

**COI DNA BARCODING AND PHYLOGENETIC RELATIONSHIPS OF WASPS
(HYMENOPTERA: VESPIDAE) FROM THE SUNDARBANS, BANGLADESH**

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Abstract: We studied the molecular identification and phylogenetic relationships of 7 Wasp species (Hymenoptera: Vespidae) found in the mangrove ecosystem of the Sundarbans region of Bangladesh, using mitochondrial COI as a marker. Wasp specimens were collected from various locations throughout the Sundarbans and all 7 were successfully amplified via PCR using COI primers. Upon sequence analysis, the 7 Wasp COI sequences had a similarity to sequences in GenBank, ranging between 94.59 - 100%. Also, MEGA11 was used to generate the phylogenetic tree, which resulted in the grouping of wasps into Clade A (*Vespa affinis*, *V. tropica*, *Rhynchium brunneum*, *R. quinquecinctum* and *Phimenes flavopictus*) and Clade B (*Polistes stigma* and *P. olivaceus*). A very strong correlation was found between Bangladeshi populations of wasps and those from other regions (bootstrap support high). The phylogenetic analysis of these wasp species can be used to help identify these wasps. In constructing a Templeton-Crandall-Sing (TCS) haplotype network, we found that the 7 species of wasps were genetically distinct from one another, with *P. stigma* being the most distantly related, separated by 68 mutation steps from all of the remaining wasp species. *R. quinquecinctum* and *R. brunneum* exhibited the largest genetic similarity, being separated from one another by only 14 and 19 mutation steps, respectively. Therefore, *P. stigma* appears to be the species that has experienced the most ecological adaptation, followed by the *Vespa*, *Rhynchium*, and *Phimenes* species. Overall, these findings contribute to the current understanding of wasp diversity in the Sundarbans. Nonetheless, broader sampling and integrative taxonomic approaches are required to fully characterize the wasp assemblage and its ecological significance in the mangrove ecosystem.

Key words: Pollinator, DNA barcoding, Mutational relationship, Sundarbans, Conservation

INTRODUCTION

Vespidae is a family under the order Hymenoptera with thousands of solitary and social species. Wasps in this family are vital to ecosystems as

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pollinators, as pest controllers and as food for other animals (Brock *et al.*, 2021, Jandt *et al.*, 2024). Also they are key indicators of ecosystem health and environmental change. Though more than 5,000 species in this family have been described across the world, their diversity and ecological impacts are still relatively unknown in many parts of the world, including the Sundarbans in Bangladesh (Kumar and Sharma 2015). The Sundarbans, a UNESCO World Heritage Site, is the largest contiguous block of mangrove forests in the world. It is an ecologically important area, containing immense plant and animal biodiversity, and is home to a variety of insects including butterflies and moths, as well as wasps, that pollinate and help control pests in the sensitive forest ecosystem (Kumar and Sharma 2014, Hossain 2014ab, Hossain *et al.*, 2015, Habib *et al.*, 2018). However, little research has been conducted on insect biodiversity in the Sundarbans, particularly on the identification and relatedness of its wasp fauna.

Identifying insect species using their morphology, especially of wasps which are extremely diverse in the Sundarbans mangrove forest, can be extremely challenging. Sexual dimorphism, cryptic species, and small size of insects often lead to ambiguity in identification (Akter *et al.*, 2023, Abedin *et al.*, 2023, Ghosh *et al.*, 2024, Islam *et al.*, 2024). In contrast, molecular identification through mitochondrial DNA sequencing offers a dependable approach for species identification (Hebert *et al.*, 2003a). The mitochondrial COI gene is very useful for phylogenetic analysis because of its intermediate evolutionary rate and its efficacy at distinguishing species boundaries (Hebert *et al.*, 2003b). The identification on hymenopterans fauna of Bangladesh based on morphology was carried out by several researchers in Bangladesh (Bhuiya and Miah 1990, Gapud 1992, Hannan 2007, Ahmed 2009, Mazumdar *et al.* 2010, Mazumdar *et al.* 2014, Akter *et al.*, 2019, Akter *et al.*, 2020). Nevertheless, only a limited study in Bangladesh has utilized molecular markers for the identification of Hymenopteran insects (Mazumdar *et al.*, 2019, Mazumdar *et al.*, 2021, Mim *et al.*, 2025).

In the Sundarbans, wasp molecular phylogenetic studies are lacking, and no comprehensive studies have been conducted on the wasp species of this unique ecosystem. This study aims to identify wasp species in the Sundarbans using the COI mitochondrial gene and to build a phylogenetic tree to explore how these species are related to one another. The findings will help to better illuminate wasp species richness in the Sundarbans, which can lead to better-informed ecological and conservation management.

MATERIAL AND METHODS

Sample collection: The collection of wasp specimens was conducted at various points within the Sundarbans mangrove forest ecosystem in Bangladesh

(Fig. 1, 2; Table 1). The wasps were collected by netting and then preserved in 70% ethanol for subsequent DNA barcoding at the DNA barcoding lab, Department of Zoology, Jahangirnagar University.

DNA extraction, PCR amplification and sequencing: The genomic DNA was extracted from seven wasp specimens. The extraction procedure was conducted using the Wizard Genomic DNA Purification Kit (Promega, USA), with adjustments based on the protocol provided by Hossain *et al.* (2022). The concentration and purity of the obtained DNA were determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). The 658 base pair region from the 5' end of the COI gene was targeted for PCR amplification. The PCR amplification was conducted with universal primers LCO1490 and HCO2198 (Folmer *et al.*, 1994). The thermal cycle for PCR amplification was as follows: an initial denaturation at 94°C for 5 minutes, followed by 35 cycles consisting of denaturation at 94°C for 1 minute, annealing at 49°C for 30 seconds, extension at 72°C for 45 seconds, and final extension at 72°C for 5 minutes. The efficiency of the amplification was checked by 1% agarose gel electrophoresis under ultraviolet light (Bio Doc Analyze, Biometra, Analytic Jena, Germany). The Promega Wizard SV Gel and PCR Clean-Up System were used to purify the amplified products. An ABI 3500 sequencer was then utilized to sequence the purified products.

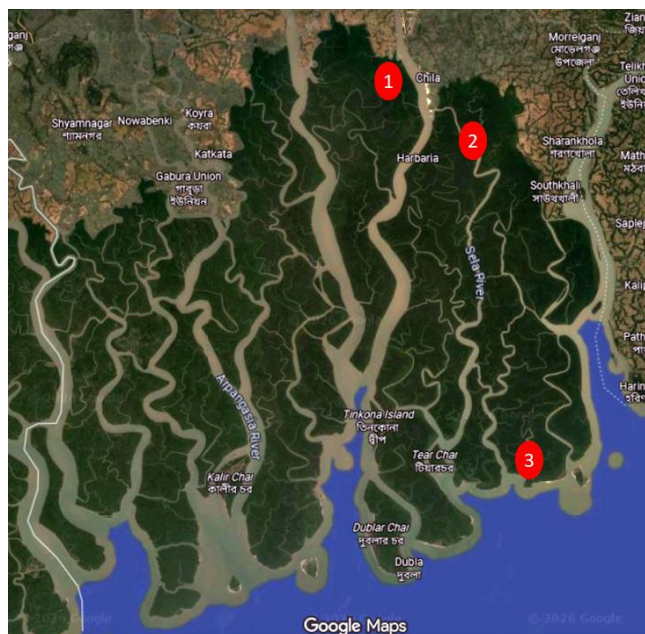


Fig. 1. Map showing the sample collection sites in Sundarbans: Karamjal (1), Andarmanik (2), Katka (3)

Phylogenetic analysis: The ClustalW program in BioEdit version 7.0 was used to perform multiple sequence alignment of the sequences (Hall 1999). The Neighbor-Joining method was used for constructing a phylogenetic tree for all species with 1000 bootstrap replicates in MEGA11. For COI gene sequence analysis of all species, different conspecific sequences were retrieved from GenBank for different geographic locations. *Aedes aegypti* species from Bangladesh was used as outgroup for phylogenetic analysis.

Haplotype network analysis: The haplotype network for all species was constructed using the POPART 1.7 program (Leigh and Bryant 2015) based on the TCS method (Clement *et al.*, 2000). This method shows the relationships between haplotypes based on mutation events.

RESULTS AND DISCUSSION

The COI gene sequences were successfully amplified for all seven wasp species collected from various locations across the Sundarbans (Fig. 1, 2, Table 1). BLAST analysis confirmed species identification, with sequence similarities ranging from 94.59% to 100% when compared to wasp sequences from other countries in GenBank (Table 1). Of the seven species, six had previously been recorded from different regions of Bangladesh, with the exception of *Phimenes flavopictus* (Ahmed *et al.*, 2009, Mazumdar *et al.* 2010, Mazumdar *et al.* 2014, Akter *et al.*, 2020, Rahman and Aslam 2025). *P. flavopictus* has not been documented in any published article from Bangladesh, with the only known record being a citizen science observation on iNaturalist from Khagrachari in 2022 (<https://inaturalist.ca/observations/137498948>). The present study thus provides the first confirmed record of *P. flavopictus* from the Sundarbans, along with its COI gene sequence which matched 98.07% with the same species from India (KM455120) in Genbank (Table 1).

Among the seven species analyzed, *V. affinis* from the Sundarbans exhibited 99.68% sequence similarity with a sample (MH036512) of the same species from the Savar region of Bangladesh. In contrast, *P. olivaceus* showed 99.69% and 98.58% similarity with two specimens (PQ795790 and AB570105) of the same species from Savar and Panchagarh, respectively (Uddin and Tsuchida 2012, Rahman and Aslam 2025). On the other hand, *P. stigma* exhibited 93.75% similarity with another specimen (PX249792) of the same species from the Savar area of Bangladesh. Although these variations are minimal, they may indicate slight genetic divergence, potentially influenced by the ecological isolation and unique environmental conditions of the Sundarbans. Nonetheless, the COI gene sequences of the remaining four species, *V. tropica*, *R. brunneum*, *R. quinquectum* and *P. flavopictus* have been submitted to NCBI's

GenBank for the first time from Bangladesh, based on specimens collected from the Sundarbans (Table 1).

Table 1. List of seven wasp taxa and their relevant information from Sundarbans areas

Scientific Name	Voucher No.	Location in Sundarbans	GPS Coordinates	Similarity in GenBank	GenBank Accession No.
<i>Vespa affinis</i>	PBV 0003	Karamjal	22°25'40.6"N 89°35'25.7"E	99.68%	PQ438547
<i>Vespa tropica</i>	PBV 0024	Andarmanik	22°18'27.5"N 89°42'06.5"E	99.18%	PV696970
<i>Polistes stigma</i>	PBV 0021	Katka	21°51'17.5"N 89°46'54.4"E	95.04%	PV576425
<i>Polistes olivaceus</i>	PBV 0029	Karamjal	22°25'38.0"N 89°35'26.6"E	99.84%	PV696999
<i>Rhynchium brunneum</i>	PBV 0027	Andarmanik	22°18'24.6"N 89°42'12.6"E	100%	PV700341
<i>Rhynchium quinquecinctum</i>	PBV 0028	Karamjal	22°25'38.7"N 89°35'26.8"E	94.59%	PV696997
<i>Phimenes flavopictus</i>	PBV 0023	Karamjal	22°25'41.7"N 89°35'22.6"E	98.07%	PV696963

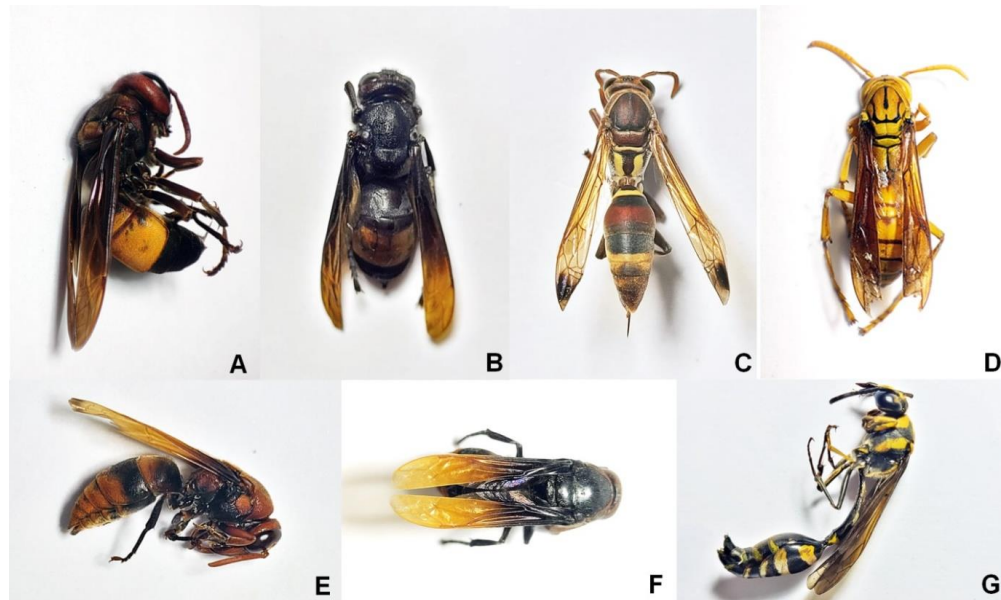


Fig. 2. Wasp voucher used for genomic DNA extraction in the investigation. A. *V. affinis*, B. *V. tropica*, C. *P. stigma*, D. *P. olivaceus*, E. *R. brunneum*, F. *R. quinquecinctum* and G. *P. flavopictus*

Phylogenetic analysis: Neighbor-Joining (NJ) analysis was employed to construct a phylogenetic tree for the seven wasp species. This analysis revealed two distinct groups, i.e., Clade A and Clade B. Bootstrap values have been added to the tree to determine the confidence level with which each branch is supported (Fig. 3). *V. affinis* from Bangladesh and India are closely related and

The analysis of phylogeny for the seven wasp species reveals variations in their genetic sequences. These wasps share an ecological niche in the Sundarbans region. A phylogenetic tree shows that social species like *Vespa* and *Polistes* are grouped separately from solitary species like *Rhynchium* and *Phimenes*, which cluster with *Vespa*. We can infer that such differences in wasp genetics correspond to different ecological functions and behaviors, in agreement

with earlier research published by Uddin and Tsuchida (2011) and Peng *et al.* (2025).

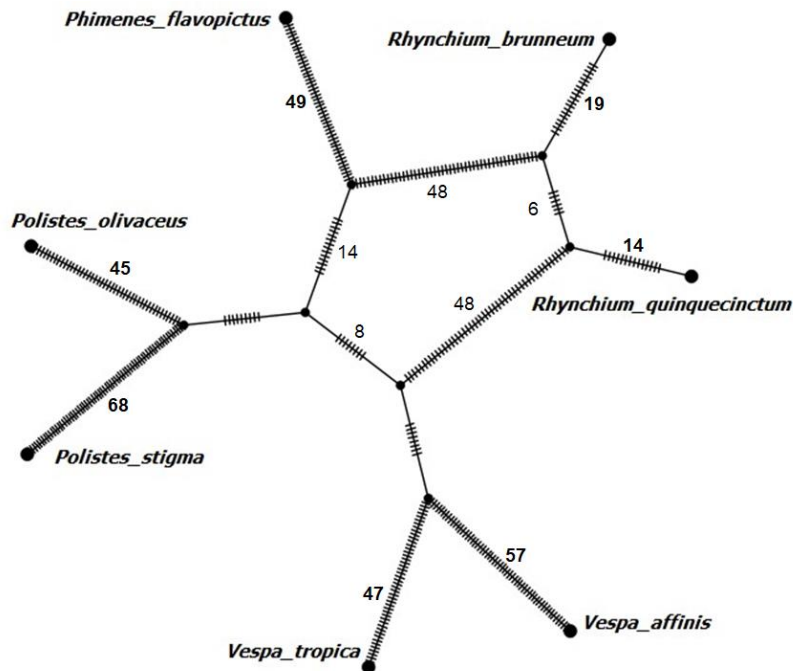


Fig. 4: TCS haplotype network of seven Vespidae species derived from the mitochondrial COI gene. Large circles denote haplotypes, while small circles indicate the immediate common ancestors. Mutational steps are shown by hatch marks and corresponding numbers.

Haplotype network: A TCS Haplotype network was constructed using mitochondrial COI gene of seven Vespidae wasp that showed mutational relationship among them (Fig. 4). Each node represents a unique haplotype, with branch lengths indicating mutational differences between them. *P. stigma* is the most genetically distant, separated by 68 mutational steps, indicating higher adaptation and evolutionary divergence (Fig. 4). While *P. olivaceus* and *P. flavopictus* follows with 45 and 49 mutational steps, showing significant genetic variation. On the other hand *V. tropica* and *V. affinis* are separated by 47 and 57 mutation steps, respectively, indicating notable divergence but not as much as the *Polistes* species. While *R. quinquecinctum* and *R. brunneum* were diverged from their immediate common ancestors by 14 and 19 mutation steps respectively. Overall, this analysis provides insight into the evolutionary history of these species, with *P. stigma* appearing to have experienced the most

adaptation or ecological changes, followed by the *Vespa* species and the *Rhynchium* and *Phimenes* (Fig. 4).

In studies involving COI gene sequences for insect phylogenetics, similar results have been observed where different species, particularly within the same family, exhibit varying levels of genetic divergence (Hebert *et al.*, 2003). For example, in the study of other insect groups like the Hymenoptera (which includes wasps) (Deodoro *et al.*, 2008), Drosophilidae (fruit flies) (Wang *et al.*, 2023) or Lepidoptera (butterflies) (Hossain *et al.*, 2022), researchers often report significant mutational differences across species within the same genus or family. These differences are measured by the number of mutational steps or sequence differences between haplotypes that aligns with the findings from the Vespidae haplotype network in the present study. Genetic studies using COI sequences have consistently shown that the number of mutational steps between haplotypes correlates with genetic differentiation and species divergence (Deodoro *et al.*, 2008, Motoki *et al.*, 2021), supporting the use of this marker for understanding evolutionary relationships across a wide range of insect taxa.

CONCLUSION

This work reports molecular identification and genetic linkage of wasp species in the Sundarbans mangrove ecosystem of Bangladesh. A total of seven wasp species were identified using the mitochondrial COI gene and their genetic diversity was estimated using a tree based on phylogenetic analysis. The phylogenetic tree revealed two distinct genetic clades. The species in Clade A are genetically closely related and are significantly distinct from the species in Clade B. This study contributes to the database on wasp species, aiding in accurate identification, and will be valuable for ecosystem management, specifically for maintaining the ecological balance in the Sundarbans mangrove forest.

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