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Research Article

# GENETIC PROFILING OF PAKISTANI LADYBIRD BEETLES (COCCINELLIDAE: COLEOPTERA) USING MITOCHONDRIAL COI DNA BARCODING

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**ABSTRACT**

Ladybird beetles (Coccinellidae: Coleoptera) are vital for pest control and ecosystem balance, but their identification is hindered by morphological similarities. This study utilizes mitochondrial COI gene barcoding to profile Pakistani ladybird beetles for species identification and biodiversity assessment, focusing on the previously unsurveyed Bannu and Dera Ismail Khan divisions of Khyber Pakhtunkhwa, Pakistan during 2021-2022. A total of 40 COI sequences representing ten species from eight genera and three tribes of ladybird beetles were analyzed for DNA barcoding and phylogenetic studies. The obtained sequences were confirmed and compared with data from GenBank and BOLD. Phylogenetic trees were constructed using the maximum likelihood method. The COI sequences showed 94% to 100% similarity with corresponding ladybird beetle sequences in the databases. Out of 687 COI gene sequence positions, 315 (46%) displayed 80% pairwise identity, while 372 (54%) were variable. Among these, 324 (47%) sites were parsimony-informative, and 48 were singleton sites. Nucleotide frequency analysis revealed a higher AT content (69.2%) compared to GC content (30.8%). The sequences showed a mean intraspecific genetic divergence of 0.60% and a mean interspecific genetic divergence of 17.46%. A distinct barcode gap was observed between conspecific and congeneric species of ladybird beetles. Most tribes within the Coccinellidae family were monophyletic, with high bootstrap values ranging from 78% to 82%, and a mean divergence threshold of 0.01 ± 0.13. In conclusion, the study area hosts diverse ladybird beetle species, and COI gene barcoding is a reliable tool for their identification and biodiversity assessment.

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## INTRODUCTION

Ladybird beetles, also known as ladybugs, play a crucial role in agricultural ecosystems as natural enemies of invasive pests such as aphids, mealybugs, sweet potato whiteflies, and scale insects. Their predatory behavior makes them highly valuable for biological pest control, helping to reduce pesticide use and improve crop yields in advanced agricultural systems such as those in the USA (Obrycki and Kring, 1998). Furthermore, these beetles serve as bioindicators, reflecting ecosystem health due to their sensitivity to environmental changes, including pollution, habitat loss, and climate impacts (Koch and Galvan, 2008). However, invasive species of ladybird beetles can threaten native populations and disrupt ecological balance (Ware et al., 2010).

The family Coccinellidae, the largest in the superfamily Coccinelloidea, includes over 6,000 described species grouped into approximately 360 genera (Ślipiński and Tomaszewska, 2010). This group exhibits remarkable habitat diversity, inhabiting a wide range of terrestrial ecosystems (Halim et al., 2017). Traditionally, identification has relied on morphological characteristics; however, morphological variation, sexual dimorphism, and interspecific similarities often complicate taxonomic efforts (Marin et al., 2010; Hodek and Evans, 2012).

To overcome these challenges, molecular approaches have become instrumental in species identification and resolving taxonomic ambiguities. Techniques such as single-stranded conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), and random amplified polymorphic DNA (RAPD) have been employed with varying success (Navajas and Fenton, 2000; Palandačić et al., 2017). Among these, DNA barcoding has emerged as a reliable method, using standardized genetic markers to identify species, including poorly preserved or morphologically ambiguous specimens (Hebert and Barrett, 2005; Kumar et al., 2009; Kumar et al., 2013).

The mitochondrial cytochrome oxidase subunit I (COI) gene is widely accepted as a standard marker for DNA barcoding due to its effectiveness in delineating species and revealing evolutionary relationships (Jalali et al., 2015). DNA barcoding has proven pivotal in assigning unidentified specimens to species, uncovering cryptic diversity, and discovering new taxa (Liu et al., 2018).

In Pakistan, 91 species of ladybird beetles, representing 39 genera, have been documented. Of these, 76 are predatory and 15 phytophagous, with distributions across Sindh, Balochistan, Khyber Pakhtunkhwa, Punjab, and Gilgit-Baltistan (Ashfaque, 2013; Urooj and Ali, 2016). However, reliance on conventional morphological methods has often resulted in misidentification, particularly among taxa with hidden diversity (Blaxter and Floyd, 2003). Molecular techniques, such as DNA barcoding, circumvent the need for expert morphological knowledge and have significantly advanced insect systematics (Caterino, 2000; Sobti et al., 2009).

Ladybird beetles are among the most abundant and diverse insect groups, widely distributed across various agro-ecological zones of Pakistan (Rafi et al., 2005; Iqbal et al., 2024a, b). However, their abundance and diversity in the Bannu and Dera Ismail Khan divisions remain poorly understood. In the present study, molecular identification of ladybird beetles was carried out to better understand their taxonomy. This research provides foundational data for barcode reference libraries and contributes to the development of integrated pest management (IPM) strategies.

## MATERIAL AND METHODS

### Study area, sampling, and specimen processing

The study was conducted in southern Khyber Pakhtunkhwa (KP), Pakistan, specifically in the Bannu and Dera Ismail Khan Divisions (Figure 1). These regions encompass diverse landscapes, including plains, hills, and agricultural lands, with elevations ranging from 150 to 1200 m. The climate is semi-arid to arid, characterized by varying temperatures and rainfall.

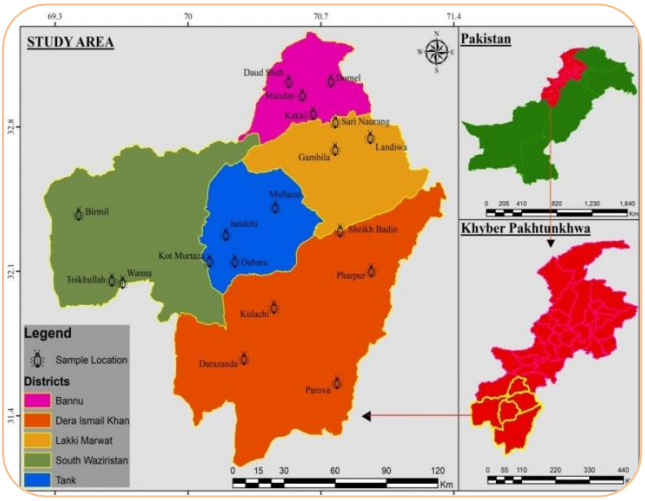


Figure 1. Distribution map showing sampling locations of ladybird beetles.

Ladybird beetles were collected from January to December 2022 using sweep netting and hand-picking techniques at randomly selected sites. Specimens were killed using potassium cyanide, labeled, and stored at the Entomology Laboratory of Gomal University. Representative specimens were preserved in 95% ethanol for DNA extraction.

**DNA extraction and PCR amplification**

Genomic DNA was extracted from four specimens of each species using the 2× EasyPure Genomic DNA Kit (TransGen Biotech, Beijing, China), following the manufacturer's protocol. DNA was extracted either from the thorax or femur (from two legs or a single leg) or from the entire body in the case of small-sized ladybird beetles (less than 3 mm in size).

A ~658 bp fragment of the mitochondrial cytochrome c oxidase subunit I (mtCOI) gene was amplified using the universal primer pair LCO1490F (GGTCAACAAATCATAAAGATATTGG) and HC02198R (TAAACTTCAGGGTGACCAAAAAATCA) (Folmer et al., 1994).

The PCR reaction was performed in a 25 µl volume, comprising 15 µl of PCR SuperMix (TransGen Biotech, Beijing, China), 4 µl of distilled water, 0.5 µl (25 pmol) of each primer, and 5 µl of template DNA. The PCR cycling conditions were as follows: an initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturation at 95°C for 45 sec, annealing at 56°C for 45 sec, and extension at 72°C for 30 sec; with a final extension at 72°C for 3 min.

The PCR products were analyzed by agarose gel electrophoresis on a 2% gel prepared in 0.5× TBE buffer. PCR products showing clear bands were selected for sequencing (Figure 2a and b). The DNA fragments were then excised from the Tris-Borate-EDTA agarose gel and purified using the EasyPure® Quick Gel Extraction Kit (TransGen Biotech, Beijing, China), according to the manufacturer's instructions.

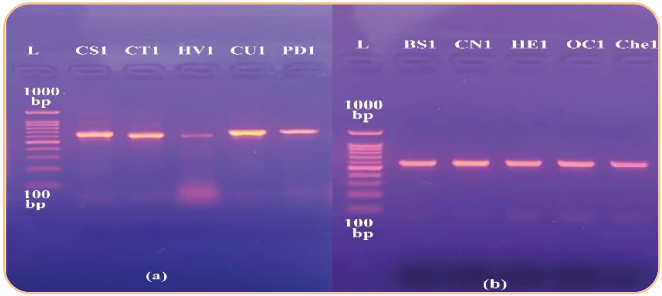


Figure 2a and 2b. Gel electrophoresis of PCR products amplified from the mitochondrial COI gene of representative ladybird beetle species.

**Sequencing and sequence analysis**

Purified samples were subjected to Sanger sequencing at Apical Scientific SDN BHD (Malaysia). All obtained COI gene sequences were translated and manually checked for the presence of stop codons and frame shifts within their open reading frames (ORFs) using MEGA 11 (Tamura et al., 2021). The sequences were then verified using the NCBI BLAST (nBLAST) tool.

The overall mean nucleotide composition and optimal genetic distance thresholds (both intraspecific and interspecific) among the COI sequences were calculated using the Kimura 2-Parameter (K2P) model in MEGA 11. Barcode gap analysis was performed using the online version of Automatic Barcode Gap Discovery (ABGD), accessible at https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html. The analysis was conducted with a relative gap width (X) of 1.0 and an intraspecific divergence (P) value ranging from 0.005 to 0.10, using the Kimura (K80) model. All other parameters were set to default values. Distance histograms were then generated and analyzed.

**Phylogenetic analysis**

A phylogenetic tree was constructed using MEGA 11 software with Maximum Likelihood (ML) analysis, based on the Kimura 2-Parameter (K2P) model. Bootstrap values indicating the confidence level of taxa clustering were shown at each branch node.

## RESULTS

**Amplification and sequence analysis**

A total of 40 mitochondrial COI barcode sequences were generated from 10 species. All sequences were confirmed using BLAST (NCBI), showing more than 90% coverage and over 93% similarity. The obtained COI sequences were aligned using both pairwise and multiple sequence alignment methods. Among the 687 sites analyzed in the COI gene sequences, 315 sites (46%) were conserved (C) with 80% pairwise identity. In contrast, 372 sites (54%) were variable (V), of which 324 (47%) were parsimony-informative (P), and 48 were singleton sites (S). The average nucleotide composition was 38.01% thymine (T), 16.05% cytosine (C), 31.14% adenine (A), and 14.75% guanine (G). Nucleotide frequency analysis revealed that the AT content (69.2%) was higher than the GC content (30.8%) (Table 1).

Table 1. Nucleotide frequencies of COI barcode sequences in study population.

| Species | T(U)% | C% | A% | G% |
|---|---|---|---|---|
| *Chilocorus nigritus* | 39.36 | 13.22 | 32.82 | 14.58 |
| *Brumoides suturalis* | 39.26 | 14.51 | 30.87 | 15.33 |
| *Henosepilachna elaterii* | 35.8 | 18.92 | 29.17 | 16.08 |
| *Oenopia conglobata* | 38.95 | 16.25 | 30.21 | 14.57 |
| *Cheilomenes sexmaculata* | 35.98 | 16.69 | 32.77 | 14.54 |
| *Coccinella septempunctata* | 38.37 | 16.44 | 30.37 | 14.81 |
| *Coccinella transversalis* | 38.21 | 16.05 | 31.3 | 14.43 |
| *Coccinella undecimpunctata* | 38.03 | 17.17 | 30.98 | 13.8 |
| *Hippodamia variegata* | 38.68 | 15.88 | 30.39 | 15.02 |
| *Propylea dissecta* | 37.54 | 15.41 | 32.6 | 14.42 |
| Average | 38.01 | 16.05 | 31.14 | 14.75 |

**Barcode gap analysis**

The complete dataset comprised newly generated sequences from the present study, along with sequences downloaded from GenBank and BOLD, totaling 70 COI sequences used for subsequent analysis. A total of 392 variable sites were identified, including 329 parsimony-informative sites and 63 singletons. The Automatic Barcode Gap Discovery (ABGD) method was employed to assess species delimitation. As summarized in Table 2, both initial and recursive partitions were used to divide the dataset.

Table 2. Partitioning of genetic cluster based on ABGD analysis at different distance threshold.

| Partition | Distance threshold (P) | Number of clusters (with recursion) | Number of cluster (without recursion) |
|---|---|---|---|
| 1 | 0.005000 | 24 | 13 |
| 2 | 0.006975 | 24 | 13 |
| 3 | 0.009729 | 24 | 13 |
| 4 | 0.013572 | 24 | 13 |
| 5 | 0.018932 | 24 | 13 |
| 6 | 0.026410 | 24 | 13 |
| 7 | 0.036840 | 24 | 13 |
| 8 | 0.051390 | 24 | 13 |
| 9 | 0.071687 | 24 | 13 |
| 10 | 0.100000 | 24 | 13 |

The results indicated that the initial partition was more stable, grouping the 70 sequences into 13 to 24 putative species depending on the applied distance thresholds (P values). In contrast, the recursive partition showed considerable fluctuations, likely leading to an overestimation of species count at lower distance thresholds (Figure 3). The barcode gap was visualized using a histogram (Figure 4), and the distribution of genetic distances, arranged from lowest to highest, was illustrated in Figure 5.

The interspecific genetic distances among 10 species of ladybird beetles are presented in Table 3. The results indicate that the interspecific genetic distance ranged from 0.132% to 0.333%, with a mean value of 0.127%. The smallest interspecific genetic distance (0.132%) was observed between *Brumoides suturalis* and another closely related species, while the largest distance (0.333%) was recorded between *Cheilomenes sexmaculata* and another species.

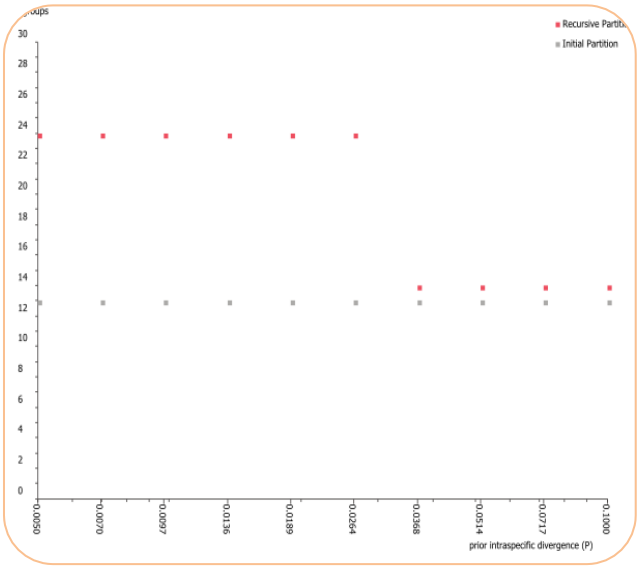


Figure 3. ABGD analysis for species delimitation based on 70 COI sequences, including newly generated data and sequences retrieved from GenBank and BOLD.

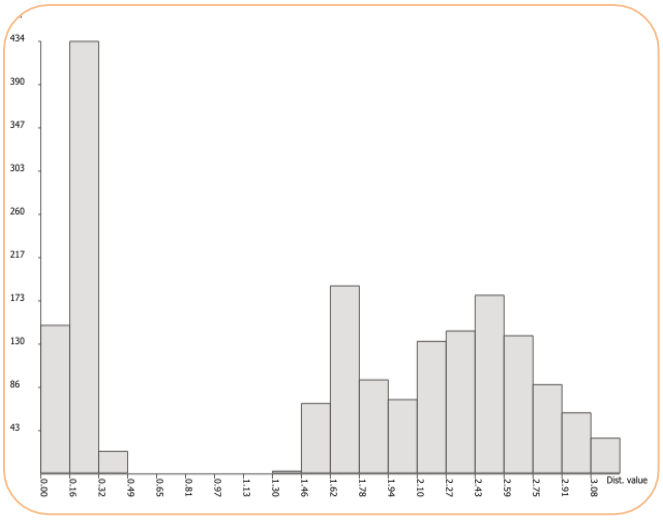


Figure 4. Histogram of genetic distances showing the barcode gap for species delimitation.

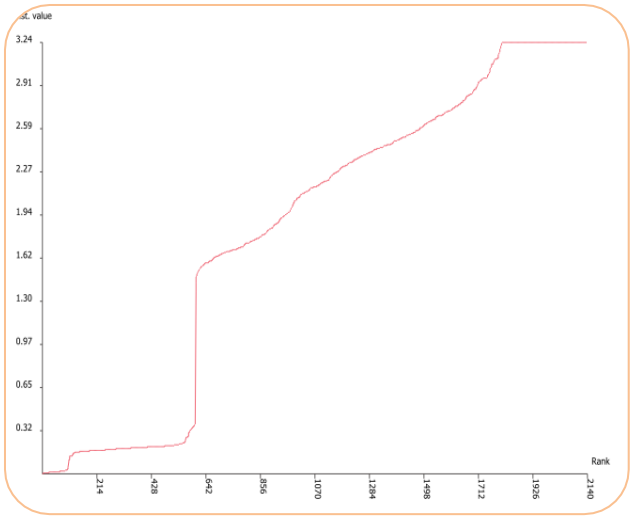


Figure 5. Ranked genetic distances of ladybird beetles from lowest to highest, generated using the ABGD online tool.

Table 3. Genetic distances (K2P) and standard deviation values.

| Ladybird beetles species | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| *C. nigritus* (1) | | 0.017 | 0.024 | 0.019 | 0.018 | 0.019 | 0.023 | 0.019 | 0.025 | 0.021 |
| *B. suturalis* (2) | 0.132 | | 0.028 | 0.021 | 0.024 | 0.024 | 0.023 | 0.022 | 0.021 | 0.022 |
| *H. elaterii* (3) | 0.259 | 0.263 | | 0.026 | 0.029 | 0.027 | 0.030 | 0.027 | 0.024 | 0.027 |
| *O. conglobata* (4) | 0.188 | 0.179 | 0.295 | | 0.020 | 0.017 | 0.023 | 0.020 | 0.021 | 0.022 |
| *C. sexmaculata* (5) | 0.177 | 0.227 | 0.333 | 0.209 | | 0.020 | 0.023 | 0.023 | 0.021 | 0.023 |
| *C. septempunctata* (6) | 0.194 | 0.225 | 0.313 | 0.158 | 0.195 | | 0.020 | 0.020 | 0.020 | 0.023 |
| *C. transversalis* (7) | 0.207 | 0.210 | 0.284 | 0.194 | 0.201 | 0.166 | | 0.022 | 0.020 | 0.024 |
| *C. undecimpunctata*(8) | 0.185 | 0.199 | 0.312 | 0.204 | 0.248 | 0.197 | 0.172 | | 0.021 | 0.022 |
| *H. variegata*(9) | 0.170 | 0.177 | 0.255 | 0.197 | 0.198 | 0.190 | 0.153 | 0.199 | | 0.022 |
| *P. dissecta* (10) | 0.180 | 0.201 | 0.261 | 0.190 | 0.210 | 0.221 | 0.215 | 0.199 | 0.194 | |

**Phylogenetic analysis**

The Maximum Likelihood (ML) method based on the Kimura 2-Parameter (K2P) model was used to determine the evolutionary positions of the specimens. The analysis produced the tree with the highest log likelihood value (-4543.86). A matrix of pairwise distances, calculated using the Maximum Composite Likelihood (MCL) method, was used to generate the initial tree(s) for the heuristic search using the Neighbor-Joining and BioNJ algorithms. The topology with the highest log likelihood was selected for the final analysis.

A total of 12 nucleotide sequences were included in the study. Codon positions considered were the first, second, third, and non-coding regions. All positions containing gaps and missing data were eliminated, resulting in a final dataset comprising 818 positions. Evolutionary analyses were conducted in MEGA 11 (Figure 6).

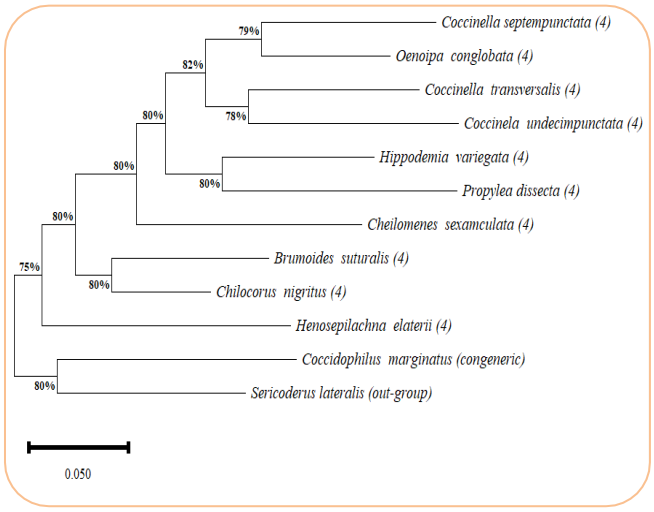


Figure 6. Phylogenetic relationships among the studied ladybird beetle species inferred using the Maximum Likelihood method and the Kimura 2-parameter model.

The results revealed two major clusters. The first cluster includes *C. septempunctata*, *O. conglobata*, *C. transversalis*, *C. undecimpunctata*, *H. variegata*, *P. dissecta*, *C. sexmaculata*, *B. suturalis*, and *C. nigritus*, all supported by high bootstrap values ranging from 78% to 82%. *H. elaterii* forms a sister clade to this cluster, with 80% bootstrap support. The second cluster comprises *C. marginatus*, also supported by 80% bootstrap value. *Sericoderus lateralis* serves as the outgroup, rooting the tree.

**DISCUSSION**

Ladybird beetles are renowned for their ecological significance, particularly within agricultural ecosystems. As natural enemies or predators, they play a crucial role in the biological control of various insect pests. All the query COI (cytochrome c oxidase subunit I) sequences showed more than 90% query coverage and over 93% similarity. The highest similarity and query coverage were observed in *C. septempunctata*, with 97% coverage and 99% identity. Conversely, the lowest query coverage and similarity were recorded in *H. elaterii*, with 90% and 93%, respectively. These variations in coverage and identity percentages may be attributed to evolutionary history, species-specific genetic diversity, or a lack of conspecific sequences in the database (Ratnasingham and Hebert, 2007; Collins and Cruickshank, 2013).

Recent studies have confirmed that COI barcoding is an effective method for differentiating closely related Coccinellidae species, even when morphological traits overlap (Hanif et al., 2023). The overall nucleotide composition exhibited a significant bias toward adenine and thymine (A+T), with the average guanine and cytosine (G+C) content at just 30.8%. This low G+C content aligns closely with the typical range of 27.7% to 39.5% reported for various insect orders (Hebert et al., 2003). Several studies have observed similar substitution patterns (Jing and Yingchun, 2006; Kartika et al., 2017; Wang et al., 2018; Poolprasert et al., 2019). Recent COI barcoding analyses in Coccinellidae further reinforce this A+T bias, supporting its reliability as a marker for species delimitation (Abdalla et al., 2022). However, the base composition of 16S rDNA gene sequences in other insects such as scale insects, kissing bugs, and mealybugs differs significantly, likely due to variations in genome composition, mutation rates, or evolutionary adaptations (Park et al., 2010; Deng et al., 2012).

According to the ABGD analysis, the initial partition remained more stable across a range of prior intraspecific divergence values compared to the recursive partition, which showed significant fluctuation and may overestimate species numbers, particularly at lower divergence thresholds. However, when the prior intraspecific divergence (P) reached 0.1000, both the initial and recursive partitions converged, supporting the presence of a barcode gap. Previous studies have shown that recursive partitioning tends to be unstable and may lead to over-partitioning, further validating the reliability of the ABGD method (Pulliandre et al., 2012; Ratnasingham and Hebert, 2013). This finding is consistent with Huang et al. (2020), who noted that the initial partition was more stable and aligned better with morphological species concepts. Similarly, recent studies on Coccinellidae COI barcodes have shown that initial partitions yield more consistent species delimitation than recursive partitions (Zhang et al., 2021).

The genetic distance between species ranged from 0.132 to 0.333, with a mean interspecific genetic distance of 0.127. The lowest interspecific distance was recorded in *B. suturalis* (0.132), while the highest was observed in *C. sexmaculata* (0.333). Similar findings were reported by Poolprasert et al. (2019), where genetic divergence among *Brumoides* species ranged from 0.11 (11%). A previous study on various ladybird beetle species also reported higher interspecific divergence for *C. sexmaculata* (0.24) and lower divergence for *Brumoides* spp. (Rain et al., 2016). More recent COI-based studies have confirmed the utility of genetic distance thresholds in identifying cryptic species within Coccinellidae (Wang et al., 2024). Similarly low interspecific divergences have been observed in butterflies of the genus *Agrodiaetus*, likely due to rapid radiation events accompanied by minimal divergence in mitochondrial DNA (Wiemers et al., 2007).

Phylogenetic analysis revealed that all lineages of ladybird beetles were reciprocally monophyletic, with strong bootstrap support (>90%) among individuals of the same species in most cases. A higher-level phylogenetic relationship was particularly evident within the tribe Coccinellini, which received the strongest bootstrap support. Previous studies have identified Coccinellini as a sister group to Chilocorini, supported by moderate to strong bootstrap values, potentially due to shared synapomorphies (Magro et al., 2010; Seago et al., 2011; Robertson et al., 2015; Escalona et al., 2017). These findings suggest that divergence

between tribes may be due to adaptive radiation, genetic variation, or differences in genetic markers. Adaptive radiation has been cited as a major factor in previous studies (Seago et al., 2011), a pattern that is also observed in the present study. Recent phylogenomic and COI-based analyses further support these relationships, emphasizing genetic variation and adaptive radiation as key drivers of tribal-level divergence (Qi et al., 2023). Our findings showed strong bootstrap support and clear lineage relationships within ladybird beetle species, aligning with results from other phylogenetic studies (Ślipiński, 2007; Poolprasert et al., 2019; Wang et al., 2019; Che et al., 2021). These genetic distance data and phylogenetic analyses confirm species identities and their relationships within the Coccinellidae family. Such findings are critical for the accurate identification of ladybird beetle species and will contribute significantly to sustainable pest management strategies.

## CONCLUSION

In conclusion, ten species of ladybird beetles were identified through both morphological and molecular methods. Genetic analysis revealed distinct intraspecific and interspecific divergences among the identified species. Phylogenetic analysis confirmed that all ladybird beetle lineages are reciprocally monophyletic. These findings highlight the need for broader studies involving extensive sampling of all Coccinellidae subfamilies across the region. Such efforts will deepen our understanding of species diversity, genetic structure, and their geographical distributions, ultimately contributing to the development of more effective and sustainable pest management practices.

## AUTHORS' CONTRIBUTIONS

AR conceptualized the study; SUK and ZI developed the methodology; AR developed the software and formally analyzed the data; AS, MW and NUK performed data archiving; AR wrote the original draft; AK, NM and ZI reviewed and edited the manuscript; AHS and SUK supervised the study.

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## 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

## REFERENCES

Abdalla, H.A., Fiteha, Y.G., Rashed, M.A.-S., Ali, R.M., Hammad, A.M.A.-S., Bream, A.S.M., Magdy, M., 2022. Molecular identification of ladybird beetles (Coccinella: Coccinellidae) using DNA barcodes. Journal of Scientific Research in Science 39(2), 179-193.

Ashfaque, M., 2013. Taxonomic studies of family Coccinellidae of Gilgit, Baltistan, Pakistan. Ph.D. Thesis, University of Agriculture, Peshawar, Pakistan, 266 pp.

Blaxter, M., Floyd, R., 2003. Molecular taxonomic for biodiversity surveys: already a reality. Trends in Ecology and Evolution 18(6), 268-269.

Caterino, M.S., Cho, S., Sperling, F.A., 2000. The current state of insect molecular systematics: a thriving Tower of Babel. Annual Review of Entomology 45(1), 1-54.

Che, L., Zhang, P., Deng, S., Escalona, H.E., Wang, X., Li, Y., Liang, D., 2021. New insights into the phylogeny and evolution of lady beetles (Coleoptera: Coccinellidae) by extensive sampling of genes and species. Molecular Phylogenetics and Evolution 156, 107045.

Collins, R.A., Cruickshank, R.H., 2013. The seven deadly sins of DNA barcoding. Molecular Ecology Resources 13(6), 969-975.

Deng, Y., Jiang, Y.H., Yang, Y., He, Z., Luo, F., Zhou, J., 2012. Molecular ecological network analyses. BMC Bioinformatics 13, 1-20.

Escalona, H.E., Zwick, A., Li, H.-S., Li, J., Wang, X., Pang, H., Hartley, D., Jermiin, L.S., Nedvěd, O., Misof, B., Niehuis, O., Ślipiński, A., Tomaszewska, W., 2017. Molecular phylogeny reveals food plasticity in the evolution of true ladybird beetles (Coleoptera: Coccinellidae: Coccinellini). BMC Evolutionary Biology 17, 151.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3, 294-299.

Halim, M., Aman-Zuki, A., Mohammed, M.A., Yaakop, S.,

2017. DNA barcoding and relationships of eight ladybugs species (Coleoptera: Coccinellidae) that infesting several crops from Peninsular Malaysia. Journal of Asia-Pacific Entomology 20(3), 814-820.

Hanif, A., Manan, A., Rehman, F.u., Ali, I., 2023. DNA barcoding as a tool for taxonomic identification of ladybird beetles (Coleoptera: Coccinellidae) from Pakistan: A review. Pak-Euro Journal of Medical and Life Sciences 6(1), 39-46.

Hebert, P.D., Barrett, R.D., 2005. Reply to the comment by L. Prendini on Identifying spiders through DNA barcodes. Canadian Journal of Zoology 83(3), 505-506.

Hebert, P.D., Cywinska, A., Ball, S.L., DeWaard, J.R., 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society of London. Series B: Biological Sciences 270(1512), 313-321.

Hodek, I., Evans, E.W., 2012. Food relationships. In: Ecology and Behavior of the Ladybird Beetles (Coccinellidae). Blackwell Publishing Ltd., Chichester, pp. 141-172.

Huang, W., Xie, X., Huo, L., Liang, X., Wang, X., Chen, X., 2020. An integrative DNA barcoding framework of ladybird beetles (Coleoptera: Coccinellidae). Scientific Reports 10(1), 10063.

Iqbal, Z., Azad, R., Jin, X., Hassan, M.A., Abbas, M., Nasir, M.F., Nie, R.E., 2024a. The review of the genus Coccinella (Coleoptera: Coccinellidae) from Pakistan. Biodiversity Data Journal 12, 137417.

Iqbal, Z., Azad, R., Nasir, M.F., Bodlah, I., Zaman, M., Hassan, M.A., Nie, R.E., 2024b. Review of ladybird tribe *Epilachnini Mulsant*, 1846 (Coleoptera: Coccinellidae) from Pakistan. Oriental Insects, 1-47.

Jalali, S.K., Ojha, R., Venkatesan, T., 2015. Molecular characterization and DNA barcoding for identification of agriculturally important insects. Insect Biochemistry and Molecular Biology 1, 317-330.

Jing, F., Yingchun, Z., 2006. Sequence analysis of mtDNA-COI gene and molecular phylogeny on twenty-seven species of coccinellids (Coleoptera: Coccinellidae). Entomotaxonomia 28(3), 179-186.

Kartika, K., Sakagami, J.I., Lakitan, B., Yabuta, S., Wijaya, A., Kadir, S., Nakao, Y., 2017. Morpho-physiological response of *Oryza glaberrima* to gradual soil drying. Rice Science 27(1), 67-74.

Koch, R.L., Galvan, T.L., 2008. Bad side of a good beetle: the North American experience with *Harmonia axyridis*. In: From Biological Control to Invasion: The Ladybird Harmonia axyridis as a Model Species, pp. 23-35.

Kumar, B., Pandey, G., Mishra, G., 2013. Predatory performance of aphidophagous ladybirds: a measure of prey suitability? International Journal of Tropical Insect Science 33(2), 120-126.

Kumar, P., Gupta, V.K., Misra, A.K., Modi, D.R., Pandey, B.K., 2009. Potential of molecular markers in plant biotechnology. Plant Omics 2(4), 141-162.

Liu, L., Guo, Z., Zhong, C., Shi, S., 2018. DNA barcoding reveals insect diversity in the mangrove ecosystems of Hainan Island, China. Genome 61, 797-806.

Magro, A., Lecompte, E., Magne, F., Hemptinne, J.L., Crouau-Roy, B., 2010. Phylogeny of ladybirds (Coleoptera: Coccinellidae): are the subfamilies monophyletic? Molecular Phylogenetics and Evolution 54, 833-848.

Marin, J., Crouau-Roy, B., Hemptinne, J.L., Lecompte, E., Magro, A., 2010. Coccinella septempunctata (Coleoptera, Coccinellidae): a species complex? Zoologica Scripta 39, 591-602.

Navajas, M., Fenton, B., 2000. The application of molecular markers in the study of diversity in acarology: a review. Experimental and Applied Acarology 24, 751-774.

Obrycki, J.J., Kring, T.J., 1998. Predaceous Coccinellidae in biological control. Annual Review of Entomology 43, 295-321.

Palandacic, A., Naseka, A., Ramler, D., Ahnelt, H., 2017. Contrasting morphology with molecular data: an approach to revision of species complexes based on the example of European *Phoxinus* (Cyprinidae). BMC Evolutionary Biology 17, 184.

Park, J.H., Wacholder, S., Gail, M.H., Peters, U., Jacobs, K.B., Chanock, S.J., Chatterjee, N., 2010. Estimation of effect size distribution from genome-wide association studies and implications for future discoveries. Nature Genetics 42, 570-575.

Poolprasert, P., Senarat, S., Nak-eiam, S., Likhitrakarn, N., 2019. Molecular taxonomic identification of predatory ladybird beetles inferred from COI sequences (Coleoptera: Coccinellidae). Malaysian Journal of Applied Sciences 4, 10-18.

Puillandre, N., Bouchet, P., Boisselier-Dubayle, M.C., Brisset, J., Buge, B., Castelin, M., Samadi, S., 2012. New taxonomy and old collections: integrating DNA

barcoding into the collection curation process. Molecular Ecology Resources 12, 396-402.

Qi, L., Xu, B., Kong, L., Li, Q., 2023. Improved phylogenetic resolution within Neritidae (Gastropoda, Nertimorpha) with implications for the evolution of shell traits and habitat. Zoologica Scripta 52, 46-57.

Rafi, M.A., Irshad, M., Inayatullah, M., 2005. Predatory ladybird beetles of Pakistan. Roohani Art Press, Blue Area, Islamabad, pp. 1-105.

Rain, F.F., Aslam, A.F., Ringki, H.S., Sultana, N., Akter, N., Howlader, A.J., 2016. Coccinellidae predators of aphids and their phylogenetic analysis using COI gene sequences. International Journal of Applied Sciences and Biotechnology 4, 408-416.

Ratnasingham, S., Hebert, P.D., 2007. BOLD: The Barcode of Life Data System. Molecular Ecology Notes 7, 355-364.

Ratnasingham, S., Hebert, P.D., 2013. A DNA-based registry for all animal species: the Barcode Index Number (BIN) system. PLoS One 8, e66213.

Robertson, J.A., Ślipiński, A., Moulton, M., Shockley, F.W., Giorgi, A., Lord, N.P., McKenna, D.D., Tomaszewska, W., Forrester, J., Miller, K.B., Whiting, M.F., McHugh, J.V., 2015. Phylogeny and classification of Cucujoidea and the recognition of a new superfamily Coccinelloidea (Coleoptera: Cucujiformia). Systematic Entomology 40, 745-778.

Seago, A.E., Giorgi, J.A., Li, J., Ślipiński, A., 2011. Phylogeny, classification and evolution of ladybird beetles (Coleoptera: Coccinellidae) based on simultaneous analysis of molecular and morphological data. Molecular Phylogenetics and Evolution 60, 137-151.

Ślipiński, A., 2007. Australian ladybird beetles (Coleoptera, Coccinellidae): their biology and classification. Australian Biological Resources Study, Canberra, Australia, pp. 1-286.

Ślipiński, A., Tomaszewska, W., 2010. Coccinellidae Latreille, 1802. In: Leschen, R.A.B., Beutel, R.G., Lawrence, J.F. (Eds.), Handbook of Zoology, Vol. 2: Coleoptera. Walter de Gruyter GmbH & Co. KG, Berlin/New York, NY, pp. 454-472.

Sobti, R.C., Kumari, M., Sharma, V.L., Sodhi, M., Mukesh, M., Shouche, Y., 2009. Sequence analysis of a few species of termites (Order: Isoptera) on the basis of partial characterization of COII gene. Molecular and Cellular Biochemistry 331, 145-151.

Tamura, K., Stecher, G., Kumar, S., 2021. MEGA11: molecular evolutionary genetics analysis version 11. Molecular Biology and Evolution 38, 3022-3027.

Urooj, Z., Ali, A., 2016. Ladybird beetles (Coleoptera: Coccinellidae) fauna of district Swabi, Nowshera and Mardan. International Journal of Agriculture and Environmental Research 2, 86-92.

Wang, Z.L., Wang, T.Z., Zhu, H.F., Wang, Z.Y., Yu, X.P., 2019. DNA barcoding evaluation and implications for phylogenetic relationships in ladybird beetles (Coleoptera: Coccinellidae). Mitochondrial DNA Part A 30, 1-8.

Wang, Z.L., Wang, X.P., Li, C.R., Xia, Z.Z., Li, S.X., 2018. Effect of dietary protein and carbohydrates on survival and growth in larvae of the *Henosepilachna vigintioctopunctata* (F.) (Coleoptera: Coccinellidae). Journal of Insect Science 18, 3.

Wang, Y., Jaworski, C.C., Zi, H., Chen, J., Desneux, N., Tan, X., 2024. Increased ladybird predation and metabolism do not counterbalance increased field aphid population growth under experimental warming. Functional Ecology 38(5), 1134-1145.

Ware, R.L., Majerus, M.E.N., Fellowes, M.D.E., 2010. Intraguild predation of immature stages of British and Japanese coccinellids by the invasive ladybird *Harmonia axyridis*. BioControl 55, 81-89.

Wiemers, M., Fiedler, K., 2007. Does the DNA barcoding gap exist? A case study in blue butterflies (Lepidoptera: Lycaenidae). Frontiers in Zoology 4, 1-16.

Zhang, H., Ning, X., Yu, X., Bu, W.J., 2021. Integrative species delimitation based on COI, ITS, and morphological evidence illustrates a unique evolutionary history of the genus Paracercion (Odonata: Coenagrionidae). PeerJ 9, e11459.