Integrative Taxonomy of Wasps: Combining Morphological and Molecular Approaches of Indian wasps based on the COI gene sequencing (Hymenoptera: Vespidae)

Mohankumar K.[1], Suja M.[1*], M. Komala[2], Kumar Krishnan[3] and Balu Prakash[4*]
1. PG and Research department of Microbiology, Hindusthan College of Arts and Science, Coimbatore - 641028, INDIA
2. Department of Pharmaceutics, School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai - 600 117, INDIA
3. Faculty of Health and Life Sciences, INTI International University, Persiaran Perdana BBN, Nilai 71800, Negeri Sembilan, MALAYSIA
4. Department of Biotechnology, School of Life Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai - 600 117, INDIA
*suja1401@gmail.com; prakazbt@gmail.com

Abstract
This study aimed to accurately identify five wasp species: Delta conoideum, Delta esuriens, Ropalidia marginata, Sceliphron distillatorium and Polybia paulista, collected from agricultural ecosystems, using both morphological characteristics and mitochondrial cytochrome c oxidase subunit I (COI) gene sequencing. Detailed taxonomic descriptions based on body size, coloration and structural features were initially used for identification. Molecular analysis of the COI gene yielded sequences ranging from 626 to 1031 base pairs, exhibiting high AT content typical of insect mitochondrial genomes.

Phylogenetic trees constructed from the COI data confirmed species identities with strong bootstrap support and revealed close relationships with existing NCBI reference sequences. The combination of morphological and molecular methods proved effective in distinguishing closely related species and understanding their evolutionary relationships. This integrative approach enhances the accuracy of wasp identification, contributing valuable data for biodiversity assessment and ecological monitoring in agroecosystems.

Keywords: Wasp identification, COI barcoding, mitochondrial DNA, phylogenetic analysis, morphological taxonomy, inland, biodiverse.

Introduction

Wasps, belonging to the order Hymenoptera, are a diverse group of insects that play vital ecological roles. Most commonly recognized wasps, such as yellow jackets and hornets, belong to the family Vespidae and are eusocial, living in colonies with a queen and sterile workers. However, the majority of wasp species are solitary, each female independently responsible for reproduction and nest building. Unlike bees and ants, which evolved from ancestral wasps, wasps do not form a monophyletic group. Morphologically, they are characterized by a narrow petiole connecting the thorax and abdomen, compound eyes and chewing-sucking mouthparts. The ovipositor in females is often modified into a sting used for defense or prey capture. Larvae are usually worm-like and develop in protected environments, either in nests or inside host organisms[8,13].

Wasps serve multiple ecological functions: some are predators or pollinators, while others are parasitoids, laying eggs on or inside other insects, eventually killing the host. Cuckoo wasps, for instance, are kleptoparasites that lay eggs in the nests of other wasps. Social wasps like *Polistes*, *Ropalidia* and *Vespula* construct various types of nests from umbrella-shaped combs under eaves to papery underground cavities[5,12]. While many wasps are beneficial as natural pest controllers, some can be nuisances when nesting close to human dwellings. Effective management includes early detection, removal of attractants and sealing entry points during spring[9].

Taxonomically, traditional morphology-based classification of wasps is labor-intensive and often limited by cryptic species. DNA barcoding has emerged as a powerful molecular tool for species identification and phylogenetic analysis. It uses a short standardized sequence of the mitochondrial cytochrome c oxidase subunit I (COI) gene which is highly conserved across eukaryotes and exhibits sufficient variability to discriminate between species[1]. This technique complements traditional taxonomy by providing rapid, reliable and cost-effective identification, particularly in morphologically ambiguous or early life stages.

The COI gene was selected for barcoding due to its ubiquity, maternal inheritance and higher evolutionary rate compared to nuclear DNA, enabling fine-scale taxonomic resolution[3]. Barcode-compliant sequences are deposited in global repositories like GenBank, EMBL and DDBJ, each linked to voucher specimens for traceability and validation (NCBI, 2023^6). DNA barcoding has proven effective in many animal groups including insects, fish and birds[10]. Though its efficacy in protists remains under investigation, studies suggest variable success depending on the group[7,11]. Integrating molecular data with morphological and ecological traits provides a comprehensive framework for wasp taxonomy and biodiversity research. This approach enhances our understanding of species boundaries,

evolutionary relationships and ecosystem roles, particularly important for ecological monitoring and pest control strategies[13].

Material and Methods

Collection of Wasps: Wasps were collected from an agricultural region at an elevation of approximately 10,926.549 meters (coordinates: 11.307317, 76.977837). No special permissions were required for collection, as the area is publicly accessible. Various nests were observed, typically containing eggs, larvae, pupae and adult wasps. Observations were made regarding the body structure, coloration and behavior of adult wasps within the nests.

Taxonomy and Identification: Morphological identification was conducted using pinned and dried specimens under a light microscope. The study followed standardized definitions for morphometric characters including the maximum width of the eye and gena (measured in lateral view), the distance between posterior ocelli and the distance between the posterior ocellus and the inner eye margin (both in dorsal view). The length of the first metasomal tergum was measured from the basal slit to the posterodorsal edge in lateral profile. The "dorsal view" of the tergum referred to the posteriorly widened surface, while the "anterodorsal view" pertained to the first metasomal sternum. Color patterns and additional morphological characters were also recorded for accurate identification.

COI/COX Gene Sequencing: Genomic DNA was extracted from wasp tissues using the EXpure DNA isolation kit (Bogar Bio Bee Stores Pvt. Ltd.). Eight wasps from each nest were preserved in 70% ethanol prior to sequencing.

PCR Protocol: Polymerase Chain Reaction (PCR) was used to amplify the mitochondrial COI gene using primers LCO1490 and HCO2198. The 25 µL reaction mixture consisted of 5 µL of DNA, 1.5 µL of each primer, 5 µL of deionized water and 12 µL of Taq Master Mix containing 2X buffer, 0.4 mM dNTPs, 3.2 mM $MgCl_2$ and 0.02% bromophenol blue. Thermal cycling conditions included denaturation at 95°C, annealing at 55°C and extension at 72°C. Unincorporated primers and nucleotides were removed using the Montage PCR Clean-Up Kit (Millipore). Sequencing was conducted with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit and AmpliTaq® DNA Polymerase FS (Applied Biosystems).

Sequencing Protocol: Single-pass sequencing was performed using 16S rRNA universal primers. The labeled DNA fragments were purified via ethanol precipitation and analyzed on an ABI 3730xl sequencer (Applied Biosystems). The resulting sequences were subjected to BLAST analysis using the NCBI similarity search tool to identify closely related species.

Phylogenetic Analyses: Phylogenetic analysis was conducted to explore evolutionary relationships among wasp species. Multiple sequence alignments were performed using MUSCLE 3.7[14] and poorly aligned regions were filtered out using Gblocks 0.91b[15]. Phylogenetic trees were constructed based on sequence similarity where closely related sequences were aligned first and progressively more divergent sequences were added. Gaps in the alignment, representing insertions, deletions, or other mutations, were managed using similarity scores from distance-based methods.

Results and Discussion

Taxonomic Identification and Morphological Characteristics: Five species of wasps were identified through detailed morphological characterization, supported by COI gene sequencing: Delta conoideum, Delta esuriens, Ropalidia marginata, Sceliphron distillatorium and Polybia paulista. Morphological traits such as body size, coloration, wing venation and sexual dimorphism provided preliminary taxonomic identification. These identifications were validated and refined through DNA barcoding using COI gene sequences.

Delta conoideum: The female of *D. conoideum* measured 23–26 mm in length with a pyriform, truncate clypeus and a smooth, shining metasoma. Males were smaller (17–19 mm) and exhibited a distinct hook-shaped terminal antennal segment and a grooved S7. The coloration included a yellow head with reddish antennae and black markings on the thorax and metasoma (Figure 1). These features align with earlier descriptions by Carpenter (1997) for the genus *Delta*.

COI sequencing yielded a 626 bp fragment (GenBank Accession No. ON502388). The high AT content (A = 31.7%, T = 44.2%) was consistent with known patterns in hymenopteran mitochondrial genomes[6]. Phylogenetic analysis placed *D. conoideum* close to *Delta conoideum* (NCBI Accession: MN344339.1), confirming the morphological identification (Figure 6).

Delta esuriens: Specimens of *D. esuriens* showed a distinctly narrowed apical portion of tergum 2 and a shallow depression on sternum 2. Females measured 20–25 mm, males 18–20 mm. The body was yellow with black patches, reddish brown appendages and reddish wings (Figure 2). Molecular analysis resulted in a 635 bp COI sequence (GenBank Accession No. ON505021) with base composition A = 32.1%, T = 40.3%. Phylogenetic analysis clustered *D. esuriens* closely with *Delta lepeletterii*, showing 100% sequence similarity, confirming species-level accuracy of the morphological data (Figure 7).

Ropalidia marginata: This species showed strong propodeal punctation and narrow yellow banding on tergum II. Females exhibited a head wider than the mesosoma and a smooth epicnemium. Coloration included a reddish-brown body with yellow markings on clypeus, scutellum and T1–T2 (Figure 3). Morphologically, these features matched previously documented R. marginata descriptions[7]. The COI

sequences (683 and 677 bp) revealed high AT richness (A = 39.6%, T = 30.6%) and confirmed the identity through alignment with NCBI sequence MN345065.1 (Figure 8). This affirms the efficiency of barcoding in differentiating closely related Ropalidiini species.

Sceliphron distillatorium: This mud dauber species was 24–28 mm long with an elongated black body and narrow yellow bands. The petiole formed nearly half the abdominal length. Yellow thoracic markings and yellow-black legs were distinctive features (Figure 4). COI sequencing (658 bp; ON505049) showed A = 35.7%, T = 38.9%. The phylogenetic tree positioned *S. distillatorium* closely with *Sceliphron curvatum* and *S. spirifex* with 100% and 98% support respectively (Figure 9). These results corroborate field identification and support previous molecular studies of Sphecidae systematics.

Polybia paulista: *P. paulista* was 16–20 mm long, with a slender black body and slightly purple iridescence. Entirely black appendages and faintly darkened wings made it morphologically distinguishable from sympatric species (Figure 5). The COI sequence was 1031 bp long (ON505023), showing a typical high AT content (A = 32.9%, T = 40.3%). Phylogenetic analysis revealed close affinity to *Polistes dominula*, with 97% sequence similarity (Figure 10), indicating strong barcoding resolution even within Vespinae genera.

This integrative approach combining traditional taxonomy and modern molecular techniques validated species identification with high confidence. Morphological characters such as punctation patterns, coloration, antennal morphology and propodeal striations were supported by COI barcoding data. COI sequencing proved highly reliable for distinguishing even closely related species, as shown by the accurate placement of *Delta* spp., *R. marginata* and *P. paulista* in phylogenetic trees with strong bootstrap support.

Figure 1: Pictures of *Delta conoideum* species and its nest: (a) Size and full view, (b) Head and Abdominal gland, (c) Venom gland, (d) Larvae, (e) Inside of the nest, (f) Nest

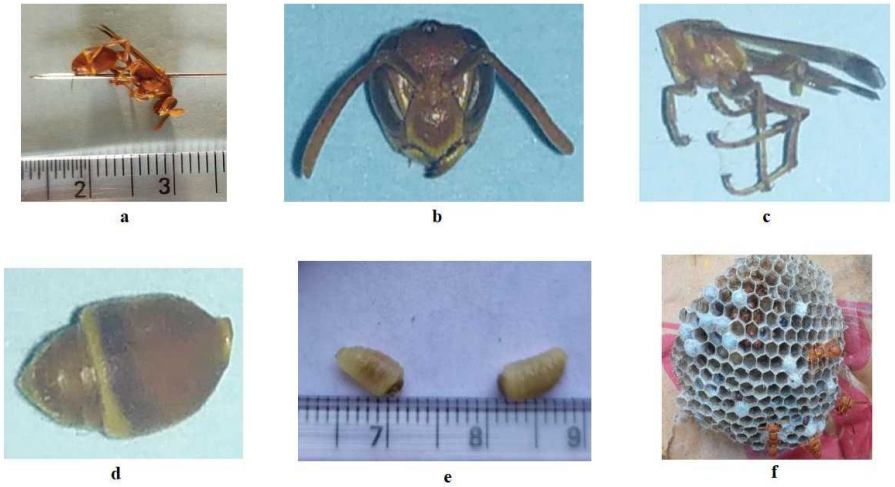

Figure 2: Pictures *Delta esuriens* and its nest: (a) and (b) Full view of the wasp, (c) Head, (d) Nest

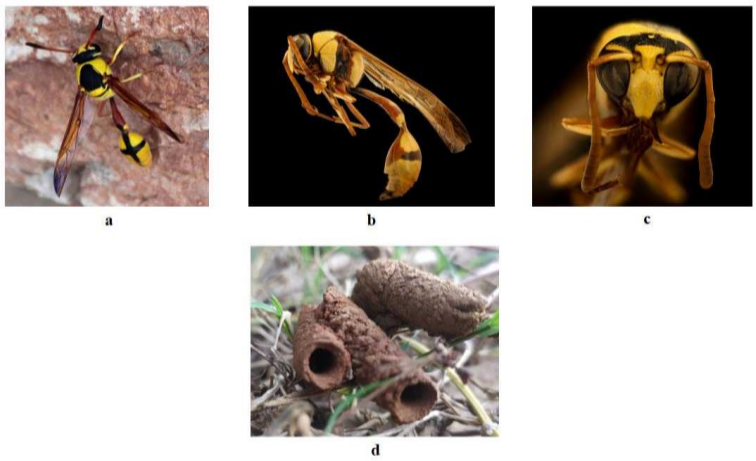

Figure 3: Pictures of *Ropalidia marginata* and its nest: (a) Size and full view of wasp, (b) Head, (c) Abdominal gland, (d) Venom gland, (e) Larvae, (f) Nest

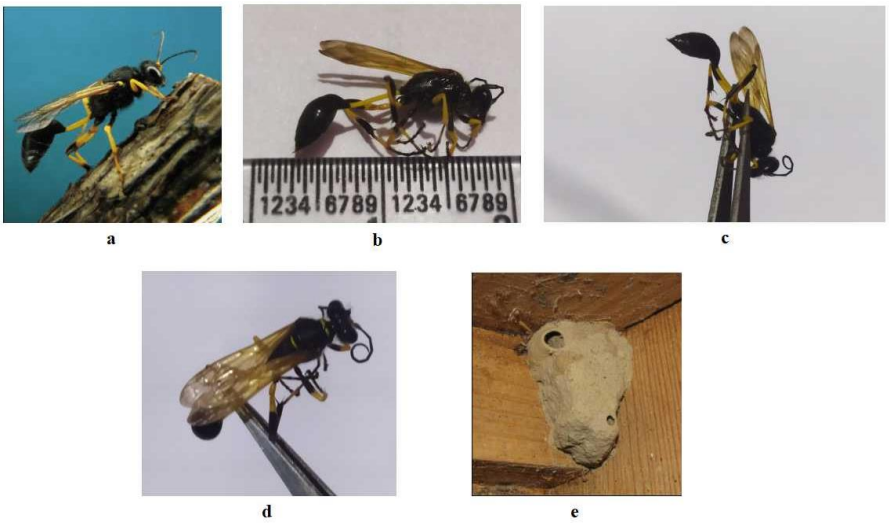

Figure 4: Pictures of *Sceliphron distillatorium* and its nest: (a) Wasp, (b) Size of the wasp, (c) Venom gland, (d) Head and Abdominal gland, (e) Nest

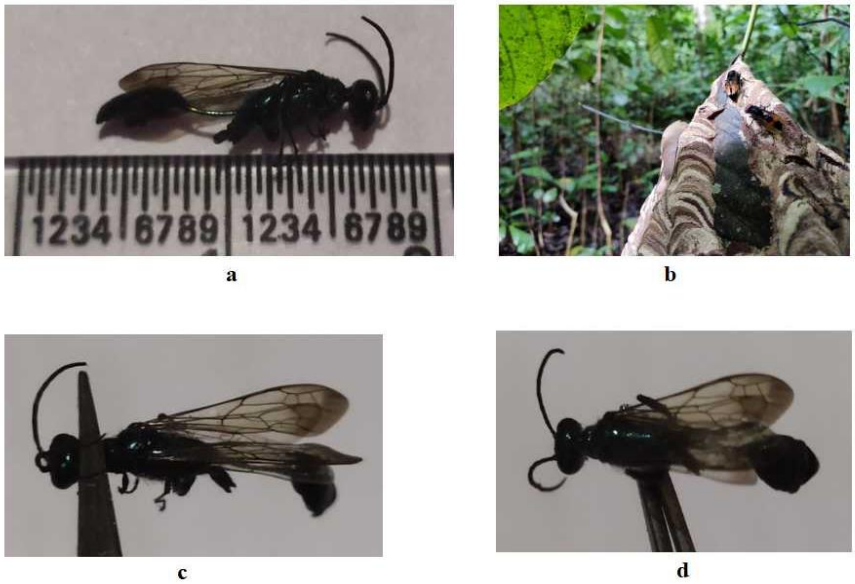

Figure 5: Pictures of *Polybia paulista* and its nest: (a) Size and Full view of wasp, (b) Nest, (c) Sting and Venom gland, (d) Antenna, Head and abdominal gland

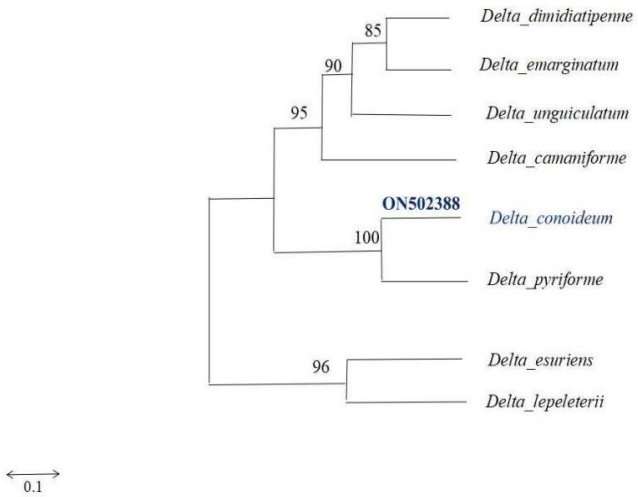

Figure 6: Phylogenetic tree of partial COI gene sequences of *Delta conoideum*

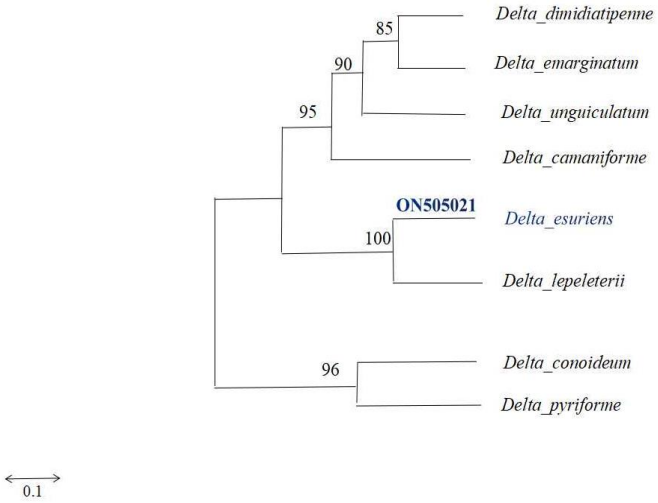

Figure 7: Phylogenetic tree of partial COI gene sequencing of *Delta esuriens*

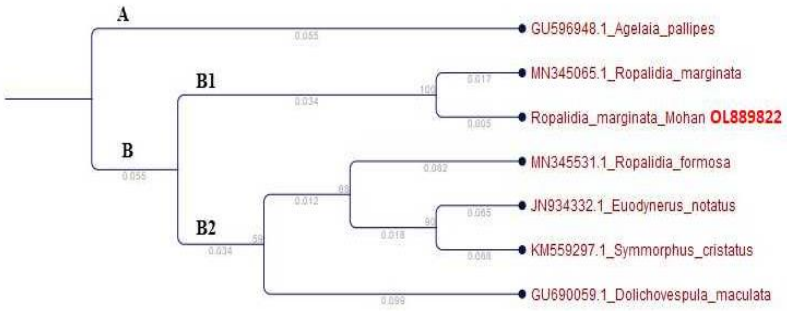

Figure 8: Phylogenetic tree of partial COI gene sequencing of *Ropalidia marginata*

These findings are consistent with Hebert et al[7] who highlighted COI barcoding's potential for species-level discrimination in animals. Minor intraspecific sequence variation was observed, suggesting local genetic drift or high gene flow among populations, particularly in agricultural habitats. The high AT content across all sequenced species reflects the conserved structure of insect mitochondrial DNA[13]. The generated phylogenetic trees (Figures 6–10) not only confirmed taxonomic placements but also provided evolutionary context. These trees highlight the relationships between the collected specimens and reference sequences, offering insight into speciation and divergence among vespid and sphecid wasps.

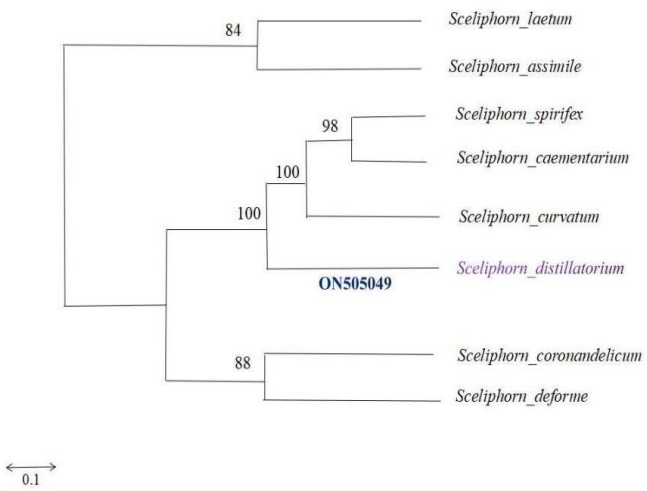

Figure 9: Phylogenetic tree of partial COI gene sequencing of *Sceliphorn distillatorium*

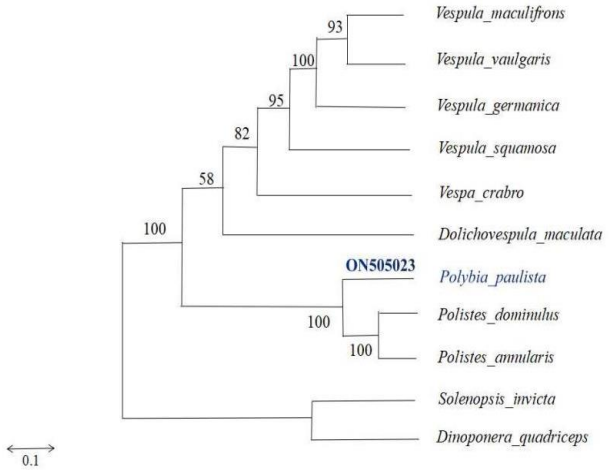

Figure 10: Phylogenetic tree of partial COI gene sequencing of *Polybia paulista*

Conclusion

This study successfully combined traditional morphological taxonomy with modern molecular techniques to accurately identify various wasp species. Detailed observations of morphological features including body coloration, nest structure and life stages (egg, larva and adult) enabled initial genus-level identification. These findings were further validated through mitochondrial COI gene sequencing.

DNA barcoding confirmed the identification of *Delta conoideum*, *Delta esuriens*, *Ropalidia marginata*, *Sceliphorn distillatorium* and *Polybia paulista*, all collected from agricultural habitats. The COI marker proved highly effective in distinguishing even closely related species, as evidenced by the strong bootstrap support in the phylogenetic trees (Figures 6–10). These trees not only confirmed species-level identification but also provided evolutionary insights by illustrating relationships between collected specimens and reference sequences from NCBI.

Minor genetic variations were observed among some populations, likely due to local gene flow or genetic drift, especially in human-modified environments. The high AT content observed across all sequenced species aligns with the conserved mitochondrial genome structure typical of insects.

The phylogenetic analysis revealed:

- *Delta conoideum* clustered closely with NCBI sequence MN344339.1.
- *Polybia paulista* showed a strong relationship with *Polistes dominula* (97% support).
- *Sceliphorn distillatorium* was closely related to *Sceliphorn curvatum* and *S. spirifex* (100% and 98% support respectively).
- *Delta esuriens* showed a 100% sequence match with *Delta lepeleterii*.
- *Ropalidia marginata* matched NCBI sequence MN345065.1 with 100% support.

This integrative approach highlights the reliability and resolution of COI barcoding in species identification and emphasizes its value in understanding wasp biodiversity and evolutionary relationships.

References

1. Antil et al, DNA barcoding, an effective tool for species identification, *Mol. Biol. Rep.*, **50**, 761–775 **(2023)**

2. Cameron Stephen L., Insect mitochondrial genomics: Implications for evolution and phylogeny, *Annu. Rev. Entomol.*, **59**, 95–117 **(2014)**

3. Du Cijie, Chen Baodan, Huang Yile, Liang Wenxi, Su Ying Hua and Liu Xingguo, Roads diverged in a wood: Proteins and RNAs encoded by cytochrome b (CYTB) gene in health and disease, *Clin. Transl. Discov.*, **5**, e70026 **(2025)**

4. Eroğlu Mücahit, Çelik Işılay, Düşükcan Mustafa, Ünal Esra Mine, Çoban Mehmet Zülfü, Gündüz Fatih and Keskin Emre, DNA barcoding of invasive *Gambusia holbrooki* Girard, 1859 and *Atherina boyeri* Risso, 1810 inhabiting Upper Euphrates River Basin, Türkiye, *Sci. Rep.*, **15**, 1907 **(2025)**

5. Gamboa James G., Nest sharing and maintenance of multiple nests by the paper wasp, *Polistes metricus* (Hymenoptera: Vespidae), *J. Kans. Entomol. Soc.*, **54**, 153–155 **(1981)**

6. Hebert Paul D.N., Cywinska Alina, Ball Shelley L. and deWaard Jeremy R., Biological identifications through DNA barcodes, *Proc. R. Soc. Lond. B Biol. Sci.*, **270**, 313–321 **(2003)**

7. Kojima Jun-ichi, Lambert Katherine, Nguyen Long T.P. and Saito Fumiko, Taxonomic notes on the paper wasps of the genus *Ropalidia* in the Indian subcontinent (Hymenoptera: Vespidae), *Entomol. Sci.*, **10**, 373–393 **(2007)**

8. Kratzer Chris Alice, The social wasps of North America, Owlfly Publishing **(2022)**

9. Kumar P.G. and Sharma G., Taxonomic studies on vespid wasps (Hymenoptera: Vespoidea: Vespidae) of Chhattisgarh, India, *J. Threat. Taxa*, **7**, 8096–8127 **(2015)**

10. Li Hongmei, Cheng Huan, Huang Renrong, Qiu Zhenya and Zhang Renyi, DNA barcoding of the genus *Discogobio* (Teleostei, Cyprinidae) in China, *Fishes*, **10**, 157 **(2025)**

11. Malaela Bibi, Tariq Nabeela, Ahmed Nazeer, Ahmed Hina Ali, Kakar Asmatullah, Kasi Sidra Khan, Ali Nida and Ahmed Nisar, Analysis of mitochondrial COI gene for species/breed level identification of the genus *Capra* (Linnaeus, 1758), *Pakistan Journal of Zoology*, **57(2)**, 811-829 **(2025)**

12. Polidori Carlo, Ferrari Andrea and Ronchetti Federico, Biology and behaviour of European wild bees, In Hidden and Wild: An Integrated Study of European Wild Bees, Springer Nature Switzerland, 49–118 **(2025)**

13. Sumana Rohini and Gadagkar Raghavendra, The structure of dominance hierarchies in the primitively eusocial wasp *Ropalidia marginata*, *Ethol. Ecol. Evol.*, **13**, 273–281 **(2001)**

14. Edgar R.C., MUSCLE: Multiple sequence alignment with high accuracy and high throughput, *Nucleic Acids Research*, **32(5)**, 1792-1797 **(2004)**

15. Talavera G. and Castresana J., Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments, *Systematic Biology*, **56(4)**, 564-577 **(2007)**.

(Received 23rd April 2025, accepted 23rd May 2025)