



**Full Length Article**

## Molecular and Phylogenetic Analysis of Family Aeshnidae of Hazara Region, Pakistan

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### Abstract

Current study was conducted on family Aeshnidae from Hazara region of Pakistan. During the survey a total of 125 members were collected and identified into 2 species under single genus. The present study focuses on molecular characterizations and phylogenetics of family Aeshnidae. Phylogenies of the analyzed taxa were elaborated with maximum likelihood, maximum parsimony and Bayesian analysis. We sequenced both mitochondrial genes *i.e.*, COI and 16S rRNA, separate and combined COI+16S data sets revealed evolutionary relationship within Aeshnidae at the species and genera level. Mean Pairwise Distances (MPD) of each species were ranged from 0.00 to 84.60%. Evolutionary rate differences among two categories Gamma distribution and Invariant were recorded as 0.07 and 1.20 substitutions per site. DNA based identification using COI, 16S and combined COI+16S data set, for all Aeshnidae species shared genetic similarities having bootstrap values MLB=70–100%, MPB= 52–100% and BPP=0.75–1% respectively. The analysis of the combined (COI+16S) data set produced trees with complete stronger bootstrap support than analyses of either gene alone. These findings had shown that the taxonomic position of Aeshnidae species based on morphological characters could be verified, further improved and confirmed by the use of modern molecular biological tools which involve the nucleotide sequences of genes used in phylogenetic investigations. © 2021 Friends Science Publishers

**Keywords:** Aeshnidae; Molecular analyses; Phylogeny; COI; 16SrRNA; Hazara region

### Introduction

Family Aeshnidae comes under sub order Anisoptera and order Odonata. Aeshnids are worldwide distributed dragonflies with a record of 441 species (Schorr *et al.* 2009). Regarding Pakistan its six genera and nine species have been reported till to date. Presently 27 species of genus *Anax* were reported worldwide and four species recorded from Pakistan (Chaudhry 2010). Aeshnid dragonflies are large in size and predators in freshwater ecosystems (Needham and Westfall 1955; Wissinger 1989). Zia *et al.* (2008) observed that they are important predators of insect pests of agriculturally important crops. Din *et al.* (2013) and Bhatti *et al.* (2013) conducted research on their Naiads (larvae) and reported they are voracious predators and consume mosquito larvae and other small crustaceans in large numbers. Besides this, Aeshnid dragonflies also be a

significant prey of birds, fishes and few invertebrates thereby playing an important component in food chain of these organisms (Chaudhry 2010).

Taxonomy is the identification of species based on the morphological characters and since it requires vast knowledge about the varied organisms and their characters. In many cases, the conventional taxonomy is difficult due to the external changes in the organisms caused by seasonal and geographical variations, whereas in the case of insects, sexual dimorphism and mimicry often leads to the misidentification of the original species (Mehmood *et al.* 2016). The incidence of sexual dimorphism is high in insects and aquatic organisms. Many organisms alter themselves physiologically and morphologically due to the unfavorable conditions in the environment. Generally, these variations accumulate in the species and bring about a drastic change in the outlook or appearance of the animals.

The most common phenomenon exhibited by the insects is to act as a mimic to the model organisms, whereby it is facilitated by anti-predatory effects and habitats survival. On the basis of the above-mentioned difficulties, by adopting manual taxonomy, misidentification of the species may arise. This problem has thus influenced the emergence of the molecular taxonomic frame work studies for the conformation and the betterment in the identification of species (Mehmood *et al.* 2016).

Although various studies have been conducted in the world to determine the phylogenetics of family Aeshnidae by several workers *i.e.*, The application of COI gene for determining genus-level evolutionary relationships among the several insect taxa (Crozier *et al.* 1989; Obrower 1994; Simon *et al.* 1994; Sperling and Hickey 1994; Sperling *et al.* 1997), including Aeshnid dragonflies (Lunt *et al.* 1996). Similarly, many workers done study on ribosomal gene, 16S rRNA and evaluated the comparison among phylogenetic relationship based on single and combined data sets of COI and 16S (Kambhampati and Charlton 1999). Many other workers have studied the molecular characterizations of various odonata species *i.e.*, (Misof *et al.* 2000; Artiss *et al.* 2001; Misof *et al.* 2001; Saux *et al.* 2003; Hasegawa and Kasuya 2006; Ware *et al.* 2007a, b, 2008; Bybee *et al.* 2008; Fleck *et al.* 2008; Dumont *et al.* 2010; Davis *et al.* 2011; Elizabeth *et al.* 2011; Kohli *et al.* 2014; Kim *et al.* 2014; Carle *et al.* 2015; Bybee *et al.* 2016; Das 2016; Suvorov 2018; Kalavanti and Jethva 2019).

The objectives of the present research work were to practice the morphometric data, and molecular data which included (COI and 16S) genes to evaluate the major evolutionary association among the species and genera of Aeshnidae dragonflies. Current study represents the Phylogenetic affiliation among the species and genera of Aeshnidae, and established the basis for subsequent comparative studies of morphological and DNA based characterization among the members of family Aeshnidae.

## Materials and Methods

### Sample collection and preservation

Specimens of family Aeshnidae were collected from throughout Hazara region of Pakistan (Fig. 1). Methods of sampling were based on Zia (2010). Method of preservation was based on Chaudhry (2010) and Zia (2010). Adult Odonates were caught with a light and strong insect collection net, during (09 am to 06 pm) on clear sunny days. Collected specimens were kept in entomological bottles having poured with cyanide; dead members were expanded on setting boards and tagged with complete information such as geographic coordinates, collector's name, date of collection and localities. All the specimens were also placed in deep freezer (-21°C) for two day for leaning from any fungal contamination. Then the dried specimens were transferred in collection boxes containing naphthalene balls in the National Insect Museum, NARC Islamabad.

### Identification

Taxonomic keys of Chaudhry (2010) and Fraser (1933, 1936) were followed to identify collected specimens up to specific level. Help in identification was also done by using reference collection of Odonata in National insect Museum, National Agricultural Research Centre (NARC) Islamabad.

### Molecular study

**Isolation of DNA:** Isolation of DNA from dried Aeshnids dragonflies was carried out using Invitrogen (PureLink Genomic DNA) kit applying spin columns with minor modifications in procedure provided by the supplier company. One leg was separated from each specimen through a forcep and placed in a labelled 1.5 mL micro centrifuge tube while leg was cut into pieces with dissection scissors. After the completion of DNA extraction process, 1% agarose/TAE gel was used to check the quantity and Quality of the extracted DNA. "UVtec" gel documentation system was used to observe DNA band under "UV" light.

### Selection of Primers and PCR amplification

Primers were selected from NCBI gene bank data available in the literature and the desired sequence of the region which has to be amplified. The isolated genomic DNA was used as template for PCR amplification based on Cytochrome C Oxidase 1 (COI), and 16S rRNA. The sequences of selected primers are presented as GGTCAACAAATCATAAAGATATGG (F), TAAACTTCAGGGTGACCAAAAAAT CA (R), CGCCTGTTTATCAAAAACAT (F) and CTCCGGTTTGAAGTCAAGATCA(R) respectively (Mehmood 2016).

### Gene sequencing

The purified DNA of the Aeshnidae dragonflies was sent for sequencing to MacroGen Korea <http://www.macrogen.com>. All amplified samples of DNA were successfully sequenced; DNA sequences were BLAST in NCBI GenBank data base for comparison sequences, 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, Maximum parsimony (MP), Maximum likelihood (ML) and Bayesian analyses (BPP). Maximum parsimony analyses were performed in PAUP4.0b10 (Swofford 2004). Maximum likelihood tree was generated through MEGA6 based on GTRGAMMA model (Tamura

*et al.* 2013). Bootstrap was considered 70% as significant. Bayesian analyses based on Markov chain Monte Carlo (MCMC) and done using the software BEAST 1.6.2 and with the application of (XSEDE) applied on the CIPRES Science Gateway v. 3.3 (Drummond and Rambaut 2007; Miller *et al.* 2010). To check the ESS value (effective sample size) TRACER 1.5 was implemented (Rambaut and Drummond 2009) Posterior probabilities values (PP) were adjusted greater than 0.95% as significant. For tree visualization, Fig Tree 1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree>), (Rambaut 2012) was used and tree annotating was done through Adobe Illustrator CS6.

## Results

### Molecular systematics and phylogenetic analysis based on 16S data

Initially 16S rRNA nucleotide sequences were BLAST in GenBank NCBI. The blast result of these species showed 98% maximum identity 99% query cover and E-value 0 and 97% maximum identity 96% query cover and E-value 0 with *A. junius* (AY282557.1) and *A. parthenope* (EU477651.1) respectively. Mean Pairwise Distances (MPD) of each species were ranged from 0.006 to 47.60%. Evolutionary rate differences among two categories Gamma distribution and invariant (+G+I) were recorded as 0.11 and 1.89 substitutions per site.

The optimal 16S rRNA tree was obtained with log likelihood -8110.6425 and its bootstrap supported values are presented below the branches (Fig. 2). The 666 positions/characters were used, out of them 1 character was conserved, 665 were variable, 661 were parsimony informative and 5 were single tone sites. The MP technique was based on TBR parameter (Nei and Kumar 2000) with heuristic search of 1000 replicates. The most parsimonious tree was obtained using MP method with length = 2985 and boot strap supported values are shown above the branches (Fig. 2). The consistency index (CI), retention index (RI) and composite index observed as 0.268342, 0.191111 and 0.051283 respectively. Bayesian Posterior Probabilities (BPPs) is presented below the branches in parenthesis (Fig. 2).

Phylogenetic tree was clustered into two main clades i.e. clade I and II. The clade I was further sub divided into four sub clades. The sub clade I consisted into four species under two different genera. Sub clade II spreaded into four species of genus *Aeshna*, while subclade III comes under three *Aeshna* species. Subclade IV comprised of four species of genus *Anax* including our local species. *A. parthenope* grouped in subclade IV and showed close relationship with *A. parthenope* (EU477651.1) and its bootstrap supported values were recorded as MLB=98, MPB=98, BPP=0.99 respectively. Clade II come up in to two i.e. subclades, V and VI. The subclade V consisted of

four species of genus *Anax*, our local species *A. immaculifrons* clustered in subclade V and showed close resemblance with *A. junius* (AY282557.1) and its supported value were recorded MLB=97, MPB=99, BPP=0.98. While subclade VI comprised of four species belonging to four different genera (Fig. 2).

### Molecular systematics and Phylogenetic analysis based on CO1 data

For molecular characterization the partial nucleotide sequence of CO1 gene were obtained for assigning the phylogenetic status of *Anax immaculifrons* (Rambur 1842) and *Anax parthenope* (Selys, 1839) belonging to genus *Anax* and family Aeshnidae. After BLAST analysis of CO1 sequences of these species showed 99% maximum identity 99% query cover and E-value 0 and 98% maximum identity 97% query cover and E-value 0 with *A. nigrofasciatus* (KF257056.1) and *A. parthenope* (KF257072.1) respectively.

Mean Pairwise Distances (MPD) within each species were ranged from 0.087–64.07%. Differences of evolutionary rate were determined among two categories using a distinct Gamma distribution (+G+I). Mean evolutionary rates in these categories were recorded as 0.07 and 1.93 substitutions per site.

The ML tree obtained with highest log likelihood (-3336.2739), having bootstrap values below the branches (Fig. 3). A total 1147 positions/characters were used, out of them 440 were conserved, 707 were variable, 351 were parsimony informative and 356 were singleton sites. The most parsimonious tree was obtained of length 910 and having supported values above the branches (Fig. 3). The consistency index (CI), the retention index (RI) and the composite index were observed as 0.841758, 0.626943 and 0.527734 respectively. Bayesian Posterior Probabilities (BPPs) is presented below the branches in parenthesis (Fig. 3).

The phylogenetic tree generated two major clades, clade I and II, clade I including four species of genus *Anax* Our local species *A. parthenope* grouped in clade I and showed close relationship with *A. parthenope* (KF257072.1) and its supported values were recorded as MLB= 97, MPB=98, BPP=97%. While local species *A. immaculifrons* showed homology with *Anax nigrofasciatus* (Gene Bank accession # KF257056.1) with supported values MLB= 95, MPB=94, BPP=0.96%. Clade II comprised of six species under four genera belonging to family Aeshnidae (Fig. 2).

### Molecular characterization and phylogenetic analysis based on combined CO1+16S data

The partial sequences of combined CO1+16S data set were selected for further study. Mean Pairwise Distances (MPD) of each species were ranged from 0.087 to 64.07%. The discrete Gamma Distribution (+G) and invariant (+I) were found 4.0905 and 0.0000% respectively.

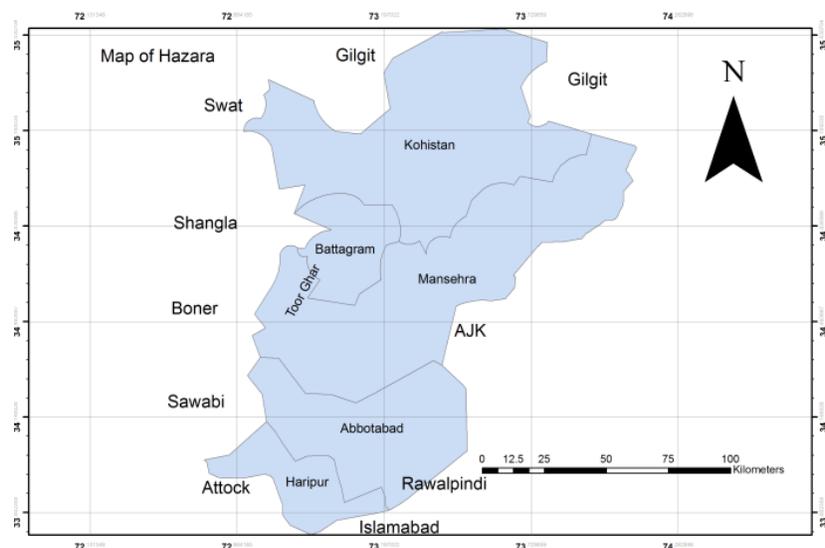


Fig. 1: GIS Map of Hazara Region-Pakistan

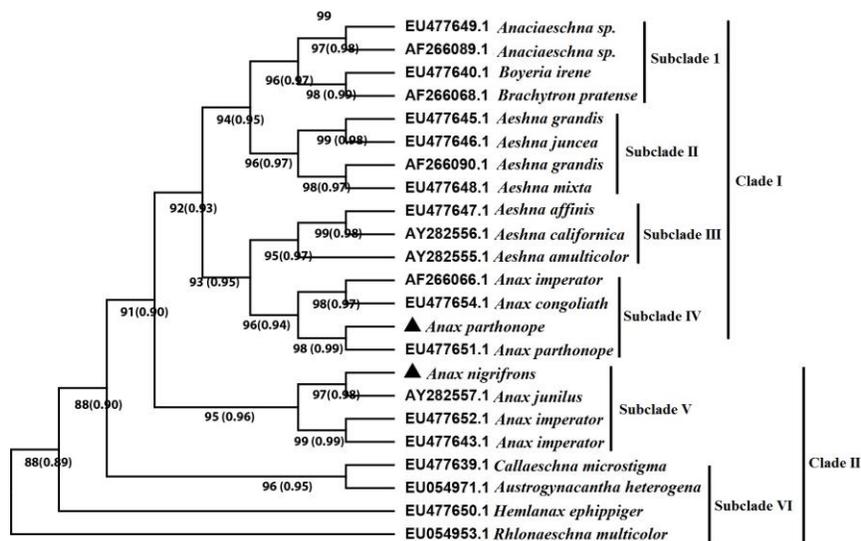


Fig. 2: Phylogenetic tree based on ML analysis of 16S gene sequence, Parsimony bootstrap values are given above the branches, Maximum likelihood bootstrap values are presented below the branches and values from posterior probabilities of Bayesian analysis are showed below the branches in parenthesis. Scale bar presented 5 changes per 100 characters (Sequences of the current study)

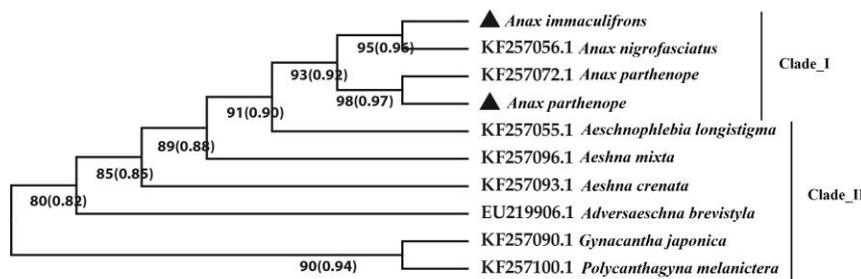
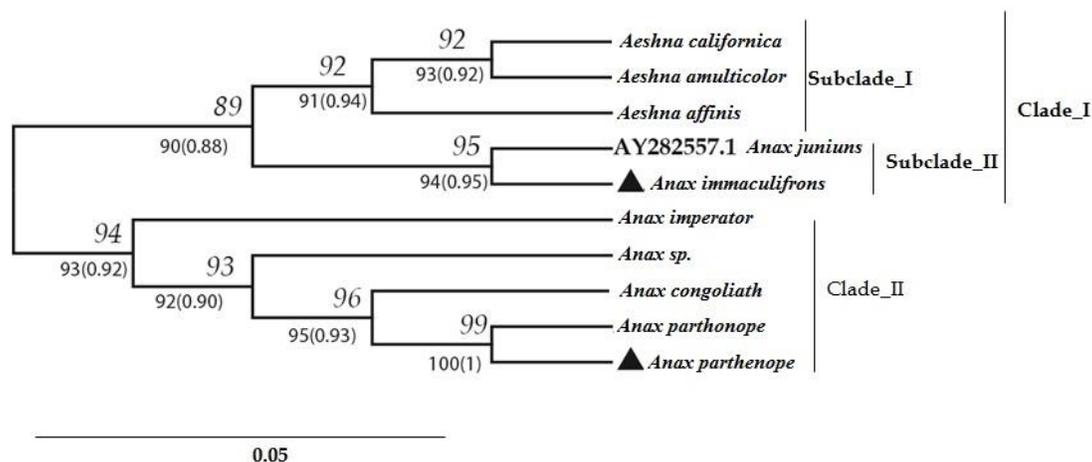


Fig. 3: Phylogenetic tree based on ML analysis of CO1 gene sequence, Parsimony bootstrap values are given above the branches, Maximum likelihood bootstrap values are presented below the branches and values from posterior probabilities of Bayesian analysis are showed below the branches in parenthesis. Scale bar presented 5 changes per 100 characters (Sequences of the current study)



**Fig. 4:** Phylogenetic based on ML analysis of COI+16S data set s, Parsimony bootstrap values are given above the branches, Maximum likelihood bootstrap values were presented below the branches and values from posterior probabilities of Bayesian analysis are showed below the branches in parenthesis. Scale bar presented 5 changes per 100 characters (Sequences of the current study)

The ML tree was obtained with highest log likelihood -10903.7856 having bootstrap values below the branches (Fig. 4). Out of 971 characters/positions, 3 conserved, 968 variable, 940 parsimony informative and 31 singleton sites. The most parsimonious tree was found having length 3228, with bootstrap values above the branches (Fig. 4). The consistency index (CI), retention index (RI) and composite index was recorded as 0.614312, 0.434348 and 0.266825 respectively. Bayesian Posterior Probabilities (BPPs) are presented below the branches in parenthesis (Fig. 4).

Phylogenetic tree generated into two major clades i.e. clade I and II. The clade I further subdivided into two subclades. Subclade I comprised of three sequences, covering three species under genus *Aeshna*, whereas subclade II consisted of two species, including our local species *Anax immaculifrons* and it showed genetic affinities with *A. junius* having supported values as MLB=94%, MPB=95%, BPP=0.95%. The result of combined COI+16S data set of *A. immaculifrons* was found similar to the outcomes of 16S but differ from COI data. Clade II included five sequences covering four species under the genus *Anax*, including our local species *A. parthonope* and this species shared genetic similarity with *A. parthonope* with supported values i.e., MLB=100%, MPB=99%, BPP=1%. The outcomes of combined COI+16S data set similar to findings of COI and 16S individual data analysis with slightly variation of bootstrap supported values (Fig. 4).

#### Genetic distance among genera of family aeshnidae

The average number of nucleotide differences (D) and the average number of nucleotide substitutions per site between genotypes (Dxy) were observed. The lowest distance was estimated between *Rhinoaeshna* and *Hemianax* (D= 8.000 and Dxy= 0.02439) followed by *Rhinoaeshna* and *Caliaeshna* (D=10.000 and Dxy=0.03049) while the highest

distances were recorded between *Anax* and *Anaciaeshna* (D=147.250 and Dxy=0.32081) followed by *Gynacanth* and *Anaciaeshna* (D=147.000 and Dxy= 0.32081) (Table 1).

#### Genetic diversity among genera of family aeshnidae

Genetic diversity among 22 genotypes of family Aeshnidae were estimated, it was revealed that 20 haplotypes resulted with Nucleotide diversity of *Anax*, *Aeshna* and other genera 0.17, 0.18 and 0.18 respectively. Haplotype gene diversity was observed i.e., *Anax*, *Aeshna* and other genera 0.95, 1.00 and 1.00 respectively. Whereas average number of nucleotide differences k resulted for *Anax*, *Aeshna* and other genera 46.76, 53.05 and 54.82 respectively. Fu's Fs test also calculated and it revealed for *Anax*, *Aeshna* and other genera 2.81, 0.77 and 0.46 respectively. The value of Tajima's D test was yielded for *Anax*, *Aeshna* and other genera -1.61\*, -1.65\* and -1.73\* respectively, (Table 2).

#### Discussion

Molecular and Phylogenetic results presented here and those of many recent workers (Fleck *et al.* 2008; Dumont *et al.* 2010) have clearly supported genus *Anax* as monophyletic. Our findings based on 16S data revealed the clustering of *A. parthonope* and *A. immaculifrons* with *A. parthonope* (EU477651.1) and *A. junius* (AY282557.1) respectively.

Whereas analysis of COI data showed homology of *A. parthonope* and *A. immaculifrons* with *A. parthonope* (KF257072.1) and *A. nigrofasciatus* (KF257056.1) respectively. The findings of the present molecular study have been justified with previous molecular works of Misof *et al.* (2001), Ware *et al.* (2007a, b), Fleck *et al.* (2008) and Davis *et al.* (2011) with minor difference of supported values. While the analysis of combined COI+16S data sets recognized the position of *A. parthonope* and *A.*

**Table 1:** Average pair wise differences between genera of family Aeshnidae

	<i>Anax</i>	<i>Aeshna</i>	<i>Anaciaeshna</i>	<i>Hemianax</i>	<i>Caliaeshna</i>	<i>Gynacanth</i>	<i>Rhinoaesc</i>	<i>Austrogyn</i>	<i>Boyeia</i>
<i>Anax</i>	-	46.571	147.250	75.667	81.833	84.833	38.833	41.333	84.500
<i>Aeshna</i>	0.16935	-	86.429	28.571	31.714	33.143	29.571	36.286	40.000
<i>Anaciaeshna</i>	0.32081	0.29199	-	140.500	145.000	147.000	97.000	99.500	144.000
<i>Hemianax</i>	0.16378	0.09653	0.29271	-	17.000	20.000	8.000	12.000	27.000
<i>Caliaeshna</i>	0.17751	0.10714	0.30146	0.03448	-	26.000	10.000	16.000	27.000
<i>Gynacanth</i>	0.18362	0.11197	0.30498	0.04040	0.05253	-	15.000	14.000	38.000
<i>Rhinoaesc</i>	0.12901	0.09990	0.30218	0.02439	0.03049	0.04573	-	19.000	19.000
<i>Austrogyn</i>	0.13508	0.12300	0.30615	0.03614	0.04819	0.04217	0.05810	-	27.000
<i>Boyeia</i>	0.19470	0.13514	0.31718	0.05806	0.05794	0.08137	0.05882	0.08385	-

Average number of nucleotide differences between populations D (above) Average number of nucleotide substitution per site between populations, Dxy (below) The data was analyzed using DnaSP 5.10 software

**Table 2:** Values of Genetic diversity of Genus *Anax*, *Aeshna* and other genera of family Aeshnidae

Species	<i>Anax</i>	<i>Aeshna</i>	Other Aeshnidae	Total
No of sequences	7	7	8	22
No of haplotypes	6	7	8	20
Nucleotide diversity	0.17	0.18	0.16	0.16
Standard deviation of nucleotide diversity	0.10	0.11	0.09	0.05
Haplotype (gene diversity)	0.95	1.00	1.00	0.99
Variance of haplotype diversity	0.01	0.01	0.00	0.00
Standard deviation of haplotype diversity	0.10	0.08	0.06	0.02
Average number of nucleotide differences k	46.76	53.05	54.82	44.28
Theta	0.23	0.25	0.25	0.20
Raggedness index	0.17	0.13	0.05	0.03
Fu's Fs test	2.81	0.77	0.46	-1.36
Tajima's D test	-1.61*	-1.65*	-1.73*	-0.85

*immaculifrons* with *A. parthenope* and *A. junius* respectively. Similar study was carried out by Bybee et al. (2008) and Carle et al. (2015) have conducted the Phylogenetic studies of Anisoptera and recorded the evolutionary relationship of *Anax* and *Aeshna* as supported values (MLB=78%, BPP=99%) and (BP =20, PP=27) respectively. Similarly, Saux et al. (2003) conducted phylogenetic study of Aeshnidae while, Kohli et al. (2014) conducted study on genus *Boyeria* using morphological and molecular markers. Whereas many previous workers studied the molecular characterizations of odonata including members of Aeshnidae i.e., (Misof et al. 2000; Artiss et al. 2001; Misof et al. 2001; Hsegawa et al. 2006; Ware et al. 2008; Elizabeth et al. 2011; Kim et al. 2014; Bybee et al. 2016; Das 2016; Suvorov 2018; Kalavanti and Jethva 2019).

The genetic distance and diversity among the genera of Aeshnidae based on molecular data is presently insufficient to entirely explain the evolutionary relationship among the genera of Aeshnidae. However, the morphological analysis has done by Ellenrieder (2002) provides a topology for evaluation. Whereas according to our findings the genera i.e., *Anaciaeshna*, *Boyeria*, *Brachytron* and *Aeshna* reusuted as the sister group in constructed phylogenetic tree (Fig. 2, Subclade I and II). While *Aeshna* also showed genetic affinities with *Anax* (Fig. 2, Subclade III, IV and V). Our results also support the genus *Brachytron* and *Aeshna* in same clade as sister group as described by Ellenrieder (2002). However, the topologies differ in that *Boyeria* and *Caliaeshna* do not

form a monophyletic group. Two genera *Anax* and *Hemianax* were not nested within same clade in our result during the present study. Whereas, Ellenrieder (2002) showed them as close related group. During the present study the genera, i.e., *Austrogynacantha*, *Rhionaeshna*, *Hemianax*, *Caliaeshna* and *Anax* were reulted group together (Fig. 2, Subclade V and VI), similar topology and evolutionary relationship also described by Carle (2012).

## Conclusion

Present work is the first molecular data of Aeshnidae from Hazara region of Pakistan. The molecular techniques of CO1, 16S and combined (CO1+16S) presented the superlative outcomes at all. Whereas investigation of (CO1+16S) data set produced the more precise consequences and Phylogenetic relationship than separate/single analysis of CO1 and 16S. Throughout the current molecular research Maximum likelihood (ML) Maximum parsimony (MP) and Bayesian analysis (BA) were used to check the reliability of each method. These methods demonstrated similar evolutionary relationship and topologies only variations were that for some nodes and with minor differences of bootstrap values.

## Author Contributions

SAM & MSA carried research work, AZ & MS, prepared the manuscript, WK & WAP data analyzed and SA proof read the final version of the manuscript for publication

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