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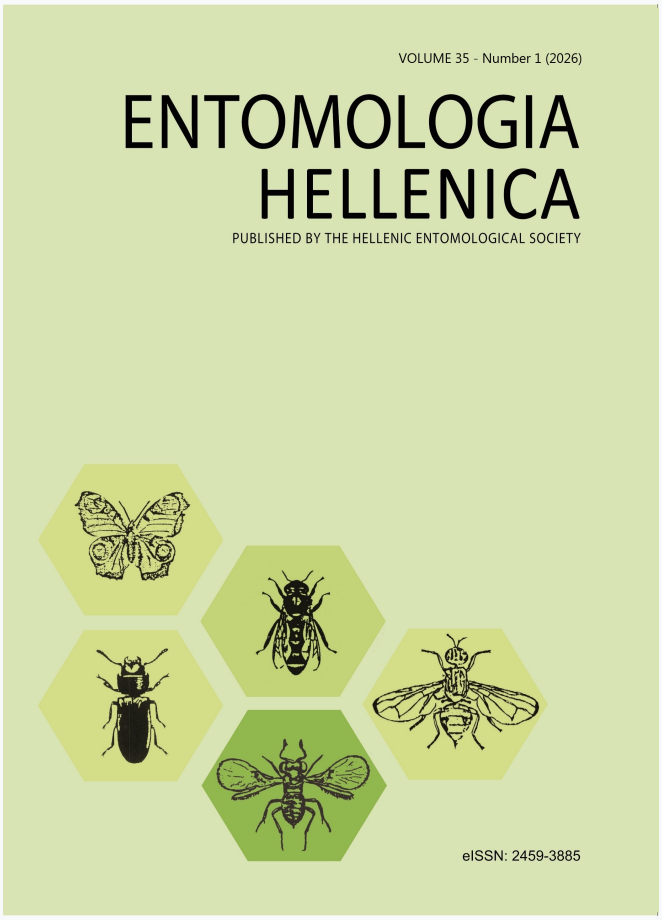

First record and DNA barcoding of the Oriental ant cricket Myrmecophilus orientalis in Rhodes, Greece (Orthoptera: Myrmecophilidae)

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First record and DNA barcoding of the Oriental ant cricket *Myrmecophilus orientalis* in Rhodes, Greece (Orthoptera: Myrmecophilidae)

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ABSTRACT

The ant cricket *Myrmecophilus orientalis* Stalling, 2010 is a recently described myrmecophilous orthopteran previously known in Greece only from the islands of Kos and Tilos. Here, we report the first confirmed record of the species from Rhodes (Dodecanese, Greece), based on both morphological examination and mitochondrial DNA barcoding of the Cytochrome c Oxidase I (COI) gene. Specimens were collected during targeted surveys of ant nests across the island. Adults were found both within nests of *Camponotus samius* and under stones without ants, while juveniles were recorded inside nests of *Pheidole koshewnikovi*, representing a new host association for the species. COI sequences (655 bp) obtained from three individuals were identical and formed a single haplotype. BLASTn searches showed ≥99% similarity to published sequences of *M. orientalis* from Kos. Maximum Likelihood analysis recovered a well-supported monophyletic clade including all Aegean specimens, clearly separated from other European congeners (*M. acervorum*, *M. balcanicus*, *M. baronii*, and *M. ochraceus*). The concordance between morphological and molecular evidence confirms the presence of *M. orientalis* on Rhodes and highlights the value of DNA barcoding for species delimitation and faunistic surveys of cryptic myrmecophilous Orthoptera in the Mediterranean region.

KEY WORDS: DNA barcoding, host associations, island biogeography, Mediterranean biodiversity, myrmecophiles.

Introduction

DNA barcoding has become an essential tool in insect systematics, biodiversity assessment, and biosecurity, relying primarily on a standardized fragment of the mitochondrial Cytochrome c Oxidase I (COI) gene to enable rapid and comparable species identification across diverse taxa (Hebert et al. 2003; Ratnasingham and Hebert 2007). In insects, COI barcodes have proven highly effective for distinguishing closely related species, uncovering cryptic diversity, and linking life stages or sexes that are otherwise difficult to associate morphologically. DNA barcoding has also been increasingly applied to Greek arthropod fauna, contributing to new faunistic records (e.g. Kalaentzis et al. 2026a, 2026b) and early detection of biological invasions (e.g. Kalaentzis et al. 2021, 2023, 2026c). By providing an objective and reproducible framework for species identification, DNA barcoding continues to enhance our understanding of insect diversity, biogeography, and host associations.

Ant crickets of the genus *Myrmecophilus* Berthold, 1827 are minute orthopterans that live inside ant nests, with most of them being considered kleptoparasitic (Hölldobler and Kwapich 2022). The genus has an almost cosmopolitan distribution, with 11 species currently recorded from Europe, with five of them being recorded in Greece (Willemse et al. 2018; Stalling et al. 2020; Zafeiriou and Stalling 2023), namely *Myrmecophilus hirticaudus* Fischer von Waldheim, 1846, *Myrmecophilus myrmecophilus* (Savi, 1819), *Myrmecophilus ochraceus* Fischer, 1853 and, quite recently, *Myrmecophilus acervorum* (Panzer, [1799]) and *Myrmecophilus orientalis* Stalling, 2010. The latter was previously known only from

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Jordan, Israel and eastern Turkey (Stalling 2010; Stalling 2024), but has been recorded from the islands of Kos and Tilos (S. Aegean, Greece; Stalling et al. 2020). In Rhodes, only *M. ochraceus* has been recorded (Willemse et al. 2018).

Herein, we report the first confirmed occurrence of *M. orientalis* from the island of Rhodes (Dodecanese, Greece), based on both morphological examination and DNA barcoding of the mitochondrial COI gene (Fig. 1). We provide new faunistic records, ecological observations on host ant associations, and the first barcode data for the species from Rhodes. The molecular results are compared with previously published sequences from other Aegean populations in order to assess genetic similarity and further corroborate species identity.

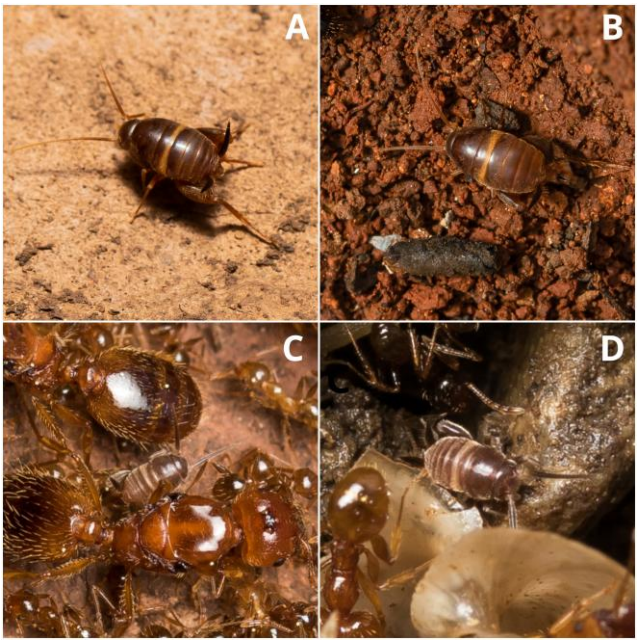

FIG. 1. Oriental ant cricket *Myrmecophilus orientalis* in Rhodes, Greece. (A) Adult specimen on Mt. Attaviros. (B) Adult specimen in *Camponotus samius* nest on Mt. Akramytis. (C) Nymph in *Pheidole koshewnikovi* nest on Mt. Attaviros. (D) Nymph in *Pheidole koshewnikovi* nest near Profitis Avakoum chapel.

Materials and Methods

Field surveys for arthropod specimen collection were carried out throughout the island of Rhodes from September 2024 to March 2025 by the first author. Ant nests were located by overturning stones, tree trunks and debris. Specimens were collected and preserved in absolute ethanol. Additional photographic records from previous field surveys are also presented here. Cricket specimens were identified following Stalling (2010), while ants were determined using the keys and criteria of Salata et al. (2020).

DNA extraction was performed at the Wellcome Sanger Institute using a minimally-destructive protocol (Korlević and Lawniczak, 2023). Briefly, the specimens were transported to Sanger in 96-well plates, with each well containing 100 µl of 100% ethanol. Ethanol was removed using a robot and 100 µl of the Lysis C buffer was added to each specimen and incubated overnight at 56°C. Extracted unpurified DNA was moved to a new lysis plate and 100 µl of 70% ethanol was put back on to the specimens in the original plate. Lysis C buffer is 200 mM Tris-HCl pH 8.0; 25 mM EDTA pH 8.0; 0.05% Tween-20 that was supplemented immediately before use with Proteinase K (0.4mg ml-1). Approximately 0.1 µl of DNA was then used for DNA barcoding of the mitochondrial Cytochrome c oxidase I (COI) gene, using a two-step PCR protocol followed by PacBio sequencing as described in Park et al. (2023). Demultiplexing and consensus sequence generation was completed using mBRAVE.

The DNA Barcode was compared with existing sequences using Barcode of Life Data Systems (BOLD) and BLASTn (GenBank) to identify the closest matches based on genetic similarity. Following identification, the sequence was submitted to the BOLD database with accompanying metadata for reference and potential future use. Pairwise genetic distances were calculated among the Rhodes specimens, among the available Kos sequences, and between the Rhodes and Kos sequences.

A total of 12 nucleotide sequences of the mitochondrial COI marker were accessed and downloaded from GenBank and BOLD databases belonging to *M. acervorum*, *M. balcanicus*, *Myrmecophilus baronii* Baccetti, 1966 and *M. ochraceus*. All sequences, including the DNA Barcodes generated in this study were aligned in MEGA (Kumar et al. 2024) and inspected manually to ensure positional homology. Maximum Likelihood phylogenetic inference was performed using the Generalised Time Reversible with invariant sites model (GTR+I), which was selected as the optimal nucleotide substitution model based on information criteria in MEGA. Node support was assessed using nonparametric bootstrap analysis with 1,000 replicates.

Results

***Myrmecophilus orientalis* Stalling, 2010**

New records. GREECE — Dodecanese/Rhodes • Mt. Attaviros; 36.20117, 27.81885; alt. 608 m; 20.X.2024; K. Kalaentzis leg.; 2 adult specimens; under a stone • Mt. Akramytis; 36.140779, 27.742245; alt. 385 m; 23.III.2025; K. Kalaentzis leg.; 3 adult specimens; under a stone, within a *Camponotus samius* nest; BOLD Sample IDs: TOL-BGEG-138_F1, TOL-BGEG-138_G1, TOL-BGEG-138_H1. • Mt. Attaviros; 36.20061, 27.81990; alt. 613 m; 12.IV.2020; K. Kalaentzis obs.; 1 nymph; under a stone, within a *Pheidole koshewnikovi* nest; photographic record • Profitis Avakoum chapel; 36.17024, 27.77282; alt. 421 m; 29.XII.2020; K. Kalaentzis obs.; 1 nymph; under a stone, within a *Pheidole koshewnikovi* nest; photographic record.

The specimens were morphologically identified as *Myrmecophilus orientalis* based on their relatively large size, with adult specimens measuring approximately 4 mm in length, their brown bodies with a distinctly pale ochreous mesonotum, and, in the females, their blunt, double-pointed dorsal valvae in lateral view.

All three sequences belonged to a single haplotype; the obtained COI sequence (655 bp) was queried against the NCBI GenBank database using BLASTn to assess sequence similarity and confirm species identity. The sequence showed a very high similarity (≥99%) to reference sequences of *M. orientalis* available in GenBank, with no close matches to other *Myrmecophilus* species. This result corroborates the morphological identification and supports the assignment of the examined specimens to *M. orientalis*, excluding potential confusion with similar species.

Pairwise COI p-distances among the examined *M. orientalis* sequences were low. The three Rhodes specimens shared a single haplotype, resulting in 0.00% within-Rhodes p-distance. The four available Kos sequences differed from each other by 0.00–0.77%, whereas the Rhodes haplotype differed from the Kos sequences by 0.92–1.38% (Table 1).

TABLE 1. Pairwise COI p-distances among *M. orientalis* sequences from Rhodes and Kos. Values are presented as percentages.

Comparison	n	p-distance (%)
Within Rhodes	3	0.00%
Within Kos	4	0.00–0.77%
Rhodes vs. Kos	3 vs. 4	0.92–1.38%

The COI-based phylogenetic analysis recovered well-supported, species-level clades within *Myrmecophilus*, corresponding to the currently recognized taxa (*M. balcanicus*, *M. acervorum*, *M. orientalis*, *M. baronii*, and *M. ochraceus*) (Fig. 3). All specimens of *M. orientalis*, including the newly generated sequences from Rhodes (TOL-BGEG-138_F1/G1/H1), formed a strongly supported and cohesive monophyletic group together with previously published sequences from Kos. The Rhodes haplotype clustered unambiguously within the *M. orientalis* clade and showed minimal divergence from the Kos material, indicating low intraspecific genetic differentiation in the COI marker across the southeastern Aegean populations. This clade was clearly separated from the remaining species, each of which formed distinct and well-supported lineages. Overall, the topology confirms the unambiguous taxonomic placement of the Rhodes specimens within *M. orientalis*.

Discussion

This is the first confirmed record of *Myrmecophilus orientalis* from Rhodes, while another specimen from Rhodes in the collection of the Natural History Museum Vienna, Austria, likely corresponds to the same species. Its geographical distribution in Greece might also include other islands of the Dodecanese, a subject for future field surveys (Fig. 2), since the close affinity between the Rhodes specimens and the available material from Kos suggests that these island populations may represent part of a broader southeastern Aegean lineage. Rather than indicating recent dispersal, the presence of the species on the two highest mountains of Rhodes may reflect older natural colonization events, possibly linked to past periods of geographical connectivity between the southeastern Aegean islands and the Anatolian mainland. However, the currently available molecular and distributional data are insufficient to infer the precise timing or direction of colonization. Additional sampling from neighbouring islands and adjacent Anatolian regions will be necessary to clarify the origin, population structure, and dispersal history of *M. orientalis* in the southeastern Aegean.

FIG. 2. Geographic distribution of *Myrmecophilus orientalis*. Literature records are shown in red circles, while the specimens collected and observed in the present study are depicted with red triangles. The type locality is indicated with a red star. Scale bar 300 km; Inset: Geographic distribution of *M. orientalis* in Greece (South Dodecanese). Scale bar 40 km.

Adult individuals of *M. orientalis* have been recorded in nests of *Camponotus* species, namely *Camponotus baldaccii* Emery, 1908 and *Camponotus samius* Forel, 1889 (Stalling et al. 2020), while juveniles have been found in nests of *Crematogaster erectepilosa* Salata and Borowiec, 2015 and *Lepisiota frauenfeldi* (Mayr, 1855) (Stalling et al. 2020). In this study, two adult specimens were found under a stone without ants, perhaps during their migration process to a new nest, while the other three specimens were found in a *C. samius* nest (as on the island of Kos; Stalling et al. 2020) (Fig. 1). Both juvenile individuals were discovered and photographed within nests of *Pheidole koshewnikovi*, thereby adding this ant species to the list of known hosts associated with the juvenile stages of *M. orientalis*.

The first DNA barcoding sequences of *M. orientalis* were recently published by Cassar et al. (2025) from material collected on Kos. Comparison of the COI sequences generated in the present study with those available from Kos revealed very high similarity, with all Rhodes specimens sharing a single haplotype. In the Maximum Likelihood analysis, the Rhodes sequences clustered tightly with the Kos material in a well-supported monophyletic clade, clearly separated from other congeners (*M. acervorum*, *M. balcanicus*, *M. baronii*, and *M. ochraceus*). Preliminary evidence indicates that the shallow divergence among Aegean populations possibly corresponds to limited time since population divergence (Fig. 3). At the same time, the clear interspecific distances

and strong bootstrap support across the tree highlight the reliability of COI barcoding for species-level delimitation within *Myrmecophilus*, although future studies combining COI with additional mitochondrial and nuclear markers, together with denser geographical sampling, will be necessary to better assess the phylogeographic structure, population connectivity, and possible dispersal routes of *M. orientalis* in the Aegean region.

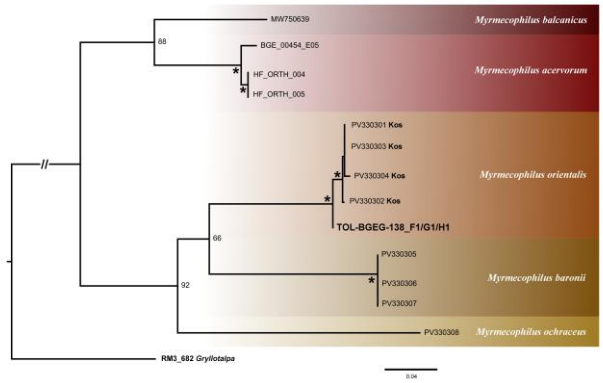

FIG. 3. COI-based phylogenetic tree of selected *Myrmecophilus* species showing the placement of the specimens from Rhodes, Greece within the *M. orientalis* clade. Numbers at nodes indicate bootstrap support; values above 95 are marked with an asterisk. GenBank accession numbers and BOLD Data Systems Sample IDs are shown for each sequence. Scale bar represents genetic distance (substitutions per site).

Overall, the concordance between morphological identification, ecological data, and molecular evidence provides robust confirmation of the occurrence of *M. orientalis* on Rhodes. The integration of DNA barcoding with traditional taxonomy proves particularly valuable in groups such as ant crickets, where small body size, morphological conservatism, and cryptic lifestyles may obscure species boundaries. Further sampling across the eastern Mediterranean, combined with broader population-level genetic analyses, will help clarify patterns of dispersal, host associations, and historical biogeography within this specialised and understudied genus.

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Author Contributions

Conceptualization: KK. Data curation: KK, TS. Formal analysis: KK. Funding acquisition: AT. Investigation: KK. Methodology: KK, TS. Resources: EK, WSIBC. Supervision: AT. Visualization: KK. Project administration: AT. Software: KK, WSIBC. Validation: KK. Writing – original draft: KK, TS. Writing – review and editing: KK, EK, AT, WSIBC, TS.

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Data Availability

All data that support the findings of this study are available in the main text and BOLD Data Systems under the Sample IDs TOL-BGEG-138_F1, TOL-BGEG-138_G1 and TOL-BGEG-138_H1.

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