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DNA barcoding of *Parotis marginata* (Hampson, 1893) from the Western Ghats using the COI gene

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Abstract

The present study provides the first integrative taxonomic assessment of *Parotis marginata* (Hampson, 1893) from the Western Ghats, based on morphological and molecular evidence. The identification was supported by external morphology and COI barcode data. Genomic DNA extracted from specimens MKP15 and MKP65 yielded a 627 bp fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene. The sequences were deposited in GenBank under accession numbers PV789710 and PZ307450. BLAST analysis showed the highest similarity (99.66–100%) with *P. marginata* sequences, confirming species identity. Phylogenetic analyses using Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods produced congruent topologies, with all *P. marginata* sequences forming a strongly supported monophyletic clade. Pairwise genetic distance analysis revealed low intraspecific divergence (0–0.34%) and clear interspecific divergence (5.8–8.4%), indicating a distinct barcode gap. The results validate the taxonomic status of *P. marginata* and demonstrate the effectiveness of COI-based DNA barcoding for accurate species identification within the genus *Parotis*.

Introduction

The genus *Parotis* Hübner, 1831 comprises a group of spilomeline moths distributed predominantly in tropical and subtropical regions. *P. marginata* (Hampson,1893) was originally described as *Cenocnemis marginata* by George Francis Hampson and subsequently transferred to the genus *Glyphodes* (Hampson,1898). Following a comprehensive phylogenetic reassessment of Crambidae, the species was later reassigned to the genus *Parotis* by Shaffer, Nielsen, and Horak (1996). The genus *Parotis*, established by Jacob Hübner, is typified by *Parotis psittacalis* Hübner.

The species is widely distributed across South and Southeast Asia, including India, Indonesia, Malaysia, and Thailand, and extends into Australia and Japan. The larval stages are known to feed on a variety of host plants, including *Alstonia scholaris* (Apocynaceae), *Gardenia jasminoides* and *Haldina cordifolia* (Rubiaceae), *Bombax ceiba* (Malvaceae), and *Tabernaemontana divaricata*, the latter recently reported from India (Robinson et al., 2010; Dabhi and Bhatt, 2019).

Accurate identification of Spilomeline moths is often challenging due to morphological similarities among species, which can obscure species boundaries and complicate taxonomic resolution. In this context, molecular approaches, especially DNA barcoding, have emerged as powerful tools for species identification and biodiversity assessment. The mitochondrial COI gene has been widely adopted as a standard barcode region because of its high discriminatory power and conserved structure across taxa (Hebert et al., 2003; Folmer et al., 1994). DNA barcoding has significantly contributed to resolving cryptic diversity, clarifying phylogenetic relationships, and supporting integrative taxonomy in Lepidoptera (Hebert & Gregory, 2005; Hajibabaei et al., 2007; Shashank et al., 2022).

Despite global advances, DNA barcode coverage for Indian Lepidoptera remains limited, with substantial gaps in many groups, including the Spilomelinae, highlighting the need for expanded molecular documentation. In this context, recent studies have made significant contributions by generating new

barcode records for several Spilomeline moths, including *Dichocrocis pyrrhalis*, *Omiodes milvinalis*, and *Rhimphalea trogusalis*, particularly from the Western Ghats region of India, thereby strengthening the regional barcode reference library and improving taxonomic resolution (Praveen et al.,2025, 2026).

Although the morphology of *P. marginata* has been described in earlier taxonomic works, COI sequences of *P. marginata* are available for some regions; barcode records from India, particularly the Western Ghats, remain absent. In this study, we provide the first COI-based DNA barcode for *P. marginata* from the Western Ghats region of India. By integrating morphological examination with molecular analysis, the present work aims to establish a reliable genetic reference for the species, contribute to the growing barcode database of Spilomelinae, and enhance understanding of species diversity and distribution in this underexplored biodiversity hotspot.

MATERIALS AND METHODS

Sample collection and preservation

Two voucher specimens of *P. marginata* (MKP15-□, MKP65- □) were collected from Kuruveli, Kerala, India (12.1858° N, 75.2533° E) on 20th July 2023 and 6th July 2025. The specimens were collected, euthanized, carefully spread, dried, and subsequently deposited as voucher material in the Department of Zoology, Payyanur College, Payyanur. Identification was based on external morphological characters and relevant literature (Hampson, 1893); genitalia were not examined. One foreleg from each voucher specimen was removed and preserved in 75% ethanol for DNA barcoding.

Genomic DNA Extraction

Genomic DNA was isolated from tissue samples using the NucleoSpin® Tissue Kit (Macherey-Nagel, Germany), following the manufacturer's instructions. Briefly, tissues were lysed with T1 buffer and proteinase K at 56°C, then RNA was digested with RNase A. The DNA was purified using B3 buffer, ethanol precipitation, and eluted in BE buffer (Praveen et al.,2026).

DNA Quality Check

The quality of the extracted DNA was assessed by electrophoresis on a 0.8% agarose gel in 0.5X TBE buffer containing ethidium bromide. Gels were visualized under UV light, and images were captured using a Bio-Rad gel documentation system.

PCR Amplification of COI Gene

The mitochondrial COI gene region was amplified using universal primers. The forward primer LCO sequence was 5'-GGTCAACAAATCATAAAGATATTGG-3', and the reverse primer HCO sequence was 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'. Each PCR reaction (10 µL) contained 5 µL 2× Phire Master Mix, 4 µL nuclease-free water, 0.25 µL of each primer, and 1 µL template DNA. PCR amplification was

performed with an initial denaturation at 98°C for 30 s, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min.

PCR Product Validation

PCR products were verified using 1.2% agarose gel electrophoresis in 0.5X TBE buffer with ethidium bromide. A 2-log DNA ladder was used as a molecular marker. The PCR products were visualized under UV light and documented.

PCR Product Purification and Sequencing

The PCR products were purified using ExoSAP-IT to remove primers and dNTPs. Sequencing reactions were performed using the BigDye Terminator v3.1 Kit according to the manufacturer's protocol. The sequencing products were purified by ethanol precipitation and analyzed on an ABI 3500 DNA Analyzer.

Sequence Analysis

Sequence quality was evaluated using Sequence Scanner Software v1, and editing was done using Geneious Pro v5.1. The sequences were aligned and compared with reference databases for species identification (Drummond et al.,2010)

BLAST analysis

The obtained mitochondrial cytochrome c oxidase subunit I (COI) sequences were subjected to similarity search using the Basic Local Alignment Search Tool (BLAST) available at the National Center for Biotechnology Information (NCBI). The sequences were queried against the nucleotide database using default parameters to identify closely related taxa. The top matching sequences, along with their percentage identity, alignment length, mismatches, and bit scores, were retrieved and used to confirm species identity and assess sequence similarity with congeners.

Phylogenetic analysis

Phylogenetic relationships were inferred using both Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods. The NJ tree (Saitou and Nei, 1987) was constructed from evolutionary distances computed under the Kimura 2-parameter (K2P) model (Kimura, 1980). The optimal tree, with a total branch length of 0.423, is shown. The robustness of the inferred topology was evaluated using bootstrap analysis with 1,000 replicates, and the percentage of replicate trees in which the associated taxa clustered together is indicated above the branches. The tree was drawn to scale, with branch lengths proportional to the number of substitutions per site.

For Maximum Likelihood (ML) analysis, the best-fitting nucleotide substitution model, General Time Reversible (GTR) with Gamma distribution (+ G), was selected based on model testing. The phylogeny was inferred using the ML method, and node support was assessed with 1,000 bootstrap replicates.

The dataset comprised 22 nucleotide sequences (Table 1), including the 1st, 2nd, and 3rd codon positions and non-coding regions. All ambiguous positions were removed using the pairwise deletion method, yielding a final dataset of 1,531 positions. All evolutionary analyses were conducted using MEGA12 (Kumar et al., 2024) with up to 7 parallel computing threads.

Table 1
List of taxa used in phylogenetic analyses with accession numbers and geographic origin

Species	**Accession Number**	**Locality**
Parotis marginata	PV789710	India
Parotis marginata	PZ307450	India
Parotis marginata	NC 087054	China
Parotis marginata	HQ952922	Australia
Parotis marginata	HQ952923	Australia
Parotis marginata	GU695317	Papua New Guinea
Parotis atlitalis	OQ660436	Bangladesh
Parotis atlitalis	MN888512	India
Parotis atlitalis	HQ952927	Australia
Parotis vertumnalis	NC 087059	China
Parotis vertumnalis	AAL5908	China
Parotis punctiferalis	HQ952932	Australia
Parotis punctiferalis	HQ952933	Australia
Parotis zambesalis	PV255021	China
Parotis ogasawarensis	NC 087056	China
Parotis confinis	NC 087050	China
Parotis amboinalis	NC 087049	China
Parotis incurvata	HQ952926	Australia
Parotis incurvata	HQ952925	Australia
Parotis fallacialis	NC 087051	China
Parotis pomonalis	NC 087057	China
Omiodes milvinalis (Outgroup)	PX067245	India

RESULTS AND DISCUSSION

Taxonomic Account and Morphological Diagnosis

Tribe: Margaroniini Swinhoe & Cotes, 1889.

Genus: *Parotis* Hübner, 1831.

Parotis marginata (Hampson, 1893).

Type locality: Sri Lanka, Kandy

Adult Wingspan 36 mm (♂), 32 mm (♀). Head green; vertex and frons bright leaf-green; compound eyes black; antennae filiform. Labial palpi long, porrect, upturned; ventral surface lemon-yellow. Thorax dorsally concolourous green; tegulae cream-edged. Abdomen green; in male with inflatable coremata bearing dark brown hair pencil. Legs slender, pale brown to yellowish; tibiae ringed with brown. Forewing bright green; costa, apex and termen with a fine reddish-brown marginal line (Fig. 1A); veins distinct, slightly raised, paler than ground colour; fringe short, greenish-white, mixed with brown-tipped scales (Fig. 1B); discal spot small, black. Hindwing paler green, more rounded than forewing; termen with reddish-brown marginal line; venation typical of Spilomelinae, M2 and M3 stalked; Sc + R1 reaching costa near apex (Fig. 1).

BLAST analysis and Phylogenetic tree analysis

Genomic DNA extracted from specimens MKP15 and MKP65 yielded a 627 bp fragment of the mitochondrial COI gene. The sequences were deposited in GenBank under accession numbers PV789710 and PZ307450, respectively, and were used in subsequent analyses (Fig. 2)

The obtained mitochondrial COI sequences were subjected to BLAST analysis against the NCBI database to confirm species identity. The query sequences showed the highest similarity to *P. marginata*, with 100% identity to the reference sequence (Accession No. NC087054) over an alignment length of 607 bp, with no mismatches. Additional matches with *P. marginata* sequences (HQ952922, GU695317, HQ952923) also showed high similarity, ranging from 99.66% to 99.67%, with only two mismatches (Table 2).

Sequences of closely related species showed comparatively lower similarity. *Parotis atlitalis* exhibited 94.23% identity, followed by *P. vertumnalis* (94.07%), *P. punctiferalis* (93.41–93.58%), and *P. incurvata* (93.52%). Other congeners, including *P. confinis*, *P. zambesalis*, *P. ogasawarensis*, *P. amboinalis*, *P. fallacialis*, and *P. pomonalis*, showed sequence similarity ranging from 92.07% to 93.41%.

The high sequence similarity (≥ 99.6%) with reference sequences of *P. marginata* and clear divergence from other congeners confirm the accurate identification of the specimens as *P. marginata*.

Table 2
BLAST similarity results of COI sequences (PV789710 and PZ307450) showing percent identity, alignment length, mismatches, and bit score of the studied specimens against reference sequences, indicating the highest similarity with *P. marginata* and clear divergence from other congeners.

Scientific name	Accession number	% of identity	Alignment length	Mismatches	Bitscore
Parotis marginata	NC087054	100.00	607	0	1122
Parotis marginata	HQ952922	99.67	607	2	1110
Parotis marginata	GU695317	99.67	607	2	1110
Parotis marginata	HQ952923	99.66	587	2	1074
Parotis atlitalis	OQ660436	94.23	607	35	928
Parotis atlitalis	MN888512	94.23	607	35	928
Parotis atlitalis	HQ952927	94.23	607	35	928
Parotis vertumnalis	NC087059	94.07	607	36	922
Parotis punctiferalis	HQ952932	93.58	607	39	905
Parotis incurvata	HQ952926	93.52	571	37	850
Parotis punctiferalis	HQ952933	93.41	607	40	900
Parotis confinis	NC087050	93.41	607	40	900
Parotis zambesalis	PV255021	93.41	607	40	900
Parotis ogasawarensis	NC087056	93.25	607	41	894
Parotis amboinalis	NC087049	93.08	607	42	889
Parotis fallacialis	NC087051	92.43	608	44	867
Parotis pomonalis	NC087057	92.07	605	48	852

The phylogenetic relationships inferred using the NJ (Fig. 3) and ML (Fig. 4) methods showed largely congruent topologies, supporting the robustness of the dataset and the reliability of the inferred evolutionary relationships. In both analyses, all *P. marginata* sequences, including the newly generated Indian samples (PV789710 and PZ307450), clustered into a strongly supported monophyletic clade with conspecific sequences from China, Australia, and Papua. The minimal branch lengths within this clade and high bootstrap support (up to 100%) indicate very low intraspecific divergence and confirm the taxonomic identity of the Indian specimens as *P. marginata*.

The *P. marginata* clade was clearly separated from other congeners, forming a distinct lineage. Closely related species, such as *P. atlitalis* and *P. vertumnalis*, formed adjacent clades with relatively short inter-

branch distances, suggesting a close evolutionary relationship. Notably, *P. atlitalis* (India, Australia, Bangladesh) clustered together with strong support (bootstrap = 100), while *P. vertumnalis* sequences from China also formed a well-supported cluster. The low genetic divergence between *P. atlitalis* and *P. vertumnalis* (~ 0.49%) may indicate recent divergence or potential cryptic speciation, warranting further investigation using additional molecular markers and morphological characters.

Other species, including *P. punctiferalis*, *P. zambesalis*, *P. ogasawarensis*, *P. confinis*, and *P. amboinalis*, formed distinct and well-separated clades, each supported by moderate to high bootstrap values. The species *P. incurvata* formed a separate lineage with strong bootstrap support (100%), indicating clear genetic differentiation from other taxa.

The more distantly related species *P. fallacialis* and *P. pomonalis* appeared as basal lineages within the genus. The outgroup taxon, *Omiodes milvinalis*, was clearly separated from all *Parotis* species, forming a distinct lineage with a long branch length, thereby validating its use as an appropriate outgroup for rooting the tree.

However, the ML tree provided improved resolution and stronger statistical support for several internal nodes compared to the NJ tree. This is likely due to the model-based nature of the ML method, which incorporates nucleotide substitution patterns and site-rate heterogeneity (GTR + G), resulting in more accurate estimates of branch lengths and evolutionary relationships. In particular, closely related taxa such as *P. atlitalis* and *P. vertumnalis* were better resolved in the ML analysis, highlighting their close evolutionary affinity. Additionally, bootstrap support values were generally higher and more consistent in the ML tree, further strengthening confidence in the inferred clades.

The pairwise genetic distance analysis based on the COI gene revealed very low intraspecific divergence within *P. marginata* (0–0.34%), indicating genetic homogeneity across geographically distant populations. In contrast, interspecific distances between *P. marginata* and other congeners ranged from 5.8% to 8.4%, demonstrating a clear barcode gap. The outgroup species *Omiodes milvinalis* exhibited high divergence (10.8–13.1%), supporting its suitability for phylogenetic rooting (Table 3**).**

Table 3
Pairwise genetic distance ranges (K2P) between *P. marginata* and other congeners based on COI sequences, showing clear interspecific divergence (≈ 5.8–8.4%) and a distinct barcode gap.

Species name	Distance range
P. atlitalis	0.058–0.061
P. vertumnalis	0.060–0.062
P. punctiferalis	0.065–0.070
P. incurvata	0.064–0.070
P. confinis	0.065–0.069
P. zambesalis	0.067–0.069
P. ogasawarensis	0.069–0.071
P. amboinalis	0.069–0.073
P. fallacialis	0.078–0.082
P. pomonalis	0.083–0.084

Overall, the congruence between NJ and ML topologies, combined with the ML tree's higher resolution, supports the validity of the phylogenetic inference. These results are also consistent with the pairwise genetic distance analysis, which revealed low intraspecific and high interspecific divergence, thereby reinforcing species delimitation within the genus *Parotis*.

CONCLUSION

The present study provides COI barcode data for *P. marginata* from the Western Ghats, India, and supports its identification through external morphology, BLAST similarity, phylogenetic clustering, and pairwise genetic distance analysis. The newly generated sequences clustered with conspecific *P. marginata* sequences, showed low intraspecific divergence, and were clearly separated from other congeners. These results strengthen the regional barcode reference library for Indian Spilomelinae and demonstrate the usefulness of COI barcoding for species-level identification in *Parotis*. Future studies should include broader geographic sampling, genitalia examination, and additional molecular markers to improve understanding of species limits and evolutionary relationships within the genus.

Declarations

CONFLICTS OF INTEREST

No conflict of interest

FINANCIAL SUPPORT

No financial support

ETHICAL APPROVAL

This study involved insect specimens only and did not require ethical approval.

AUTHOR CONTRIBUTION STATEMENT

MKPK and MPU conceived and designed the research. AMV and PVM conducted the experiments and collected data. SJ contributed experimental materials. MKPK and AMV analyzed and interpreted the data. MKPK and SJ wrote the manuscript. All authors read and approved the manuscript.

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Figures

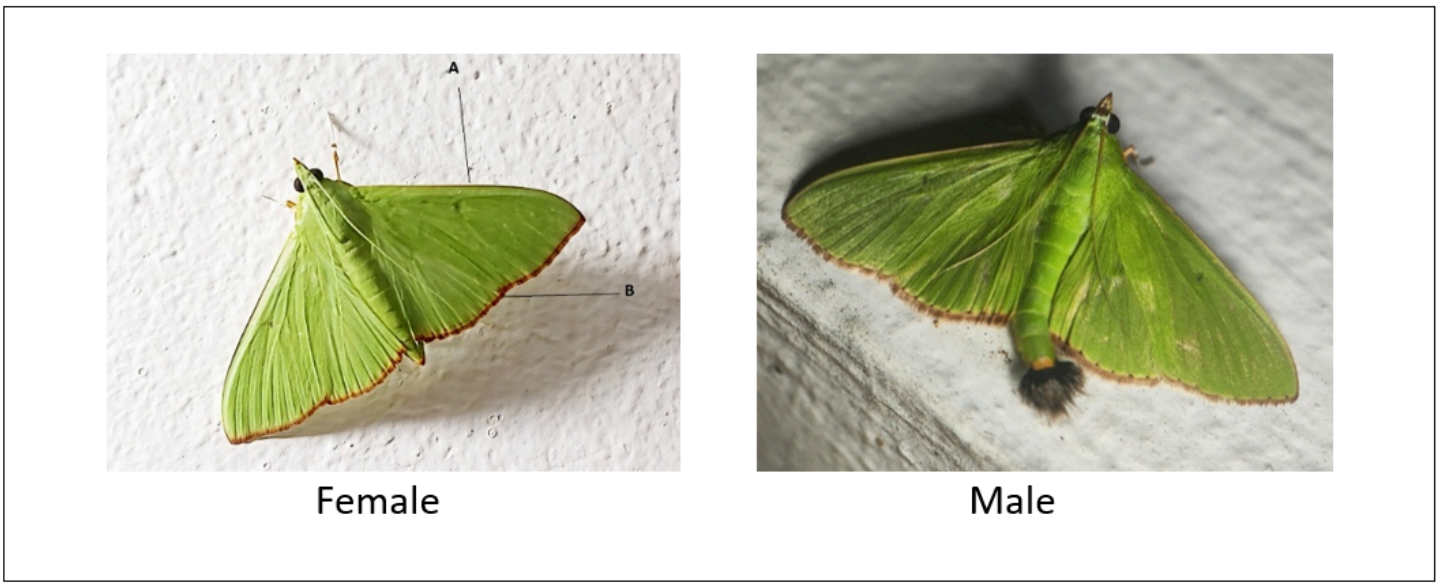
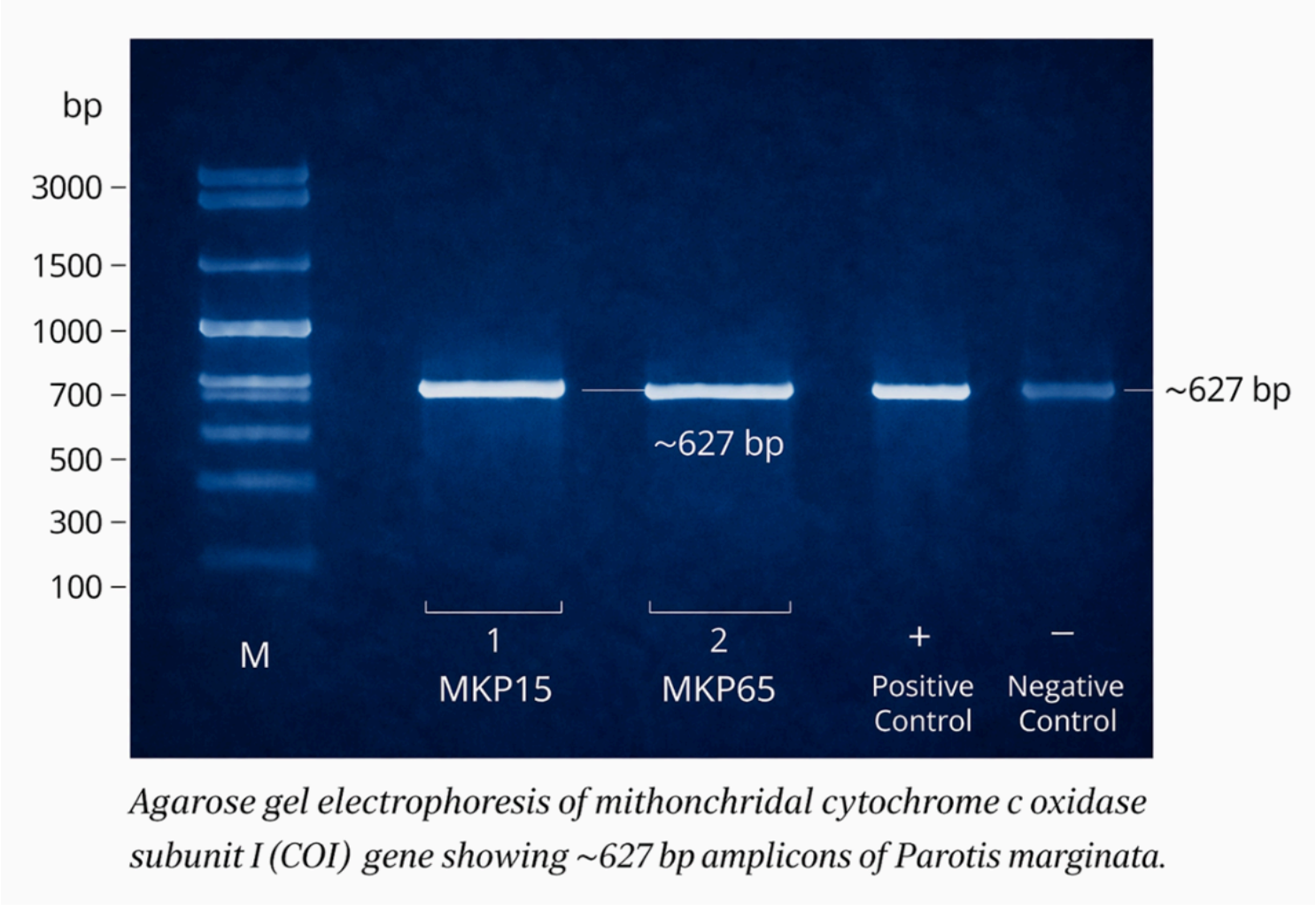


Figure 1

Live habitus image of *Parotis marginata* (Hampson, 1893)



Agarose gel electrophoresis of mithonchridal cytochrome c oxidase subunit I (COI) gene showing ~627 bp amplicons of Parotis marginata.

Figure 2

Agarose gel electrophoresis showing amplification of the mitochondrial COI gene fragment of *P. marginata*. Lane M: DNA ladder; Lane 1: MKP15 (accession PV789710), showing a 627 bp amplicon; Lane 2: MKP65 (PZ307450), showing a similar ~627 bp COI amplicon. M: DNA ladder (100–3000 bp).

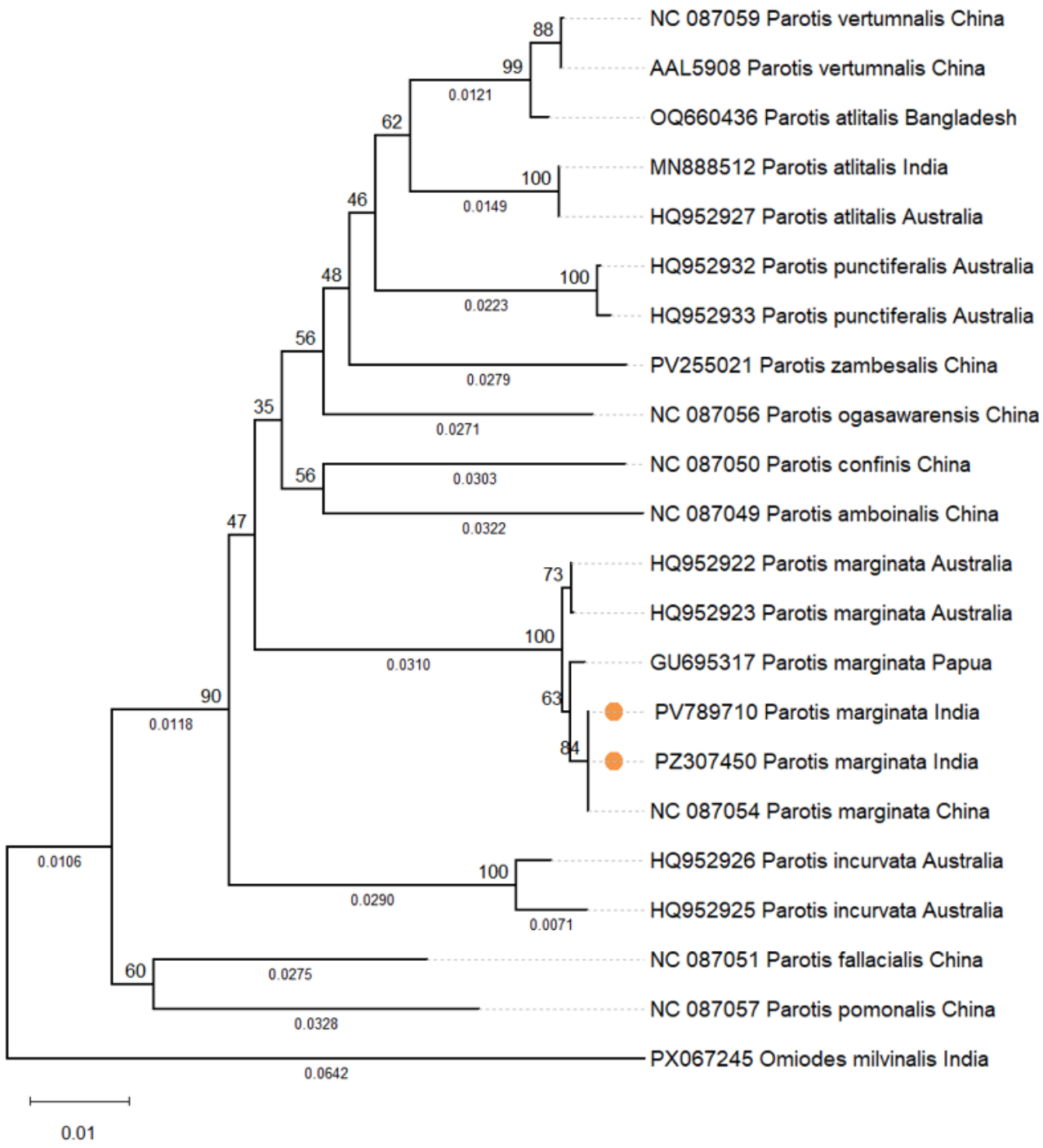


Figure 3

Neighbor-Joining (NJ) tree based on COI sequences showing relationships among *Parotis*species. Bootstrap values are indicated at nodes, and branch lengths represent genetic distances (scale bar = 0.01 substitutions/site). Newly generated *P. marginata* sequences are highlighted; *Omiodes milvinalis* was used as the outgroup.

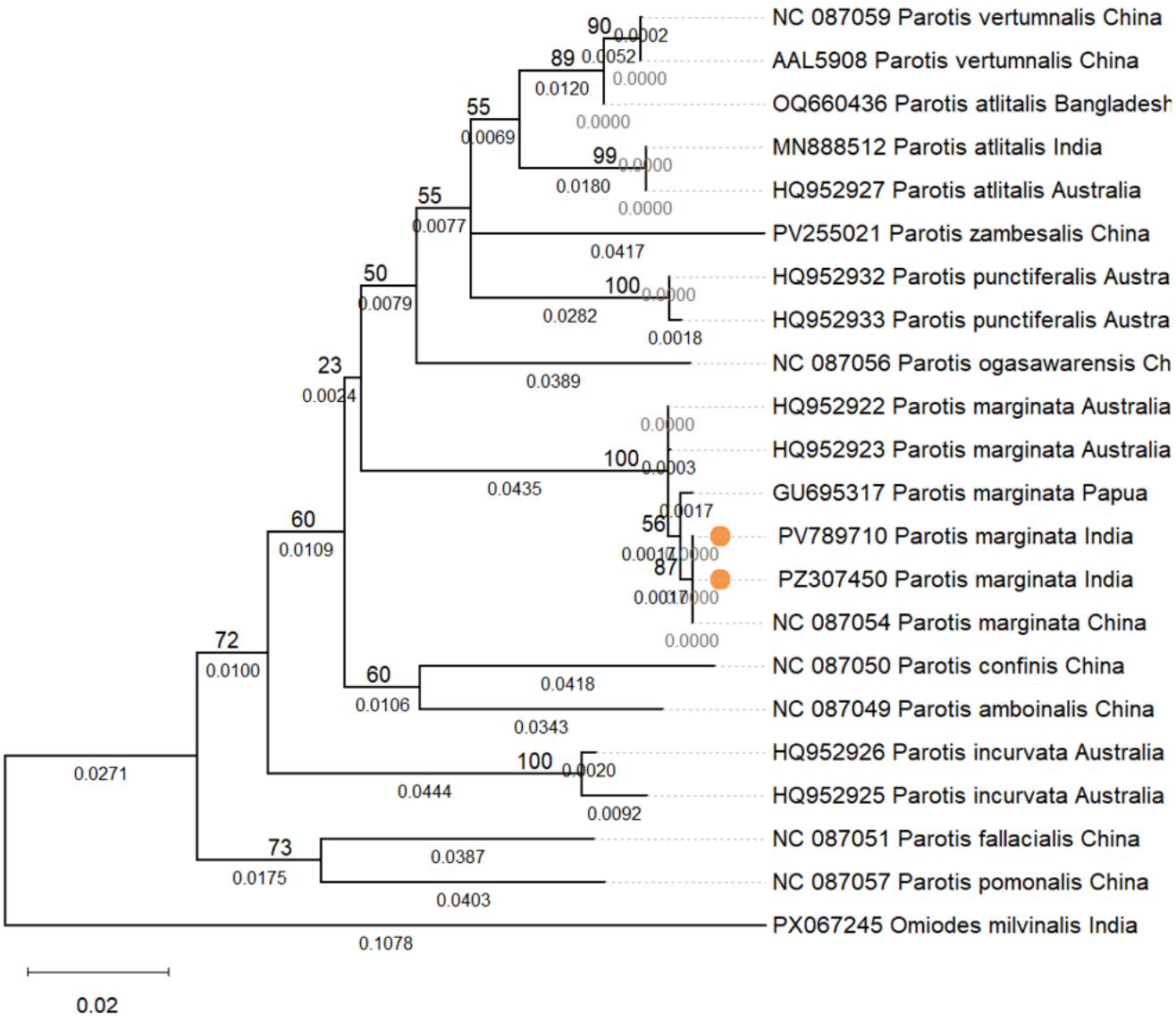


Figure 4

Maximum Likelihood (ML) phylogenetic tree based on mitochondrial COI gene sequences showing relationships among *Parotis*species. The tree was constructed using the GTR+G model with 1,000 bootstrap replicates. Bootstrap values are indicated at nodes, and branch lengths represent substitutions per site (scale bar = 0.02). Newly generated *P. marginata*sequences are highlighted; *Omiodes milvinalis* was used as the outgroup.