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16S rRNA GENE BASED IDENTIFICATION AND GENETIC RELATIONSHIP OF FOUR DAMSELFLY SPECIES OF BANGLADESH

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ABSTRACT: The mitochondrial 16S ribosomal RNA (16S rRNA) gene has emerged as a reliable alternative to morphological identification for identifying damselflies. In this study, an average of 478 bp 16S rRNA sequences was generated from four damselfly species. A GenBank BLAST search found that three of the four damselfly species had sequence similarity ranging from 98% to 99%. However, the only species that did not show a significant match to Genbank was identified morphologically as *Aristocypha quadrimaculata*. The sequences of *A. quadrimaculata* and three other matching species were then submitted to Genbank, where they were assigned accession numbers PP464226 (*Pseudocopera ciliata*), PP464231 (*Euphaea ochracea*), PP464251 (*A. quadrimaculata*), and PP464253 (*Pseudagrion rubriceps*). In the present study, *A. quadrimaculata* sequences were the first submission to the NCBI GenBank database. After analysis of the four sequences, the interspecific genetic divergence between the four species ranged from 0.203 to 0.977%. Later, a phylogenetic tree was constructed and the results showed that the four damselfly species came from a common ancestor. The phylogenetic tree presented a single major group where *P. rubriceps, P. ciliata* and *A. quadrimaculata* resided. On the other hand, *E. ochracea* exists in a remote area where this species has a longer branch, indicating significant genetic divergence from the other three damselfly species. Additionally, a TCS haplotype network of 16S rRNA gene sequences was constructed. Among the four damselfly species, *E. ochracea* has the largest number of mutation sites (193 sites). This reveals that *E. ochracea* has become genetically distant from the other three damselfly species. Finally, 16S rRNA gene-based identification is rapid and accurate, and barcodes can validate unknown species, monitor biodiversity, and determine evolutionary relationships.

Key words**:** Identification, Diversity, 16S rRNA gene, Damselfly, Genbank, Bangladesh

INTRODUCTION

Damselflies belong to the order Odonata which are divided into suborders

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Anisoptera: dragonflies and suborder Zygoptera: damselflies. Zygoptera have a greater focus on evolutionary, behavioral, biomechanical, and ecological research due to their phylogenetic location and distinct behavior (Zwick 2001). They are found all over the world and are known for their vibrant hues and unique wing patterns. Within the Odonata, there are 6,426 described extant species, grouped into three suborders: Anisozygoptera, Anisoptera (dragonflies) and Zygoptera (damselflies), with 2,967 species of dragonflies and 3,332 species of damselflies (Kohli *et al.*, 2021). In Bangladesh alone, a total of 160 species of Odonata have been recorded, including 82 species of Zygoptera (damselflies) and 88 species of Anisoptera (dragonflies) (Kabir *et al.*, 2008, Bashar *et al.*, 2014, Habib *et al.*, 2016, Kalkman *et al.*, 2020). Another online database describes 45 species of damselflies from 6 families along with their photographic documentation from Bangladesh (Shah and Khan, 2020). It has long been known that damselflies play a crucial role in controlling the population of harmful insects, including flies and mosquitoes, while the immature stages of damselflies feed on mosquito larvae, worms and other stages immature insect pests in aquatic habitats (Córdoba-Aguilar *et al.*, 2021). Additionally, damselflies are excellent indicators of freshwater habitat quality and could be used regionally and nationally (Kutcher and Bried, 2014).

Accurate identification of damselfish species is essential for ecological studies, environmental monitoring and sustainable pest control (Kaunisto *et al.*, 2019, Palacino-Rodríguez *et al.*, 2020). However, it can be difficult to recognize these insects because they have similar physical characteristics and the coloration of their first instars, called tenerals, is the same in most species (Merritt *et al.*, 1996). Nevertheless, many taxa remain difficult to identify due to the confusing state of taxonomy (Kalkman *et al.,* 2020). Traditional identification methods based on morphology can be ambiguous and time-consuming, due to their cryptic appearance, sexual dimorphism and teneral stage, leading to confusion and misidentification (Xiao *et al.*, 2010). In contrast, DNA-based species identification using mitochondrial DNA is a more accurate and efficient method that can mitigate misidentification and facilitate biodiversity monitoring and research. evolution (Hebert *et al.*, 2003ab). DNA barcodes can aid in the identification of cryptic species and the differentiation of populations that appear identical but genetically unique (Wang 2020). The mitochondrial COI gene is used as a marker gene to identify groups of insects because its effectiveness is well accepted in the scientific field. In some groups of insects, such as damselflies, the effectiveness of the COI gene for species identification may be hampered by primer binding problems, leading to a preference for the 16S rRNA gene, which can be more easily amplified with primers (Papakostas 2005, Dijkstra 2013). Therefore, the present study aimed to generate using

mitochondrial 16S rRNA gene sequences for four damselfly species, collected from four different geolocations of Kaptai National Park (Kaptai NP) and Jahangirnagar University campus (JU), Bangladesh. The results obtained will enable the correct and precise identification of these four damselfish species, thereby contributing to pest control efforts and improving our molecular database with the broader aim of conserving biodiversity in Bangladesh and beyond.

MATERIAL AND METHODS

Study area and sample collection: Four species of damselflies were collected from four geographical locations in Bangladesh (Fig. 1, Table 1). Specimens were collected by hand in the field. Voucher specimens were prepared from the collected samples. The collected damselflies were transferred to the DNA barcoding laboratory of the Department of Zoology, Jahangirnagar University for further processing. The taxonomic keys of Fraser (1933, 1934, 1936), Asahina (1967), Mitra (1983), Lahiri (1987), Mitra (2006) were used for the preliminary morphological identification of damselflies.

DNA extraction, amplification and sequencing: The genomic DNA of the four damselflies was extracted from their body parts in accordance with the Wizard Genomic@ DNA Purification Kit's protocol. PCR amplification of the mitochondrial 16S rRNA gene region was performed using the primers 16Sar-L (5'-CGCCTGTTTATCAAAAAACAT-3') and 16Sbr-H (5'-CCGGTCTGAACTCAGATCACGT-3'). The PCR reaction was carried out using 20 µL of Q2 Green PCR Master Mix and a Veriti thermal cycler (USA). Cycling conditions were maintained: initial denaturation (95 °C for 4 min), 35 cycles of denaturation (95 °C for 30 s), primer annealing (49 °C for 30 s), and primer extension (72°C for 45 seconds). dry), and a final extension (72°C for 5 min). The PCR-amplified product was evaluated by 1% agarose gel electrophoresis under ultraviolet light (Bio Analyzer) (Fig. 2). To sequence the amplification product, a sequencer with model number ABI 3500 was used.

Sequence editing, genetic divergence and phylogenetic analysis: The sequences of these four different damselfish species were edited using Chromas, version 2.6.2. The assembled sequences were aligned using the ClustalW multiple alignment function of BioEdit version 7.0 (Hall 1999). The Kimura2 Parameter (K2P) model was used in MEGA10 software to calculate and summarize nucleotide compositions, and it was used to determine pairwise distances between nucleotides (Kimura 1980, Kumar et al., 2018). Based on Neighbor-Joining (NJ) methods (Saitou and Nei 1987, Kumar *et al.*, 2018) with 1000 bootstrap replications, a phylogenetic tree was constructed using MEGA10. The TCS network was built using PopART version 1.7.

Fig. 1. Four Damselfly species collected from four geo locations of Bangladesh: *Pseudagrion rubriceps* (A), *Pseudocopera ciliata* (B), *Euphaea ochracea* (C), and *Aristocypha quadrimaculata* (D).

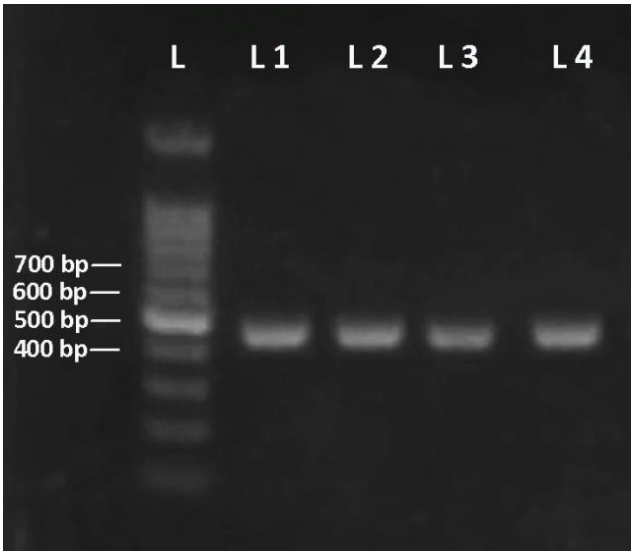

Fig. 2. Agarose gel electrophoresis of 16S rRNA from *P. ciliata* (L 1), *E. ochracea* (L 2), *A. quadrimaculata* (L 3) and *P. rubriceps* (L 4). L= 100 bp DNA Ladder.

RESULTS AND DISCUSSION

In this investigation, four different species of damselfish were used to generate mitochondrial 16S ribosomal RNA (16S rRNA) gene sequences averaging 478 bp. Gel electrophoresis revealed DNA ladder bands of approximately 500 bp in all four PCR samples, which is close to the 16S rRNA gene length of 478 bp, confirming the success of the amplification of the target gene (Fig. 2). A GenBank BLAST search found 98-99% sequence similarity for three of the four damselfly species, and another did not match significantly. Sequences from one species that did not match and three that did match were then submitted to GenBank and assigned accession numbers as follows: PP464226 (*Pseudocopera ciliata*), PP464231 (*Euphaea ochracea*), PP464251 (*Aristocypha quadrimaculata*), and PP464253 (*Pseudagrion rubriceps*) (Table 1). In the present study, *A. quadrimaculata* was identified morpho-taxonomically and the sequences are the first submitted to NCBI GenBank. The taxonomic data of *A. quadrimaculata* (female) show that the body color is black. The head is black; the thorax is black with a yellow band. The abdominal segments have a yellow color laterally. The wings are transparent, while the pterostigma and legs are black (Fig. 1, D).

Table 1. List of four damselflies, their geo-location, voucher, homology and GenBank accession numbers

Scientific Name	Family	Voucher No.	Geo Locations	Similarity in GenBank	GenBank Accession No.
Pseudagrion rubriceps	Coenagrionidae	DMBV012	22°29'32.5"N 92°08'56.3"E (Kaptai NP)	98.94-98.53%	PP464253
Pseudocopera ciliata	Platycnemididae	DMBV032	23°52'33.9"N 90°16'06.3"E (JU Campus)	99.38-99.16%	PP464226
Euphaea ochracea	Euphaeidae	DMBV026	22°29'35.5"N 92°09'46.9"E (Kaptai NP)	98.74%	PP464231
Aristocypha quadrimaculata	Chlorocyphidae	DMBV035	22°29'40.6N 92°08'54.8E (Kaptai NP)	First Submission	PP464251

Multiple Sequence Alignment: Bio Edit was used to perform multiple sequence alignments of four different species of damselflies, namely *P. rubriceps*, *P. ciliata*, *E. ochracea* and *A. quadrimaculata*, using genetic sequences from '16S rRNA. In the datasheet, letters represented non-conserved regions, while dots denoted identical or conserved sections (Fig. 3). In contrast, spaces represented by dashes in a sequence indicate insertions or deletions relative to other sequences (Fig. 3). These gaps may reflect nucleotide insertions or deletions that occurred

during the evolution of species (Seo 2022). This multiple sequence alignment is a valuable tool for understanding the genetic makeup and evolutionary relationships of these damselfly species. By analyzing these alignments, researchers can identify conserved functional elements, explore genetic diversity, and infer the evolutionary history of damselflies (Robinson 2014).

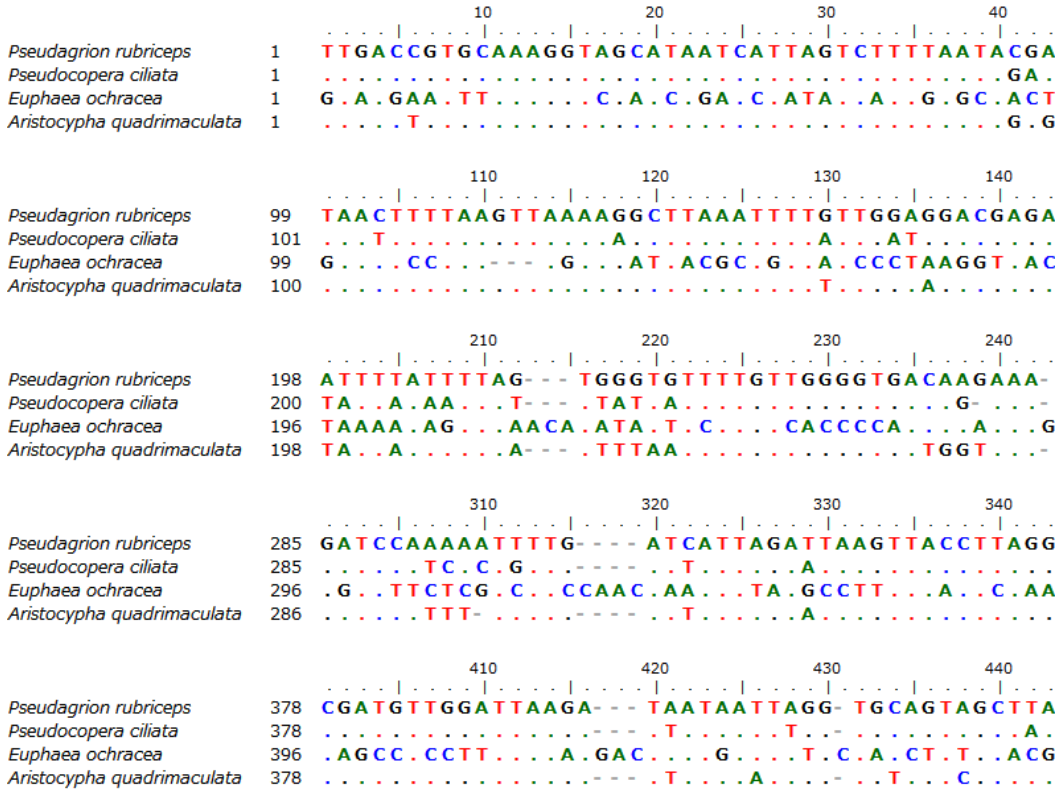

Fig. 3. Multiple sequence alignment based on 16S rRNA gene sequences of four damselfly species. Letters represents non-conserved regions, while dots denoted identical or conserved regions among these four nucleotide sequences.

Genetic distance analysis: Pairwise genetic distances between damselfish species based on 16S rRNA nucleotide sequences were assessed using the K2P model in MEGA10 (Table 2). The genetic divergence of the four species was found to range between 0.203 and 0.977. The genetic gap between *A. quadrimaculata*, *P. rubriceps* and *P. ciliata* was determined to be 0.203, indicating a close genetic relationship between these three species. The largest distance of 0.977 was observed between *A. quadrimaculata* and *E. ochracea*, this high value suggests that these two species have accumulated significant genetic

differences over time. Nevertheless, *E. ochracea* has the highest genetic divergence compared to all others. Values range from 0.891 (compared to *P. ciliata*) to 0.937 (compared to *P. rubriceps*), indicating substantial evolutionary distance. While *P. rubriceps*, *P. ciliata* and *A. quadrimaculata* appear to be more closely related to each other as their pairwise genetic distances are lower (0.203 - 0.217) (Table 2). Pairwise genetic divergence analysis is a useful starting point for understanding the relationships between damselflies and their genetic makeup. However, the data can be used to prioritize species for new conservation efforts, particularly those with high genetic divergence and potentially unique evolutionary histories (Alves 2023).

Table 2. Percentage pairwise distances among four Damselfly species

Species	1	2	3	4
Pseudagrion rubriceps				
Pseudocopera ciliata	0.217			
Euphaea ochracea	0.937	0.891		
Aristocypha quadrimaculata	0.203	0.203	0.977	-

Phylogenetic tree analysis: Construction of a phylogenetic tree using the NJ method in MEGA10 revealed that all four damselfish species share a common ancestor (Fig. 4). The phylogenetic tree presented a single major group where *P. rubriceps*, *A. quadrimaculata* and *P. ciliata* resided. These species share a more recent common ancestor due to their shorter branch lengths. On the other hand, *E. ochracea* exists alone, distant from the main group (Fig. 4). This species has a longer branch, indicating significant genetic divergence from the other three damselfly species. The three Bangladeshi species bear similarities to their respective species from various geographical areas; this suggests that these species from Bangladesh and different countries are closely related. While *E. ochracea* from Bangladesh and Malaysia belong to the same group, this suggests that the geographically separated populations may still be relatively similar genetically, although *E. ochracea* shows significant genetic divergence from the other three damselfly species (Fig. 4). The current phylogenetic tree provides a useful starting point for understanding relationships among damselflies, and further investigations are needed to encompass a larger number of damselfly species sequences to obtain a more complete scenario (Nakhleh 2013).

Haplotype network analysis: Based on the TCS haplotype network of the mitochondrial 16S rRNA gene, a mutational link appears between four damselfly species (Fig. 5). The present results showed the close relationships between *P. ciliata*, *A. quadrimaculata* and *P. rubriceps*, indicating that they were more closely related. Although a distinct evolutionary lineage was shown by *E.*

ochracea with substantial genetic divergence from the other three species, these data provide a more quantitative measure of the genetic differences observed in the haplotype network.

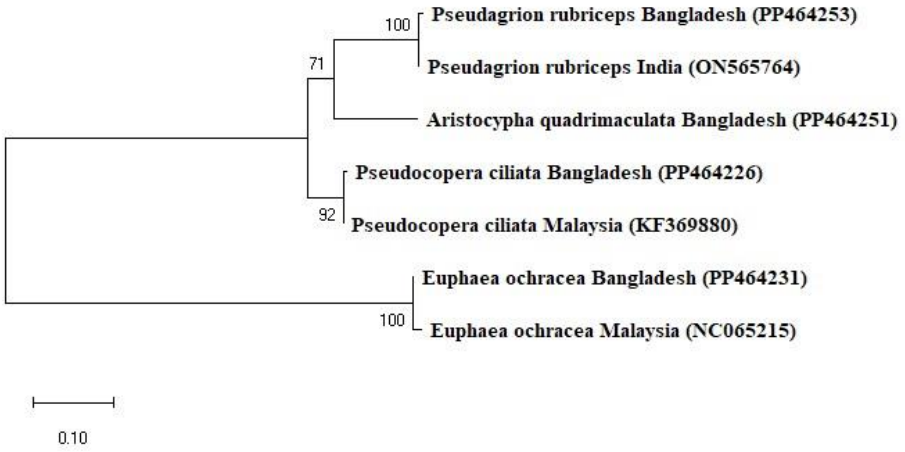

Fig. 4. Molecular phylogenetic tree of four damselfly species by Neighbor-Joining (NJ) Method Using MEGA10

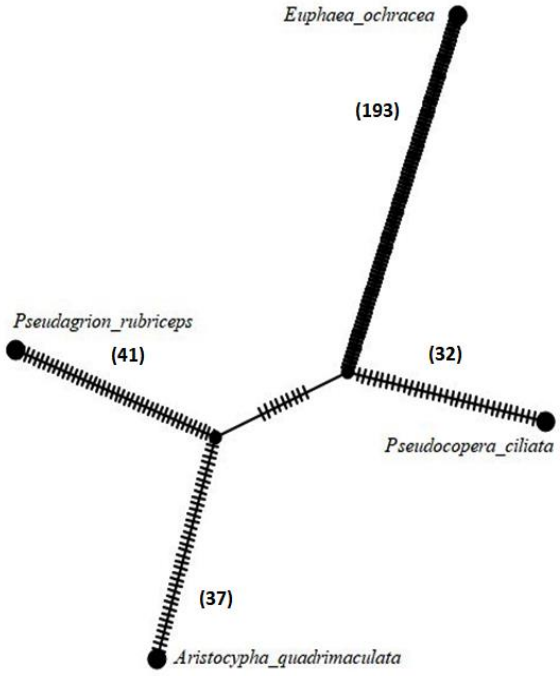

Fig. 5. Mitochondrial 16s rRNA gene haplotype analysis of four damselfly species using Popart 1.7 on the basis of the TCS network. Black circles represent haplotypes, while small black circles denote common ancestors. Mutational steps are represented by hatch marks and numbers.

E. ochracea has 193 mutation steps compared to other damselflies (32–41 mutation steps), indicating much higher genetic diversity within its population or older divergence from its common ancestor compared to the three other species (Fig. 5). This high number of mutational steps suggests that *E. ochracea* has had a longer evolutionary history or greater mutational accumulation in its mitochondrial 16S rRNA genes. The high number of mutations in *E. ochracea* could otherwise indicate that it has been under strong evolutionary pressure. This pressure can come from various factors such as predation, disease and habitat changes (McPeek 2008). Damselfly conservation preserves genetic diversity, essential for the adaptability and survival of species in changing environments (Strange 2007). Bangladesh, with its diverse ecosystems, is home to a rich variety of damselfly species (Kabir *et al.*, 2008, Chowdhury and Mohiuddin 2011, Bashar *et al.*, 2014, Khan 2015ab, Habib *et al.*, 2016). Using methods such as DNA barcoding (specifically targeting the 16S rRNA gene), molecular research has emerged as an important approach to studying these insects. This has resulted in advances in a variety of areas, including accurate species identification and biodiversity assessment, a better understanding of evolutionary links, improved conservation and management tactics, and a knowledge base for future research. Through continued investment in molecular research on damselflies, Bangladesh can ensure the preservation of its distinct and ecologically important insect, damselfly fauna, while making valuable contributions to the creation of molecular databases in the country.

CONCLUSION

The damselfly species are similar, which is confusing. Thus, traditional identification based on morphology is imprecise and time-consuming. DNA-based species identification (using mitochondrial 16S rRNA) is more accurate but reduces identification errors. In this study, the mitochondrial 16S rRNA genes of *P. ciliata*, *E. ochracea*, *A. quadrimaculata*, and *P. rubriceps* were sequenced and submitted to the NCBI GenBank, with *A. quadrimaculata* being the first to be submitted. The average sequence length was 478 bp. This data will help identify these four damselfly species and will be used for biodiversity monitoring, environmental impact assessment and evolutionary studies. This research will also contribute to the creation of a genomic database that will preserve biodiversity in Bangladesh and beyond.

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