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Research Article

MOLECULAR PHYLOGENETIC ANALYSIS OF THE SUBFAMILY CHILOCORINAE (COLEOPTERA: COCCINELLIDAE) FROM THE MALAKAND REGION, PAKISTAN

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ABSTRACT

The subfamily *Chilocorinae* plays an active role in biological control, being predominantly predatory in nature and distributed worldwide, including in Pakistan. They feed on soft-bodied insects such as scale insects, aphids, leafhoppers, mealybugs, and psyllids. In this research, three species have been molecularly identified for the first time, belonging to three different genera: Brumoides, Exochomus, and Parexochomus. The study was conducted from April 2022 to April 2023, during which a total of 375 samples were collected from the Malakand region of Pakistan, specifically from seven districts. The collected specimens were preserved in insect boxes and tagged. DNA extraction was performed from the legs of ladybird beetles, followed by PCR amplification using the primers LCO1490 and HCO2198. Sanger sequencing was carried out using BioEdit software (version 7.2), and phylogenetic analyses were performed using MEGA 11 software to construct a phylogenetic tree. Three species belonging to three genera of the subfamily *Chilocorinae* were molecularly identified and phylogenetically analyzed from the Malakand region of Pakistan: *Brumoides suturalis* (Fabricius, 1789), *Exochomus nigripennis* (Erichson, 1843), and *Parexochomus nigromaculatus* (Goeze, 1777). Overall, this study underscores the importance of phylogenetic analysis for the accurate taxonomy of the subfamily *Chilocorinae* and their relationship with closely related species.

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INTRODUCTION

The predatory nature of ladybird beetles is well known. Most lady beetles feed on aphids, psyllids, whiteflies, mealybugs, scale insects, thrips, spider mites, leaf beetle larvae, and other small arthropods. Among them, the subfamily Chilocorinae holds significant importance due to its strong predatory behavior against scale insects, aphids, mealybugs, and psyllids (Kundoo et al., 2018). A single ladybird beetle can consume aphids equivalent to its body weight daily, while a larva can consume up to 50 aphids per day. These beetles play a crucial role in controlling insect pests, including leafhoppers, scale

insects, and other soft-bodied insects (Khalil et al., 2021).

The Coccinellidae family, which includes ladybird beetles, is one of the most well-known insect families. Despite their ecological and economic significance, the evolutionary relationships within Coccinellidae remain poorly understood (Li et al., 2021). The tribe Chilocorini described by Mulsant, (1846), comprises approximately 250 species within 27 genera (Łączyński and Tomaszewska, 2012; Biranvand et al., 2017; Li et al., 2017). Ladybird beetles belong to the family Coccinellidae, which is one of the dominant beetle groups in the biosphere, consisting of more than 6,000 species classified into 360 documented genera (Magro et al., 2010; Bodlah et al., 2021). The major subfamilies within Coccinellidae include Chilocorinae, Coccinellinae, Coccidulinae, Scymninae, Sticholotidinae, and Epilachninae (Saeed et al., 2016). Coccinellidae is considered one of the most diverse beetle families, encompassing over 375 genera and 6,000 species (Bajracharya et al., 2024).

The phylogenetic relationships within Chilocorini remain poorly understood. A phylogenetic reconstruction of the subfamily Chilocorini, which includes all 27 genera, has been conducted based on five molecular markers and 86 adult morphological characters. Moreover, 16 morphological characters were selected for reconstructing ancestral states using maximum parsimony and maximum likelihood methods (Li et al., 2020). Over 1.9 million specimens, representing approximately 172,000 species, have been barcoded (Pereira et al., 2013). Hebert's DNA barcoding method, first proposed in 2003, has proven to be a valuable tool for species identification (Hebert et al., 2003; Schindel and Miller, 2005). In recent years, DNA sequence data have become an essential resource for beetle phylogenetics. Studies on beetle phylogeny have primarily utilized nuclear rDNA genes (28S, 18S), mitochondrial genes (COX I, COX II, 16S), and, more recently, mitochondrial genomes (Shull et al., 2001; Caterino et al., 2002).

The Molecular Evolutionary Genetics Analysis (MEGA) software has been developed for comparative analyses of DNA and protein sequences, allowing researchers to infer molecular evolutionary patterns of genes, genomes, and species over time (Kumar et al., 1994; Tamura et al., 2011). DNA barcoding not only facilitates species identification but also aids in reconstructing evolutionary relationships across different taxonomic ranks (Yan et al., 2015; Akram et al., 2017). A standard DNA barcode for animals has been established, consisting of a 658-bp fragment of the Cytochrome C Oxidase I (COX I) mitochondrial gene (Hebert et al., 2004). Extensive DNA barcode reference libraries have been developed for various beetle families, including Carabidae, Chrysomelidae, and Elateridae (Raupach et al., 2010; García-Robledo et al., 2013; Oba et al., 2015). However, DNA barcoding studies on ladybird beetles remain in their early stages.

To date, over 100 species of Coccinellid beetles have been identified in Pakistan (Saeed et al., 2016). A recent molecular phylogenetic study on the subfamily Chilocorinae in Pakistan analyzed three species from three genera namely *Brumoides*, *Exochomus*, and *Parexochomus* using the COX I gene. However, knowledge regarding the phylogenetic relationships of Pakistani Chilocorinae beetles remains limited. Therefore, the primary objective of this study was to sequence mitochondrial Cytochrome Oxidase I (COX I) and assess previously proposed phylogenetic hypotheses for three ladybird beetle species belonging to three genera within the subfamily Chilocorinae under the family Coccinellidae, collected from Pakistan.

MATERIALS AND METHODS

Study area

Malakand division consists of the districts of Shangla, Swat, Lower Dir, Upper Dir, Chitral, Buner, and Malakand. The region's maximum altitudinal range extends from 3,049 meters in the west to 4,876 meters in the northeast, where Afghanistan is situated. Malakand division, with a total land area of 29,872 km^2, is bordered by Afghanistan to the north, Hazara to the east, Mardan to the southeast, Charsadda to the southwest, and the Mohmand and Bajaur agencies to the west. Figure 1 represents the Map of Malakand region Pakistan while Table 1 shows the selected localities for the collection of Ladybird Beetles of Malakand region Pakistan. Its geographical coordinates range from 35.5°N to 72°E (Ullah et al., 2016).

Sample collection

During the active seasons of ladybird beetles, from April 2022 to April 2023, a total of 375 samples were collected from the Malakand region of Pakistan Extensive year-round surveys were conducted at various intervals in different fields as well as in common vegetation to assess the presence of ladybird beetles. For morphological identification and DNA

extraction, the samples were preserved in 70% ethanol and 5% glycerol and stored at the Molecular Lab, Department of Zoology, University of Swabi; the Museum, University of Buner; and Kaisee Dreams Research Laboratory, Dargai, Malakand, Khyber Pakhtunkhwa.

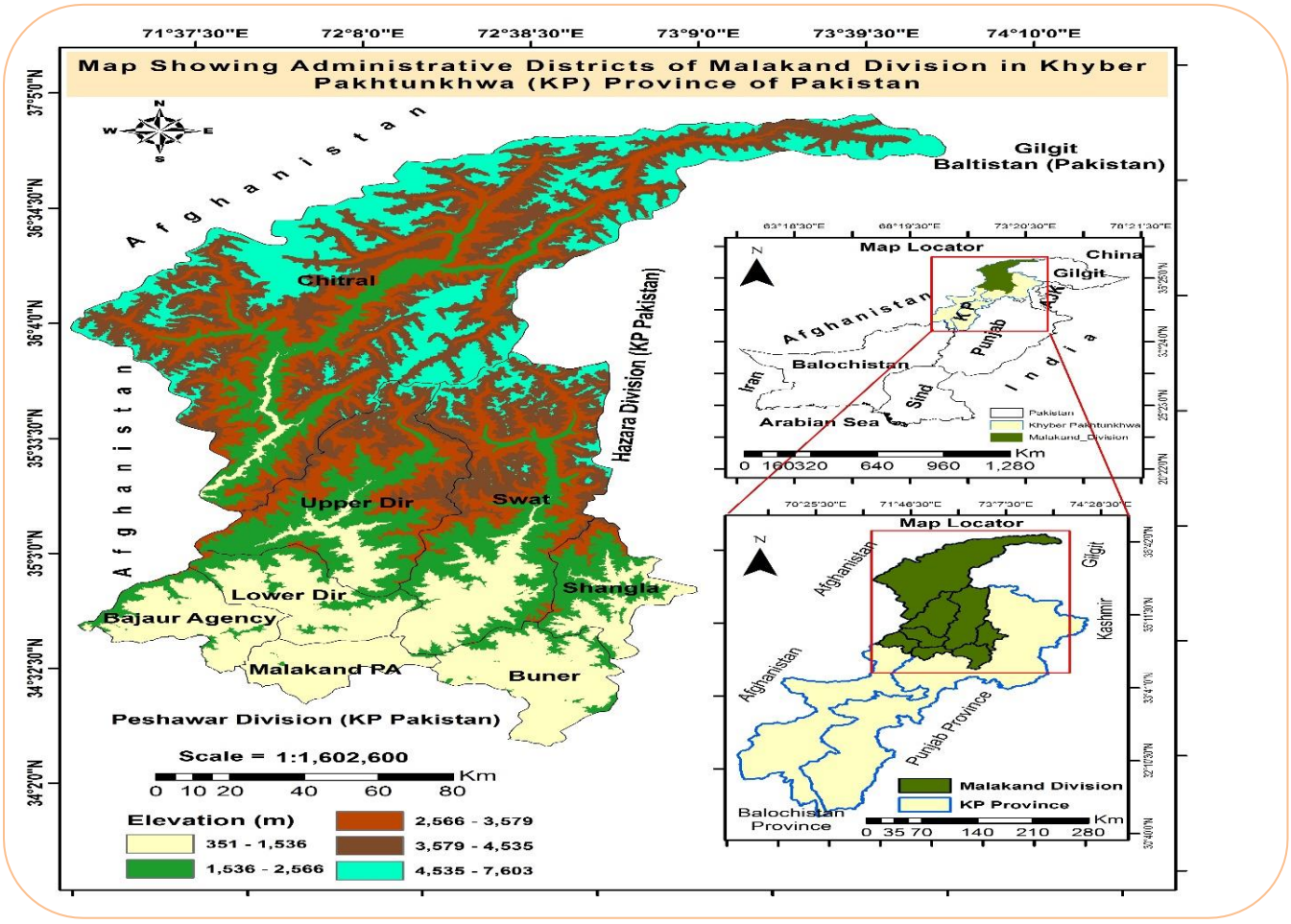

Figure 1. Map of Malakand region Pakistan.

Table 1. Selected localities for the collection of Ladybird Beetles of Malakand region, Pakistan.

Sr. No.	District	Longitude	Latitude
1	Buner	72.6151° E	34.3943° N
2	Swat	72.4258° E	35.2227° N
3	Dir Lower	71.8097° E	34.9161° N
4	Dir Upper	72.0468° E	35.3356° N
5	Chitral	72.1416° E	36.1113° N
6	Shangla	72.7570° E	34.8872° N
7	Malakand	71.9046° E	34.5030° N

Morphological identification

The preserved samples were processed for morphological identification following the protocols described by Kapur (1958), Canepari and Milanese (1997), Poorani (2003, 2004. For morphological identification, a stereomicroscope (SZX12, Olympus, Japan) was used to count dorsal dots, examine patterns and colors, assess fine taxonomically important details, and capture photographs.

DNA extraction and PCR

DNA extraction was carried out from the leg of a ladybird beetle. First, the leg was cut, crushed, and placed in an Eppendorf tube. Lysis buffer was then added, and the sample was incubated at 56°C for 1 h. After incubation, PCI (phenol-chloroform-isoamyl alcohol) was added, and the mixture was kept at room temperature for 20 to 25 min. This was followed by centrifugation at 10,000 rpm for 20 min. Subsequently, 500 µl of cold isopropanol was added, and the sample was stored in a freezer for 24 h. Gel electrophoresis was then performed.

DNA extraction was conducted using the QIAamp DNA

Extraction Kit (QIAGEN, USA) according to the manufacturer's instructions, while DNA quantification was performed using a NanoVue™ Plus spectrophotometer (GE Healthcare, UK).

PCR amplification was carried out using the primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Figure 2) on a T-Professional Biometra PCR machine (Analytik Jena, Germany).

Sequencing

The PCR amplified purified products were Sanger Sequenced from Macrogen Seoul South Korea (www.macrogen.com). Sanger Sequencing was carried out by using BioEdit software version 7.2.

BLAST

The .ab1 files were analyzed using NCBI-BLAST, and sequences with more than 90% similarity to known organisms were identified. The corresponding data, including FASTA sequences, Gen Bank IDs, authors, affiliations, and reported countries, were downloaded for further analysis. In BLAST, the 100 most closely related sequences were retrieved along with their associated metadata, such as the reported country and author information.

Phylogenetic analysis

Phylogenetic analyses were conducted using MEGA 11 software to construct a phylogenetic tree and assess evolutionary relationships.

RESULTS

The present study focused on the molecular phylogenetic analysis of the subfamily Chilocorinae in the Malakand region of Pakistan, conducted from April 2022 to April 2023. A total of 375 specimens belonging to the subfamily Chilocorinae were collected from the study area. During the study, three different species from three distinct genera within this subfamily were molecularly reported for the first time. The identified species include *Brumoidessuturalis* (Fabricius, 1789) (Figure 2), *Exochomus nigripennis* (Erichson, 1843) (Figure 3), and *Par exochomus nigromaculatus* (Goeze, 1777) (Figure 4).

Phylogenetic trees were constructed for each species, illustrating their genetic similarity and evolutionary relationships within the subfamily Chilocorinae. Moreover, the morphological characteristics, host plants, seasonal occurrence, and distribution of the reported species were thoroughly examined.

Subfamily: Chilocorinae (Chapin, 1965)

Distinguished characters

Body

Broadly oval, rounded, and dorsally convex. The head is transverse, finely and irregularly punctate, and broad. The clypeus is expanded on each side in front of the eyes, forming broad plates that cover the bases of the antennae. The antennae are short, consisting of 7 to 11 segments. The terminal segment of the maxillary palpi is nearly cylindrical and slightly expanded towards the apex.

Legs

The femora are robust or swollen, sometimes strongly flattened. The tibiae are expanded and toothed on the lower margin. The tarsal claws may be toothed or toothless.

Thorax and Elytra

The pronotum is deeply emarginated at the anterior margins, with its lateral sides descending vertically. The base of the elytra is slightly wider than that of the pronotum. The elytra are black or marked with red spots, with relatively short epipleura.

Remarks

The subfamily *Chilocorinae* is represented by only the genus *Brumoides* in the Malakand region. Members of this subfamily are primarily predators of scale insects, though some species also feed on aphids, mealybugs, and psyllids (Kuznetsov, 1997).

Genus *Brumoides* Chapin, 1965a

Type species: *Brumoides suturalis*

Distinguished characters

The body is oval, convex, and glabrous dorsally. The antennae are 8-segmented, with a 3-segmented club; the apical segment is partly embedded in the penultimate segment. The maxillary palpus has a securiform terminal segment. The elytral margin is relatively reflexed. The abdomen has six visible sternites in males and five in females. The postcoxal line is complete. The legs have non-inflated femora, and the claws are slightly compact at the base, without an angular basal tooth.

Remarks

The genus *Brumoides* is cosmopolitan. In the Malakand region, this genus is represented by a single species, *Brumoides suturalis*, which is found in most districts of the region.

***Brumoides suturalis* (Fabricius, 1789)**

Distinguished characters

The body is oval-shaped, with a brown head that is not deeply inserted. The eyes are large and brownish-black.

The antennae are small (8-segmented), with the basal segment being triangular, broad, and longer than the second segment. The maxillary palpus is 4-segmented, with the terminal segment having an oblique cut at the apex. The pronotum is yellowish-brown, slightly projected on each anterior lateral side, and finely pitted. The elytra are yellow, with three brownish-black longitudinal stripes, one on each elytron and one along the mid-dorsal junction of the elytra, not touching the posterior margin. The first and second abdominal sternites are densely covered with yellow hairs. Ventrally, the body is yellowish-brown.

Host plants

General vegetation, *Tripolium* spp.

Remarks

B.suturalis is an important predator that feeds on various hosts, including mites, psyllids, coccids, and aphids, thereby protecting cereal crops from pest damage.

B.suturalis its presence on *Aleurocanthus husaini* Corbett, *Aphis craccivora* Koch, *Aphis fabae* Scopoli, and *Aphis gossypii* Glover. Kapur (1942) documented *B.suturalis* feeding on three species of aphids, one species of mite, and six species of coccids Ullah et al. (2016) and Saeed et al. (2016) reported this species from district Buner in the Malakand region.

Seasonal occurrence

Collection data indicate that this species is active from May to November in Shangla, Swat, Buner, Malakand, and Lower Dir districts of the Malakand region. In the present study, it was found to be most abundant during October and November.

General distribution

India, Afghanistan, Iran, Iraq, Israel, Jordan, Lebanon, Oman, Saudi Arabia, and Syria (Rafi et al. 2005).

Molecular and phylogenetic analysis of *B. suturalis*

For developing the dendrogram of *B.suturalis*, all similar sequences were downloaded in FASTA format from NCBI BLAST and aligned using MUSCLE software. The aligned sequences were saved in Omega format for processing through Omega 11. The sequences were automatically clustered into clades using the BioNJ and Neighbor-Joining algorithms with a bootstrap replication value of 100. The tree with a maximum likelihood value of -1927.91 was obtained. The substitution value was also initially assigned to the tree. The analysis involved 11 DNA sequences, with a total of 150 positions in the final dataset.

Figure 5 represented the phylogenetic tree of *B. suturalis* based on COX1 using the maximum likelihood method.

Figure 2. *Brumoides suturalis* (Fabricius, 1789).

Figure 3. *Exochomus nigripennis* (Erichson 1843).

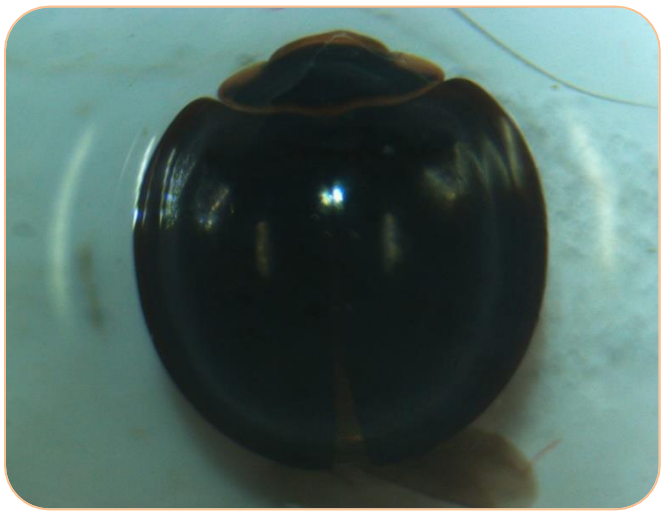

Figure 4. *Paraxochormus nigromaculatus* (Goeze, 1777).

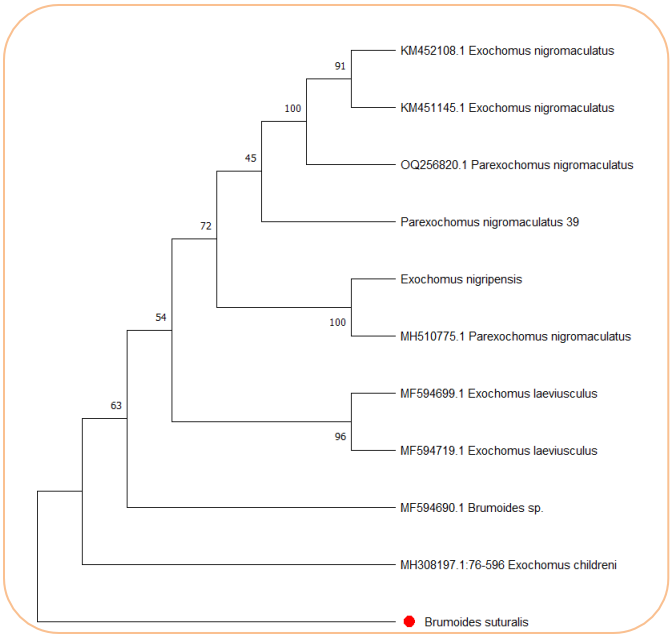

Figure 5. Phylogenetic tree of *B. suturalis* based on COX1 using the maximum likelihood method.

***Exochomus nigripennsis* (Erichson, 1843)**

Distinguished characters

The body measures 3.5-4.5 mm in length and 2.6-3.5 mm in width. It has an oval, somewhat convex shape, with a shiny and glabrous dorsal surface. The head is black, while the mouthparts, antennae, and legs are yellow. The pronotum is yellow, and the elytra are entirely black and shiny (Raimundoand van Harten, 2000).

Host

This species is not commonly found. It is an active predator of psyllids (Rakhshani and Saeedifar, 2013) and specifically preys on the common pistachio psylla (*Agonoscenapistaciae*), which feeds on pistachio trees (*Pistaciavera*) **(**Mehrnejad, 2010).

General distribution

In Pakistan, this species has been recorded in the Buner and Malakand districts of Khyber Pakhtunkhwa. It is not a widely distributed coccinellid species locally but has been reported from Afghanistan China **(**Ren et al., 2009**),** India (Pushpendraand Prakash, 2010), Iran (Zare Khormizi et al., 2013), Indonesia (Muniappan, 2012), Russia (Ren et al., 2009).

Seasonal occurrence

This species is observed from June to August.

Molecular and phylogenetic analysis of *Exochomus nigripennis* (Erichson, 1843)

To develop the dendrogram of *E.nigripennis*, all similar sequences were downloaded in FASTA format from NCBI BLAST and aligned using MUSCLE software. The aligned sequences were saved in Omega format for processing through Omega 11. Clustering into clades was performed automatically using the BioNJ and Neighbor-Joining algorithms with a bootstrap replication value of 100. The tree with the maximum likelihood value of -1927.91 was obtained. The substitution value was also initially assigned to the tree. The analysis involved 11 DNA sequences, with a total of 533 positions in the final dataset. Figure 6 represented the phylogenetic tree of *E. nigripennis* based on COX1 using the maximum likelihood method.

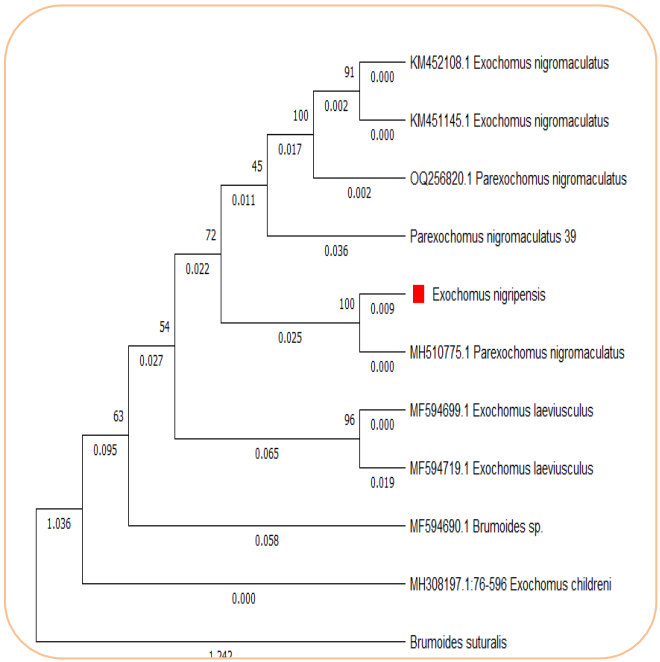

Figure 6. Phylogenetic tree of *E. nigripennis* based on COX1 using the maximum likelihood method.

***Paraxochomus nigromaculatus* (Goeze, 1777)**

Distinguishing characteristics

This species is a known predator of mealy bugs and aphids (Pope 1983). It has been collected from wild vegetation and natural habitats. Specimens have been recorded from February to May and in September (Al Ansi et al., 2020).

General distribution

Afghanistan, India, Pakistan, Nepal, Saudi Arabia, Iran, Iraq, China, Uzbekistan, Kazakhstan, and Turkmenistan in Asia, as well as the United Kingdom (Al Ansi et al., 2020).

Host plants

General vegetation

Seasonal occurrence

May to September

Molecular and phylogenetic analysis of *P. nigromaculatus*

For the development of the dendrogram of P. *nigromaculatus*, all similar sequences were downloaded in FASTA format from NCBI BLAST and aligned using MUSCLE software. The aligned sequences were saved in Omega format for processing through Omega 1. The sequences were automatically clustered into clades using the BioNJ and Neighbor-Joining algorithms with a bootstrap replication value of 100. The tree with a maximum likelihood value of -3550.57 was obtained. Substitution values were also initially assigned to the tree. The analysis involved 33 DNA sequences, with a total of 598 positions in the final dataset. Figure 7 represented the phylogenetic tree of *P. nigromaculatus* based on COX1, constructed using the maximum likelihood method.

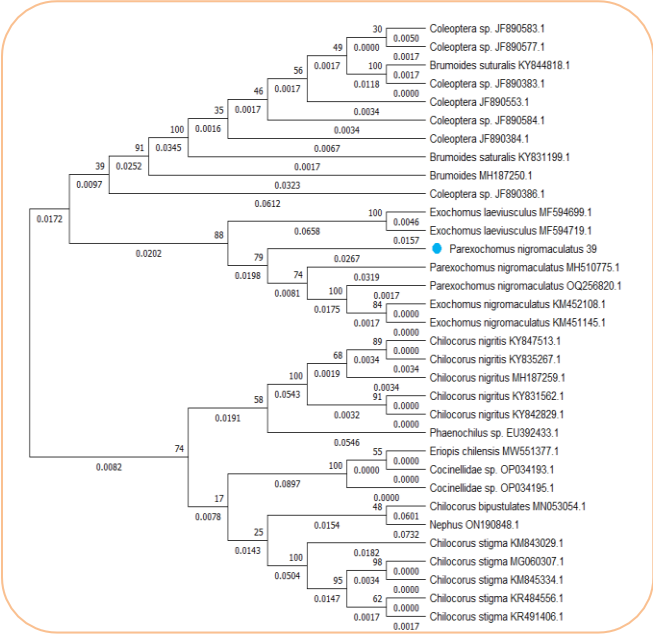

Figure 7. Phylogenetic tree of *P.nigromaculatus* based on COX1, constructed using the maximum likelihood method.

DISCUSSION

The present study was conducted from April 2022 to April 2023. A total of 375 samples were collected from the Malakand region, Pakistan, and were morphologically identified. DNA extraction and PCR amplification using the primers LCO1490 and HCO2198 were performed, followed by Sanger sequencing and phylogenetic analysis using different software tools.

Based on morphological characteristics, the collected samples were identified as *B. suturalis*, *E. nigripennis*, and *P. nigromaculatus*. The molecular phylogenetic analysis confirmed the morphological identification.

In the study of the *B. suturalis* COXI sequence, the species exhibited an A+T content of 68% and a G+C content of 32%. When compared with the COXI sequence of a clustered species [ON715439] reported from India, which also had an A+T content of 68% and a G+C content of 32%, the intraspecific p-distance was found to be 0.00000000. These results suggest that both species are closely related and belong to the same clade.

Similarly, *P. nigromaculatus* showed a pairwise genetic distance of 0.06346 to 0.04974 from its clustered group members. According to reports by Vella et al. (2018) and Al-Saadi (2023) from Iraq, the species had an A+T content of 69-70.2% and a G+C content of 31-29.8%. Our study found *P. nigromaculatus* to have an A+T content of 70.5% and a G+C content of 31.3%, indicating genetic similarity and stability.

The third identified species, *E. nigripennis*), exhibited a pairwise genetic distance of 0.020927 from its clustered group members. A previously reported sequence (MH510775) from Malta had an A+T content of 68.9% and a G+C content of 31%. In comparison, our identified species had an A+T content of 69.5% and a G+C content of 30.4%, demonstrating their similarity and genetic stability.

Li et al. (2020) studied the phylogenetic relationships within the tribe Chilocorini, which remain poorly understood. Their study reconstructed the phylogeny of Chilocorini, incorporating all 27 genera using five molecular markers and 86 adult morphological characters. However, in our study, we report three species belonging to three different genera of the subfamily Chilocorinae (*B. suturalis*, *E. nigripennis*, and *P. nigromaculatus*), which were, for the first time, molecularly identified and phylogenetically analyzed from the Malakand region, Pakistan. Moreover, we documented their morphological characteristics, seasonal occurrence, host plants, and distribution.

Magro et al. (2010) found that the recovered phylogenies were congruent and indicated that the subfamily Coccinellinae is monophyletic, whereas Coccidulinae, Epilachninae, Scymninae, and Chilocorinae are paraphyletic. They identified the tribe Chilocorini as the sister group of Coccinellinae for the first time. However, our study suggests that the subfamily Chilocorinae is paraphyletic.

Poolprasert et al. (2019) attempted to use molecular taxonomic identification for Coccinellidae and provided

the first phylogenetic reconstruction of the relationships within the family based on the analysis of a 658-bp fragment of mitochondrial DNA from the 5' region of the COXI gene. Their study revealed that Coccinellidae species shared 99-100% identity. Eleven coccinellid species (belonging to 10 genera across four subfamilies: Coccinellinae, Chilocorinae, Sticholotidinae, and Scymninae) and relevant outgroup species were reconstructed using the Neighbor-Joining (NJ) method with 1,000 bootstrap replicates. The recovered phylogeny demonstrated that most subfamilies (except Sticholotidinae) within Coccinellidae were reciprocally monophyletic. In the present study, *Brumoides* species showed similarity to previously reported species, while *Chilocoruspolitus* and *C. nigritus* were not found in our study area.

CONCLUSION

The current study concluded that only three species and three genera of the subfamily Chilocorinae were molecularly and phylogenetically identified from the study area. The reported species were analyzed molecularly and phylogenetically using different software programs, such as BioEdit 7.2 and MEGA11. DNA extraction and PCR amplification were performed using the LCO1490 and HCO2198 primers, followed by Sanger sequencing. Based on morphological characteristics, the collected samples were identified as *Brumoides suturalis* (Fabricius, 1789), *Exochomus nigripennis* (Erichson, 1843), and *Parexochomus nigromaculatus* (Goeze, 1777), with molecular phylogenetic analysis confirming the morphological identifications. Phylogenetic trees for each species of the subfamily Chilocorinae were constructed.

AUTHORS' CONTRIBUTIONS

KS and MSA conceptualized the study; QZ, KS, and KM developed the methodology; QZ and KS worked on the software; QZ conducted the formal analysis. KS carried out the investigation; UR provided the necessary resources; KS and QZ curated the data, whereas KS and IAK prepared the original draft; KS reviewed and edited the manuscript; MSA and KM supervised the project; MSA, KM, KS, and AJA managed its administration.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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