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43/2 Varadarajulu Nagar, 5th Street West, Ganapathy, Coimbatore, Tamil Nadu 641006, India
Registered Office: 3A2 Varadarajulu Nagar, FCI Road, Ganapathy, Coimbatore, Tamil Nadu 641006, India
Ph: +91 9385339863 | www.threatenedtaxa.org
Email: sanjay@threatenedtaxa.org

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Cover: Coromandal Sacred Langur *Semnopithecus priam* - made with acrylic paint. © P. Kritika.

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COMMUNICATION

Description of a new species of the genus *Anthaxia* (*Haplanthaxia* Reitter, 1911) from India with molecular barcoding and phylogenetic analysis

S. Seena [1], P.P. Anand [2] & Y. Shibu Vardhanan [3]

[1,2,3] Biochemistry & Toxicology Division, Department of Zoology, University of Calicut, Kozhikode, Kerala 673635, India.
[1] seena.s1984@gmail.com, [2] anandpp633@gmail.com (corresponding author), [3] svardhanan@gmail.com (corresponding author)
[1,2] These authors contributed equally to this work.

Abstract: This paper deals with the description of a new *Anthaxia* (subgenus *Haplanthaxia* Reitter, 1911) species from southern India, which belongs to the *Anthaxia* (*H.) winkleri* Obenberger, 1914 species-group: *Anthaxia* (*H.*) *keralensis* **sp. nov.** In addition to a morphological description, we also generated mt. COI DNA sequences and discuss the results of a phylogenetic analysis of the new species with previously deposited COI DNA sequences of *Anthaxia* spp. In a maximum-likelihood phylogenetic analysis, the new species shared the same hypothetical ancestor node with *A. melancholica* Gory, 1841 and similar molecular characteristics (~48% similarity) with *A. tenella* Kiesenwetter, 1858 and *A. corinthia* Reiche & Saulcy, 1856. More systematic studies are required to understand the species diversity, distribution, biology, and evolutionary significance of the *Anthaxia* (H.) species groups.

Keywords: Beetle, Buprestidae, CO1 gene, Coleoptera, molecular phylogeny, oriental region, southern India, Western Ghats.

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Author details: Dr. Seena, S. completed her PhD from University of Calicut and the thesis dealt with the morphology, molecular, morphometric study of Jewel beetles of Kerala, South India with special emphasis on antennal sensilla structure and light reflection mechanisms of Buprestid. P. P. Anand doing Ph. D research (University of Calicut) on molecular aspects of mussel foot proteins. Dr. Y. Shibu Vardhanan working as associate professor in Zoology, University of Calicut. His lab focused diverse aspects such as geometric morphometrics, toxicology, Biochemistry, molecular biology, biomaterial characterization and waste management.

Author contributions: Field level collection: SS; Description: SS and PPA; molecular analysis: SS and PPA; Supervision: YSV.

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ज्ञान - विज्ञानं विमुक्तये

INTRODUCTION

Buprestidae is one of the world's largest coleopteran families, with nearly 15,000 extant species in 522 genera (Bellamy 2008). The genus *Anthaxia* Eschscholtz, 1829, is a diversified taxon with a wide distribution; this genus includes 697 species worldwide (Bellamy 2008; Kubáň 2016). The genus *Anthaxia* comprises eight subgenera (Bílý 2019), of which *Haplanthaxia* Reitter, 1911 is the largest, comprising 70% of species of the genus. There are currently 20 defined species-groups in the subgenus *Haplanthaxia* and many more awaiting definitions (Bílý 2017, 2019). Due to its worldwide distribution and the extreme morphological similarity of some species, it is considered as the taxonomically most challenging group in Buprestidae (Bílý 2019). Anthaxiini from the Oriental region, particularly from the Indian subcontinent, have received little attention. Southern Indian *Anthaxia* (*H.*) has not yet been studied; in this work, we discuss the new species from *Anthaxia* species group.

In addition to the morphological description, we discuss the molecular phylogenetic position of our new species among relative species. Due to limited sampling, the Buprestidae group's molecular identification, classification, and phylogenetic analysis are not yet well developed. At present, species identification and classification are primarily based on morphological characteristics. Compared to other buprestid genera, *Agrilus* Curtis, 1825, which has received the most attention in molecular barcoding and phylogenetic analysis. Kelnarova et al. (2018) investigated and developed the first DNA reference library for ~ 100 *Agrilus* species from the Northern Hemisphere using three mitochondrial markers: *cox1-5'* (DNA barcode fragments)*, cox1-3', and rmL.* Rapid detection and taxonomic identification of buprestid species is the first step, especially if the species is economically significant. Recently, mitochondrial DNA-based species identification methods have become increasingly important as a practical alternative to classical morphology-based identification (Herbert et al. 2003; Riedel et al. 2013a, b; Ashfaq & Herbert 2016). Here, we present the first molecular mt. CO1 barcoding sequence of the genus *Anthaxia* from India, with the first mt. CO1 phylogeny analysis of all known *Anthaxia* species available in NCBI and BOLD databases.

MATERIALS AND METHODS

Specimens studied here were collected with yellow pan traps from the Aralam wildlife sanctuary (11.9505^{0}N 75.8231^{0}E, 238 m) in Kannur district, southern Western Ghats, Kerala, India. Images were taken with a Carl Zeiss SteREO Discovery.V20 microscope with a 6MP CCD sensor camera 506 attached and processed with Adobe Photoshop CS8 to standardize background and remove artifacts formed during stacking. In addition, measurements of body parts of holotype specimen were taken with Carl Zeiss SteREO Discovery V20 inbuild software. The holotype and paratype are deposited in the Department of Zoology, University of Calicut (DZUC) and will be transferred to the National Collections of Zoological Survey of India, Western Ghat Regional Centre, Kozhikode, Kerala (ZSIK).

The body length was measured in the middle of the body following the elytral suture (the same for the pronotal and elytral length); width of the body was measured at the maximum body width (usually the maximum span between lateral pronotal margins or span between the outer margin of humeral callosities) (Bílý 2020). The terminology used to describe surface sculpture is based on Harris (1979).

DNA extraction, amplification, sequencing, and phylogenetic analysis

Genomic DNA was extracted from the thoracic leg using Nucleospin® Tissue Kit (Macherey-Nagel) following the manufacturer's instructions. The extracted DNA was subjected to PCR amplification. PCR was performed in a reaction mixture containing 6.25 µL master mix (PCR master mix: Phire Hot Start II PCR Master Mix, Thermofisher, Cat. No: F125S), 1.25 µL forward and reverse primer, 1 µL extracted DNA sample and 3.25 µL water. The total volume of the reaction mixture is 13 µL. For performing PCR mitochondrial cytochrome c oxidase subunit 1 (CO1) amplification, we used Lep primer (LepF1 5' ATTCAACCAATCATAAAGATATTGG 3' and LepR1 5' TAAACTTCTGGATGTCCAAAAAATCA 3') (Herbert et al. 2004; Wilson 2012). The thermal profiles of CO1 amplification were 5 min at 95° C, 40 cycles of 10 sec at 94° C, 1 min at 52° C, and 45 sec at 72° C, followed by a final extension of 10 min at 72° C. The purified PCR products were sequenced at Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram, Kerala, India, using the dideoxy chain termination method (Sanger & Coulson 1975). The forward and reverse strands were aligned using Clustal W in MEGA X to ensure the sequences were clear without any mismatches, frameshift regions, premature stop codons, etc.

The sequences were checked in the NCBI BLAST tool to find similar sequences in the NCBI database. All mt. CO1 DNA sequences of *Anthaxia* species were retrieved from NCBI and BOLD database and aligned in MEGA X,

MUSCLE alignment method (Kumar et al. 2018), and the aligned sequences were used for phylogeny construction analysis. To find out the best model for phylogeny analysis, we performed maximum likelihood fits of 24 different nucleotide substitution models. Models with the lowest BIC scores (Bayesian information criterion) are considered to describe the substitution pattern the best. For each model, AICc value (Alkaline information criterion, corrected), Maximum likelihood value (*InL*), and the number of parameters (including branch lengths) are also validated (Nei & Kumar 2000). A total of 30 nucleotide sequences (including new species CO1) were used for phylogenetic analysis. GTR+G+I (General Time Reversible model + Gamma Distributed with Invariants Sites) model is the best model for the phylogeny construction analysis of the genus *Anthaxia* (Parameters = 67; BIC = 10045.924; AICc = 9554.572; *InL* = -4709.885).

Phylogenetic relationship of taxa was analysed by using maximum likelihood and neighbour-joining method. The evolutionary history was inferred using the maximum likelihood method and the General Time Reversible model (Nei & Kumar 2000). The bootstrap consensus tree inferred from 1,000 replicates (Felsenstein 1985) is taken to represent the evolutionary history of the taxa analysed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein 1985). Initial tree(s) for the heuristic search were automatically obtained by applying neighbour-join and BioNJ algorithms to a matric of pairwise distances estimates using the maximum composite likelihood (MCL) approach, then selecting the topology with superior log likelihood value. A discrete Gama distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2661)). The rate variation model allowed some sites to be evolutionarily invariable ([+*I*], 18.07% sites). This analysis involved 30 nucleotide sequences (including new species CO1). Codon positions included were 1^{st}+2^{nd}+3^{rd}+Noncoding. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There was a total of 384 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

RESULTS

Anthaxia (*Haplanthaxia*) *winkleri* Obenberger, 1914 species group

Small to medium-sized species (4.0–6.0 mm). The head is wide, the forehead is flat, wide, the eyes projecting beyond head contour; upper lobe of eye more obtuse. On the broad vertex, the inner rims of the eyes are far apart, diverging towards vertex. Frons rather variable, from flat, slightly grooved in the middle, to widely depressed; frontal pubescence thicker, more sparse, rather reclined; clypeus almost flat. The forehead is always dark copper or green in colour, as is the rest of the body, only rarely a little lighter. The pronotum is almost twice as wide as long, fairly flat, depressed, the posterior angles not protruding backwards at all. It is widest in the anterior third, from there to the base and to the anterior margin finely and weakly, equally narrowed. Anterior margin deeply bisinuate, with pronounced central lobe. The posterior angles are rectangular. The structure of the pronotum is regular; it consists of low cells similar to those of the head; these are half extinct, very fine, only the central granules protrude somewhat more clearly, the walls of the cells are almost distinct. Scutellum slightly wider than long. The elytra are flat, without depressions, finely granulated, margins deep & wider, not shagreened, dark coppery, slightly wider in the shoulders than pronotum, individually tapered, and rounded at the apex. Metatibiae proportionally shorter, stronger, inner edge usually more strongly sinuate, incised, acutely serrate, with stronger, more acute apical spur. Aedeagus narrower, less sinuate; apex median lobe subparallel, more angulate, acutely pointed.

TAXONOMY

Anthaxia (*Haplanthaxia*) *keralensis* sp. nov. (Image 1,2; Figure 1)

urn:lsid:zoobank.org:act:AF553762-19DC-438D-8BBA-8EE282C7130D

Material examined

Holotype: DZUC BLAK001, male, 10.vi.2019, Aralam Wildlife Sanctuary, Kerala, India, (11.9505^{0}N, 75.8231^{0}E, 238 m), coll: S. Seena".

Paratype: DZUC BLAK002, male, same as holotype.

Measurement (Holotype): total length 5.71 mm, the width of head 1.65 mm, length of pronotum 1.08 mm, the width of pronotum 1.89 mm, length of elytra 3.87 mm, and width of elytra 1.92 mm.

Diagnosis: Medium-sized (5.7 mm) (Image 2A), robust;

frons, vertex and pronotum bright green metallic with bronze lusters; elytra bronze with bright green lusters; ventral surface, antennae and legs bronze-green metallic, metepimera and abdominal ventrites green with bronze lusters; pronotum with distinct deep posterolateral depressions; lateral sides of 1st abdominal segment with tomentose spot; metatibiae straight, with dense hispid bristles externally; entire body covered with setose, golden yellow, small erect pubescence (Image 2).

Description of the holotype

Head slightly wider than anterior pronotal margin; frons convex, vertex weakly depressed, 0.5 times as wide as width of eye; frontoclypeus anteriorly slightly convex; eyes large, narrowly reniform, slightly projecting beyond the outline of the head; inner ocular margins parallel, feebly converging toward vertex; sculpture of head consisting of very small, dense, polygonal cells with central grains; short erect yellow pubescence uniformly distributed; clypeus roughly micro-sculptured (Image 2D); antennae long, almost reaching posterior pronotal angles when laid alongside; scape claviform, about 4 times as long as wide, pedicel suboval, about 1.5 times as long as wide; third antennomere triangular, about twice as long as wide, antennomeres 4–10 trapezoidal, slightly longer than wide, terminal antennomere rhomboid, twice as long as wide.

Pronotum weakly convex, 1.8 times as wide as long, with wide, distinct deep postero-lateral depressions; anterior margin bisinuate, posterior margin almost straight; lateral margins widely, regularly rounded, posterior angles obtuse-angled, maximum pronotal width at midlength; pronotal sculpture consisting of a simple, fine, network of subpolygonal cells with weakly raised borders, slightly denser on latero-posterior areas; cell bottom strongly micro-sculptured, with distinct central grain; bearing short, erect, golden yellow pronotal pubescence. Scutellum small, finely micro sculptured, pentagonal, as wide as long (Image 1A).

Elytra regularly convex and tapering posteriorly, 2.9 times as long as wide; basal, transverse depressions shallow, not reaching scutellum, humeral callosities small, only weakly projecting beyond elytral outline; elytral epipleura rather wide, parallel-sided, almost reaching elytral apex; lateral preapical serrations very fine, the apex of each elytron broadly rounded; elytral sculpture almost homogeneous, consisting of fine, dense, simple punctures with small erect golden pubescence; apex of elytra weakly dentate (Image 2A, 2C).

Ventral surface lustrous with finely ocellate sculpture, cell borders weakly raised; abdominal ventrites almost glabrous; prosternal process wide, subparallel, with well-developed and acute lateral angles; anal ventrite weakly truncate apically, slightly angulate and rather strongly serrate laterally (Image 2B). Legs long and slender, protibiae weakly curved, meso- and metatibiae straight, with dense hispid bristles externally (Image 2E); tarsal claws delicate, hook-shaped, not enlarged at base.

Aedeagus long, slender, weakly spindle-shaped, dorso-ventrally flattened, and the median lobe sharply

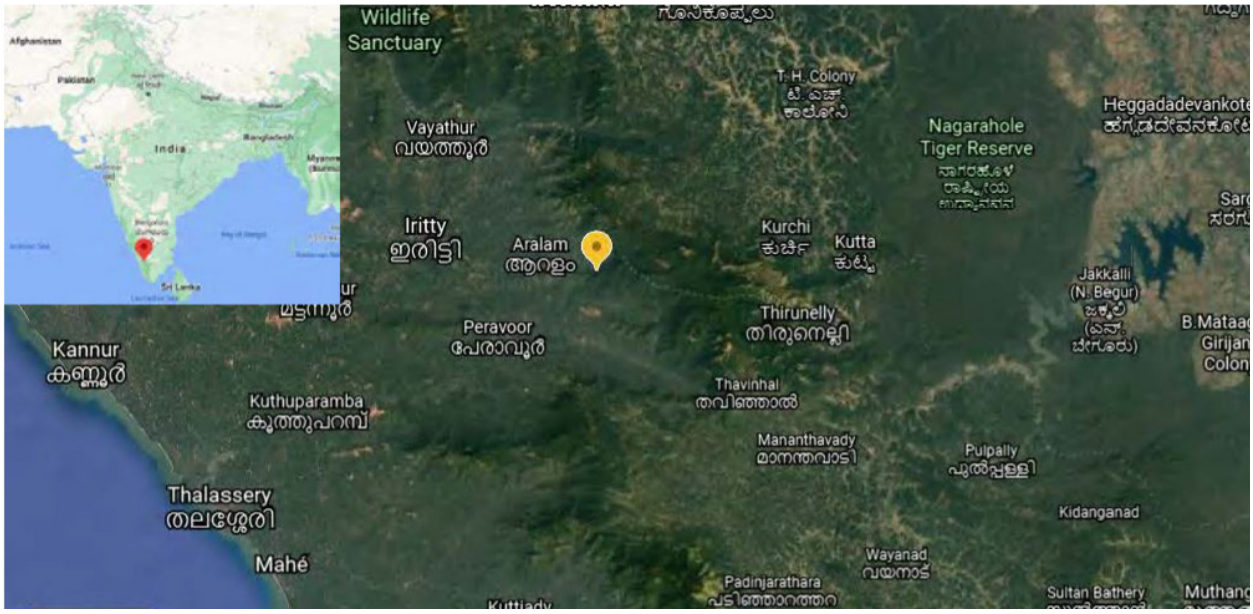

Image 1. Aralam Wildlife Sanctuary with Holotype collection locality (yellow mark).

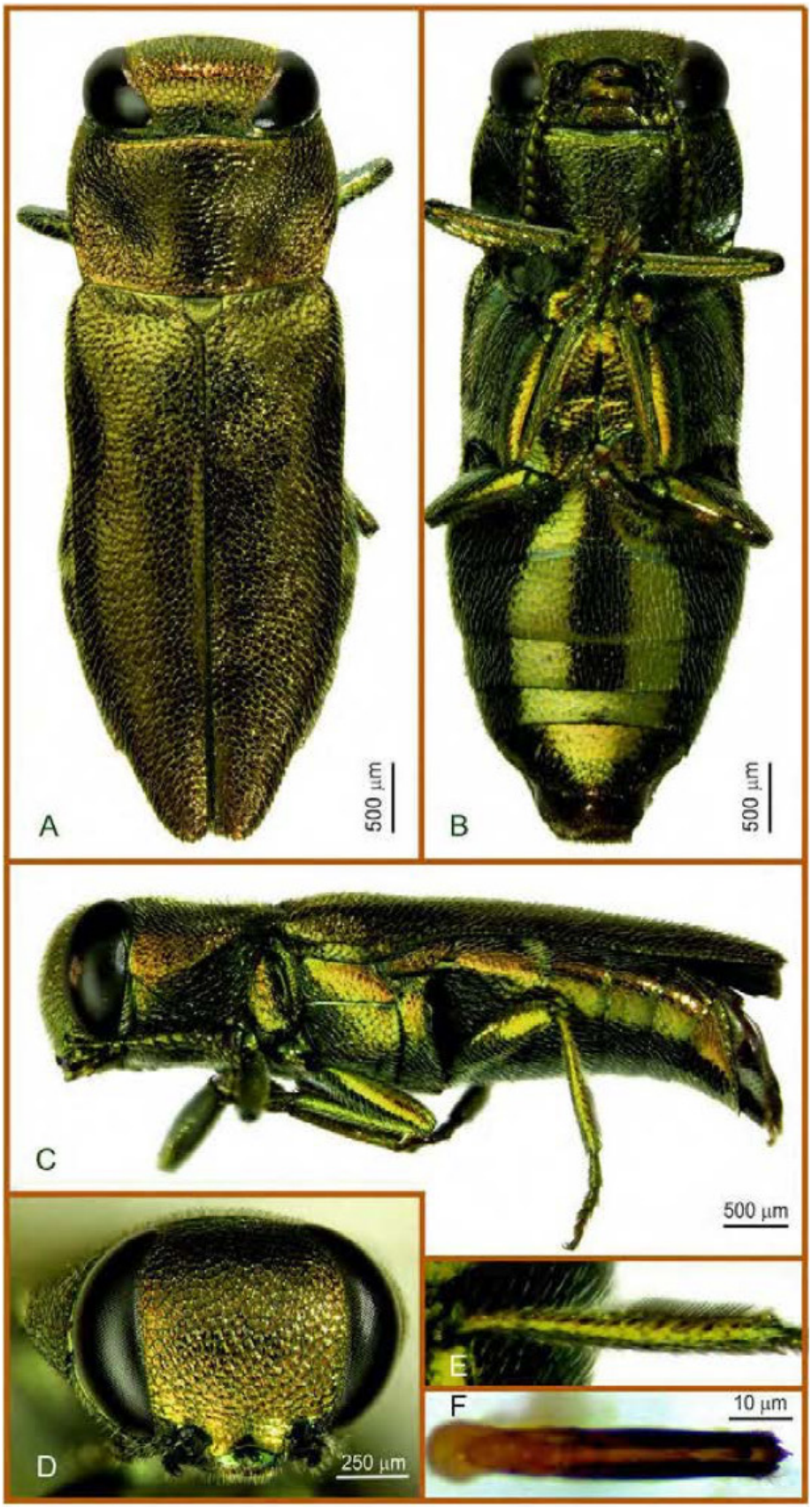

Image 2. ***Anthaxia* (*Haplanthaxia*) *keralensis*** **sp. nov. holotype DZUC BLAK001 (male): A—dorsal aspect | B—ventral aspect | C—lateral view | D—frontal aspect of head | E—Metatibia | F—Aedeagus. © Y. Shibu Vardhanan.**

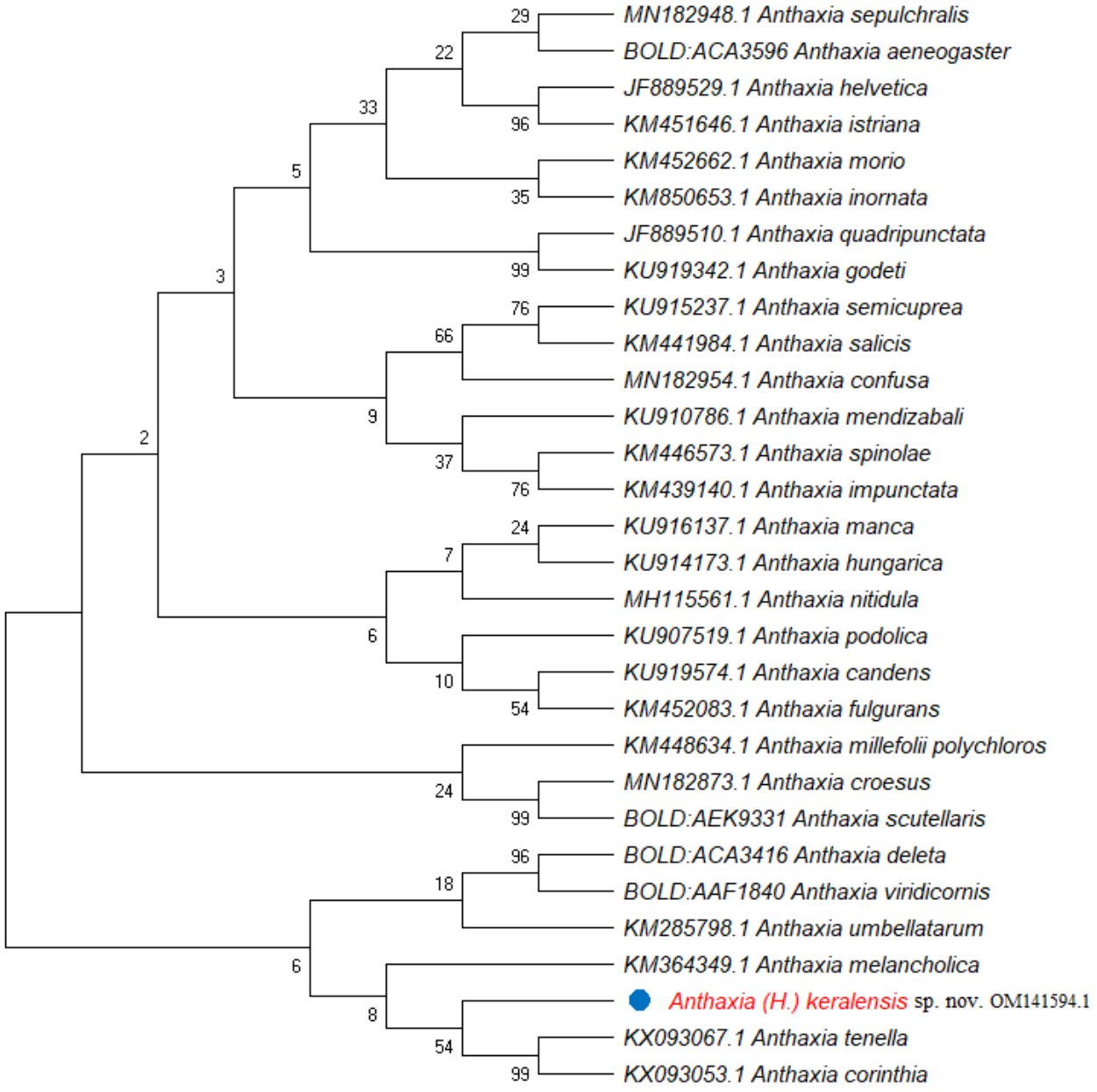

Figure 1. Maximum-likelihood phylogeny analysis. Red color indicated new species *Anthaxia* (*Haplanthaxia*) *keralensis* sp. nov. MUSCLE alignment, 1,000 bootstrap, developed in MEGA X.

pointed apically (Image 2F).

Female: unknown.

Etymology: The new species is named after the Indian state Kerala where the holotype was collected.

Distribution: India, Kerala State, known only from the type locality.

Differential diagnosis: This species is similar to *A. marshalli* Stebbing, 1914 and *A.* (*H.*) *tanjorensis* Obenberger, 1938 by size and general habitus but it distinguishes by its setose body, uniformly distributed golden yellow short erect pubescence, tomentum on the lateral side of 1st ventrite (Image 2C), and aedeagus shape (Image 2F). *A.* (*H.*) *keralensis* sp. nov. is easily distinguished from *A.* (*H.*) *tanjorensis* Obenberger, 1938 by its pronotonal sculpture, since *A.* (*H.*) *keralensis* sp. nov. has a pronotal sculpture usually regularly polygonal on the whole pronotal surface, but in longitudinally stretched on discal area as in *A.* (*H.*) *tanjorensis.*

Molecular phylogeny analysis

A total of 29 mt.CO1 barcoding sequences of the genus *Anthaxia* available in NCBI and BOLD database.

In the ML phylogenetic analysis (Figure 1), the tree divides into two major clades, one clade containing seven species and the other clade containing 23 species. *Anthaxia (H.) keralensis* sp. nov. (OM141594.1) is positioned in a distinct clade. *A.* (*H.*) *keralensis* sp. nov. and *A. melancholica* diverged from the same hypothetical ancestor node. *A.* (*H.*) *keralensis* sp. nov. showed a molecular relationship (~48% of similarity) with *A. melancholica, A. tenella,* and *A. corinthia.* The resulted molecular phylogeny of *Anthaxia* has a strongly preliminary character because all main clades and subclades have very low nodal support. Basal clade which includes new species, is formed by representatives of the subgenera *Haplanthaxia* (*A. deleta* [= *A. caseyi*] - *A. melancholica*) and *Melanthaxia* (*A. tenella* and *A. corinthia*). In the same time all other species of subgenus *Melanthaxia* form a monophyletic most distant subclade (*A. sepulchralis* - *A. godeti*). Intermediate subclades are also mainly polyphyletic and include representatives of subgenera *Anthaxia* s. str., *Haplanthaxia* and *Cratomerus*. Only basal subclade of the second clade comprises species of *Haplanthaxia.* It's important to remember that one of the factors contributing to the preliminary uncorrelated relationship of some *Anthaxia* spp. was a lack of data in genebanks. For the purpose of studying the molecular phylogenetic link among the *Anthaxia*, multilocus-based gene barcoding and the development of phylogenies with extremely comparable taxa will be helpful. More molecular and morphological systematic studies are required to understand the phylogenetic relationship among the *Anthaxia* spp.

CONCLUSION

The lack of proper revision of species from the Indian subcontinent and the high degree of morphological variability in the *A. winkleri* species group are significant impediments in assigning and describing a new species from India. The lack of appropriate molecular barcode sequences in GenBank databases makes mt. CO1 barcoding ineffective for species identification at the moment. Nevertheless, we can use the barcode for molecular phylogeny and genetic similarity analysis. *A. (H.) keralensis* sp. nov. showed no close similarity with previously studied *Anthaxia* spp. A multiple gene sequencing studies are required to confirm the species group belonging of newly described species and to build the molecular phylogeny and their evolutionary origin of the genus *Anthaxia*.

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