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DIVERSITY AND MITOCHONDRIAL CO1-BASED BARCODING OF ORTHOPTERAN SPECIES OF BANGLADESH

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Abstract

Recognizing the ecological and economic significance of Orthoptera species in Bangladesh, maintaining an up-to-date inventory of the species within the country becomes crucial. Building upon previous records, the current investigation places a central focus on documenting the taxonomic identity, species distribution, and evolutionary relationships of Orthoptera species in Bangladesh, employing the mitochondrial COI-based barcoding technique. The findings reveal a total of 13 orthopteran species across 13 genera and 9 families, with three species identified through barcoding. Notably, *Phlaeoba sikkimensis* is recorded as a first for the Bangladeshi entomofauna, highlighting its importance in local biodiversity. The comprehensive genetic data presented in this study significantly enhance our understanding of orthopteran diversity, facilitating the development of effective measures for their conservation and sustainable management, thus fostering a harmonious coexistence with these ecologically and economically valuable species.

Keywords: Orthopterans, DNA barcoding, Phylogenetic relationships, Haplotype diversity, Bangladesh.

Introduction

Orthopterans (Orthoptera: Insecta), encompassing grasshoppers, locusts, and crickets, constitute a vast and diverse group with over 28,000 species worldwide (Tan and Wahab, 2018). The global and regional ecosystems and economies are significantly influenced by this diversity, as highlighted by studies (Naskrecki, 2013; Sun *et al*., 2015; Paul *et al*., 2016). Their impact is twofold, as they can cause extensive crop damage, potentially leading to widespread famine (Appert and Deuse, 1982; Resh and Cardé, 2009; Sun *et al*., 2015; Le Gall *et al*., 2019), and concurrently serve as crucial bio-indicators for

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various ecological habitats, including forests (Armstrong and van Hensbergen, 1997). However, the rapid changes in species distribution and habitat degradation due to globalization and climate change pose significant challenges to their conservation (Kumar and Usmani, 2015). Noteworthy, in the Chittagong Hill Tracts of Bangladesh, large acridids among orthopterans play a vital role in sustaining indigenous communities, as documented by Mazumdar in 2019a. Moreover, Bangladesh offers abundant opportunities for biofuel research, utilizing phytophagous pests like acridids through cellulose extraction and application technology, as emphasized by Mazumdar in 2019b. Alarmingly, certain acridid species in Bangladesh are at risk of being classified as near-threatened, underscoring the urgency of conservation efforts (Mazumdar, 2020). As Bangladesh proactively prepares to repel a potential locust invasion, the government has undertaken measures to raise awareness, formulate a robust long-term strategy, and alleviate panic, all while addressing the associated risks of climate change (CABI, 2020). To effectively manage and respond to potential outbreaks, it is imperative to comprehend the current distribution and identity of local and regional orthopteran species.

The application of accurate, rapid, and cost-effective identification methods, such as mitochondrial DNA barcoding, is gaining prominence for enhancing biodiversity assessment, understanding species distributions, and formulating conservation plans (Hebert *et al*., 2003; Li *et al*., 2017, 2020). Taxonomic studies on acridids have been ongoing since the mid-sixties, with researchers like Alam (1962, 1967, 1970) pioneering efforts to compile information on agro-arthropod pests, including acridids. Subsequent contributions from Gapud (1992), Das (2004), Ahmad *et al*. (2009), and Aktar *et al*. (2018) have significantly enriched our understanding of orthopteran diversity and their interactions with crops and grasslands. In 2022, Mazumdar *et al*. reported on orthopteran species found on the Chittagong University Campus, contributing valuable insights to the ongoing exploration of these insects in various ecosystems.

The presentation of DNA barcoding data, comprehensive references, and discussions on Orthopterans in Bangladesh, including ecologically and economically important species records, aims to establish a baseline for future monitoring and management efforts. The current study adopts a comprehensive approach to compile an updated list of Orthopteran species within Bangladesh. Utilizing the Mitochondrial CO1-based barcoding technique, the research provides valuable insights into phylogenetic relationships, haplotype diversity, and distribution patterns of Orthopterans in both Bangladesh and its surrounding region. This holistic understanding is pivotal for informing conservation strategies, enabling the development of well-informed and effective measures to ensure the preservation and sustainable management of these species and their ecosystems.

Materials and Methods

Sample Collections and Preparation: Townes Malaise trap deployed for a two-week duration between an agriculture and grassland field adjacent to the Noakhali Science and Technology University (NSTU) campus (22°47′31″N; 91°06′07″E). The collected samples, consisting of 23 orthopterans, underwent initial screening for subsequent taxonomic identification, and seven candidates were chosen for molecular analysis. The taxonomic identification process drew upon references including Bhowmik (1985), Mandal *et al.* (2007), Shishodia *et al.* (2010), Srinivasan and Prabakar (2013), Kundu *et al.* (2020) and BIP (2022) to ensure accuracy in identifying *Phlaeoba sikkimensis*, which emerged as a first country record in this study. Furthermore, articles containing descriptions of the original collections of orthopterans were scrutinized, and species were identified through DNA barcoding. Notably, only articles presenting firsthand collections were included. The reference lists of collected articles were thoroughly searched to identify additional relevant articles, ensuring a comprehensive and rigorous approach in documenting the orthopteran species in the specified location.

Molecular analysis: Samples were prepared for DNA barcoding by following the guidelines of Standard Operation Procedures provided by BIO (2017) (www.dnabarcoding.ca). Specifically, the hind femoral muscles of each specimen (somatic tissue rich in mitochondria) were selected for DNA extraction, and the rest of the body of each specimen was stored as a voucher specimen at the department museum of the Zoology Department, NSTU.

All genomic steps from DNA extractions to PCR product generation were done at a molecular lab in the Poultry Research and Training Centre, Chattogram Veterinary and Animal Sciences University, Chattogram-4225. Tissue lysis, DNA extraction, and polymerase chain reaction (PCR) amplification for DNA Barcoding were conducted following the Monarch Genomic DNA Purification Kit Protocol (NEB#T3010, New England BioLabs Inc.). Vouchers were recovered after DNA extraction for imaging and curation. PCR amplification of the target region of cytochrome oxidase 1 (COI) using *Forward Primer*: LCO1490: 5'-ggtcaacaaatcataaagatattgg-3' and *Reverse Primer*: HC02198: 5'-taaacttcagggtgaccaaaaaatca-3'. The thermo cycle protocol included and initial denaturing stage of 95°C (5 min) followed by 30 cycles of 94°C (45 s), 48°C (45 s) and 70°C (1 min), and a final extension stage of 72°C (10 minutes). Finally, PCR products were checked via electrophoresis using a 1% agarose gels and visualized a gel visualized using a gel recording system (BioDoc Analyzer, Biometra, Germany). PCR products were then purified and quality checked prior to submission to Macrogen Inc. for

sequencing. Once sequenced, the resulting partial mitochondrial cytochrome oxidase 1 DNA sequence chromatograms were assessed for quality using Bio Edit v.7.0.5 software. Each sequence was examined through the *Basic Local Alignment Search Tool* (*BLAST)* (https://blast.ncbi.nlm.nih.gov) and *Open Reading Frame Finder* (ORF finder) (https://www.ncbi.nlm.nih.gov/orffinder/) to diminish the indels, mismatches, and start-stop codons. The Obtained COI sequences were analyzed using sequence scanner version 2 of the applied biosystem. A total of 7 DNA barcode sequences of 2 subfamilies were submitted to the *National Center for Biotechnology Information* (NCBI) after finalization, and the accession numbers were obtained from the GenBank for every species we uploaded (Table 1).

Phylogenetic Network Constructions: A total of 56 Orthopteran sequences distributed in Bangladesh were obtained from BOLD database (Bold Systems v4) for overall analysis. The final dataset was aligned using the Clustal X program (Thompson *et al*., 1997), and the genetic divergences were estimated by Kimura 2 parameter (K2P) in Molecular Evolutionary Genetics Analysis (MEGA) programming 10 or MEGA10 (Kumar *et al*., 2016, 2018).

Haplotype network constructions: For constructing a haplotype network, the sequences of orthopterans were obtained from the BOLD database which includes 56 sequences of Bangladesh, each 10 sequences from India, Pakistan, China, Spain, Germany, Canada, etc. used for haplotype network construction purpose include MEGA, DNA SP, Popart, and TCS network. Besides, the Haplotype of each contributed species including *P. sikkimensis*, *A. crenulate,* and *O. fuscuvittata* along with their BOLD repository of available locations analyzed for a proper understanding of the distribution patterns of these species.

Results and Discussion

In the examination of 23 specimens, we partially sequenced the COI gene of 7 samples. These sequences were identified as any one of the three orthopteran species – *Atractomorpha crenulata* (one sequence: OQ842338, 644bp), *Oxya fuscovittata* (four sequences: OQ842271, 564bp; OQ842336, 651bp; OQ845424, 598bp; OQ842339, 652bp), and *Phlaeoba fuscovittata* (four sequences: OQ842271, 564bp; OQ842336, 651bp; OQ845424, 598bp; OQ842339, 652bp), and *Phlaeoba sikkimensis* (two sequences: OQ844086, 657bp; OQ845423, 631bp). A total of 17 of the specimens were morphologically identified as *Aulacobothrus luteipes, Chondracris rosea*, *Choroedocus violaceipes*, *Oxya hyla, Phlaeoba tenebrosa, Trilophidia annulata* (Acrididae),

Atractomorpha lata (Pyrgomorphidae), *Euparatettix nigritibis* (Tetrigidae), *Conocephalus exemptus*, *Ducetia japonica*, and three up to genus such as *Furcilarnaca* sp. (Gryllacrididae), *Tetrix* sp. (Tetrigidae) and *Homoeoxipha* sp. (Trigonidiidae). The remaining three specimens included unidentified species.

Of the barcoded samples, our *O. fuscovittata* sequences exhibit a strong association with previously documented records from Bangladesh, including GMBCD3305, GMBCC1761, and GBMNB59901 (Fig. 1). Phylogenetic analysis reveals a noteworthy connection between the Indian isolate GBMND81283-21 and our newly submitted *O. fuscovittata* sequences, providing enhanced clarity in understanding the species distribution. Additionally, at a divergence level of 0.06, neighboring species such as *O. hyla*, identified by accession numbers GMBCD3304, GMBCI3080, and GMBCN749, contribute to the intricate genetic landscape. Notably, *A. crenulata* (OQ842338) exhibited

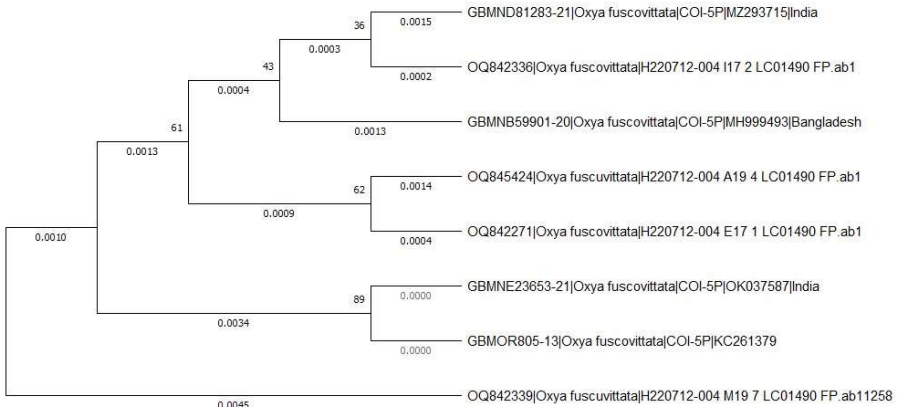

Fig. 1. Phylogenetic tree showing genetic relationships of *Oxya fuscuvittata*, rooted at the midpoint.

a close association with other Bangladeshi specimens of *Atractomorpha* (GMBCH3829-15, GMBCC1789-15, GMBCI3081-15, and GBMOR6888-19) in the BOLD repository specimen displaying slight divergence and a moderate to high confidence level. In our current investigation, the newly submitted Bangladeshi sequence OQ842338 demonstrates a close relationship with Pakistani isolates MTINS062-18, as illustrated in Fig. 2. Similarly, our contributed *P. sikkimensis* specimens (OQ844086 and OQ845423) exhibit associations with accession numbers GBMOR7400-19, GMBCB1681-15, GMBCC1764-15, GMBCJ2773-15, and GMBCN748-15, boasting a high confidence level and a divergence level of approximately 0.04 (Fig. 3). Notably, although *P.*

sikkimensis isolates from Bangladesh are absent in the BOLD database, our recent submission demonstrates a close relationship (CI = 99) with other isolates from India. The Bangladeshi isolates' closest neighbors are identified as GBMND7038-21 and GBMND7036-21, both originating from Indian isolates. These findings collectively contribute valuable insights into the genetic relationships and distribution patterns of the studied orthopteran species in the South Asian region.

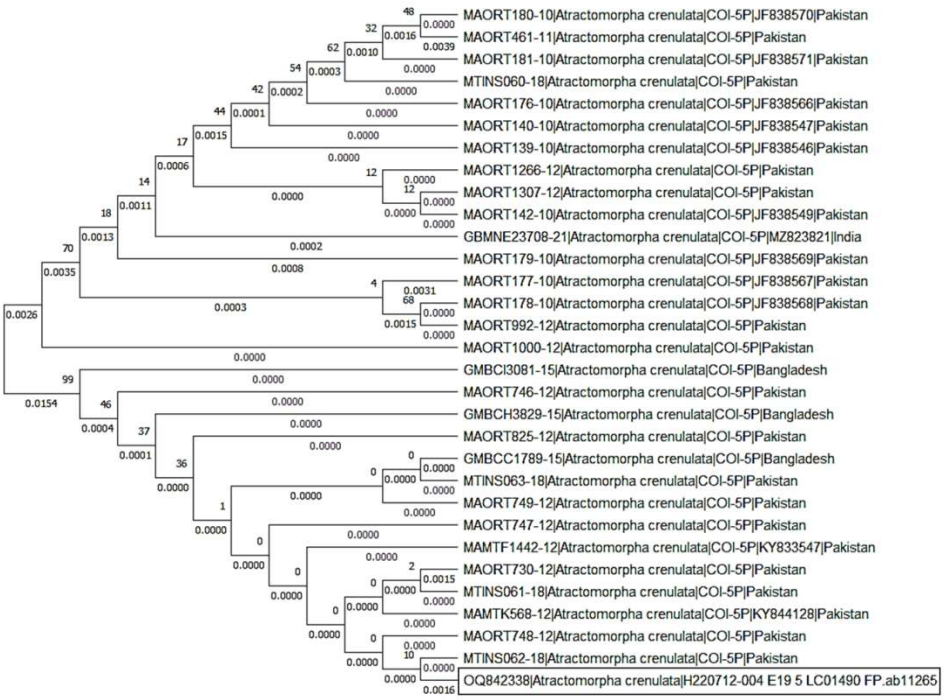

Fig. 2. Phylogenetic tree showing genetic relationships of *Atractomorpha crenulata*, rooted at the midpoint.

The haplotype analysis of Orthopteran species reveals intriguing patterns of interconnectedness among global populations. Australian haplotypes exhibit links with various Bangladeshi counterparts, marked by linker nodes with a divergence level of 18 nucleotide substitutions in single haplotype cases. Canadian haplotypes show connections with intermediate nodes, forming associations with German sequences. The Chinese haplotype is associated with both Pakistani and Spanish haplotypes through

interconnected nodes. German haplotypes display associations with Bangladeshi counterparts, featuring a relatively minor divergence level and interconnected nodes. Notably, they show a slight connection with Australian sequences after three interconnected nodes. Spain's haplotypes exhibit substantial diversity, with some linked to Indian, Australian, Canadian, Chinese, and Bangladeshi interconnected nodes (Fig. 4). Furthermore, the individual haplotype analysis of *P. sikkimensis* reveals eight haplotype nodes, with Hap_8 containing two Bangladeshi sequences contributed in this study. Other sequences from Indian isolates and the Bangladeshi haplotype closely relate to Indian

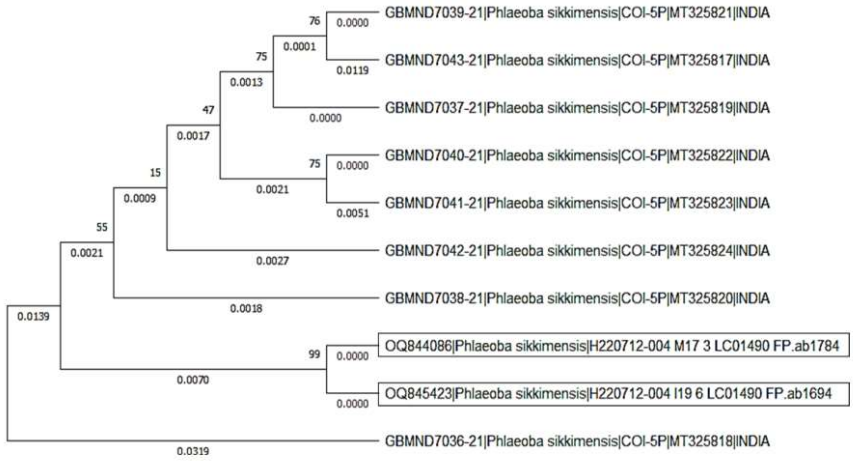

Fig. 3. Phylogenetic tree showing genetic relationships of *Phlaeoba sikkimensis*, rooted at the midpoint.

Hap_6 (Accession no of GBMND7042-21) with an interconnected node and a six-mutational divergence level, consistent with the phylogenetic analysis of this species (Fig. 5A). In the case of *O. fuscovittata*, one overlapping haplotype is identified with Indian isolates, suggesting a potential migration of the Indian species (GBMNE23653-21) to Bangladesh and close association with BD isolates of GBMOR805-13 (Fig. 5B). This finding is corroborated by phylogenetic analysis, highlighting a strong correlation between the two sequences from Indian GBMNE23653-21 and Bangladeshi isolates GBMOR805-13 (Fig. 1). In the case of *A. crenulata*, haplotype analysis indicates that Indian, Bangladeshi, and Pakistani species share only two haplotypes, suggesting a wide distribution with low divergence and de novo mutations. Bangladeshi sequences are closely associated with partial sequences of Pakistani nodes, showcasing a minor divergence level between them (Fig. 5C). These results offer valuable insights into the

genetic connectivity and migration patterns of Orthopteran species across different geographical regions.

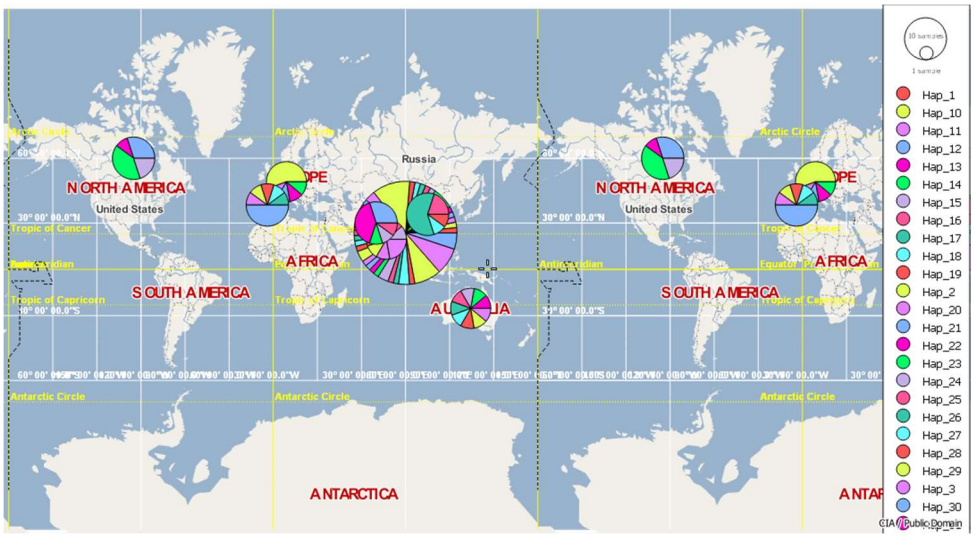

Fig. 4.A map displaying the haplotype distribution of Orthoptera species across various geographical regions

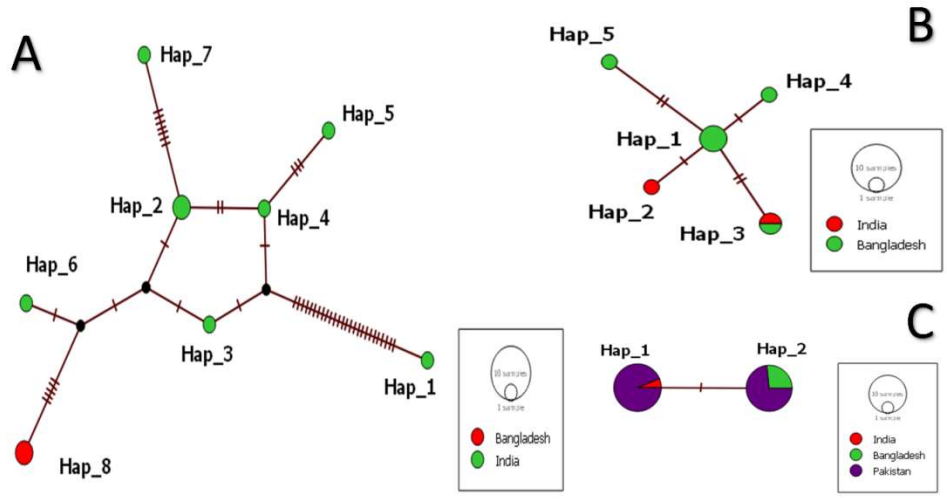

Fig. 5 (A, B, C). Haplotype analysis of the present studied orthopterans *P. sikkimensis, O. fuscuvittata, A. crenulate.*

However, in the case of *A. crenulate* species haplotypes of Indian, Bangladeshi, and Pakistani species are found to be distributed in just two haplotypes. This indicates the wide distribution of this species with a low level of divergence and de novo mutations. Our Bangladeshi sequences are found associated with the partial sequences of the Pakistani nodes (Fig. 5C). Phylogenetic relationships further confirmed this finding as Pakistani isolates MTINSO62 are closely related to our contributed species with a minor divergence level (Fig. 2).

Again, in the case of *P. sikkimensis* most of the BD sequence is placed on the group of Hap_1 and the center nodes that interconnect with all Indian and Bangladeshi sequences. Here, Hap_1, Hap_3, Hap_4, Hap_5, Hap_6, Hap_7 contain individual sequence of GBMND7036-21, GBMND7038-21, GBMND7040-21, GBMND7041-21, GBMND7042-21, GBMND7043-21 respectively. Where Hap_2 includes two sequences of GBMND7037-21 and GBMND7039-21. Similarly, Hap_8 contains two sequences of H220712-004_M17_3_LC01490_FP_*P. sikkimensis* and H220712-004_I19_6_LC01490 _FP_*P. sikkimensis*. Among the 8 haplotype variants, Hap_1 to Hap_7 are of Indian origin and Hap_1 only is of Bangladeshi origin. In addition, Hap_2 is the ancestor type, and Hap_1, Hap_5, Hap_7, Hap_6 & Hap_8 are recently evolved types. Hap_3 differs from Hap_2 with a few mutations, whereas Hap_4 is distinct by two times more mutations. Hap_7 differs from Hap_2 with 7-time mutations than Hap_3 and Hap_1 with a maximum of 26 times more mutations. The two Bangladeshi sequences incorporated in Hap_8 evolved from Hap_2 or Hap_3 with two unknown internodes and total mutations of 7 times. Hap_6 and Hap_8 are connected with a common internode and their complete divergence is about a few mutations for Hap_6 and 5 times for Hap_6 from the common unknown internodes

Most specifically, *P. sikkimensis* Ramme, 1941, a member of the Acrididae family, Acridinae subfamily, and Phlaeobini tribe, was confirmed first country record through DNA barcoding in the present study, was documented in various faunal surveys across South Asia. Described by Kirby in 1914 and Ramme in 1941, this species is characterized by straight and continuous lateral carinae of the pronotum, with greenish basally tinted hind wings. The distribution of *P. sikkimensis* spans Bangladesh, where it is also recorded in India, Nepal, and Bhutan according to Bhowmik (1985), Shishodia *et al*. (2010) and BIP (2022). This species is still restricted in South Asia, eight COI gene sequences of *P. sikkimensis* are present in the global DNA barcode database (BIP 2022). Kundu *et al.* (2020) had previously confirmed the species through DNA barcoding in India. This short note highlights the importance of molecular techniques in confirming the identity and

distribution of species, adding valuable data to the understanding of South Asian biodiversity.

Conclusions

The present study builds upon the foundations laid by Das *et al.* (2022) and Mazumdar *et al.* (2022) in the field of DNA barcoding, contributing to an updated catalog of orthopteran species in Bangladesh. The orthopteran fauna in Bangladesh remains incompletely explored, with numerous species yet to be discovered. There is a pressing need for advanced studies to unravel the intricacies of their biology and potential applications. The present study sheds light on the evolutionary relationships within various Orthoptera species documented in Bangladesh, offering valuable insights through phylogenetic analyses. Noteworthy outcomes include the identification of unique haplotypes, elucidation of distribution patterns, and documentation of first country records. This comprehensive update enhances our understanding of the orthopteran diversity in Bangladesh, shedding light on the intricate ecological dynamics within the region. These findings collectively contribute to a deeper understanding of the distribution and identity of this understudied yet ecologically significant species group. The gaps filled by this research provide a foundation for further investigations, emphasizing the importance of continued exploration and study of orthopteran biodiversity in the context of Bangladesh.

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