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Morphological and Phylogenetic Confirmation of *Polistes* Wasps (*Polistes perplexus* and *Polistes stigma*) in District Shangla, Khyber Pakhtunkhwa, Pakistan

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Abstract

Paper wasps (Hymenoptera: Vespidae: Polistinae) are ecologically significant insects; however, their species-level identification is often challenging due to morphological similarities and the scarcity of regional molecular data, particularly from northern Pakistan. This study aimed to confirm species identity and reconstruct phylogenetic relationships of *Polistes* species from District Shangla, Khyber Pakhtunkhwa, Pakistan, using an integrative taxonomic approach combining morphological examination and DNA barcoding. A total of 393 specimens of the genus *Polistes* (Latreille 1802) were collected from multiple localities and identified using morphological characteristics. Molecular validation was performed using the mitochondrial cytochrome c oxidase subunit I (*COI*) gene. BLAST analysis revealed high sequence similarity for *Polistes perplexus* (99.84%) and *Polistes stigma* (100%) with reference sequences available in GenBank. Phylogenetic analysis using the Maximum Likelihood method (Kimura 2-parameter model, 1,000 bootstrap replicates) showed strong bootstrap support (95–100%) for distinct species-level clades. *Polistes perplexus* showed very low genetic divergence, indicating genetic homogeneity across populations, whereas *Polistes stigma* showed relatively higher intraspecific variation, suggesting population differentiation and potential population structuring. This study provides the first phylogenetic data for *Polistes* species from District Shangla and contributes reference sequences to the global barcode database. These findings show the effectiveness of *COI* barcoding for species identification and support the use of integrative taxonomy in resolving species boundaries within the genus *Polistes*.

Keywords: *Polistes*; *COI* barcoding; Phylogenetics; Polistinae; Pakistan

Introduction

The subfamily Polistinae (Hymenoptera: Vespidae) comprises ecologically and evolutionarily significant eusocial insects that are common in tropical and temperate regions. The genus *Polistes* is one of the most species-rich and well-investigated genera within the subfamily due to its ecological interactions, social behavior, and geographic distribution (Schmid-Egger *et al.* 2017a). *Polistes* species are primarily insect predators and therefore aid in natural pest control, which makes them valuable elements in land-based ecosystems (Brito *et al.* 2024). Although they are important, proper species-level identification within *Polistes* remains challenging. Morphological similarities among species, the presence of cryptic traits, and high intraspecific variation often limit the reliability of morphology-based identification (Nguyen *et al.* 2017). These challenges are particularly evident in south and central Asia, including Pakistan, where taxonomic coverage is incomplete and molecular data are limited (Bashir *et al.* 2022). In recent years, molecular techniques have been extensively incorporated into both taxonomic and phylogenetic research to address the shortcomings of morphology-based approaches. The mitochondrial cytochrome c oxidase subunit I (*COI*) gene used in DNA barcoding has demonstrated its usefulness in taxonomic delimitation of

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species, the detection of cryptic diversity, and reconstruction of evolutionary relationships in insects (Lin *et al.* 2017). *COI*-based studies have effectively resolved species boundaries and clarified evolutionary relationships among *Polistes* in various biogeographic groups (Rodriguez-Perez *et al.* 2021).

Despite these advances, molecular phylogenetic data on *Polistes* species in Pakistan remain scarce, particularly from mountainous regions such as District Shangla, where entomological studies are limited despite high habitat diversity. Therefore, the present study aimed to confirm the identity of *Polistes* species from District Shangla, Khyber Pakhtunkhwa, Pakistan, using morphological and molecular approaches. Additionally, this study aimed to reconstruct the phylogenetic relationships of *Polistes perplexus* and *Polistes stigma* based on mitochondrial *COI* gene sequences and to assess genetic variation between species to quantify patterns of intraspecific and species-level divergence.

Materials and Methods

Study area

The study was conducted in District Shangla located in northern Khyber Pakhtunkhwa, Pakistan, which occupies an area of about 1,586 km^2 positioned between 34.4° N to 35.0° N and 72.4° E to 72.8° E (Ahmad *et al.* 2021). District Shangla is subdivided into five tehsils: Alpuri, Besham, Chakisar, Martung, and Puran. Its elevation ranges from 448 m to over 4,500 m above sea level, with prominent mountains, such as Spinghar (4,464 m) and Takht Ghar (4,332 m) (Iqbal *et al.* 2022). The climate is montane, and the area experiences cold winters (the temperature falls below -5°C), hot summers (up to 38°C) (Nazir *et al.* 2021). The natural vegetation includes *Cedrus deodara*, *Abies pindrow*, *Pinus wallichiana*, and *Picea smithiana*, providing suitable habitats for hymenopteran diversity (Bashir *et al.* 2022; Iqbal *et al.* 2022). The district is characterized by humid and highland climatic conditions with a total precipitation of 52 inches (1,320 mm) and a fluctuation of 134 millimeters (1961-2014) (Khan *et al.* 2019).

Specimen collection and morphological identification

Field surveys were conducted in three tehsils of District Shangla Khyber Pakhtunkhwa, Pakistan, to collect wasp species belonging to subfamily Polistinae. A total of 393 specimens were collected from 10 sampling sites across tehsil Alpuri (4 sites), tehsil Besham (3 sites), and tehsil Puran (3 sites), comprising 19 to 66 specimens per site. Sampling sites were selected based on habitat diversity, accessibility and safety considerations, ensuring representation of different habitat types, including forests, gardens, agricultural fields, and semi-urban areas. Sampling was conducted through repeated visits (3-4 times) during the study period to ensure adequate coverage. Specimens were collected using sweep nets during the day (8:00-17:00) and some nests were collected during the night by capturing the whole hive in plastic bags. All environmental data such as temperature, humidity, and GPS coordinates, among other parameters, were recorded at each collecting location. Specimens were preserved in 98% ethanol in sterile conical centrifuge tubes and stored at -20ºC for subsequent DNA extraction, while selected specimens were pinned and preserved for morphological identification. Morphological identification was performed using a stereomicroscope (Lambomet KCZM4-4X) and standard taxonomic keys (Reed *et al.* 1988; Nguyen *et al.* 2017). Identification was based on diagnostic morphological characters including eye shape, wing venation, leg structure, body coloration, abdominal segmentation patterns, antennal length and segmentation, and stinger morphology.

DNA extraction, PCR amplification and sequencing

DNA was extracted using the Qiagen DNeasy Blood and Tissue kit, following the manufacturer's protocols. DNA quality and concentration were measured using NanoDrop spectrophotometry (Fig. 1; Table 5). Some DNA samples showed relatively high A260/A280 ratios, which may be associated with low DNA concentration, residual RNA, or spectrophotometric variation commonly observed in NanoDrop measurements of insect DNA extracts. However, the DNA quality was considered adequate because successful PCR amplification and high-quality *COI* sequences were obtained from all analyzed specimens.

PCR amplification was performed in a 25 μL reaction mixture containing:

- 12.5 μL of PCR master mix
- 1 μL of each primer (10 μM) (Table 1)
- 2 μL of template DNA
- 8.5 μL of nuclease-free water.

PCR amplification was performed under the following thermal cycling conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 52°C for 35 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min (Table 2).

PCR products were sequenced using Sanger sequencing method. Initial DNA extraction and PCR amplification were carried out at Kaisee Dreams Research Laboratory, Dargai, Pakistan. Sanger sequencing was performed by Macrogen Inc., a biotechnology and precision medicine company headquartered in Seoul, South Korea. Due to limited financial resources and budget constraints, molecular sequencing was performed on only one representative specimen. The selected specimen was carefully chosen based on its clear morphological characteristics and good preservation condition to represent the studied population. The remaining specimens were identified and confirmed through detailed morphological examination and comparison with available taxonomic keys and published literature.

Table 1: Primer sequences used for PCR amplification of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene, including primer names, nucleotide sequences, orientation and target regions (Folmer *et al*. 1994)

Primer name	Sequence	Orientation	Target region	Reference
LCO1490	5′-GGTCAACAAATCATAAAGATATTGG-3′	Forward	*COI* (5' end)	(Folmer *et al*. 1994)
HCO2198	5′TAAACTTCAGGGTGACCAAAAAATCA-3′	Reverse	*COI* (3' end)	(Folmer *et al*. 1994)

Table 2: PCR thermal profile for amplification of the mitochondrial *COI* gene (Lorenz 2012)

	Cycle	Temperature	Time
Stage 1	Initial Denaturation	95°C	5 min
Stage 2	Denaturing	94°C	45 sec
35 Cycles	Annealing	52°C	35 sec
	Extension	72°C	1 min
Stage 3	Final Extension	72°C	7 min
Stage 4	Hold	10°C	Infinity

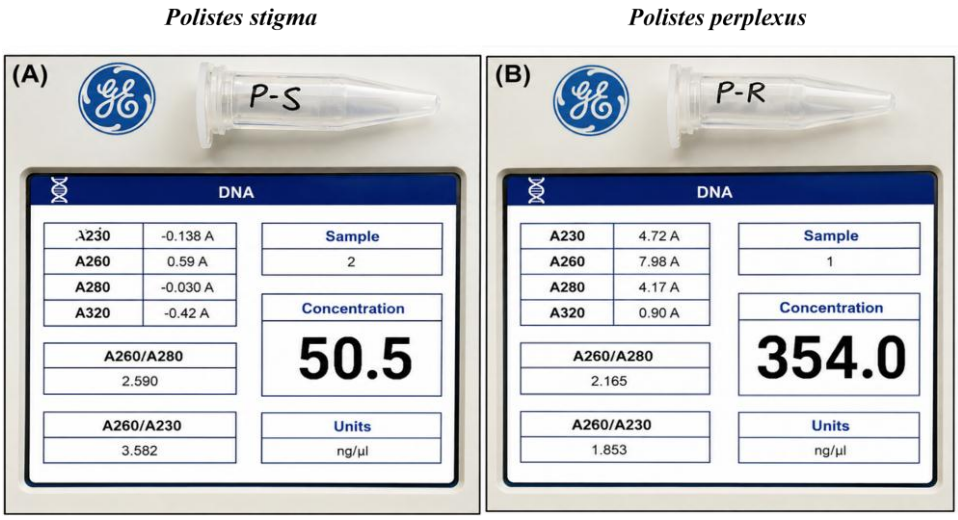

Fig. 1: Nanodrop spectrophotometer output showing DNA concentration and purity profiles of *Polistes perplexus* and *Polistes stigma* samples used for *COI* gene amplification

Since all collected specimens showed highly similar diagnostic characters, sequencing of one representative specimen was considered adequate for preliminary molecular support of the morphological identification in the present study. It is acknowledged that sequencing multiple specimens could provide stronger molecular evidence, and broader molecular analyses are planned for future studies when resources become available.

Raw sequences were edited and analyzed using BioEdit (v. 7.2.5) and MEGA (v. 12). The ambiguous bases were edited manually.

Species confirmation

BLASTn searches were performed against the NCBI GenBank database. Species identification was considered reliable when sequence similarity exceeded 98% (Rodriguez-Perez *et al.* 2021).

GenBank selection criteria

Twenty-four reference sequences were selected from GenBank based on high sequence similarity (> 96%), correct species identification and geographic representation. Only well-annotated sequences with complete or near complete regions were included. Outgroup taxa (*Vespula* species) were selected based on previous phylogenetic studies to ensure proper tree rooting. Details of all reference sequences used in the phylogenetic analysis, including accession numbers and geographic origin, are provided in Table 3.

Phylogenetic analysis

Phylogenetic relationships were reconstructed using the Maximum Likelihood method implemented in MEGA (v. 12) (Kumar *et al.* 2024). Sequences were aligned using the MUSCLE algorithm. The Kimura 2-parameter (K2P) model was applied to compute evolutionary distances, as it is commonly used in *COI*-based DNA barcoding studies. The node support was estimated with 1,000 bootstrap replicates.

Results

All of the 393 specimens were collected from different sites

Table 3: Details of all reference sequences used in the phylogenetic analysis, including accession numbers and geographic origin

Species	Accession number	Country
Polistes stigma	PV993975	Pakistan
Polistes stigma	HQ567776	Canada
Polistes stigma bernardi	GU596890	USA
Polistes stigma	PV576425	Bangladesh
Polistes stigma tamula	MG733323	Vietnam
Polistes japonicus	OM991953	South Korea
Polistes formosanus	AB917976	Japan
Polistes lepcha	MG733329	Vietnam
Polistes reliciniclypeus	MG733332	Vietnam
Polistes snelleni	OM991964	South Korea
Polistes bellicosus	GU596858	USA
Polistes carolina	EU649532	Canada
Polistes carolina	EF136427	USA
Polistes metricus	EF136447	USA
Polistes perplexus	PV993977	Pakistan
Polistes perplexus	EF136452	USA
Polistes perplexus	GU596886	USA
Polistes rothneyi	OM991972	South Korea
Polistes jokahamae	OL343182	South Korea
Polistes olivaceus	PQ795790	Bangladesh
Polistes wattii	MT891261	India
Vespula alascensis	HM435869	Canada
Vespula vulgaris	PV993975	Pakistan
Vespula flavopilosa	HQ567776	Canada

Table 4: Species Composition and Relative Abundance of *Polistes* Wasps in District Shangla, Khyber Pakhtunkhwa, Pakistan

Scientific name	Local name	Subfamily	No. of specimens	Relative abundance
Polistes stigma	Ware Dandaari	Polistinae	173	44.02%
Polistes perplexus	Sre Dandaari	Polistinae	220	55.98%

Table 5: Extracted DNA concentration and purity assessment of *P. perplexus* and *P. stigma* samples measured using a Nanodrop spectrophotometer

Sr. No.	Sample ID	DNA concentration ng/μL	Absorbance ratio A260/A280	Absorbance ratio A260/A230
1	P.R	354 ng/μL	2.165	1.853
2	P.S	50.5 ng/ μL	2.590	3.582

in three tehsils (Alpuri, Besham and Puran). All of 393 specimens were examined and identified as belonging to two species within the genus *Polistes*. These included *Polistes perplexus* and *Polistes stigma*. Among the collected specimens, *Polistes perplexus* was more abundant (55.98%), whereas *Polistes stigma* accounted for 44.02% of the total specimens collected (Table 4). For molecular analysis, a single representative specimen from each of the two identified species, *Polistes perplexus* and *Polistes stigma*, was selected for DNA sequencing to confirm species identity.

Polistes perplexus

Material examined

Kund, Besham 14 ♀ and 8 ♂, 2.viii.2024, Asaf Khan leg.; Lilowni, Alpuri 40 ♀ and 26 ♂, 11.ix.2024, Asaf Khan leg.; Lalkhani, Alpuri 32 ♀ and 20 ♂, 8.xi.2024, Asaf Khan leg.; Kotkay, Puran 37 ♀ and 24 ♂, 5.iv.2025, Asaf Khan leg.; Machkanday, Puran 12 ♀ and 7 ♂, 15.v.2025, Asaf Khan leg.

Body measurements

Body length ranged from 16-22 mm, and forewing length ranged from 14-19 mm (Fig. 2).

Diagnostic characters

Body predominantly reddish-brown with yellow markings on the clypeus and metasomal segments. Antennae elongated, with dark apical segments. Wings translucent brownish with distinct venation. Pronotum comparatively broad, and metasomal tergites with narrow yellow posterior bands. Legs slender and yellowish-brown. The species can be distinguished from *P. stigma* by its comparatively larger body size and darker brown thoracic and abdominal coloration (Fig. 2).

Distribution

Nepal, Iran, India and Pakistan.

Remarks

Polistes perplexus is reported here from District Shangla.

Polistes stigma

Material examined

Faridabad, Alpuri 24 ♀ and 14 ♂, 25.vii.2024, Asaf Khan leg.; Sundovi, Puran 27 ♀ and 15 ♂, 6.x.2024, Asaf Khan leg.; City, Besham 21 ♀ and 12 ♂, 15.iii.2025, Asaf Khan leg.; Shung, Besham 17 ♀ and 9 ♂, 11.iv.2025, Asaf Khan leg.; Shahpur, Alpuri 22 ♀ and 12 ♂, 13.v.2025, Asaf Khan leg.

Body measurements

Body length ranged from 13-14 mm, and forewing length ranged from 11.1-11.3 mm (Fig. 3).

Diagnostic characters

Body smaller and comparatively slender, predominantly yellowish-brown with distinct yellow markings on the face and thorax. Clypeus broad and yellow. Antennae moderately elongated with lighter basal segments. Wings lightly infuscate with clear venation. Abdomen with distinct yellow apical bands on metasomal tergites. This species differs from *P. perplexus* by its smaller body size, lighter coloration, and more prominent yellow markings (Fig. 3).

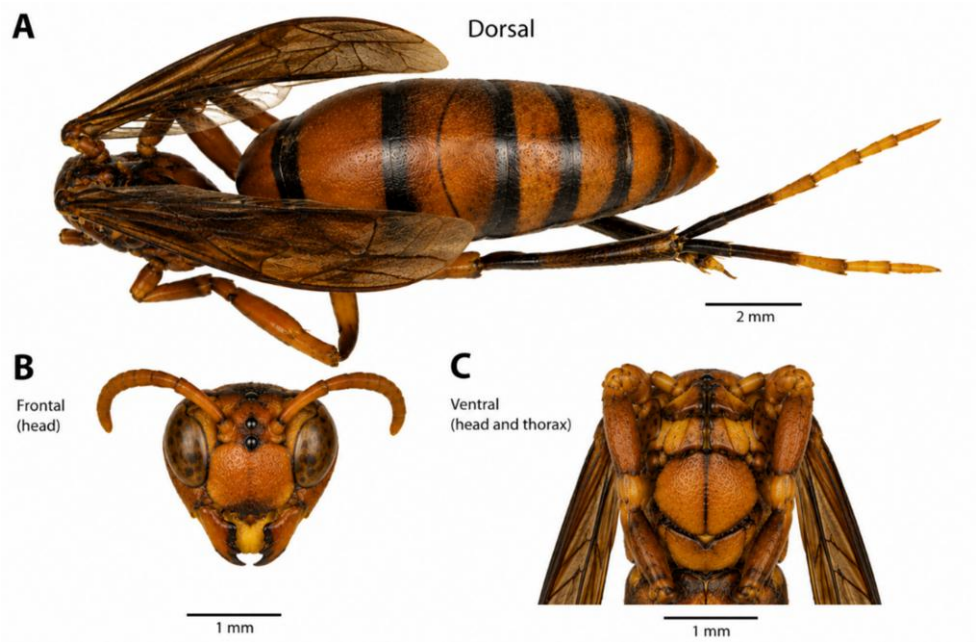

Fig. 2: Morphological characteristics of *Polistes perplexus* showing (**A**) dorsal view, (**B**) abdominal view, and (**C**) Ventral and head view, highlighting key diagnostic features used for species identification

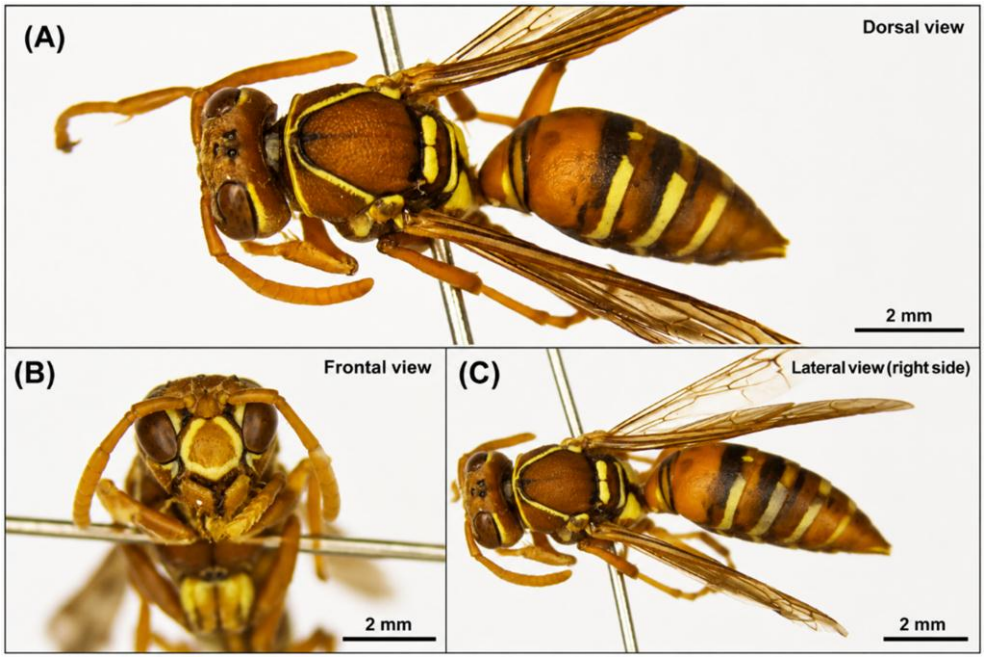

Fig. 3: Morphological characteristics of *Polistes stigma* showing (**A**) dorsal view, (**B**) abdominal view and (**C**) ventral and head view, highlighting key diagnostic features used for species identification

Distribution

Pakistan, China, Europe, Iran, Australia, Russia, Africa, Turkey, USA, Chile, Afghanistan, Uzbekistan, Israel, India, Turkmenistan, Mongolia and Argentina (Rafi *et al.* 2017).

Remarks

Polistes stigma has previously been recorded from Khyber Paktunkhwa: Swat (Rasool *et al.* 2017), Dir Valley (Khan *et al.* 2018), Swat, Miadam and Siadu Sharif (Mahmood *et al.* 2012), Punjab: Murree, Sindh: Karachi (Rafi *et al.* 2017),

Table 6: Nucleotide composition and GC content (%) of mitochondrial *COI* gene sequences of *Polistes perplexus* and *Polistes stigma*

Species	Adenine (A)	Thymine (T)	Cytosine (C)	Guanine (G)	GC content
P. perplexus	27.5%	32.4%	19.3%	20.8%	40.1%
P. stigma	28.0%	30.0 %	22.0%	20.0%	42.0%

Table 7: Top BLASTn matches for the mitochondrial *COI* sequence of *Polistes perplexus* from District Shangla, Pakistan, showing sequence similarity with reference sequences available in GenBank

Accession no	Specie name	Max Score	Total Score	E value	Query Cover	% Identity
EF136452	*Polistes perplexus*	1153	1153	0.0	100%	99.84%
GU596858	*Polistes bellicosus*	1153	1153	0.0	100%	99.84%
HQ578094	*Polistes rubiginosus*	1129	1129	0.0	97%	99.84%
EU649463	*Polistes perplexus*	1123	1123	0.0	97%	99.84%

Table 8: Top BLASTn matches for the mitochondrial *COI* sequence of *Polistes stigma* from District Shangla, Pakistan, showing sequence similarity with reference sequences available in GenBank

Accession no	Specie name	Max Score	Total Score	E value	Query Cover	% Identity
HQ567776	*Polistes stigma*	1149	1149	0.0	99%	100%
HM433936	*Polistes stigma*	1138	1138	0.0	99%	99.68%
GU596890	*Polistes stigma bernardi*	1120	1120	0.0	100%	98.88%
EF136458	*Polistes stigma*	1105	1105	0.0	100%	98.56%

Table 9: Summary of intra- and interspecific Kimura 2-parameter (K2P) genetic distance ranges observed among *Polistes* species and *Vespula* outgroup taxa using mitochondrial *COI* barcode sequences

Comparison Type	K2P Distance Range
Intraspecific	0.00–0.01
Interspecific (*Polistes*)	0.03–0.18
Outgroup comparisons	0.20–0.25

Azad Jammu and Kashmir: Paniola, Rawalakot, Khaigala, Chaprian, Banjosa and Mandhol (Khan *et al.* 2017).

Molecular procedure

COI sequences of *Polistes perplexus* and *Polistes stigma* were successfully amplified. The obtained sequences were approximately 680 bp in length. After trimming low-quality ends and removing ambiguous regions, a final aligned sequence length of 628 bp was obtained and used for subsequent analysis. Newly generated sequences were submitted to GenBank under accession numbers PV993977 and PV993975. The GC content was approximately 40.1% (*P. perplexus*) and 42% (*Polistes stigma*) (Table 5 and 6).

A BLAST search revealed high similarity (> 99.50%), with 99.84% identity for *Polistes perplexus* and 100% identity for *Polistes stigma* (f). Morphological identification was consistent with molecular results.

Phylogenetic analysis

The phylogenetic dataset comprised 24 *COI* nucleotide sequences, including 21 ingroup (*Polistes*) and 3 outgroups (*Vespula*). The tree was rooted using *Vespula alascensis*, *Vespula vulgaris* and *Vespula flavopilosa* as outgroup taxa (Fig. 4). The Maximum-Likelihood method was used to construct the phylogenetic tree. The optimal tree with a sum of branch length equal to 1.302 is shown in Fig. 4. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. Evolutionary distances calculated using the Kimura 2-parameter (K2P) model are expressed as the number of base substitutions per site. The analytical procedure encompassed 24 nucleotide sequences. All of the unclear positions in each pair of sequences were treated using the pairwise deletion feature, and the final dataset contained 1,646 positions. Evolutionary analyses were conducted using MEGA version 12 with up to four parallel computing threads.

The Maximum Likelihood phylogeny showed that *Polistes* sequences formed well-supported and distinct clades clearly separated from outgroup taxa (Fig. 4; Table 7). The *Polistes perplexus* clade included the Pakistani sequence (PV993977), which clustered with sequences from the USA with bootstrap support (93–100%) indicating low genetic divergence. Similarly, the *Polistes stigma* clade grouped the Pakistani sequence (PV993975) with sequences from Canada, USA, Bangladesh, and Vietnam, supported by high bootstrap values (98–100%). Other *Polistes* species formed distinct lineages with moderate to strong bootstrap support.

Outgroup taxa (*Vespula* species) formed a separate and well-supported clade, confirming correct tree rooting and deep evolutionary divergence from *Polistes*.

Genetic divergence

Pairwise genetic distance (K2P) supported the phylogenetic relationships observed in the maximum likelihood tree (Table 9).

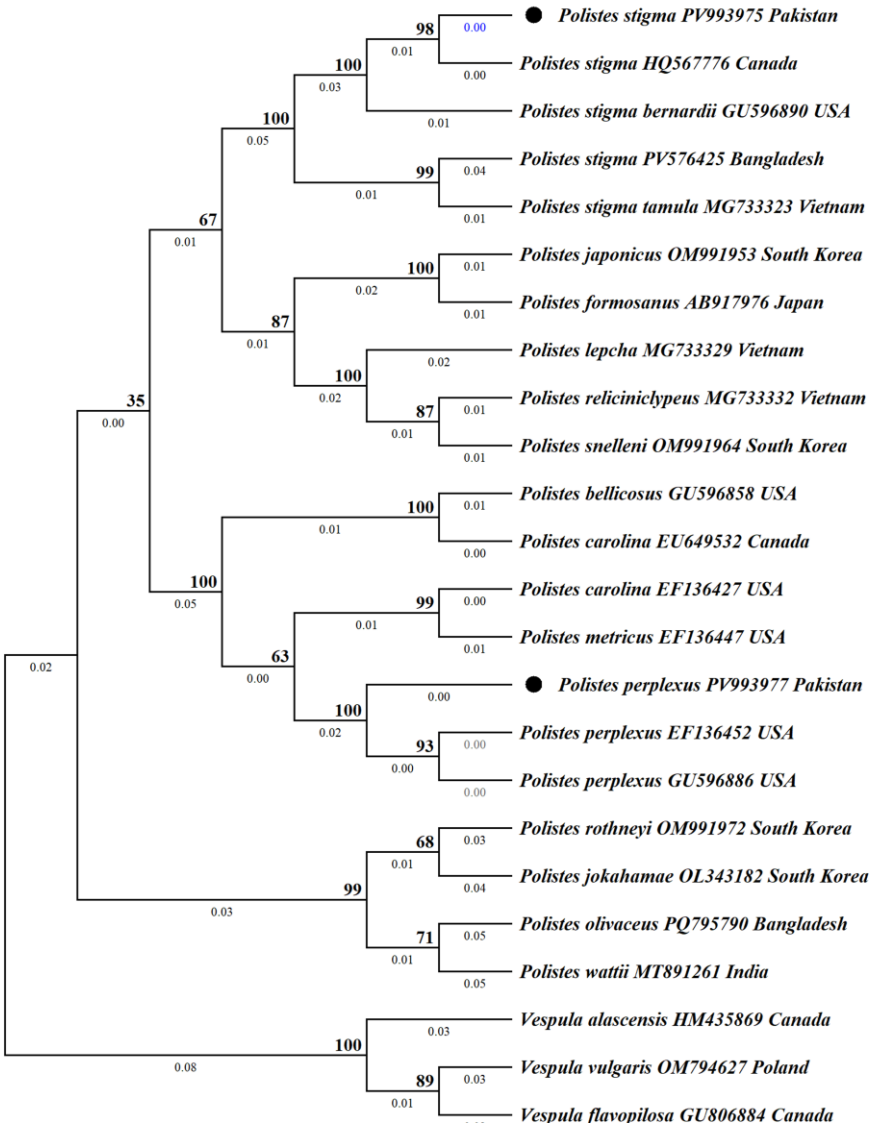

Fig. 4: Maximum Likelihood phylogenetic tree based on mitochondrial *COI* gene sequences showing the evolutionary relationships of *Polistes perplexus* and *Polistes stigma* from District Shangla, Pakistan, along with related taxa retrieved from GenBank

The K2P distances between the two *P. perplexus* from Pakistan (PV993977) and the USA (EF136452) were 0.00, indicating no detectable genetic divergence. In comparison, interspecific distances among closely related *Polistes* species ranged from approximately 0.03-0.18. Higher genetic distances (0.20-0.25) were observed between *Polistes* and outgroup taxa (*Vespula*). These values are consistent with commonly reported *COI* barcode thresholds (> 2%). The very low divergence observed among the analyzed *P. perplexus* sequences supports their conspecific status and indicates high similarity among the compared *COI* sequences. Comparatively higher divergence observed among some *P. stigma* reference sequences may reflect geographic variation; however, additional sampling and multilocus analyses are required before conclusions regarding intraspecific structuring can be made. The relatively high distances between certain sequences labeled *P. stigma* (and the associated internal lengths in the ML tree) may suggest possible intraspecific structuring; however, additional sampling and multilocus analyses are required to confirm this pattern (Table 8 and 9).

Discussion

The present study integrates morphological examination with mitochondrial *COI* sequence data to confirm the identity and phylogenetic relationships of *Polistes perplexus* and *Polistes stigma* collected from District Shangla, Khyber Pakhtunkhwa, Pakistan. The combined use of classical taxonomy and molecular evidence produced consistent

results, reinforcing the reliability of species identification and highlighting the usefulness of integrative approaches for resolving taxonomic uncertainty within *Polistes*. Phylogenetic reconstruction using the Maximum Likelihood (ML) method revealed that both *P. perplexus* and *P. stigma* form distinct and well-supported clades. The Pakistani *P. perplexus* sequence clustered closely with a conspecific sequence from North America and showed very low detectable genetic divergence. This observation suggests high similarity among the analyzed *COI* sequences from geographically distant regions. However, because only a single representative specimen from Pakistan was sequenced, conclusions regarding mitochondrial uniformity or population-level patterns should be considered preliminary. Similar findings have been reported in other *Polistes* species, where *COI* variation remains low even across broad distributional ranges, which may reflect recent range expansion, historical connectivity, or slow rates of mitochondrial evolution (Schmid-Egger *et al.* 2017b; Rodriguez-Perez *et al.* 2021). Such genetic stability indicates that *COI* barcodes are effective for confirming species identity in *P. perplexus*, even when samples originate from distant regions.

In contrast, *P. stigma* displayed comparatively higher levels of intraspecific genetic divergence. Sequences from Pakistan, Bangladesh and Vietnam grouped within a single clade but exhibited longer internal branches and higher pairwise K2P distances. This level of divergence may reflect geographic structuring among populations or historical isolation across different parts of its range. Similar patterns have been documented within the *P. stigma* species group, where wide distribution and ecological variability have been associated with increased genetic heterogeneity (Nguyen *et al.* 2017; Brito *et al.* 2024). While the observed divergence does not necessarily indicate the presence of separate species, it suggests that *P. stigma* may represent some level of population differentiation that warrants further investigation.

The genetic distance analysis generally supports the phylogenetic findings. Intraspecific distances were clearly lower than interspecific distances, producing a visible barcode gap between species. This pattern confirms the effectiveness of *COI* barcoding for species-level discrimination within *Polistes*, as reported in recent molecular studies on Vespidae (Rodriguez-Perez *et al.* 2021). Nevertheless, the elevated divergence observed within *P. stigma* also highlights the limitations of relying exclusively on mitochondrial markers. Recent integrative studies emphasize that mitochondrial data should be complemented with nuclear markers and detailed morphometric analyses to accurately resolve species boundaries, particularly in groups with complex evolutionary histories (Nguyen *et al.* 2017).

The strong genetic separation observed between *Polistes* and the outgroup taxa included in the analysis further supports the robustness of the phylogenetic framework. The deep divergence between *Polistes* and non-*Polistes* taxa is consistent with established vespid phylogenies and confirms that *COI* sequences retain sufficient phylogenetic signal for resolving relationships at both species and genus levels (Schmid-Egger *et al.* 2017b). Although 393 specimens were collected and morphologically examined, molecular analyses were limited to a single representative specimen from each identified species. Therefore, the present dataset is suitable for preliminary species confirmation and phylogenetic placement but remains insufficient for robust population-level interpretations. Consequently, conclusions regarding genetic homogeneity, population differentiation, and intraspecific structuring should be interpreted cautiously. Future studies incorporating larger molecular sample sizes from multiple geographic localities across Pakistan will provide a more comprehensive understanding of genetic diversity and population dynamics within *Polistes* species.

From a regional perspective, this study represents the first molecular phylogenetic assessment of *Polistes* species from District Shangla. The generation of *COI* barcode data from this mountainous and poorly studied region contributes to filling a significant gap in the molecular documentation of Pakistan's hymenopteran fauna. Given the ecological importance of paper wasps and the limited molecular data currently available from northern Pakistan, expanded sampling across additional localities and the inclusion of multiple genetic markers would provide a more comprehensive understanding of *Polistes* and wasp diversity and population structure in the region.

Conclusion

The present study combined morphological identification and *COI*-based DNA barcoding to confirm the occurrence of *Polistes perplexus* and *Polistes stigma* in District Shangla, Pakistan. Phylogenetic analysis supported clear species-level differentiation and showed high similarity with reference GenBank sequences. However, because molecular analysis was limited to a single representative specimen from each species, conclusions regarding population structure and evolutionary relationships should be considered preliminary. Further studies using larger sample sizes and additional molecular markers are recommended.

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Authors' Contribution

Specimens were collected by Asaf Khan, Riaz Ahmad, Mukhtar Ahmad and Farzana Bibi, while Riaz Ahmad, Asaf Khan, Ikram Ullah, Muhammad Ismail and Muhammad Sayaf carried out the identification and molecular work. Riaz Ahmad, Asaf Khan, Abo Saeed, Nida Hayat, Said Miraj ud Din and Sajid Ullah assisted in the preparation of manuscript.

Conflict of Interest

Authors declared no conflict of interest.

Data Availability

Data presented in this study will be available upon a fair requst to the corresponding author.

Declaration of Generative AI Use

No generative AI tools were used in the preparation of this manuscript, including for content generation, language improvement, editing, or translation. The authors take full responsibility for the content, accuracy, originality, and integrity of the manuscript.

Ethics Approval

Ethical approval was not applicable to this study, as it involved only insect specimens and did not invole human or vertabrate animals.

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