INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
25–390/2026/35:350103
http://www.fspublishers.org

Full Length Article

Morphological and Molecular Based Assessment of Two *Anax* Species (Odonata: Aeshnidae) from Islamabad Capital Territory, Pakistan

Ahmed Zia[1], Sardar Azhar Mehmood[2], Maryum Ilyas[1], Muhammad Inam[3*], Waqas Ahmad[1], Muhammad Naeem Riaz[4] and Rukhshanda Saleem[5]

[1]*National Insect Museum, National Agriculture Research Centre, Islamabad, Pakistan*
[2]*Department of Zoology, Hazara University, Mansehra, Pakistan*
[3]*Department of Zoology, Abdul Wali Khan University, Mardan, Pakistan*
[4]*National Institute for Genomics and Advanced Biotecnology, NIGAB, Islamabad, Pakistan*
[5]*University of Sialkot, Punjab, Pakistan*
[*]For correspondence: inam8294@gmail.com
Received 21 April 2025; Accept4d 08 September 2025; Published online 29 October 2025

Editor: Javaid Iqbal

Abstract

The present investigation focused on the morphological and molecular assessment of the genus *Anax* from the Islamabad Capital Territory of Pakistan. A total of 25 specimens were collected during the survey, which were classified into two species within the genus *Anax*. The phylogenies of the analysed taxa were constructed using maximum parsimony, Bayesian analysis and maximum likelihood approaches. The sequencing of mitochondrial genes and 16S rRNA data sets uncovered evolutionary associations at both the species of Aeshnidae and their genus levels. The Mean Pairwise Distances (MPD) for every species varied from 0.00 to 84.60%. Differences in evolutionary rates between the two groups, Gamma distribution and Invariant, were noted at 0.07 and 1.20 substitutions per site, respectively. The molecular characterisation of *Anax* species through a DNA-based approach utilising the 16S dataset revealed shared genetic similarities, with bootstrap values of MLB ranging from 70 to 100%, MPB from 52 to 100%, and BPP between 0.75 and 1%, respectively. The analysis of the integrated (16S) data set yielded trees with significantly enhanced bootstrap support compared to the analyses of each gene individually. The findings demonstrated and verified the taxonomic position of *Anax* species, based on morphological characters, and further enhanced it through the application of tools of molecular biology, specifically involving the nucleotide sequences utilised in evolutionary lineage analysis.

Keywords: Molecular analyses; Phylogeny; 16SrRNA; Species identification; Taxonomy

Introduction

Dragonflies serve as vital indicators for assessing the health of freshwater wetland ecosystems (Chaudhry *et al*. 2013). The Aeshnidae family is categorised under the suborder Anisoptera of the Odonata order. Aeshnids are globally distributed, with 510 species documented under 53 genera (Paulson *et al*. 2025). Aeshnid dragonflies are large predators within the aquatic food chain (Needham and Jr 1955). According to Zia *et al*. (2008), their importance as key natural enemies of insect pests significantly affects agronomic yields. Additionally, Aeshnid dragonflies serve as valuable prey for fishes, birds, and some invertebrates; that is why they play a significant part in the food chain (Chaudhry 2010).

Anax Leach, 1815, is a diversified genus of dragonflies, with species employed as perfect animals for many environmental, behavioural, and physiological investigations because of their considerable size and prominent behaviours (Sharkey *et al*. 2015; Bybee *et al*. 2016; May *et al*. 2017). From Pakistan, six genera representing ten species of family Ashnidae are known (Kalkman *et al*. 2020). Presently 37 species of genus *Anax* are reported worldwide (Paulson *et al*. 2025), while five species are known from Pakistan (Chaudhry 2010). Taxonomy involves identifying species through their morphological characteristics, requiring a comprehensive understanding of diverse organisms and their characteristic features. The establishment of molecular taxonomic frameworks enhances the understanding and improvement of species identification (Mehmood *et al*. 2016, 2021).

To cite this paper: Zia A, SA Mehmood, M Ilyas, M Inam, W Ahmad, MN Riaz, R Saleem (2026). Morphological and molecular based assessment of two *Anax* species (Odonata: Aeshnidae) from Islamabad capital territory, Pakistan. *Intl J Agric Biol* 35:350103. https://doi.org/10.17957/IJAB/15.2418

Anax parthenope is known from various parts of the world: Britain (Phillips 1997; Parr 2011), Africa (Chelmick 1999; Mitra and Clausnitzer 2018), Ireland (Nelson *et al*. 2003), the West Siberian Plain (Kosterin 2007), France (Rochelet and Maillard 2009), Latvia (Kalniņš 2009), Pakistan (Akbar *et al*. 2017; Sahito *et al*. 2017; Kalkman *et al*. 2020; Ahmed *et al*. 2023), India (Somal and Walia 2022), Galicia, Spain (Martínez *et al.* 2011), southwestern Saudi Arabia (Monnerat and Dhafer 2016), northeastern Algeria (Boucenna 2018), the West Palearctic (Schneider *et al*. 2023), south of Iraq (Ahmed and Kareem 2024) and Croatia (Štih and Koren 2022).

Anax immaculifrons is also earlier reported globally: India (Sangal and Kumar 1970), Greece (Battin 1990), Iran (Lohmann 1990), China (Wan *et al*. 2011), Cambodia (Kosterin 2012), Pakistan (Khaliq *et al*. 1994; Usman *et al*. 2017; Attaullah *et al*. 2021; Hayat *et al*. 2023), India (Tiple *et al*. 2012; Rohmare *et al*. 2015), Bangladesh, Afghanistan, China, Cyprus, Greece, Hong Kong, Iran, India, Nepal, Pakistan, Thailand, Sri Lanka, Turkey and Vietnam (Mitra 2010).

Although, despite worldwide studies, thorough morphological and molecular characterization of *Anax* species from Pakistan's capital territory is still insufficient. Moreover, no previous investigation has integrated 16S rRNA molecular analysis with comprehensive morphological analysis of *Anax* species from the Islamabad region.

The current study aimed to evaluate the morphology and phylogeny of the Odonate species, *Anax parthenope* and *Anax immaculifrons*. The phylogenetic analysis employed (16S) rRNA gene sequences to evaluate the evolutionary relationship among *Anax* species. This study also has established the basis for comparable studies of integrating both morpho-based characterization and molecular analysis within the genus *Anax* recorded from Islamabad, Pakistan.

Methods and Materials

Sampling and preservation

Specimens of both species were recorded from several locations within the Islamabad region of Pakistan (Fig. 1). The sampling was conducted following Zia (2010) and the mounting was carried out following Chaudhry (2010) and Zia (2010). Specimens were caught through insect nets from 9 am to 5:30 pm on clear, sunny days. Afterward, these were preserved in entomological jars containing ethyl acetate-soaked swabs and spread on setting boards and labelled with details, including coordinates, name of the collector, collection date, and positive sites information. The samples were stored at freezing temperature (-21°C) for about two days in order to prevent any fungal growth. The dried samples were transferred to collecting jars, which kept naphthalene balls. The specimens are housed at the National Insect Museum, Islamabad.

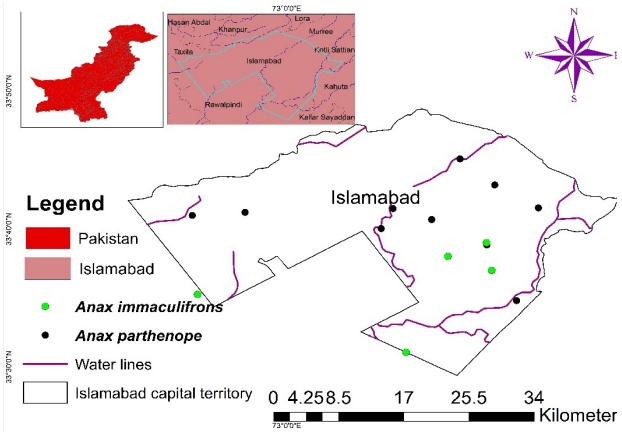

Fig. 1: showing collection spots for *A. parthenope* and *A. immaculifrons*

Identification of the specimens

The identification of the collected specimens was done following Fraser (1936) and Chaudhry (2010) and also validated using the reference specimens of *Anax* at the National Insect Museum (NIM), Islamabad.

Molecular analysis

Isolation of DNA: DNA was extracted following standard Guryev protocols (Heesch *et al*. 2013). DNA was isolated from dried *Anax* species. Legs were detached from each specimen using forceps and placed in a labelled 2 mL Eppendorf tube, while the legs were sectioned with dissection scissors. Following completing the procedure for extracting DNA, a 1% agarose/TAE gel and Nanodrop were employed for evaluating the quality and quantity of DNA extracted. The DNA bands were visualised in ultraviolet light by employing the "UV technology" gel documentation system.

Primer selection and PCR amplification

Primers were selected at NCBI at the gene bank database found in scientific literature, targeting specific sequence regions to be amplified. Genomic DNA has been obtained and used as a template for the PCR amplification of 16S rRNA. The selected primer sequences are as follows: CGCCTGTTTATCAAAAACAT (forward) and CTCCGGTTTGAACTCAGATCA (reverse) (Mehmood *et al.* 2016).

Gene sequencing

The purified samples of DNA for *Anax* species were sent to Macrogen Korea (http://www.macrogen.com) for sequencing. Each sample was sequenced successfully; these sequences were subjected to Basic Local Alignment Search Tool (BLAST) analysis in the NCBI database to compare them, along with their verification and phylogenetic analysis.

Molecular taxonomy and evolutionary phylogenetics

Alignments of DNA sequences were done employing MUSCLE alignment (Edgar 2004), CLUSTAL X 2.1 (Larkin *et al*. 2007) and BioEdit software 7.2.5 (Hall 1999). Phylogenetic relationships were analysed using the neighbor-joining method, with analyses conducted in PAUP4.0b10 (Saitou and Nei 1987). A neighbor-joining tree was constructed using MegaXI. Bootstrap was regarded as 70% significant. Bayesian analyses utilising Markov chain Monte Carlo (MCMC) methods were performed using BEAST 1.6.2, assisted by XSEDE on the CIPRES Science Gateway v. 3.3 (Drummond and Rambaut 2007; Miller *et al*. 2012). The implementation of TRACER 1.5 was utilised to assess the ESS value (effective sample size) (Rambaut *et al*. 2009). Values for posterior probabilities (PP) were adjusted to exceed 0.95% to quantify significance. For the visualisation of trees, FigTree 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree; Drummond *et al*. 2012) was used, and Illustrator CS6 was used for the annotation of trees.

Results

Materials examined

Islamabad, Pakistan: *Anax parthenope*: 1♂; (73.110667 E, 33.667278 N), 06.vi.2024, leg. Zia. 1♂; (73.124583 E, 33.691528 N), 05.vi.2024, leg. Zia. 2♀; (73.169972 E, 33.678194 N), 07.vi.2024, leg. Zia. 2♂; (73.234639 E, 33.647389 N), 07.vi.2024, leg. Ahmad. 1♀; (73.269444 E, 33.58025 N), 06.vi.2024, leg. Ilyas. 1♂; (73.243917 E, 33.720056 N), 06.vi.2024, leg. Inam. 1♂; (73.203167 E, 33.751611 N), 19.v.2024, leg. Mehmood. 2♀; (72.951139 E, 33.686972 N), 20.vi.2024, Leg. Ilyas. 2♀; (72.889194 E, 33.68325 N), 22.vi.2024, leg. Zia. 2♂; (73.295056 E, 33.692306 N), 25.vi.2024, leg. Mehmood, (Fig.1).

Anax immaculifrons: 2♀; (72.895472 E, 33.587722 N), 29.vi.2024, leg. Ilyas. 2♂; (73.188806 E, 33.633722 N), 29.vi.2024, leg. Ahmad. 2♂; (73.23425 E, 33.650278 N), 29.vi.2024, leg. Ilyas. 2♂; (73.240278 E, 33.616861 N), 29.vi.2024, leg. Mehmood. 2♂; (73.139806 E, 33.517861 N), 29.vi.2024, leg. Ahmad (Fig. 1).

Measurements

Anax parthenope: Abdomen 47.5-48.5 mm, hindwing 46.5-50 mm, anal appendages 5.5 mm.
Anax immaculifrons: Abdomen 54-55 mm, hindwings 46.6-50 mm, anal appendages 5.5 mm.

Diagnostic characteristics

***Anax parthenope*:** Head: labrum and labium golden-pale; frons and face yellow to greenish-pale; Frons without a dark T-shaped spot, frons yellow-bluish stripe above; eyes dark-bluish; occiput blackish. Prothorax: black-brownish, yellowish laterally. Thorax: bluish green without black broad markings. Legs: dark black, femora Red-tinged brown. Wings: translucent, enfumed with Yellow-tinged brown from apices closely to discoidal triangle, but pale apices; pterostigma Red-tinged brown, long and narrow, spreading to 3 cells; costal margin pale, going beyond pterostigma outer margin; membrane black; discoidal cell longer in forewing and narrow than those of hindwings, made of six cells, with only five in the hindwing; 4 or 5 cubital veins in four wings; 13 to 14 celled anal loop. Hind wing base notched; hind wing tornus rounded; anal triangle lacking. Two layers of cells present between the origins of *IA* and *Cuii* of hindwings. Abdomen: abdomen with bluish-coloured markings, segment 1 olive-brown, segment 2 blue-green, segment 3 with a large triangular blue cover at both sides at base, segment 4 to 9 with jugal and ridges black finely, segment 10 dark-black, with its sides and apical border scarcely bluish-grey, sometimes almost entirely bluish. Anal appendages: inferior very short, paler and broader than superior; superior anal appendages reddish-brown; superior anal appendages of male obtuse at apex (Fraser 1936) (Fig. 2A).

***Anax immaculifrons*:** Head: labium yellow ochraceous; labrum green-yellowish bordered with dark chocolate brown; frons and face pale cyan, with narrow blackish margin towards base of frons; eyes azure blue when alive; occiput pale blue. Prothorax: deep reddish-brown, lighter at sides, posterior lobe carrying a long hairy fringe; thorax light turquoise blue dorsally, mid-dorsal carina finely brownish-black; blue-green at sides. Thorax: blue-green on sides broadly striped with deep black. Legs: darkish. Wings: transparent, tinged with golden yellow from terminal to base of triangle, palish at apex, deep toward wing base; pterostigma red-brown, spreading to 3 cells approximately; membrane dark; discoidal cell of the forewing with six cells, five in hind; six cubital nervures in forewing, four in hind; anal loop 12-celled; hind wing base notched; tornus rounded; no anal triangle present; two rows of intercalation present between the origins of *Cuii* and *IA* in hindwings. Abdomen: segment one dark blackish; segment two bluish green; segment three with its base broadly turquoise-blue on sides; segments four to eight with apical half black; segment nine dark dorsally; segment ten red-brown, with black limited to its base. Anal appendages: constantly shaped, orange-brown; inferior scarcely triangular, apex notched, bearing two small spines at sides; superior anal appendages of male obtuse apically (Fraser 1936) (Fig. 2B).

Systematics and phylogenetic based on 16S gene data

According to BLAST analysis of 16S rRNA in the GenBank NCBI, it shows highly significant sequence similarities. The results show that *A. imperator* (NC031821.1) has 98% maximum identity and 99% query coverage with an E-value of 0, and that *A. congoliath* (EU477654.1) shows 97% identity and 96% query coverage

Fig. 2: A: *Anax parthenope*) and **B**: *Anax immaculifrons*

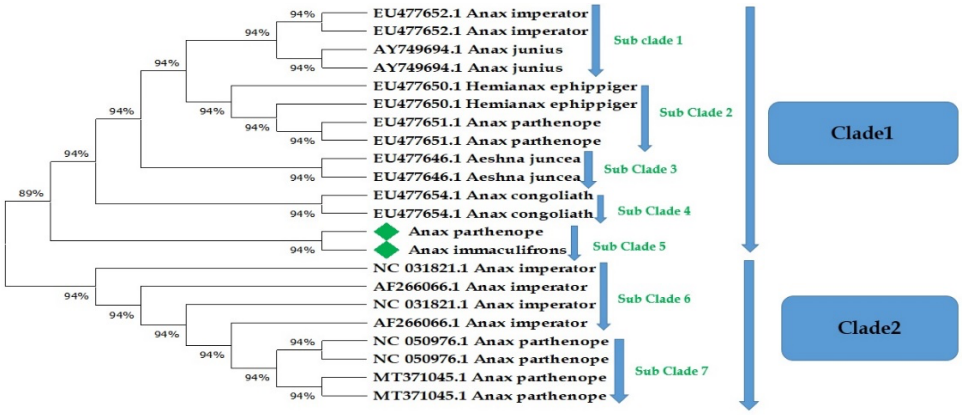

Fig. 3: Neighbor-Joining analysis of 16S gene sequence: Parsimony bootstrap, Maximum likelihood bootstrap and Bayesian analysis. The scale bar indicates 5 changes per 100 characters (sequences for the present study)

with an E-value of 0. The mean pairwise distance (MPD) ranged from 0.38 to 15.00% among species, while evolutionary rate analysis revealed invariant site parameters (+G+I) and gamma distributions of 1.89 and 0.11 substitutions per site, respectively. The 16S rRNA tree was made with a log likelihood of -944.217, and its bootstrap values are indicated under the branches (Fig. 3). Of the 340 analysed positions, 169 were conserved, and 171 were variable, and parsimony was informative, with 0 single-tone sites. The maximum parsimony technique was employed based on TBR parameters (Saitou and Nei 1987) with 1000 replicate heuristic searches, resulting in a most parsimonious tree length of 2985, with bootstrap values as indicated in the branches. The resulting phylogenetic tree clustered into two major clades (1 and 2), with Clade 1 further subdivided into 5 subclades and Clade 2 consisting of two subclades. The subclade 1 comprises two species under the same genus. Subclade 2 contains two Aeshna species under two genera, while subclade 3 includes two Aeshna species. Subclade 4 contained two *Anax* species, and subclade 5 presented our local species. *Anax parthenope* clustered with *Anax congoliath* (EU477654.1) in subclade 4, with bootstrap values documented as MPB (98), MLB (98) and BPP (0.99) correspondingly. Clade 2 is divided into two subclades: subclade 6, containing the same species of genus *Anax* (*A. imperator*) and subclade 7, which also comprises the same species of genus *Anax* (*A. parthenope*).

Maximum likelihood inference of rate heterogeneity among sites in aeshnidae mitochondrial DNA

The predicted value for the shape parameter of discrete Gamma distributions is 1.7810. The evaluation of substitution patterns and rates was conducted utilising the Tamura and Nei (1993) model. A discrete Gamma distribution was employed to model variations in evolutionary rates within locations (five classes, +G). The average evolutionary rates observed in these groups were 0.23, 0.52, 0.82, 1.23 and 2.19 substitutions per site. The frequencies of nucleotides are as follows: A = 35.51%, T/U = 37.19%, C = 12.17% and G = 15.13%. A tree topology was constructed automatically for the estimation of ML values. The greatest log-likelihood for this calculation was -846.832. The study included 22 sequences of nucleotides. All entries with gaps and missing data were removed. The ultimate dataset included 298 positions.

Pairwise distance of the aeshnidae family

Base substitutions per site between sequences are provided. Analyses were performed utilising the Maximum

Table 1: Evolutionary Divergence Estimation in Aeshnidae Mitochondrial Genomes Using Maximum Likelihood

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1. Anax parthenope																						
2. NC 031821.1:14301-14636 Anax imperator	1.2																					
3. EU477652.1:183-518 Anax imperator	0.0	1.2																				
4. AF266066.1:295-630 Anax imperator	1.2	0.0	1.2																			
5. NC 050976.1:14089-14424 Anax parthenope	1.2	0.0	1.2	0.0																		
6. EU477654.1:185-521 Anax congoliath	0.0	1.1	0.0	1.1	1.2																	
7. MT371045.1:14088-14423 Anax parthenope	1.2	0.0	1.2	0.0	0.0	1.2																
8. EU477651.1:183-518 Anax parthenope	0.0	1.2	0.0	1.2	1.2	0.0	1.2															
9. EU477650.1:183-518 Hemianax ephippiger	0.0	1.2	0.0	1.2	1.2	0.0	1.2	0.0														
10. EU477646.1:184-518 Aeshna juncea	0.0	1.1	0.0	1.1	1.2	0.0	1.2	0.0	0.0													
11. AY749694.1:40-360 Anax junius	0.0	1.2	0.0	1.2	1.2	0.0	1.2	0.0	0.0	0.0												
12. Anax immaculifrons	0.0	1.2	0.0	1.2	1.2	0.0	1.2	0.0	0.0	0.0	0.0											
13. NC 031821.1:14301-14636 Anax imperator(2)	1.2	0.0	1.2	0.0	0.0	1.1	0.0	1.2	1.2	1.1	1.2	1.2										
14. EU477652.1:183-518 Anax imperator(2)	0.0	1.2	0.0	1.2	1.2	0.0	1.2	0.0	0.0	0.0	0.0	0.0	1.2									
15. AF266066.1:295-630 Anax imperator(2)	1.2	0.0	1.2	0.0	0.0	1.1	0.0	1.2	1.2	1.1	1.2	1.2	0.0	1.2								
16. NC 050976.1:14089-14424 Anax parthenope(2)	1.2	0.0	1.2	0.0	0.0	1.2	0.0	1.2	1.2	1.2	1.2	1.2	0.0	1.2	0.0							
17. EU477654.1:185-521 Anax congoliath(2)	0.0	1.1	0.0	1.1	1.2	0.0	1.2	0.0	0.0	0.0	0.0	0.0	1.1	0.0	1.1	1.2						
18. MT371045.1:14088-14423 Anax parthenope(2)	1.2	0.0	1.2	0.0	0.0	1.2	0.0	1.2	1.2	1.2	1.2	1.2	0.0	1.2	0.0	0.0	1.2					
19. EU477651.1:183-518 Anax parthenope(2)	0.0	1.2	0.0	1.2	1.2	0.0	1.2	0.0	0.0	0.0	0.0	0.0	1.2	0.0	1.2	1.2	0.0	1.2				
20. EU477650.1:183-518 Hemianax ephippiger(2)	0.0	1.2	0.0	1.2	1.2	0.0	1.2	0.0	0.0	0.0	0.0	0.0	1.2	0.0	1.2	1.2	0.0	1.2	0.0			
21. EU477646.1:184-518 Aeshna juncea(2)	0.0	1.1	0.0	1.1	1.2	0.0	1.2	0.0	0.0	0.0	0.0	0.0	1.1	0.0	1.1	1.2	0.0	1.2	0.0	0.0		
22. AY749694.1:40-360 Anax junius(2)	0.0	1.2	0.0	1.2	1.2	0.0	1.2	0.0	0.0	0.0	0.0	0.0	1.2	0.0	1.2	1.2	0.0	1.2	0.0	0.0	0.0	

Table 2: Tajima's D values for Aeshnidae genetic sequences

Sr. No.	Configuration	Count
1	Identical locations present in all three sequences	140
2	Distinct locations in all three sequences	2
3	Distinct variations in Sequence A	4
4	Distinct variations in Sequence B	153
5	Distinct variations in Sequence C	3

Composite Likelihood model. The data presents the frequency of base substitutions per site across the analysed sequences. Analyses were performed utilising the Maximum Composite Likelihood model. The study included 22 nucleotide sequences. Entries with gaps and incomplete information were removed. The comprehensive dataset comprised 298 locations, as presented in Table 1.

Tajima's test for *Anax sequences*

The equivalence of evolutionary rates between sequences A: *Anax parthenope*, B: *NC031821.1:14301-14636 Anax imperator* and sequence C: *EU477652.1:183-518 Anax imperator* serves as an outgroup in Tajima's relative rate test (Tajima 1993). The χ^2 test statistic calculated was 141.41 with a *P* value of 0.00000, based on 1 degree of freedom. A p-value below 0.05 is commonly used to reject the null hypothesis concerning equal rates among lineages. The analysis consisted of three nucleotide sequences. Positions with gaps and incomplete information were eliminated. The final dataset presented in Table 2 comprises a total of 302 positions.

Discussion

Islamabad is situated at 33.674000N 73.136833E on the north side of the Pothohar Plateau. The elevation measures approximately 1,770 feet (www.Tutiempo.net.com). The area of Islamabad is 906 km² (Butt *et al*. 2011). An additional area of 2,717 square kilometres is designated as the Specified Area, bordered by the Hills of Margalla to the north and northeast. Its southern section features a series of rolling plains. The Kurang River drains the area, and the Rawal Dam is situated along its course (Encyclopedia Britannica 2023). The climate exhibits characteristics of warmth and temperateness. Islamabad experiences significant rainfall even during its driest month, with an average annual temperature of 68.5°F. The average amount of rainfall in this area measures approximately 39.8 inches. (Climate-Data.org 2024).

Molecular analysis using 16S rRNA sequences revealed significant phylogenetic relationships. The BLAST results indicated a significant sequence similarity with previously described Aeshnidae species, where *A. parthenope* exhibited a maximum identity of 98% (99% query cover) and *A. immaculifrons* demonstrated a maximum identity of 97% (96% query cover) when compared to reference sequences of *A. imperator* and *A. congoliath*, respectively. The elevated similarity values validate the precision of morphological identification and indicate the validity of 16S rRNA as a molecular marker for species identification in Anax species. Two main clades resulted from phylogenetic analysis through both bootstrap and Bayesian probability testing (MLB = 70-100%, MPB = 52-100% and BPP = 0.75-1%).

The phylogenetic association between *A. parthenope* in subclade IV and *A. congoliath* (EU477654.1) is supported by MLB = 98, MPB = 98 and BPP = 0.99, which indicates a

close evolutionary relationship. The findings validate the taxonomic structure described in previous morphological analyses, which positions these species under the *Anax* genus. The Gamma distribution (0.11) and invariant sites (1.89) parameters indicate variable evolutionary rates across different sites in the 16S rRNA gene. The presence of 171 variable and parsimony-informative characters out of 340 total positions suggests significant genetic diversity within the studied taxa, providing a strong phylogenetic signal for resolving relationships at both species and genus levels. The Mean Pairwise Distances (MPD) ranging from 0.38 to 15.00% indicate changing levels of genetic divergence among the studied species. Tajima's relative rate test ($\chi^2 = 141.41$, $P < 0.05$) indicates significant differences in evolutionary rates between families. The presence of 140 identical sites across all three sequences, along with unique differences in each sequence, suggests varying evolutionary pressures on different lineages. The present study represents morphological characterization and molecular analysis of *the Anax* genus from Islamabad Capital Territory, Pakistan. The molecular techniques of 16S yielded outstanding findings. The analysis of the (16S) data set yielded more accurate results and a clearer understanding of phylogenetic relationships compared to a single analysis of 16S. In the present molecular investigation, Maximum Parsimony (MP) was employed to assess the accuracy of every approach used. The techniques exhibited comparable evolutionary relationships and topologies, with deviations occurring only at certain node locations and slight variations in bootstrap values.

This study opens several avenues for future research. Further sampling across different geographic regions and inclusion of additional molecular markers would provide a more comprehensive understanding of the phylogenetic relationships and evolutionary history of Aeshnidae in Pakistan. Integration of ecological data with molecular findings could also help understand the factors influencing species distribution and population dynamics.

Conclusion

This study successfully documented and recognised two *Anax* species in Islamabad's capital territory using integrative morphological and genetic methodologies. This study arranges the basis for upcoming biodiversity evaluations in Pakistan's freshwater environments. The incorporation of standard taxonomy with DNA barcoding proves the efficacy of employing several procedures for accurate species identification. Further studies are required to extend the sampling across the different places in Pakistan and combine additional DNA markers to construct a more profound understanding of Aeshnidae diversity and evolution in South Asia.

Acknowledgements

Authors thankfully acknowledge the National Insect Museum (NIM), National Agricultural Research Centre (NARC), Islamabad, Pakistan for providing laboratory facilities and support during the morphological and molecular identification of the specimens.

Author Contributions

AZ and MI conducted molecular and morphological identification and supervision, methodology and resources. SAM designed the study and collected the specimen. MI and WA Writing - original draft and software. NR assisted with molecular identification, visualisation and methodology. RS investigated and proofread the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest regarding the publication of this manuscript.

Data Availability

The data for the study is physically available in the form of speciemen as well as extracted DNA at National Insect museum, NARC, Islamabad, Pakistan.

Ethical Approval

This study did not involve any experiments on human participants or animals.

References

Ahmed B, M Amin, A Zia, M Qasim, M Khan, Z Ullah, M Khan (2023). Dragonflies' fauna (Odonata: Anisoptera) of Kech Balochistan, Pakistan. *J Kash J Agric* 3:215–220

Ahmed HK, DK Kareem (2024). Morphological identification of dragonflies' naiads (Odonata: Anisoptera) from temporary ponds in Basrah Province, South of Iraq: Identification of dragonflies from Basrah, Iraq. *Iraq J Aquacult* 21:89–109 https://doi.org/10.58629/ijaq.v21i1.513

Akbar A, SA Mehmood, WA Panhwar, S Ahmed, S Tabassum, S Rajput, M Ali (2017). Collection and identification of genus Anax (Odonata, Aeshnidae) from district Swat. *J Entomol Zool Stud* 5:1440–1442

Attaullah M, I Ullah, M Ali, F Maula, I Ilahi, B Ahmad, S Khwaja, Z Ullah, M Siraj, SMM Raheel (2021). Diversity of the Anisoptera & Zygoptera (Odonata: Insecta) of Swat, Pakistan. *Braz J Biol* 83:1-8 https://doi.org/10.1590/1519-6984.251958

Battin T (1990). *Anax immaculifrons Rambur, 1842 from the island of Karpathos, Greece: An oriental representative in the European dragonfly fauna (Odonata: Aeshnidae).* Flumsberg Scientific

Boucenna N (2018). Biologie et écologie des Aeshnidés (genre Anax) dans le bassin versant de la seybouse. *Ph.D. Thesis.* Université Mouloud MAMMERI, Tizi-Ouzou, Algeria

Butt MJ, A Waqas, MF Iqbal, G Muhammad, MAK Lodhi (2011). Assessment of urban sprawl of Islamabad metropolitan area using multi-sensor and multi-temporal satellite data. *Arab J Sci Eng* 37:101–114 https://doi.org/10.1007/s13369-011-0148-3

Bybee S, A Córdoba-Aguilar, MC Duryea, R Futahashi, B Hansson, MO Lorenzo-Carballa, R Schilder, R Stoks, A Suvorov, EI Svensson, J

Swaegers, Y Takahashi, PC Watts, M Wellenreuther (2016). Odonata (dragonflies and damselflies) as a bridge between ecology and evolutionary genomics. *Front Zool* 13:1-20 https://doi.org/10.1186/s12983-016-0176-7

Chaudhry MT, AU Mohsin, MI Bhatti, RA Javed, G Abbas (2013). First record of Anaciaeshna jaspidea and *Epophthalmia vittata vittata* (Odonata: Anisoptera) from Pakistan. *Iran J Sci Technol* 37:445–448

Chaudhry T (2010). Systematics of dragonfly (Anisoptera; Odonata) of Pakistan. *Ph.D. Thesis*. Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan

Chelmick DG (1999). Larvae of the genus Anax in Africa (Anisoptera: Aeshnidae). *Odonatologica* 28:209–218

Climate-Data.org (2024). Climate data for Islamabad, Pakistan. Available at: https://en.climate-data.org/asia/pakistan/islamabad-capital-territory/islamabad-32/ (Accessed: 29 July 2025)

Drummond AJ, MA Suchard, D Xie, A Rambaut (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Mol Biol Evol* 29:1969–1973. https://doi.org/10.1093/molbev/mss075

Drummond AJ, A Rambaut (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* 7:1-8 https://doi.org/10.1186/1471-2148-7-214

Edgar RC (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucl Acids Res* 32:1792–1797 https://doi.org/10.1093/nar/gkh340

Encyclopedia Britannica (2023). *Islamabad. Archived from the original on 3 May 2015*. Available at: https://www.britannica.com/place/Islamabad (Accessed: February 11, 2025)

Fraser FC (1936). Fauna of British India. *Odonata* 3:142–146

Hall TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser* 41:95–98

Hayat D, N Tariq, SA Zia, G Makai, S Wali, F Shahwani, A Hakeem (2023). Guardians of Quetta's skies: Aeshnidae dragonflies explored. *Pak-Eur J Med Life Sci* 6:295–302

Heesch SV, WP Kloosterman, N Lansu, FP Ruzius, E Levandowsky, CC Lee, S Zhou, S Goldstein, DC Schwartz, TT Harkins, V Guryev, E Cuppen (2013). Improving mammalian genome scaffolding using large insert mate-pair next-generation sequencing. *BMC Genom* 14:1-11 https://doi.org/10.1186/1471-2164-14-257

Kalkman VJ, R Babu, M Bedjanič, K Conniff, T Gyeltshen, MK Khan, KA Subramanian, A Zia, AG Orr (2020). Checklist of the dragonflies and damselflies (Insecta: Odonata) of Bangladesh, Bhutan, India, Nepal, Pakistan and Sri Lanka. *Zootaxa* 4849:1–84 https://doi.org/10.11646/zootaxa.4849.1.1

Kalniņš MĀ (2009). Lesser emperor Anax parthenope (Selys, 1839) (Odonata: Aeshnidae) - a new dragonfly species in Latvia. *Latvijas Entomol* 47:16–20

Khaliq A, S Aslam, SA Anjum (1994). Description of the last instar naiad of Anax immaculifrons Rambur (Aeshnidae: Odonata). *Pak Entomol* 16:75–76

Kosterin OE (2012). Odonata of the Cambodian coastal regions in late rainy season of 2011. *In: International Dragonfly Fund-Report*, Vol. 45, pp:1–102

Kosterin OE (2007). The first record of Anax on the West Siberian Plain: *A. parthenope* Selys in Omsk (Anisoptera: Aeshnidae). *Notul Odonatol* 6:112–115

Larkin MA, G Blackshields, NP Brown, R Chenna, PA McGettigan, H McWilliam, F Valentin, IM Wallace, A Wilm, R Lopez, JD Thompson, TJ Gibson, DG Higgins (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* 23:2947–2948

Lohmann H (1990). Anax immaculifrons Rambur, 1842 in Iran (Odonata: Aeshnidae). *Opusc Zool Flumin* 54:9–10

Martínez E, GD Silva, CD Romay (2011). Primeiras citas de Anax parthenope (Odonata: Aeshnidae) para Galicia e datos sobre o seu hábitat e comportamento. *Chioglossa* 3:7–13

May ML, JA Gregoire, SM Gregoire, MA Lubertazzi, JH Matthews (2017). Emergence phenology, uncertainty, and the evolution of migratory behavior in *Anax junius* (Odonata: Aeshnidae). *PLoS One* 12:1-27 https://doi.org/10.1371/journal.pone.0183508

Mehmood SA, MS Ahmad, A Zia, S Ahmed, M Shah, W Khan, WA Panhwar (2021). Molecular and phylogenetic analysis of family Aeshnidae of Hazara region, Pakistan. *Intl J Agric Biol* 25:109–116 https://doi.org/10.17957/IJAB/15.1644

Mehmood SA, MS Khan, A Zia, FA Shaheen (2016). Morphological evaluation of Gomphidae dragonflies of Hazara region Pakistan through principle component analysis. *J Entomol Zool Stud* 4:183–188

Miller MA, W Pfeiffer, T Schwartz (2012). The CIPRES science gateway: enabling high-impact science for phylogenetics researchers with limited resources. *In: Proceedings of the 1st Conference of the Extreme Science and Engineering Discovery Environment: Bridging from the eXtreme to the campus and beyond*, pp:1–8. ACM, New York, USA. https://doi.org/10.1145/2335755.2335836

Mitra A (2010). *Anax immaculifrons*. The IUCN Red List of Threatened Species 2010:e.T165463A6022090. Available at: https://dx.doi.org/10.2305/IUCN.UK.2010-4.RLTS.T165463A6022090.en (Accessed: February 6, 2025)

Mitra A, V Clausnitzer (2018). *Anax parthenope*. The IUCN Red List of Threatened Species 2018:e.T165488A72312377. Available at: https://dx.doi.org/10.2305/IUCN.UK.2018-1.RLTS.T165488A72312377.en (Accessed: February 9, 2025)

Monnerat C, HMA Dhafer (2016). Odonata records from southwestern Saudi Arabia. *Not Odonatol* 8:203–245

Needham JG, MJW Jr (1955). *A Manual of the Dragonflies of North America (Anisoptera): Including the Greater Antilles and the Provinces of the Mexican Border*. University of California Press, California, USA

Nelson B, C Ronayne, R Thompson (2003). Colonization and changing status of four Odonata species, *Anax imperator*, *Anax parthenope*, *Aeshna mixta* and *Sympetrum fonscolombii*, in Ireland 2000-2002. *Irish Nat J* 27:266–272 https://www.jstor.org/stable/25536492

Parr AJ (2011). Migrant and dispersive dragonflies in Britain during 2010. *J Brit Dragonfly Soc* 27:69–79

Paulson D, M Schorr, J Abbott, C Bota-Sierra, C Deliry, KD Dijkstra, F Lozano (2025). *World Odonata List*. Odonata Central, University of Alabama. Available at: https://www.odonatacentral.org/app/#/wol/ (Accessed: April 2, 2025)

Phillips J (1997). Lesser Emperor Dragonfly Anax parthenope (Selys) in Gloucestershire; the first British record. *J Brit Dragonfly Soc* 13:22–24

Rambaut A, SY Ho, AJ Drummond, B Shapiro (2009). Accommodating the effect of ancient DNA damage on inferences of demographic histories. *Mol Biol Evol* 26:245–248 https://doi.org/10.1093/molbev/msn256

Rochelet B, W Maillard (2009). Redécouverte d'Anax parthenope (Selys 1839) en Sarthe et état des connaissances sur la présence de l'espèce en Pays de la Loire (Odonata, Anisoptera, Aeshnidae). *Martinia* 25:79–84

Rohmare VB, DM Rathod, SG Dholu, BM Parasharya, SS Talmale (2015). An inventory of odonates of central Gujarat, India. *J Threat Taxa* 7:7805–7811 https://doi.org/10.11609/JoTT.o4292.7805-11

Sahito HA, SR Bhutto, T Kousar, FA Jatoi, WM Mangrio, G BD, ZH Shah (2017). Morhphotaxanomic characteristics of dragonfly, Lesser Emperor, Anax parthenope (Selys, 1839) (Odonata: Aeshnidae) at region Sukkur, Sindh. *Sindh J Adv Bot Zool* 5:1–6 https://doi.org/10.3390/d15090950

Saitou N, M Nei (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425

Sangal SK, A Kumar (1970). Studies on the taxonomy of larvae of Doon Valley Odonata II: 3. *Anax guttatus* (Burmeister); 4. *Anax immaculifrons* Rambur (family Aeshnidae). *J Nat Hist* 4:305–313

Schneider T, A Vierstraete, OE Kosterin, D Ikemeyer, FS Hu, N Snegovaya, HJ Dumont (2023). Molecular phylogeny of Holarctic Aeshnidae with a focus on the West Palaearctic and some remarks on its genera worldwide (Aeshnidae, Odonata). *Diversity* 15:1-40 https://doi.org/10.3390/d15090950

Sharkey CR, JC Partridge, NW Roberts (2015). Polarization sensitivity as a

visual contrast enhancer in the emperor dragonfly larva, *Anax imperator*. *J Exp Biol* 218:3399–3405 https://doi.org/10.1242/jeb.122507

Somal DS, GK Walia (2022). Cytological study of family Aeshnidae (Odonata: Anisoptera) from India: A review. *Biosci Biotechnol Res Asia* 19:843–855 http://dx.doi.org/10.13005/bbra/3035

Štih A, T Koren (2022). Dragonfly fauna (Insecta: Odonata) of the Brijuni National Park, Croatia. *Nat Croat* 31:19–30 https://doi.org/10.20302/NC.2022.31.2

Tajima F (1993). Simple methods for testing molecular clock hypothesis. *Genetics* 135:599–607

Tamura K, M Nei (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* 10:512–526

Tiple AD, S Paunikar, SS Talmale (2012). Dragonflies and damselflies (Odonata: Insecta) of tropical forest research institute, Jabalpur, Madhya Pradesh, central India. *J Threat Taxa* 4:2529–2533 https://doi.org/10.11609/JoTT.o2657.2529-33

Tutiempo.net (2025). Climate data: Islamabad Airport (1973 - 2025). Available at: https://en.tutiempo.net/climate/ws-415710.htm (Accessed: February 11, 2025)

Usman K, K Pervaiz, HU Rehman, W Mohammad (2017). Exploring of dragonfly fauna in lower region of district Karak KP, Pakistan. *J Entomol Zool Stud* 5:708–710

Wan F, YH Jiang, J Wan (2011). Descriptions of *Anax immaculifrons* Rambur and Tetracanthagyna waterhousei McLachlan exuviae from China (Anisoptera: Aeshnidae). *Odonatologica* 40:339–345

Zia A (2010). Biosystematics of damselflies (Zygoptera: Odonata) of Pakistan. *Ph.D. Thesis*. Arid Agriculture University, Rawalpindi, Pakistan

Zia A, M Naeem, MA Rafi, SA Hassan (2008). A list of damselflies (Zygoptera: Odonata) recorded from Azad Jammu and Kashmir, Pakistan. *Biol Sci Pak J Sci Ind Res* 51:329–332