottom with Cyanide) to kill them (Mehmood *et al*. 2020b). The samples were brought to Department of Genetics, Hazara University Mansehra, Khyber Pakhtunkhwa Pakistan. Then the samples were properly pinned on boards for further identification. The study was composed of two parts *i.e*. morphological and molecular study.

Identification and Morphometric Analysis

Samples were identified using identification key developed by Chaudhry (2010) and Fraser (1934).

Morphometry of abdomen, hind and fore wings were measured with the help of scale. Morphometric analysis was done through STATISTICA^ 7 v. 7 (Hilbe 2007) and PASTv. 3.40 software Based on recorded information, the Principal Component Analysis and Cladogram analysis were constructed. Alpha diversity among all species was measured based on three morphometric characters (length of fore wing, hind wing and abdomen). The analysis was performed based on their morphometric parameters *i.e*., FW, HW and abdomen. The alpha diversity was estimated through PAST software. Moreover, Dominance D, Simpson D, Shannon-H and Evenness were calculated through PAST software.

Morphological characters region (the color and frames of eye and structure of clypeus) were targeted from head region, while wing venation, number of nervier and shape of the cells in the wing were studied for morphological characterization of wings. The morphological characters of head and wings were recorded and converted into binary data (1, 0) matrix and these binary characters were analyzed through PAUP software V. 10.4.

Molecular Analysis

DNA Extraction

One leg was separated from each specimen of dragonfly through a forceps and placed in a labelled 1.5 mL micro centrifuge tube. The leg was cut into pieces with dissection scissors and ground into fine powder.

The genomic DNA was extracted from the fine powder of legs of collected specimens by using phenol chloroform method (Watts *et al*. 2001). The quality of extracted DNA was checked on 1% agarose/TAE gel and the quantity was observed using spectrophotometer. The agarose gel profiles of DNA were documented under UV light using UVtec" gel documentation system (Mehmood *et al*. 2016).

Selection of Primers and PCR amplification

The extracted DNA was used for PCR amplification of 12S rRNA gene: The conserve region of 12 S rRNA gene was used for amplification with the primers *i.e*., 5'AAA CTA GGA TTA GAT ACC CTA TTA T3´ 12S F, 5' AAG AGC GAC GGG CGA TGT GT3' 12S R that were adopted from the previous literature (Saux *et al*. 2003).

For the amplification, 20–30 ng of genomic DNA along with other the components (1.5 μL $MgCl_2$, 1.5 μL DNTPs, 0.2 μL *Taq Polymerase*, 2 μL buffer, 2 μL of forward and reverse primers and 8.8 μL of PCR water with final volume of 20 μL) of PCR mixture was used (Saiki *et al*. 1988).

Gene sequencing

The purified DNA of nine specimens of the Libellulidae

dragonflies were sent for sequencing to Macrogen Korea http:/www.macrogen.com. All amplified samples of DNA were successfully sequenced and the retrieved sequences were BLAST in NCBI GenBank database for sequences comparison, identification and further phylogenetic study.

Molecular characterization and Phylogenetic analysis

Sequences of DNA were aligned applying Muscle alignment (Edgar 2004) and CLUSTAL X 2.1 (Larkin *et al.* 2007). Aligned data was edited in BioEdit 7.2.5 (Hall 1999). Analyses of Phylogenetic relationship were executed using three methods namely Maximum parsimony (MP), Maximum likelihood (ML) and Neighbor-Joining (NJ). Maximum parsimony analyses were performed in PAUP4.0b10 (Swofford 2004). Maximum likelihood and Neighbor-Joining trees were generated through MEGA6 based on GTRGAMMA model (Tamura *et al*. 2013). Bootstrap was considered 70% as significant. For tree visualization, Fig Tree 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree) was used and tree annotating was done through Adobe Illustrator CS6.

Results

In this study, 233 specimens of Libellulidae, Odonata were collected from district Attock. Initially, the collected specimens were sorted out into 4 genera (*Crocothemis, Orthetrum, Sympetrum* and *Zygonyx*) and 10 species (*Crocothemis erythraea, C. servilia, Orthetrum pruinosum, O. brunneum, O. sabina, O. taeniolatum, O. cancellatum, O. glaucum, Sympetrum fonscolombii* and *Zygonyx torridus*).

Morphometric and Morphological Analysis of Wings and Abdomen

The length of three factors *i.e*., fore wing, hind wing and abdomen was selected for morphometric analysis. The morphometric data were analyzed to construct Cladogram/Dendrogram among the Libellulids species to understand the evolutionary relationship. Cladogram tree constructed three clades *i.e*., I, II and III. In clade I, four species had been clustered while II and III clades contain three species each (Fig. 1). In clade I, the species Sympetrum fonscolombi showed similarity with *Crocothemis erythraea* having bootstrap value 73%. In clade II, *O. pronosium* showed resemblance with *O. bruneum* having bootstrap value 75%. In clade III, *O. gluacum* showed correlation with *Z. torridus* having bootstrap value 67% (Fig. 1).

The result of Principal Component Analysis was based on three morphometric parameters *i.e*., length of fore wing, hind wing and abdomen. Among these, one species namely *O. teniolatum* showed a variable trend and it was plotted in the region of 0 to -20 toward component 2. While, other 8 species were observed in the same plot at 0 – to +20.

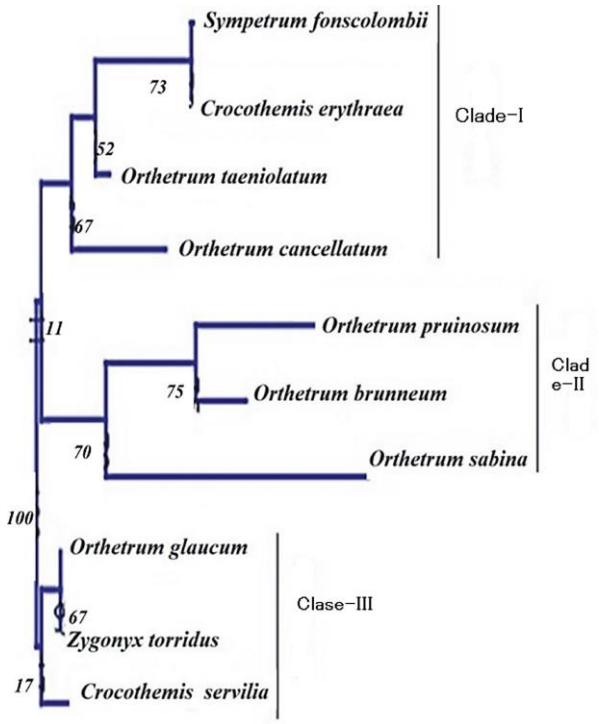

Fig. 1: Dendrogram constructed based on three morphometric parameter showing evolutionary relationships among Libellulidae species

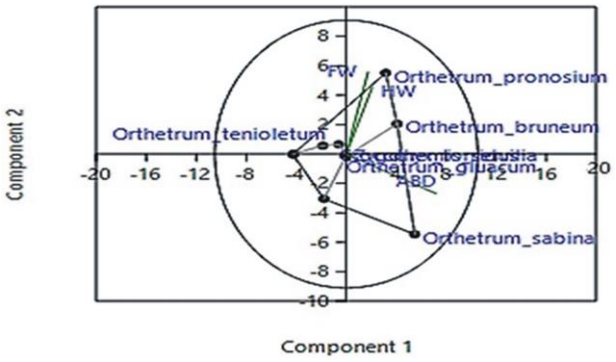

Fig. 2: Principal component plot constructed based on three morphometric parametric parameters showing relationships among Libellulidae species. PASTv. 3.40 software was used to construct this plot

Similarly, *O. sabina* also showed variable trends and plotted in the region of 0 to -20. So, high variation was observed for *O. sabina* and *O. teniolatum.* Fore wing (FW) and hind wing (HW) showed correlation toward 0 to + 20 at component 1 and 0 to +10 at component 2, while abdomen (ABD) showed a variable trend (0 to +20 at component 1 and 0 to -10 at component 2) (Fig. 2).

Alpha diversity among ten species of dragonflies was estimated on the bases of three morphometric factors *i.e*., fore wing, hind wing and abdomen. Various components of

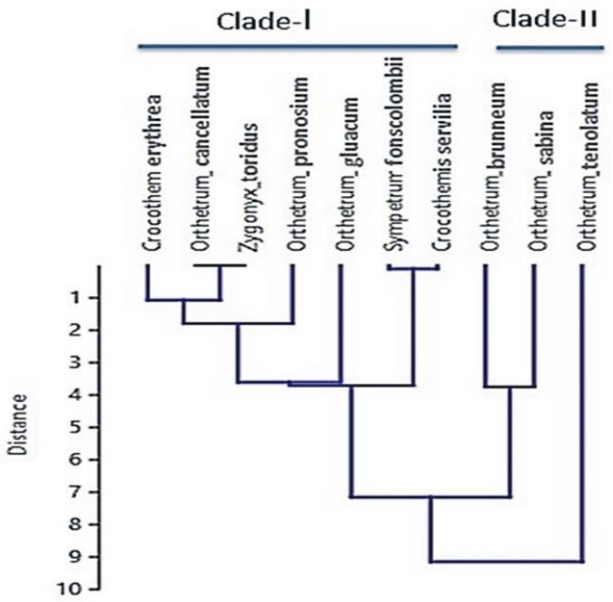

Fig. 3: Phylogenetic tree constructed based on morphological characters

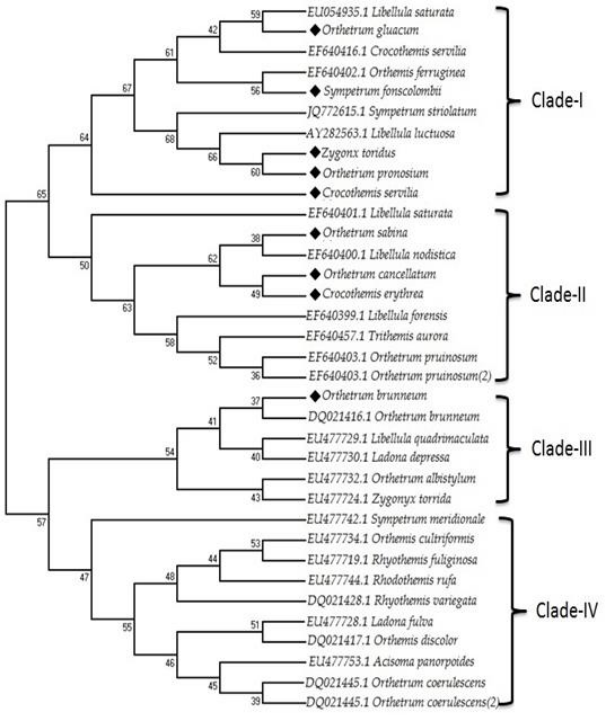

Fig. 4: Phylogenetic Tree based on Maximum Likelihood Method of 12S gene sequence of Libellulidae family, Maximum likelihood bootstrap values are presented above the branches

the Alpha diversity were resulted *i.e*. Dominance; D, Simpson_1–D, Shannon H, Evenness_e^H/S, Brillouin, Menhinick, Margalef, Equitability_J, Fisher_alpha, Berger-Parker and Chao-1. The highest value (2.3) of Shannon_H while the lowest (0.1) value of Berger-Parkerwas were observed for fore wings. Similar results were obtained for hind wings. However, in case of abdomen the highest (2.29) value of Shannon_H and the lowest (0.11) of Berger-Parker were recorded. Further, the same trend was observed in Dominance_D, Evenness_e^H/S and Equitability_J. In simpson_1-D the least value was 0.8926 of HW and maximum was 0.9 of FW (Table 1). In Brillouin 2.18 was the lowest value was observed in FW and 2.234 was the highest value in ABD. In Menhinick 0.5067 was the lowest calculated value in ABD and 0.586 was maximum value was observed in HW and same trend was calculated in Margalef and Fisher alpha. 0.113 was the least value of ABD and 0.1564 was the maximum value of FW in Berger-parker (Table 1).

Morphological characters were recorded from all identified species and then these characters were converted into numerical/statistical (binary matrix) data *i.e*. 0 for absent characters and 1 for present. The binary matrix 0,1 data analyzed to construct the phylogenetic tree. The phylogenetic figure constructed two clades *i.e*. clade I and II. Clade I composed of seven species (*Crocothemis erythraea^, O. cancellatum^, Z. toridus^, O. pruinosum^, O. gluacum, S. fonscolombii* and *C. servilia)*. Whereas, clade II was constructed into three species (*O. brunneum, O. sabina* and *O. teniolatum)* (Fig. 3).

Molecular Analysis

Evolutionary relationship of species under the family Libellulidae was done on molecular basis. A total of 35 nucleotide sequences were analyzed among them 9 sequences of the current research work, while other 26 sequences were retrieved from GenBank NCBI, related to family Libellulidae. These sequences were aligned using MUSCLE and BioEdit. The data was used for further analysis of Phylogenetic relationship.

The Maximum Likelihood (ML) analysis was done and constructed phylogenetic tree with length of -10733.49. The Maximum Likelihood Phylogenetic tree constructed four clades *i.e*. I, II, III and IV. The clade I consisted of 5 sequences and among them *Orthetrum*gluacum* showed similarity with (EU054935.1) *Libellula saturate* having 59 bootstrap value. *Zygonyx torridus* had similarity with (EF640403.1) *Orthetrum pruinosum* which have 60 bootstrap value, while *Crocothemis servilia* formed a separate branch. The clad II distributed into 9 sequences, among them *Orthetrum sabina* had close resemblance with (EF640400.1) *Libellula nodistica* with having 38 bootstrap value. *Orthetrum cancellatum* showed closeness with *C. erythraea* and *O. brunneum* has resemblance with (DQ021416.1) *Orthetrum brunneum* having 37 bootstrap value (Fig. 4).

Further, the molecular analysis was also confirmed with Neighbor-Joining (NJ) based method. For NJ analysis, a total of 35 sequences were applied among them 9 sequences were from the present study while others were

Table 1: Alpha diversity of Libellulidae family based on three morphometric characters

	FW	Lower	Upper	HW	Lower	Upper	ABD	Lower	Upper
Taxa_S	10	10	10	10	10	10	10	10	10
Individuals	299	299	299	291	291	291	389	389	389
Dominance_D	0.1005	0.1	0.1062	0.1004	0.1009	0.1074	0.1006	0.1006	0.1059
Simpson_1-D	0.8995	0.8938	0.9	0.8996	0.8926	0.8991	0.8994	0.8941	0.8994
Shannon_H	2.3	2.259	2.29	2.301	2.264	2.296	2.299	2.27	2.296
Evenness_e^H/S	0.9976	0.9574	0.9877	0.998	0.9623	0.9938	0.9969	0.9682	0.9938
Brillouin	2.195	2.18	2.21	2.22	2.188	2.219	2.231	2.209	2.234
Menhinick	0.5768	0.5768	0.5768	0.586	0.586	0.586	0.5067	0.5067	0.5067
Margalef	1.579	1.579	1.579	1.586	1.586	1.586	1.509	1.509	1.509
Equitability_J	0.9989	0.9811	0.9946	0.9991	0.9833	0.9973	0.9987	0.986	0.9973
Fisher_alpha	1.99	1.993	1.993	2.006	2.006	2.006	1.872	1.872	1.872
Berger-Parker	0.1164	0.1131	0.1564	0.1133	0.1133	0.158	0.1181	0.113	0.1515
Chao-1	10	10	10	10	10	10	10	10	10

FW: fore Wings, HW: Hind Wings, ABD: Abdomen, Taxa: S, group of species, Individual: number of specimens of each species, Dominance: D, Simpson:1-D, Shannon:H and Evenness: e-H/S

retrieved from GenBank data. The NJ phylogenetic tree was constructed of optimal length 22.41725 and having 325 characters. The NJ Phylogenetic tree was consisted of three clades *i.e.* I, II, and III. The clade I consisted on 14 sequences in which one sequence *(Orthetrum brunneum*) of the present study has close resemblance with (DQ021416.1) *Orthetrum, brunneum.* Six nucleotide sequences were gathered in clade II. *Sympetrum fonscolmbii*, had similarity with the sequences of *Orthemis ferruginea* and *Crocothemis servilia* with bootstrap value 44 and 15 respectively. The clade III consisted of 7 nucleotide sequences. In this clade *Orthetrum gluacum* showed affinity with *Libellula luctuosa* having bootstrap value 45. Threes sequences (*Zygonyx torridus, Orthetrum pruinosum* and *O. Sabina)* were gathered in this clade with bootstrap value 54. *Libellula nodistica* showed affinity with *Orthetrum sabina* having bootstrap value 39. Two sequences *Orthetrum cancellatum* and *Crocothemis erythreae*, expressed similarity with sequence of *Libellula forensis, Trithemis aurora* and *Orthetrum pruinosum*, showing bootstrap value 53 (Fig. 5).

Maximum Parsimony (MP) tree constructed 4 clades *i.e*. I, II, III and IV. Sixteen nucleotide sequences were clustered in clade I. *Orthetrum brunneum* clustered with 15 sequences having bootstrap value 64 (which extracted from GenBank data). Three sequences *i.e*., *Orthetrum cancellatum, Crocothemis erythraea* and *O. Sabina*were gathered in clade II and showed relationship with 3 species of *Libellula* genus. Nine sequences were clustered in clade III. *Zygonyx torridus, Orthetrum pruinosum* and *O. gluacum* had affinity with *Libellula luctuosa* with bootstrap number 42 while *Sympetrum fonscolombii* reflected relationship to *Libellula luctuosa* with bootstrap value 43. *Sympetrum fonscolombii* came into neighbor branch of *Orthemis ferruginea* and *Crocothemis servilia* and expressed 55 bootstrap number. While, *Crocothemis servilia* formed an isolated branch (Fig. 6).

Evolutionary tree was also constructed based on Maximum Parsimony Method (tree length of 3440 which had consistency index 0.275000, 0.494528 was its retention index and the composite index was 0.135995$ for all sites)

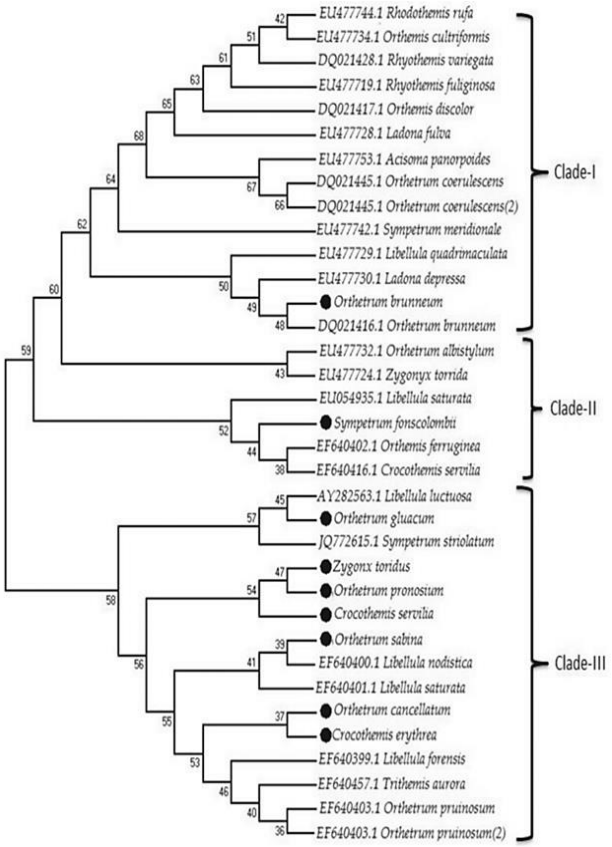

Fig. 5: Phylogenetic tree based on Neighbor-Joining Method of 12S gene sequence of Libellulidae family, Neighbor-Joining bootstrap values are presented above the branches.

and Neighbor-Joining Method (tree length 22.41725) (Fig. 6).

Discussion

Libullid Dragonflies are members of family Libellulidae. Being very sensitive to the water conditions (Kietzka *et al*.

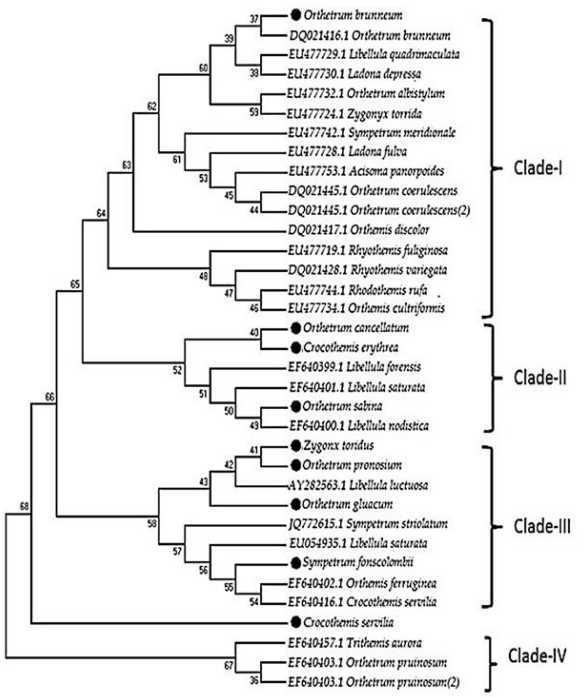

Fig. 6: Phylogenetic tree based on Maximum Parsimony method of 12S gene sequence of Libellulidae family, Maximum Parsimony bootstrap values are presented above the branches.

2017), the dragonflies are considered as eco-friendly insects which serve as the indicators of environmental pollution, especially water pollution (Simon *et al*. 2019). Dragonflies also provide an attractive alternative to the chemical control of mosquitos in the water bodies (Norma-Rashid and Saleeza 2014). Worldwide changes in the environment with enhanced metal content and pollution have seriously disturbed the distribution of dragonflies; many species have become endangered (Kadoya *et al*. 2009; Ferreira *et al*. 2014; Beaune and Sellier 2021). The present study was conducted to record the species of dragonflies in the water bodies of Attock, an area at the transection of two Pakistani provinces. The morphological characters including the pattern of wings venation were used for the identification of and characterization of taxa in various groups and subgroups. This research represents the first report of its type in the study area, based on molecular and morphological characterization of family Libellulidae. On the basis of morphological characters, the specimens were characterized into ten species with four genera. The systematic study of dragonfly, which was given by previous workers, the results of current study support the work (Fraser 1936; Tillyard and Fraser 1940; Carle 1995; Bechly 1996; Zia 2010). Previously, the integrative approach (morphological and molecular) have been used to find out the phylogenetic patter of Odonate fauna from various regions of world (Mehmood 2016; Huang *et al*. 2020).

The outcomes and the results of the present work could be compared with the morphological and morphometric study of Chaudhry (2010) and Raza (2016). Findings of the morphometric and morphological of the present study were found similar with the earlier results of different workers (Manwar *et al*. 2012; Pilgrim and Dohlen 2012; Eslami *et al*. 2015). The estimation phylogeny of Odonate would be useful to understand interspecific relationship and integration of morphological and molecular data could be more important for better phylogenetic estimation among the members of dragonfly (Huang *et al*. 2020).

Results of present work comprised of phylogenetic analysis using 12S DNA primer sequences yielded phylogenetic affiliation of group Libellulidae (Dumont *et al*. 2010; Bastos *et al*. 2021). The remarkable results were yielded by Rach *et al*. (2008). During phylogenetic analysis, the close relationship with other members of Libellulidae has been recorded and the reported members have been collected from various regions of world. Recently, the morphological and phylogenetic characters have been discussed to explore distribution and migration pattern of different species of Odonata (Huang *et al*. 2020; Bastos *et al*. 2021).

The amplified 12S RNA of mitochondrial region showed that *Crocothemis erythraea* have genetic similarity to *Orthetrum cancellatum* (Maximum Likelihood Bootstrap =49%, Neighbor Joining Bootstrap =37% Maximum Parsimony Bootstrap =40%) while *Crocothemis servilia* made isolated division. Our study confirmed the previous work of researchers and strongly supported the monophyly of Libellulidae dragonflies with some variation of sequences at several clades (Ware *et al*. 2007; Bybee *et al*. 2008; Fleck *et al*. 2008; Dumont *et al*. 2010; Bastos *et al*. 2021). The current phylogenetic work comprised of 12S information expressed the genetic resemblance of *O. brunneum* with *O.brunneum* (DQ021416.1), *Libellula quadirmaculata* (EU477729.1) and *Ladona depressa* (EU477730.1). Similarly, Ware *et al*. (2007) had recorded the relationship of family Libellulidae with Acisoma with *Bradinopyga*, *Crocothemis* and *Palpopleura.* Using similar techniques, on Anisoptera, the evolutionary relation genus *Crocothemis*, *Rhyothemis* and *Palpopleura* had been reported (Carle *et al*. 2015). Current study based on molecular basis identified that earlier study was with Accordace as Pilgrim and Dohlen (2008) and they comprised of *Brachyothemis* with *Deielia phaon*. Same as, genetic relation was identified with *Tholymis* and *Zyxomma* (Ware *et al*. 2007; Dijkstra and Schröter 2020). However, Carle *et al*. (2015) have elaborated relationship (evolutionary) of *Brachyothemis* with *Tramea*, *Psuedothemis* and *Tholymis*.

Conclusion

The present work investigated the morphological and molecular identification of dragonflies. The wings and abdomen were targeted in morphological identification and the nucleotide sequences of 12S rRNA were retrieved from

the morphologically characterized specimens of dragonfly. During morphological and morphometric analysis, ten species were identified belonging to four genera. The molecular based analysis recorded that *Crocothemis erythraea* have genetic similarity to *Orthetrum cancellatum*, while *Crocothemis servilia* made isolated division. Further, it was revealed that *O. Sabina* grouped with *Libellula nodistica* (EF640400.1) and *O. glaucum* grouped with *Libellula saturate* (EU054935.1) and *L. luctuosa* (AY282563.1). Similarly, *O. brunneum* had genetic similarity with *O. bruneum* (DQ021416.1) and *Sympetrum fonscolombii* with *Orthemis ferruginea* (EF640402.1) and *Zygonyx torridus* with *O. pruinosum*. However, molecular techniques of 12S gene presented the superlative outcomes at all and it could help to resolve the phylogenetic of unknown dragonfly.

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Author Contributions

Malika Mehreen Nisar performed main experiments and applied molecular analysis, Khushi Muhammad supervised research and provided guidelines for writing manuscript, Sardar Azhar Mehmood helped in sample collection and species identification, Shabbir Ahmed helped in writing and data analysis, Bibi Nazia Murtaza helped in experimentation design and proofreading, Muhammad Shahid Nadeem helped in the revision of manuscript and data analysis. All authors have read and approved the final manuscript.

Conflict of Interest

The authors declared that they have no conflict of interest.

Data Availability

The data presented in this study are available on request from the corresponding author.

Ethics Approval

There is no direct involvement of animals or humans. However, the study was approved by the 20th meeting of the Advanced Studies and Research Board, Hazara University, Mansehra, Pakistan.

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