



Available Online at EScience Press

Plant Protection

ISSN: 2617-1287 (Online), 2617-1279 (Print)
http://esciencepress.net/journals/PP

Research Article

DNA Barcoding and Phylogenetic Analysis of the Subfamily Epilachninae (Coleoptera: Coccinellidae) from the Malakand Region, Pakistan

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ARTICLE INFO

Article history

Received: 18th March, 2025

Revised: 17th July, 2025

Accepted: 19th July, 2025

Keywords

Family Coccinellidae

Subfamily Epilachninae

DNA barcoding

Molecular Phylogenetic analysis

Malakand

Pakistan

ABSTRACT

The taxonomic identification of the family Coccinellidae, including the Ladybird beetles, remains ambiguous due to considerable morphological variability. In recent years, molecular phylogenetic analysis and DNA barcoding have played a crucial role in validating species taxonomy. This study, therefore, aimed to investigate the DNA barcoding and phylogenetic relationships of the subfamily Epilachninae in the Malakand region of Khyber Pakhtunkhwa, Pakistan. The survey was conducted from April 2022 to April 2023. Morphological identification was carried out using various standard taxonomic keys. DNA extraction and PCR amplification were performed using the universal primers LCO1490 and HCO2198, followed by Sanger sequencing. Phylogenetic analysis was conducted using various bioinformatics tools. A total of 510 specimens belonging to the subfamily Epilachninae were collected, comprising four species: *Henosepilachna vigintioctopunctata* (Fabricius, 1755), *H.septima* (Dieke, 1947), *Afidentula manderstjernae* (Mulsant, 1853), and *Subcoccinella vigintiquatuorpunctata*, representing three genera within the subfamily. This study underscores the significance of molecular phylogenetics in resolving the taxonomy and elucidating the evolutionary relationships of Epilachninae species in the Malakand region.

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Introduction

The subfamily Epilachninae is divided into four tribes: Epilachnini, Cynegetini, Epivertini, and Eremochilini (Gordon and Vandenberg, 1987; Jadwiszczak and Węgrzynowicz, 2003). The tribe Cynegetini Thomson (= Madaini Gordon) includes ten genera, comprising between

2 to 45 species, while the tribe Epilachnini consists of the remaining 11 genera and exhibits the greatest species diversity within Epilachninae (Tomaszewska and Szawaryn, 2016). With over 1,000 described species, Epilachninae accounts for nearly 20% of all species in the family Coccinellidae, which until recently were grouped

into 25 genera (Jadwiszczak and Węgrzynowicz, 2003; Szawaryn and Tomaszewska, 2013).

Species of *Epilachna* primarily feed on members of the families Cucurbitaceae and Solanaceae. For example, *E. vigintioctopunctata* is a significant pest of solanaceous crops such as potatoes and eggplants in Asia. Ladybird beetles are voracious predators, with adults capable of consuming their own body weight in aphids each day, while larvae may consume up to 50 aphids per day.

The Adonis ladybird (*Hippodamia variegata*), an important predator in agricultural ecosystems, plays a crucial role in biological control and is also a valuable model for evolutionary and genomic studies within the family Coccinellidae.

The DNA barcoding method proposed by Hebert et al. (2003) has proven to be an effective tool for rapid species identification (Hebert et al., 2003; Schindel and Miller, 2005). In recent years, DNA sequence data have become increasingly important for beetle phylogenetics. Molecular studies have typically utilized nuclear rDNA genes (28S, 18S), mitochondrial genes (COXI, COXII, 16S), and more recently, complete mitochondrial genomes (Shull et al., 2001; Caterino and Vogler, 2002).

The Molecular Evolutionary Genetics Analysis (MEGA) software is widely used for comparative analysis of DNA and protein sequences to infer molecular evolutionary patterns across genes, genomes, and species over time (Kumar et al., 1994; Tamura et al., 2011). MEGA is available in two editions: a graphical user interface (GUI) version for visual data analysis (Tamura et al., 2011) and a command-line edition (MEGA-CC) designed for automated pipeline analysis (Kumar et al., 2012).

Saeed et al. (2025) conducted a study to develop a Phylogenetic tree of three species *Brumoides suturalis* (Fabricius, 1789), *Exochomus nigripennis* (Erichson, 1843), and *Parexochomus nigromaculatus* (Goeze, 1777) of subfamily Chillocorinae from Malakand region Pakistan. To date, over 1.9 million specimens representing approximately 172,000 species have been barcoded (Pereira et al., 2013). The standard barcode region for animals is a 658-bp fragment of the mitochondrial cytochrome c oxidase subunit I gene (COXI) (Hebert et al., 2004). Comprehensive reference libraries of DNA barcodes have been developed for beetle families such as Carabidae, Chrysomelidae, and Elateridae (Raupach et al., 2010; García-Robledo et al., 2013; Oba et al., 2015).

Twelve phytophagous ladybird beetle species from Pakistan were described by Naz et al. (2012). According

to Saeed et al. (2016), thirteen species were reported from District Buner. In the Malakand division, Ullah et al. (2016) identified 28 species belonging to 19 genera across the subfamilies Coccinellinae, Chillocorinae, Scymninae, and Epilachninae.

Jamal et al. (2025) assessed the diversity of ladybird beetle species in the Swat District using morphometric and DNA barcoding techniques. Ladybird beetles, a diverse group within Coccinellidae, exhibit significant variation in morphological characteristics. However, due to taxonomic ambiguity arising from morphological identification, molecular phylogenetic analysis and DNA barcoding play a crucial role in authenticating species identity.

This study aims to explore the DNA barcoding and phylogenetic relationships of the subfamily Epilachninae in the Malakand region of Khyber Pakhtunkhwa, Pakistan. The molecular and phylogenetic aspects of the Epilachninae fauna in this region have remained largely unexplored. Therefore, the current research underscores the importance of employing DNA barcoding and phylogenetic analysis to advance the taxonomy and evolutionary understanding of Epilachninae (Coleoptera: Coccinellidae) in the Malakand region of Pakistan.

Materials and Methods

Sample collection

During the active season of ladybird beetles, spanning from April 2022 to April 2023, a total of 510 specimens were collected from various regions within Malakand division, including the plains and hilly areas of Malakand, Buner, Swat, Shangla, Dir, and Chitral, covering the upper parts of Khyber Pakhtunkhwa, Pakistan (Figure 1). Extensive year-round surveys were conducted at regular intervals, focusing on habitats associated with Poaceae, Cucurbitaceae, Solanaceae, as well as general vegetation, vegetable fields, and surrounding flora for the presence of Ladybird beetles. For morphological and molecular analysis, collected specimens were preserved in 70% ethanol supplemented with 5% glycerol.

Morphological identification

Preserved specimens were processed for morphological identification using standard taxonomic keys and diagnostic characters. Identification was carried out based on established morphological features, including body shape, color patterns, number and arrangement of dorsal spots, and structure of taxonomically important parts such as the pronotum and elytra. A

stereomicroscope (SZX12, Olympus, Japan) was used for detailed examination and photographic documentation of these features. The identification process relied heavily on comprehensive regional and sub-regional taxonomic literature, including Coccinellidae of Nepal (Kapur, 1958), Predatory Ladybird Beetles of Pakistan (Rafi et al., 2005), Coccinellidae (Coleoptera) from Nepal (Canepari, 1997), An Annotated Checklist of the

Coccinellidae (Coleoptera) of the Indian Sub-region (Poorani, 2003), and Notes on the Coccinellidae (Coleoptera) of the Indian Sub-continent, Including New Synonymies (Poorani, 2004). These references provided critical comparative descriptions and diagnostic keys for the identification of both common and rare species of ladybird beetles (Coccinellidae) occurring in the study region.

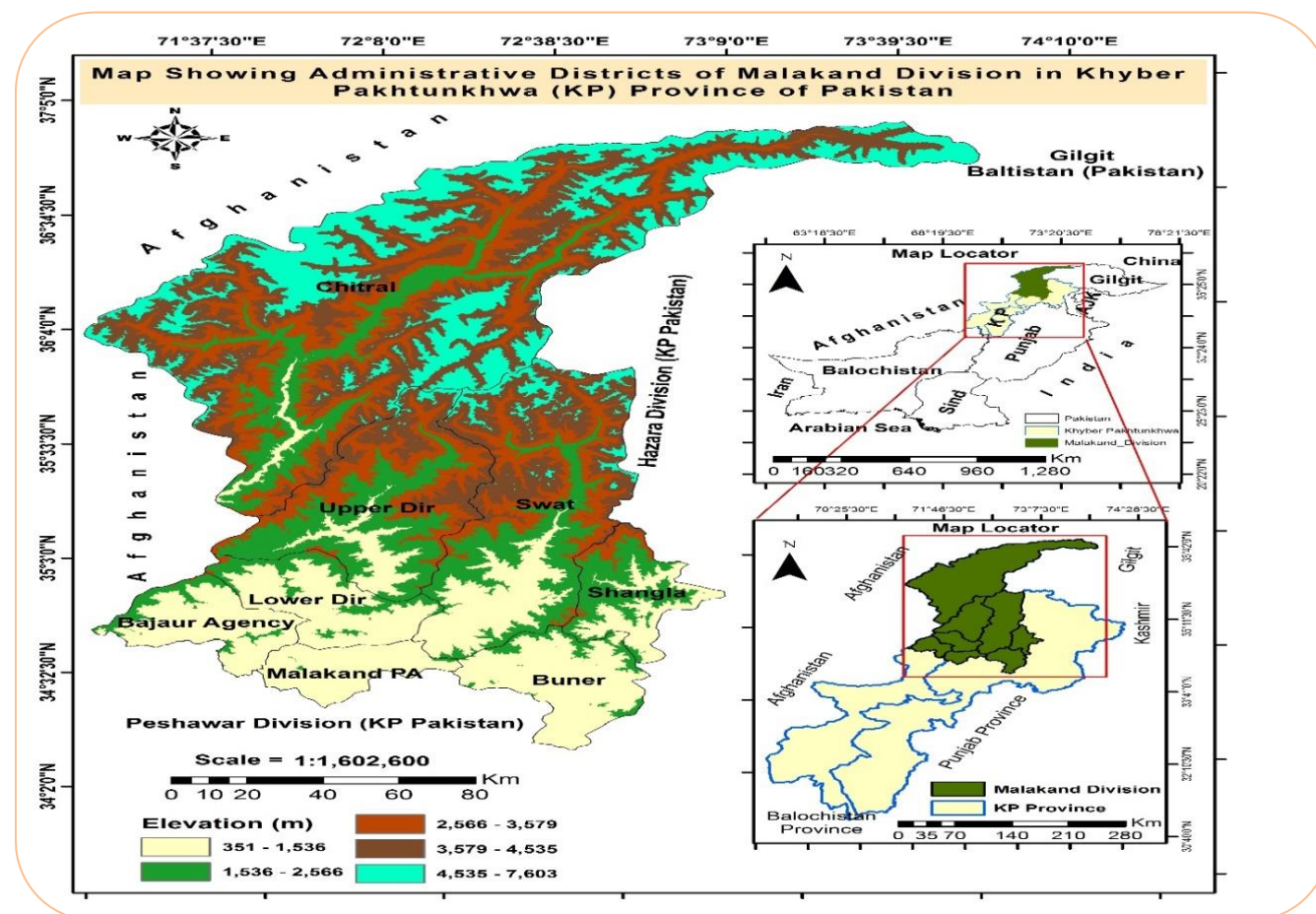


Figure 1. Map of Malakand division (prepared by using ArcGIS software).

DNA extraction, PCR, and sequencing

For molecular identification, DNA was extracted from selected specimens using the QIAamp DNA Mini Kit (QIAGEN, USA) following the manufacturer's protocol. DNA concentration and purity were assessed using a NanoVue™ Plus spectrophotometer (GE Healthcare, UK). Polymerase Chain Reaction (PCR) amplification of the mitochondrial COI gene was performed using the universal primers:

1. LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3'
2. HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'

PCR was conducted using a T-Professional Biometra thermocycler (Analytik Jena, Germany). The amplified PCR products were purified and subjected to Sanger sequencing by Macrogen Inc., Seoul, South Korea.

Agarose gel electrophoresis

A 1.5% agarose gel was prepared to confirm the presence and size of PCR-amplified products (200-1000 bp). Specifically, 1.8 g of agarose powder was dissolved in 120 ml of 1× TAE buffer (Tris-acetate-EDTA, pH 6.8) by heating in a microwave at 80°C and swirling intermittently for 1-2 min. The solution was allowed to

cool to ~60°C before adding 2 µl of ethidium bromide for DNA staining. The mixture was then poured into a gel tray fitted with a comb and allowed to solidify at room temperature for approximately 30 min.

Following centrifugation, 6 µl of each PCR product was loaded into the wells along with a 100 bp DNA ladder for size reference. Electrophoresis was run for 25-30 min at 110 V. The gel was subsequently visualized and photographed under UV illumination.

BLAST and phylogenetic analysis

Resulting chromatogram files (.ab1) were analyzed using NCBI's BLAST tool to identify sequences with ≥90% similarity. The top 100 closest matches were selected and corresponding metadata including FASTA sequences, GenBank accession numbers, authorship, affiliations, and countries of origin were compiled. Phylogenetic analyses were conducted using both Maximum Likelihood (ML) and Neighbor-Joining (NJ)

methods, employing a combination of online and offline tools to construct phylogenetic trees.

Results

A total of 510 specimens belonging to the subfamily *Epilachninae* were collected. These specimens comprised four distinct species classified under three genera. Accordingly, molecular and phylogenetic analyses were conducted to confirm their taxonomic placement. The identified species were *Henosepilachna vigintioctopunctata* (Fabricius, 1755), *Henosepilachna septima* (Dieke, 1947), *Afidentula manderstjerna* (Mulsant, 1853), and *Subcoccinella vigintiquatuorpunctata*. Table 1 showed the list of accession numbers and sequence divergence percentages of the reported species belonging to the subfamily *Epilachninae*. The DNA bands obtained through gel electrophoresis are presented in Figure 2.

Table 1. List of accession numbers and sequence divergence percentages of reported species of subfamily *Epilachninae*.

Sr. No.	Species	Accession No.	Divergence Percentage (Intraspecific)
1	<i>Henosepilachna vigintioctopunctata</i>	PQ475999	0.00000000
2	<i>Afidentula manderstjerna</i>	PQ893836	0.00954
3	<i>Henosepilachna septima</i>	PQ476004	0.00000000
4	<i>Subcoccinella vigintiquatuorpunctata</i>	PQ476000	0.00000000

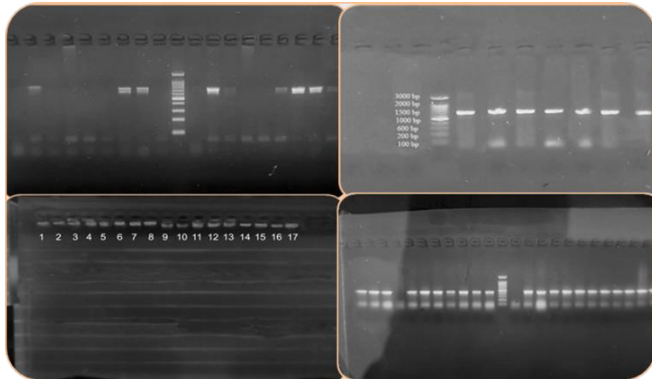


Figure 2. DNA bands obtained through gel electrophoresis.

Henosepilachna vigintioctopunctata (Fabricius, 1755)

Distinguishing characters

The body was rounded and dorsally convex, with yellowish-red ground coloration. The pronotum was reddish-brown, typically bearing up to seven spots, although spotless individuals were also observed. The elytra were pale brown to reddish-brown, displaying between 12 and 28 spots, with 26 being the most common.

Elytral spots were generally arranged in a straight line, and the elytral apex was distinctly angled (Figure 3).



Figure 3. *H. vigintioctopunctata*.

Molecular phylogenetic analysis of *H. vigintioctopunctata*

The phylogenetic analysis based on the mitochondrial cytochrome c oxidase subunit I (COX1) gene was conducted using the Maximum Likelihood (ML) method to determine the evolutionary relationships of *H. vigintioctopunctata*. The resulting phylogenetic tree (Figure 4) clearly clustered *H. vigintioctopunctata* with other closely related *Henosepilachna* species,

forming a well-supported monophyletic clade. The bootstrap values at the nodes indicated strong statistical support for most of the branches, confirming the reliability of the inferred phylogenetic relationships. The tree also revealed clear genetic divergence between *H. vigintioctopunctata* and other genera within the Coccinellidae family, further validating the species' distinct taxonomic position based on COX1 sequences.

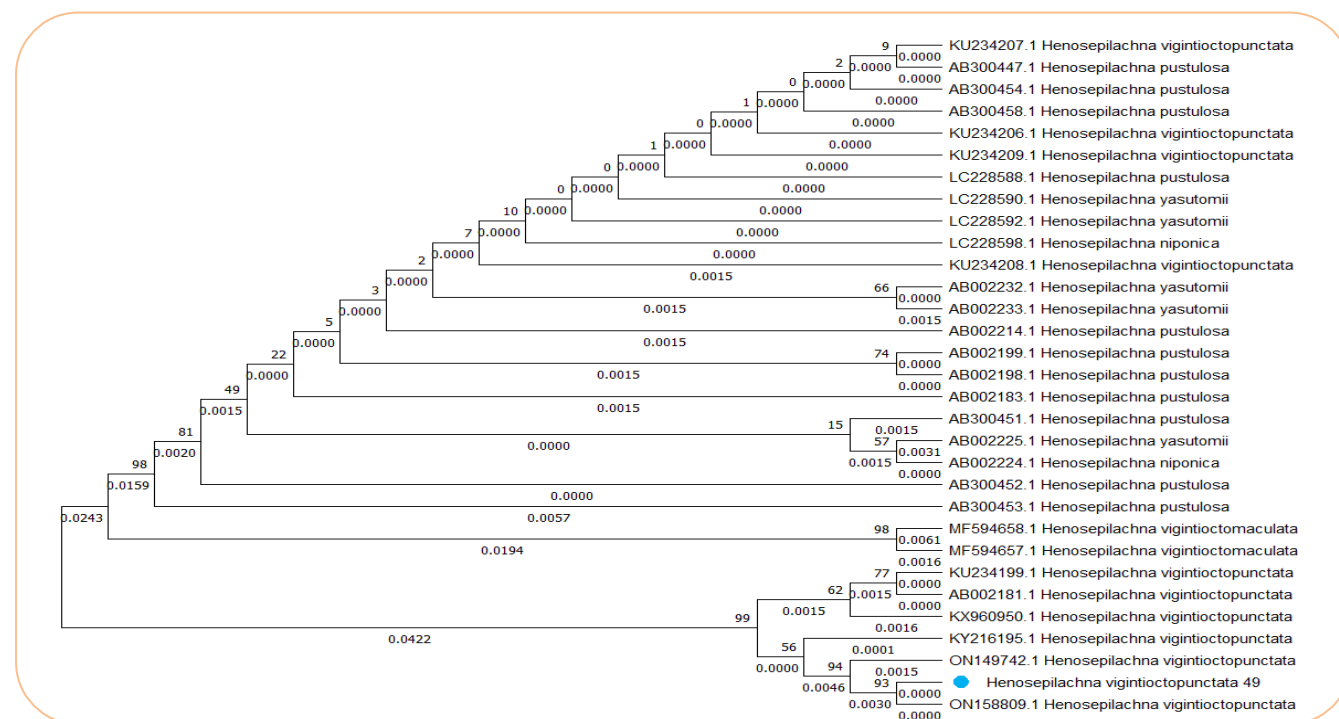


Figure 4. Phylogenetic tree of *H. vigintioctopunctata* based on COX1 gene using the ML method.

DNA barcode analysis of *H. vigintioctopunctata*

The COX1 gene sequences of *H. vigintioctopunctata* obtained in this study were analyzed to assess nucleotide composition and divergence. DNA sequences for this species previously deposited in GenBank by other researchers were also retrieved for comparative analysis. The final alignment comprised 31 sequences, each 656 base pairs in length. A distinct barcode gap was observed among species, with no overlap in sequence divergence.

The aligned dataset consisted of 656 nucleotide positions, of which 583 were conserved, 73 were variable, 67 were parsimony-informative, and 6 were singleton sites. An increase in mean Kimura 2-Parameter (K2P) genetic divergence was observed with increasing taxonomic level. The mean intraspecific divergence was 0.0000% (Table 2). In the Neighbor-Joining tree,

conspecific sequences clustered together with 93% bootstrap support, confirming species-level resolution.

Table 2. Maximum likelihood estimates of the substitution matrix of *H. vigintioctopunctata*.

	A	T/U	C	G
A	-	4.38	4.38	16.24
T/U	4.38	-	16.24	4.38
C	4.38	16.24	-	4.38
G	16.24	4.38	4.38	-

Each entry represented the probability of substitution (r) from one nucleotide base (row) to another (column). Substitution patterns and rates were estimated using the Kimura 2-parameter model (Kimura, 1980). Rates of transitional substitutions were indicated in bold, while those of transversal substitutions were shown in

italics. The relative values of instantaneous substitution rates (r) were considered for interpretation. For simplicity, the sum of all r values was normalized to 100. The nucleotide frequencies were assumed to be equal, with A = 25.00%, T/U = 25.00%, C = 25.00%, and G = 25.00%. For the estimation of maximum likelihood (ML) values, the tree topology was computed automatically. The maximum log-likelihood value obtained for this computation was -1461.950.

The analysis included 31 nucleotide sequences, with codon positions comprising 1st 2nd 3rd and non-coding regions. A total of 656 positions were retained in the final dataset. Evolutionary analyses were performed using MEGA11 (Tamura et al., 2021).

***Henosepilachna septima* (Dieke, 1947)**

Distinguishing characters

The body was large and highly convex dorsally. The head lacked spots. The pronotum bore 2 to 6 spots, occasionally 4 or 7, with the 7th spot appearing hazy. The elytral spot pattern was variable but not as complex as that of *H. vigintioctopunctata*. All 14 elytral spots were present and generally rounded, with the persistent spots being larger than the non-persistent ones. The spots did not touch the suture or the margin (Figure 5).



Figure 5. *H. septima*.

Molecular Phylogenetic Analysis of *H. septima* (Dieke, 1947)

The phylogenetic tree (Figure 6), constructed using the Maximum Likelihood method based on the COX1 gene, revealed the evolutionary relationship of *H. septima* with closely related species. The analysis showed that *H. septima* clustered distinctly within its clade, indicating a clear genetic divergence from other species included in the study. Bootstrap values at the nodes supported the reliability of the branching pattern, confirming the robustness of the inferred phylogenetic relationships.

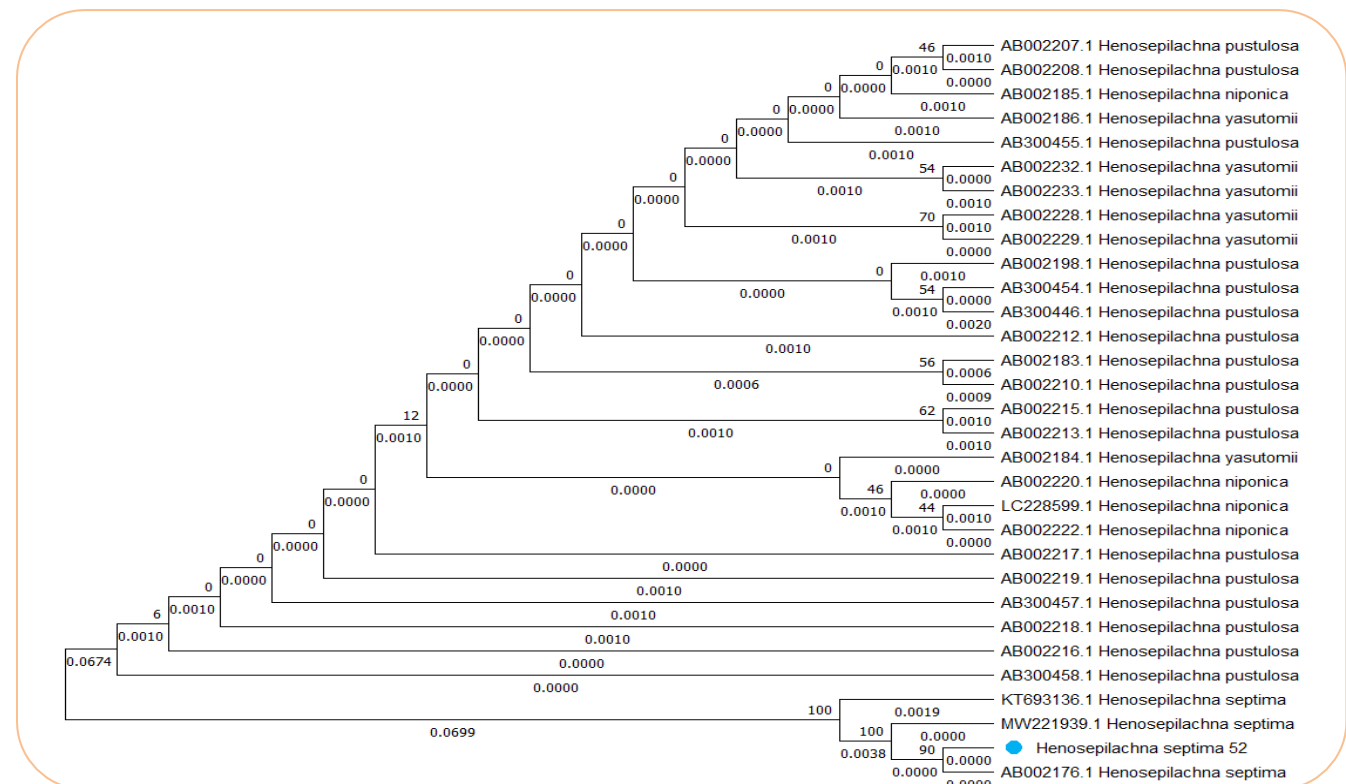


Figure 6. Phylogenetic tree of *H. septima* based on COX1 using the ML method.

DNA barcode analysis of *H. septima*

The COX1 gene sequences of *H. septima*, belonging to the family Coccinellidae, obtained in this study were analyzed for nucleotide divergence and composition. DNA sequences of related species were retrieved from GenBank, having been previously deposited by other researchers, for comparative analysis. The final aligned dataset comprised 31 sequences, each 981 base pairs in length. A distinct barcode gap was observed between species, with no overlap in sequence divergence. The alignment consisted of 981 nucleotide characters, of which 849 were conserved, 132 were variable, 127 were parsimony-informative, and 5 were singleton sites. An increase in mean Kimura 2-Parameter (K2P) divergence was observed across different taxonomic levels. The mean intraspecific divergence was 0.0000% (Table 3). In the Neighbor-Joining tree, conspecific sequences clustered together with a bootstrap support of 90%.

Table 3. Maximum likelihood estimates of the substitution matrix of *H. septima*.

	A	T/U	C	G
A	-	5.67	5.67	13.66
T/U	5.67	-	13.66	5.67
C	5.67	13.66	-	5.67
G	13.66	5.67	5.67	-

Each entry represents the probability of substitution (r) from one nucleotide base (row) to another (column). Substitution patterns and rates were estimated using the Kimura 2-parameter model (Kimura, 1980). Rates of transitional substitutions are shown in bold, while those of transversional substitutions are indicated in italics. The relative values of instantaneous substitution rates (r) were considered for comparative evaluation. For simplicity, the sum of all r values was normalized to 100. The nucleotide frequencies were A = 25.00%, T/U = 25.00%, C = 25.00%, and G = 25.00%.

To estimate ML values, the tree topology was inferred automatically. The highest log-likelihood value obtained was -2131.355. The analysis included 31 nucleotide sequences, incorporating codon positions 1st, 2nd, 3rd, and noncoding regions. A total of 981 positions were retained in the final dataset. Evolutionary analyses were performed using MEGA11 (Tamura et al., 2021).

Afidentula manderstjernae

Distinguishing characters

The body was shortly oval and generally small. The

antennae were nearly equal in width to the head, with a compact club. The clypeus and labrum were narrow. The mandibles were compact and sub-triangular in shape. The pronotum exhibited a transverse median spot. The elytra bore six spots, five located near the suture, while the sixth appeared as a break in the middle. The tarsal claw was bifid, with the inner division shorter than the outer and bearing a sub-triangular basal tooth (Figure 7).



Figure 7. *A. manderstjernae*.

Molecular phylogenetic analysis of *A. manderstjernae*

The phylogenetic tree of *A. manderstjernae*, constructed using the COX1 gene and the ML method (Figure 8), revealed the evolutionary relationships of the species with closely related taxa. The analysis showed that *A. manderstjernae* clustered distinctly within its clade, indicating a clear genetic divergence from other species. The tree topology supported the monophyly of the species and provided strong bootstrap values, confirming the robustness of the inferred phylogenetic relationships.

DNA barcode analysis of *A. manderstjernae*

COX1 gene sequences of *A. manderstjernae* obtained in this study were analyzed for nucleotide divergence and composition. DNA sequences of the species were also retrieved from GenBank, as deposited by other researchers, for comparative analysis. The final aligned dataset comprised 21 sequences, each 745 base pairs in length. A clear barcode gap was observed among the species, with no overlap in sequence divergence.

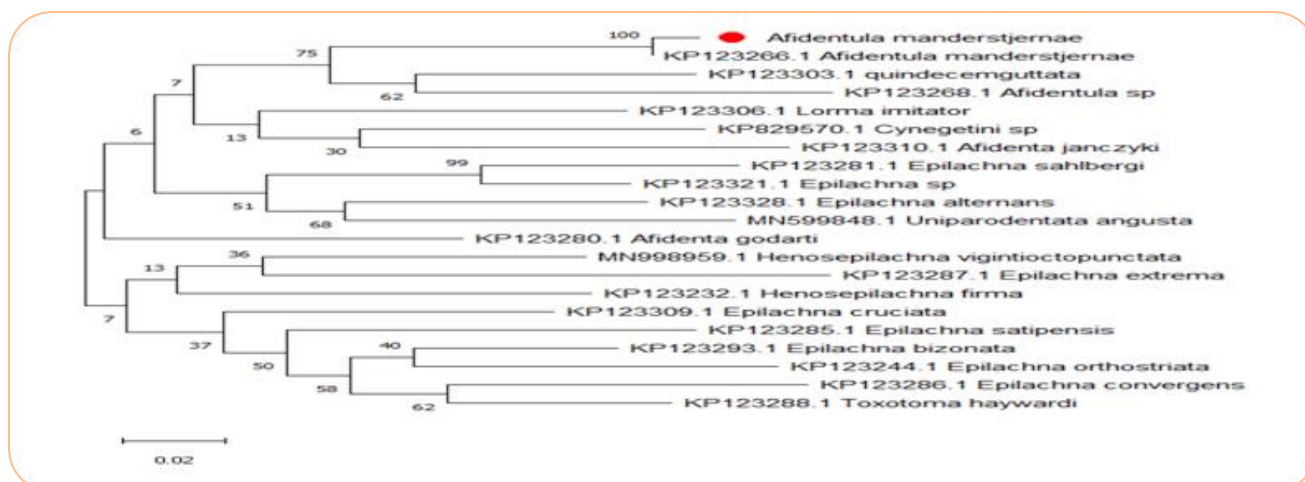


Figure 8. Phylogenetic tree of *A. manderstjernae* based on COX1 sequences using the ML method.

The aligned dataset included 745 genetic characters, of which 438 were conserved sites, 307 were variable sites, 241 were parsimony-informative, and 66 were singleton sites. An increase in mean Kimura 2-parameter (K2P) divergences was observed across different taxonomic levels. The mean intraspecific divergence was 0.00954% (Table 4). The average nucleotide composition consisted of 70.4% A+T and 29.7% G+C content. In the NJ tree, conspecific sequences clustered together with 100% bootstrap support.

Table 4. Maximum likelihood estimates of the substitution matrix of *A. amanderstjernae*.

	A	T/U	C	G
A	-	5.51	5.51	13.97
T/U	5.51	-	13.97	5.51
C	5.51	13.97	-	5.51
G	13.97	5.51	5.51	-

Each matrix entry represents the probability of substitution (*r*) from one nucleotide (row) to another (column). Substitution patterns and rates were estimated using the Kimura 2-parameter model (Kimura, 1980). Rates of transitional substitutions are shown in bold, while those of transversions are shown in italics. Relative instantaneous substitution rates should be interpreted based on their proportional values. For simplicity, the sum of *r* values was normalized to 100. The assumed nucleotide frequencies were A = 25.00%, T/U = 25.00%, C = 25.00%, and G = 25.00%.

ML values were estimated using an automatically computed tree topology, yielding a maximum log-

likelihood value of -6598.985. This analysis included 21 nucleotide sequences, with codon positions comprising 1st, 2nd, 3rd, and noncoding regions. A total of 745 positions were included in the final dataset. Evolutionary analyses were performed using MEGA11 (Tamura et al., 2021).

Subcoccinella vigintiquatuorpunctata

Distinguishing characters

S. vigintiquatuorpunctata, commonly known as the 24-spot ladybird beetle, belonged to the family Coccinellidae and was the only member of the genus *Subcoccinella*. It exhibited the typical, nearly hemispherical ladybird shape, characterized by a domed body and spotted elytra. Adults measured approximately 3 to 4 mm in length (Figure 9).



Figure 9. *S. vigintiquatuorpunctata*.

The species had a distinctively domed body, with a smooth curvature extending from the head through the pronotum to the elytra. The elytra were covered with short, pale hairs, which, though difficult to observe without magnification, imparted a characteristic matte appearance. The beetle was dark orange in color, including the legs and antennae. Black spots were present on the elytra, typically ranging from 20 to 24 in number, occasionally up to 26. These spots varied in size and could sometimes be fused or even absent. Melanic forms were extremely rare, and a very rare form with yellow spots was also reported.

Unlike many commonly known ladybird species, *S. vigintiquatuorpunctata* was neither shiny nor predatory on aphids. Its elytra appeared velvety, and it primarily fed on fungal molds growing on plants.

Molecular phylogenetic analysis of *S. vigintiquatuorpunctata*

The phylogenetic tree (Figure 10) of *S. vigintiquatuorpunctata*, constructed using the COX1 gene and the Maximum Likelihood method, revealed its distinct genetic placement within the family Coccinellidae. The species formed a well-supported clade, indicating clear genetic divergence from closely related taxa.

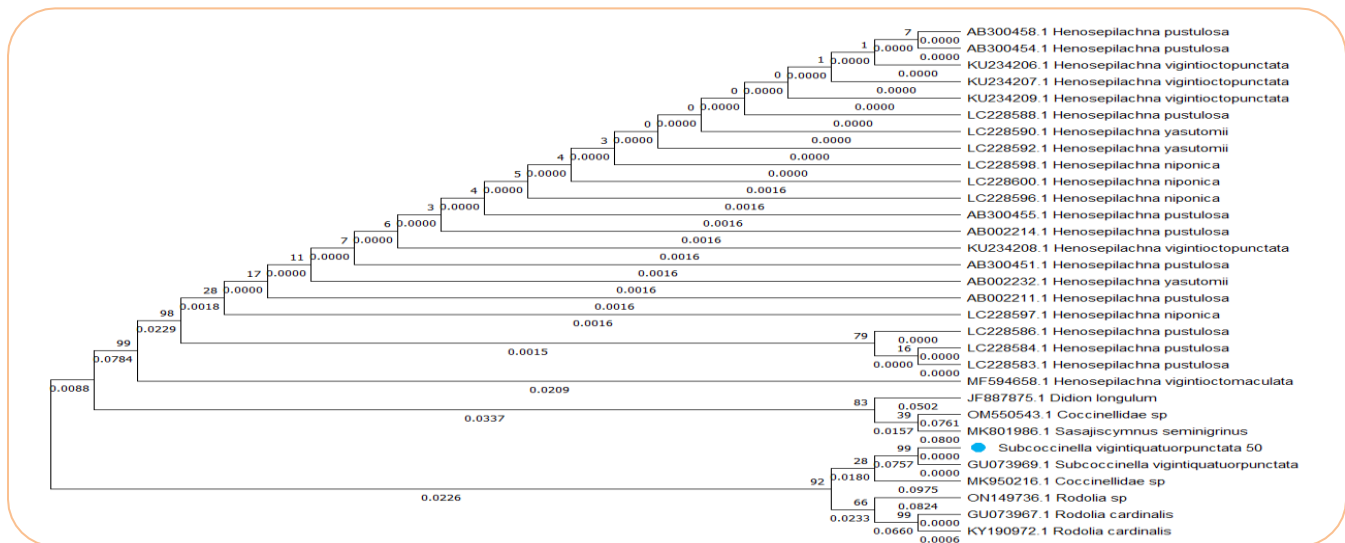


Figure 10. Phylogenetic tree of *S. vigintiquatuorpunctata* based on COX1 gene using the ML method.

DNA barcode analysis of *S. vigintiquatuorpunctata*

COX1 gene sequences of *A. manderstjernae*, belonging to the family Coccinellidae, obtained in this study, were analyzed for nucleotide divergence and composition. DNA sequences of this species, previously deposited in GenBank by other researchers, were retrieved for comparative analysis. The final aligned dataset comprised 21 sequences, each 745 base pairs in length. A distinct barcode gap was observed between species, with no overlap in sequence divergence. The aligned data consisted of 745 genetic characters, including 438 conserved sites, 307 variable sites, 241 parsimony-informative sites, and 66 singleton sites. An increase in the mean Kimura 2-parameter (K2P) divergences was noted across different taxonomic levels. The mean intraspecific divergence was calculated as 0.00954% (Table 5). The average nucleotide composition revealed

an A+T content of 70.4% and a G+C content of 29.7%.

In the Neighbor-Joining tree, conspecific sequences clustered together with 100% bootstrap support, indicating strong genetic similarity.

Substitution patterns and rates were estimated under the Kimura 2-parameter model (Kimura, 1980). Each entry represented the probability of substitution (r) from one base (row) to another base (column). Rates of transitional substitutions were shown in bold, while those of transversional substitutions were shown in italics. The relative values of instantaneous substitution rates (r) were normalized to a sum of 100 for simplicity. The nucleotide frequencies were A = 25.00%, T/U = 25.00%, C = 25.00%, and G = 25.00%.

For estimating ML values, a tree topology was automatically computed, yielding a maximum log-likelihood of -6598.985. The analysis included all 21

nucleotide sequences, covering codon positions 1st, 2nd, 3rd, and non-coding regions. The final dataset consisted of 745 positions. All evolutionary analyses were conducted using MEGA11 (Tamura et al., 2021).

Table 5. Maximum likelihood estimates of the substitution matrix of *S. vigintiquatuorpunctata*.

	A	T/U	C	G
A	-	6.34	6.34	12.31
T/U	6.34	-	12.31	6.34
C	6.34	12.31	-	6.34
G	12.31	6.34	6.34	-

Discussion

The family Coccinellidae comprises six subfamilies, most of which are either predatory or phytophagous and play an important role in biological control. These beetles are distributed worldwide, including in Pakistan. In the present study, four species from the subfamily Epilachninae were molecularly identified, belonging to the genera Henosepilachna, Afidentula, and Subcoccinella. This research was conducted from April 2022 to April 2023, during which a total of 510 samples were collected. DNA extraction and PCR amplification were carried out using primers LCO1490 and HCO2198, followed by Sanger sequencing. Phylogenetic analysis was performed using various bioinformatics tools.

Based on morphological characteristics, the collected specimens were identified as *H. vigintioctopunctata*, *H. septima*, *A. manderstjernae*, and *S. vigintiquatuorpunctata*. Molecular phylogenetic analysis supported the morphological identifications.

Katoh et al. (2014) reconstructed the phylogeny of 46 species of Epilachnini from Asia, Africa, the America, and the Australian region, including 16 species of Epilachna, 24 of Henosepilachna, and one species each from Adira, Afidentula, Afidentula, Afissula, Chnootriba, and Epiverta. Their analysis revealed that Epilachna and Henosepilachna were reciprocally polyphyletic. Asian Epilachna species formed a monophyletic group, except for the inclusion of *Afissula* sp. Similarly, Asian and Australian Henosepilachna species formed a monophyletic group, excluding *H. boisduvali*. African Epilachna and Henosepilachna species did not cluster with their Asian and American congeners; instead, they were paraphyletic or formed distinct clades, such as a monophyletic group with *Chnootribasimilis*. American Epilachna species formed a monophyletic clade with *A.*

clarkii (America) and *A. manderstjernae bielawskii* (Asia), which was a sister group to the Asian and Australian Henosepilachna clade but distinct from Asian Epilachna.

In the present study, four species of the subfamily Epilachninae were molecularly and phylogenetically reported for the first time from the Malakand region, Pakistan: *H. vigintioctopunctata*, *H. septima*, *A. manderstjernae*, and *S. vigintiquatuorpunctata*.

According to Szawaryn et al. (2015), a comprehensive dataset comprising four DNA markers (18S and 28S rRNA, 16S, and COX1 mtDNA) and a matrix of 104 morphological characters for 153 Epilachnini species (~11% of known species) from all previously recognized genera was assembled. Phylogenetic analyses were conducted using maximum likelihood, parsimony, and Bayesian inference. Among the 25 genera studied, only 14 were recovered as monophyletic. Genera such as Afidentula, Afidentula, Afissula, Epilachna, Henosepilachna, Toxotoma, and Mada were identified as poly- or paraphyletic. Meanwhile, Chnootriba, Subafissa, Lalokia, and Papuaepilachna formed monophyletic groups and were redefined accordingly.

In our study, we constructed phylogenetic trees for four species of the subfamily Epilachninae from the Malakand region and submitted their sequences to the NCBI GenBank database.

Pairwise comparisons of the *COX1* gene sequences of *A. manderstjernae* revealed a low genetic divergence of 0.00954, indicating minimal variation between haplotypes. A sequence reported from China (accession no. KP123266.1) showed an A+T content of 70.5% and G+C of 29.5%, compared to our findings of 70.4% A+T and 29.7% G+C, demonstrating high similarity and clustering within the same clade. Additional pairwise analysis of *A. manderstjernae* revealed a genetic difference of 0.00000 in one instance, further supporting low intra-species variation. A study from Pakistan (accession no. JF890400, IBOL 2011b) reported A+T and G+C contents of 64.1% and 35.5%, respectively, which were similar to our results of 68.7% and 35.3%. Another COX1-based study from Pakistan (accession no. JF890470, IBOL 2011c) reported A+T and G+C contents of 64.4% and 35.9%, respectively, confirming consistent clustering in the same clade.

Conclusion

In this study, four species from three genera of the subfamily Epilachninae were molecularly and

phylogenetically reported for the first time from the Malakand region of Pakistan. These species include *H. vigintioctopunctata*, *H. septima*, *A. manderstjernae*, and *S. vigintiquatuoropunctata*. DNA extraction and PCR amplification were carried out using the universal primers LCO1490 and HCO2198, followed by Sanger sequencing. Phylogenetic analyses were conducted using software such as BioEdit 7.2 and MEGA11. The morphological identifications of the collected specimens were strongly supported by molecular and phylogenetic data. The sequences of all four species have been submitted to the NCBI GenBank database. This research represents the first comprehensive molecular and phylogenetic investigation of the subfamily Epilachninae from Pakistan.

Authors' Contributions

KS and MSA conceptualized the study; QZ, KS and KM developed the methodology; FN and FZ performed the identification of the specimens; QZ and KS handled software-related tasks; QZ conducted the formal analysis; KS undertook the investigations; UN provided the resources; KS and QZ managed data; KS, AJA, and IAK prepared the original draft of the manuscript; KS, AJA, and UR reviewed and edited the manuscript; MSA and KM supervised the studies; MSA, KM, and KS administered the project.

Research Funding

This research did not receive any grant from funding agencies.

Conflict of Interest

The authors declare no conflict of interest.

Sustainable Development Goals Targeted

SDG 2: Zero Hunger
SDG 13: Climate Action
SDG 15: Life on Land

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