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Arecanut white grubs *Leucopholis* species (Melolonthinae: Scarabaeidae: Coleoptera) morphological, molecular identification and phylogenetic analysis

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ABSTRACT

Arecanut (*Areca catechu*) is one of the most important commercial crops in India, which grows extensively in the malnad and coastal areas. White grubs are the most important pests on this crop and cause considerable yield loss. Surveys were conducted to record species that are infested with white grubs of Karnataka and Kerala states. Three species of white grubs were collected and described using morphological and molecular characters. A detailed study of male genitalial structures such as aedeagus and endophallus in three species of *Leucopholis* Dejean, 1833 (Coleoptera: Scarabaeidae: Melolonthinae) *viz.*, *Leucopholis lepidophora* Blanchard, 1850, *Leucopholis burmeisteri* Brenske, 1894 and *Leucopholis coneophora* Burmeister, 1855 showed substantial differences. The mitochondrial cytochrome oxidase subunit I gene was sequenced for molecular identification and phylogenetic analysis in this respect. *L. lepidophora*, *L. burmeisteri* and *L. coneophora* species were successfully identified by employing COXI gene. Phylogenetic analyses revealed that the COXI DNA barcode fragment of *Leucopholis* species and out-group taxa, available in genetic databases, confirms the species identity.

Introduction

Many white grubs in India are well-known pests of a number of cultivated crops. They belong to four sub families of Scarabaeidae *viz.*, Dynastinae, Cetoniinae, Rutelinae and Melolonthinae, which includes all the phytophagous species (Veeresh, 1983). The arecanut root grubs belong to the Melolonthinae subfamily and the genus *Leucopholis* in the malnad and costal belt of Karnataka is considered to be the main pest of Arecanut (Veeresh et al., 1982). This *Leucopholis* genus consists of three important species *viz.*, *Leucopholis lepidophora*, *L. coneophora* and *L. burmeisteri*. Among these *L. lepidophora* and *L. burmeisteri* take two years for completion of life cycle, whereas *L. coneophora* takes one year (Kumar, 1999). *L. lepidophora* is distributed throughout the Karnataka hilly and high rainfall regions. Whereas, *L. burmeistri* and *L. coneophora* are confined to coastal regions. The grubs of these species cause damage to the roots by feeding, which leads to the appearance of symptoms such as leaf yellowing, stem tapering and nut decrease, which ultimately leads to reduced vigor and yield (Kumar, 1999), lose anchorage and toppling of palms when disturbed (Nair and Daniel, 1982). A few grubs (6–8) suffice to kill palms (Prakash et al., 2011). Kalleshwaraswamy et al. (2015) reported that there was 27.86–36.97% damage by *L. lepidophora* with a yield reduction of 39.79–41.60% in different districts of hilly regions of Karnataka. *L. burmeisteri*, found to be restricted to the coastal region and reported causing 28.80% damage with a 39.79–41.16% yield reduction. Adults of *L. lepidophora* emerge from the soil for mating at dusk between 18:30 to 19:30 h during June to October (Kumar, 1999; Adarsha, 2014; Kalleshwaraswamy et al., 2016). Whereas, *L. coneophora* (Prathibha et al., 2013) and *L. burmeisteri* (Kalleshwaraswamy et al., 2017) emerge during May to August and May to July, respectively.

For taxonomic and systematic purposes, external and internal structures of male genitals were used. These structures are very important for the description of taxa's phylogenetic hypothesis and development. Male genitalia often provide an important taxonomically useful morphological characteristic for distinguishing organisms at the species level. As in coleopteran beetles, where males have been widely used for species differentiation (Medina et al., 2013). D'Hotman and Scholtz, 1990prepared a description of the external structures of male genitalia in 12 families of Scarabaeidae. Male genitalia have been used in different taxonomic studies of *Leucopholis* spp. *viz.*, *L. fontainei* Brenske, 1894; *L. hirtiventris* Frey, 1963; *L. jacquinoti* Blanchard, 1851; *L. pollens* Sharp, 1876; and *L. semperi* Brenske, 1896, (Calcetas and

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Adorada, 2017) and *L. zollantans* (Suparno, 2015) in other parts of the world.

Field entomologist rely on body colour or mostly colour on elytra, but its reliability is in question. There is a necessary of searching for more reliable character which can be used for identification. However, there is currently no published taxonomic information on *Leucopholis* species and also their distribution in India. Male genitalia's morphological characteristics include aedeagus and endophallus, providing more accurate information on the identification of the *Leucopholis*. The present study was therefore carried out to study the aedeagal and endophallus structures of three species of *Leucopholis viz.*, *L. lepidophora*, *L. burmeisteri* and *L. coneophora*.

Addition of molecular identification to morphological character always gives better understanding for delineating species (Mehle and Trdan, 2012). DNA barcoding is a molecular method of species discrimination aimed at identifying biological specimens using a 658 bp (Folmer region) fragment of the 5′ end of the mitochondrial oxidase-1 cytochrome (CO-I) gene (Hebert et al., 2003). The species identification is achieved by comparing the unknown sequence of the sample to the reference database. It is evident from the literature that DNA barcoding is a reliable tool for species discrimination in various types of insects *viz.*, Lepidoptera (Burns et al., 2008), Coleoptera (Greenstone et al., 2005), Hemiptera (Foottit et al., 2010), Diptera (Smith et al., 2006, 2007), Hymenoptera (Smith et al., 2008; Fisher and Smith, 2008), Thysanoptera (Glover et al., 2010) *etc.* Given the importance of crops and pest damage, it is very important to understand and generate the basic molecular identification information for *Leucopholis* spp. In this study, an attempt was made to identify the species of *Leucopholis* at molecular level by cloning and sequencing the gene of 5′ mitochondrial cytochrome oxidase c subunit 1 (COXI) and its phylogenetic analysis.

Materials and methods

Sampling of adult beetles

The adult beetles of three *Leucopholis* species *viz.*, *L. lepidophora*, *L. burmeisteri* and *L. coneophora* were collected from different parts of Karnataka and Kerala during 2013–2018. *L. lepidophora* emerged from June to September and other two species like *L. burmeisteri* and *L. coneophora* emerged during May–June. Hand collection of adult beetles were made during the emergence period immediately after dusk *i.e.*, between 18:30 to 21:00 h by visually searching throughout the arecanut garden. *L. lepidophora* was collected from Hosanagara taluk, Shivamogga district (13°52′ N; 75°12′ E, 692 msl), Thirthahalli taluk, Shivamogga district (13°53′ N; 75°13′ E, 720 msl) and Sagara taluk, Shivamogga district, Karnataka (14°06′ N; 74°52′ E), *L. burmeisteri* was collected from Bramhavara taluk, Udupi district, Karnataka (13°26′ N; 74°48′ E,250 msl). Whereas, *L. coneophora* was collected from Nileshwaram taluk, Kasaragod district, Kerala (13°26′ N; 74°48′ E, 250 msl) in coconut garden (Fig. 10). The collected beetles were brought to the laboratory, Department of Agricultural Entomology, UAHS, Shivamogga and sorted on the basis of male and female. For genitalia dissection, sorted male beetles were prepared.

Specimen preparation for genitalia dissection

For male genitalia dissection and study, dried male specimens were placed in 70% alcohol for genitalia extraction. The abdomen was removed with a pointing needle from the thorax. Aedeagus and spiculum gastrale were carefully removed from the dissected abdomen by means of forceps and placed in the cavity slides. Spiculum gastrale was carefully removed with forceps from aedeagus. Aedeagus structures were placed in a test tube in 10% KOH solution for 6–8 h until all fat bodies and tissues were macerated and aedeagus became translucent. The endophallus was drawn by injecting water from the base of the aedeagus using a syringe attached to the hollow needle (D'Hotman and Scholtz, 1990; Sreedevi and Sakshi, 2016). The terminology of the structures of aedeagus follows D'Hotman and Scholtz (1990). The photographs were taken from the Leica DFC450 camera mounted on a Leica M205C trinocular stereo microscope with a digital imaging system connected to the Leica Application Suite (LAS) from Leica. All the measurements of the aedeagus and spiculum gastrale were taken from photographs using Leica application software.

Aedeagus measurements were recorded by measuring the length and width of the phallobase and paramere as well as the length and width of the stem and the length of the spiculum gastrale arms. Aedeagus and spiculum gastrale were then transferred to a glycerin-containing microvial and preserved after the study.

Molecular analysis

The representative specimens identified morphologically were used for molecular analysis. The specimens were directly soaked into 99.5% ethanol and stored at 4 °C. Ethanol-preserved voucher specimens are deposited in the Department of Agricultural Entomology, UAHS, Shivamogga.

Deoxyribonucleic acid preparations

Total genomic DNA was extracted with modified Cetyl Trimethyl Ammonium Bromide (CTAB) from the legs of a single specimen. Legs dissected from ethanol-preserved specimens were washed twice with distilled water and dried. The legs were grinded with liquid nitrogen to fine powder. Samples were homogenized in an extraction buffer of 300 μl (100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 0.02 m ethylenediaminetetraacetic acid, 2× CTAB, 2× PVP, 2 μl β-mercaptoethanol). The homogenized mixture was incubated at 65 °C for 30 min. During the incubation process, tubes were inverted for occasional mixing. Equal volume of (300 μl) of Chloroform: Isoamyl alcohol (24:1) were added. The homogeneous product was centrifuged for 10 min at 8000 rpm. The final aqueous phase obtained was transferred into a new eppendorf tube. An equivalent volume of ice cold isopropanol was added. The tubes were centrifuged for 15 min at 13000 rpm. The pellet was dried and dissolved in milli Q water/TE buffer (30 μl). NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to quantify DNA.

DNA barcode amplification, cloning and sequencing analysis

The 'barcode/folmer' region of cytochrome oxidase subunit I gene (COXI) (658 bp consensus sequence) was amplified using the primer pair LCO1480 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′). The PCR mixture contained 3 μl of genomic DNA, 0.7 μl of 10 mM dNTPs, 0.7 μl primers (10 p moles), 2.5 μl of 10× PCR reaction buffer, 0.7 μl of 25 mM $MgCl_2$, 0.3 μl of DMSO, 0.5 μl of 3 unit Taq DNA polymerase (Genei) and was filled up to 25 μl with sterile nuclease free water. All PCR amplification reactions were conducted in Thermal cyclers, peqSTAR (UK). Thermo cycling conditions of the PCR included an initial denaturation at 94 °C (5 min), followed by 35 cycles at 94 °C (denaturation, 45 s), 48 °C (annealing, 45 s), 72 °C (extension, 60 s), and a final extension step at 72 °C (10 min). For each PCR reactions positive (MtCOI plasmid obtained from previous studies in our lab). Contamination was checked for using negative controls (water instead of genomic DNA and without water) controls were included. An aliquot (20 μl) of each amplification reaction was analyzed on 1.5% w/v agarose gels cast and run in 1× TAE buffer (40 mM Tris, 20 mM Acetate and 1 mM EDTA, pH 8.6) at about 100 V until the dye marker was near the end of the gel. The DNA bands photographed under transmitted UV light.

The *Leucopholis* COXI gene PCR products were eluted and gel purified by manual instructions using NucleoSpin ® Gel and PCR Clean - up

Kit (Macherey-Nagel, Düren, Germany). The eluted products were ligated and cloned in to pTZ57R/T vector (Thermo Scientific™ InsTAclone™ PCR Cloning Kit, USA) according to kit guidelines. The cloned and transformed cells (20 µl) were spread on LB agar plates containing X-gal (270 µg/ml), IPTG (120 µg/ml) and ampicillin (100 µg/ml). The plates were then incubated at 37 °C for overnight to screen blue and white colonies. Cloning was confirmed by the colony PCR, the plasmid mobility check and the restriction analysis of recombinant plasmid DNA containing *Leucopholis* mtCOI gene. Plasmids were isolated using GenJET™ plasmid MiniPrep kit (Thermo Scientific, USA), according to the manufacturer's guidelines from the overnight culture of positive clones cultured in LB broth containing suitable antibiotic preferably ampicillin. Sequencing was performed in triplicates of the above clones in an automated sequencer (ABI prism® 3730 XL DNA Analyzer; Medauxin, Bengaluru) using M13 Forward and M13 Reverse primers following standard operating protocols.

Sequences on the 5′ and 3′ ends were trimmed to remove unwanted vector sequences using the GATT and AATC sequences. Forward and reverse primers were highlighted and for further analysis 658 bp consensus sequences were selected. NCBI Basic local alignment search tool (BLASTn) was subjected to edited putative sequences to perform a similarity search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and at DNA barcode sequence repository of the BOLD database (http://www.boldsystems.org/index.php/IDS_OpenIdEngine). Sequences were aligned using ClustalW (Thompson et al., 1997) with default settings in BioEdit 7.2.5 (Hall, 1999).

Base composition

MEGA version 6.0 was used to calculate the A, T, G and C base frequency (Tamura et al., 2013).

Phylogenetic analysis

In MEGA 6.0 (Tamura et al., 2013), data sets were compiled using sequences generated in this study and sequences from previous studies obtained from the NCBI GenBank and BOLD databases. Using all sequences generated in the study and the reference sequences, a neighbor-joining (NJ) and maximum likelihood (ML) analysis based on the K-2 parameter model (Kimura, 1980) with complete gap deletion and resampling with 1000 bootstrap replications was done.

Results

The present investigation was based on the molecular identification, morphological characteristics and the structure of male genitals as well. The described taxa were provided with nucleotide sequences, photographs for key identification characters and male genitalia.

Key to species of Leucopholis Dejean 1833

1. Body is black, scales on body and elytra large, less dense, subovate and offwhite, scales emerge from the base of broad punctuations (Fig. 1), prosternal process smooth and sub-rectangular, paramere and phallobase are uniformly light brown coloured, paramere arms are symmetrical, close together and lobe-like (Fig. 4A), endophallus membranous, broad and a pair of lateral basal sclerite present only at dorsal region (Fig. 7A & C) …………………………………………………………………… *L. lepidophora* Blanchard.

- Body is brownish in appearance, Body covered with elongated yellowish to brownish scales, scales emerge out of the body and pointed towards posterior (Figs. 2 & 3), prosternal process triangular with pointed and covered with a number of scales and hairs, paramere and phallobase are dark brown coloured, paramere arms are symmetrical, separated widely and lobe-like (Fig. 8A), endophallus membranous, broad and short chitinized spines are present in both dorsal and ventral region (Figs. 8C & 9C) ………………………………………………………………….2

2. Elytra lighter than pronotum (Fig. 2); Endophallus fleshy and contains single row of short chitinized spines running from ventral anterior to mid region (Fig. 8C) ………………………………………… ……………………*L. burmeisteri* Brenske.
   - Body uniformly brown coloured (Fig. 3); Endophallus fleshy and contains a mass of short chitinized spines in ventral mid anterior region (Fig. 9C)……………….*L. coneophora* Burmeister.

Description of male genitalia structure of Leucopholis spp.

The genitalia characters of three species of arecanut white grubs *viz.*, *L. lepidophora* (Fig. 1), *L. burmeisteri* (Fig. 2) and *L. coneophora* (Fig. 3) along with aedeagus and endophallus structures are mentioned below.

Descriptions of male genitalia are discussed under subheads, tegmen, endophallus/internal sac and speculum gastrale.

1. *Leucopholis lepidophora* Blanchard (Fig. 1)

Material examined: Karnataka: 350♂ 50♀, Shivamogga, Hosanagara, 13.vii.2016, Coll. Kalleshwaraswamy, C.M., Adarsha, S·K & Kavita Hegde; 56♂ 30♀, Shivamogga, Thirthahalli, 29.viii.2017, Coll. Adarsha, S·K & Kalleshwaraswamy, C.M; 39♂ 15♀, Shivamogga, Sagara, 02.viii.2014, Coll. Adarsha, S.K.

Tegmen

Aedeagus uniformly light brown coloured. Phallobase is larger than paramere, sub-circle, evenly sclerotized measuring the length of 6.81 mm and width 4.28 mm, broader anterior and slightly narrow at the base. Dorsal anterior margin of phallobase connected to the paramere with membranous tissue. Paramere ventral inner posterior margin elongate inverted; inner anterior region elongate, spindle-shaped, narrower and lobe-like. Paramere measuring length of 6.61 mm and width of 3.74 mm, broader at the base and narrower at apical region. Paramere ventral inner posterior margin is connected by sclerotized pointed arms. Paramere dorsal posterior margin inverted Y shaped and connected by membranous integument (Fig. 4A & B). The dorsal mid-posterior region of paramere is bell-shaped. Ventral mid posterior margin of paramere possesses hook-like structure (Fig. 4C). Paramere possess laterally with the horizontal groove on mid posterior half of dorsum to the ventral and both sides of paramere bounded by an elevated ridge (Fig. 4D).

Enophallus/internal sac

Endophallus long and membranous, extracted endophallus longer than aedeagus. Endophallus (Fig. 7A & B). Endophallus membranous, broad and a pair of lateral basal sclerite present at dorsal. Temones long and connected to the base (Fig. 7C).

Spiculum gastrale

Y shaped sclerite, stem long measuring length of 4.04 mm and width of 0.71 mm. Arms are symmetrically length (3.88 mm) and pointed towards the apex and connected by membranous structure (Fig. 4E).

Remarks

This species can be distinguished from other two species of

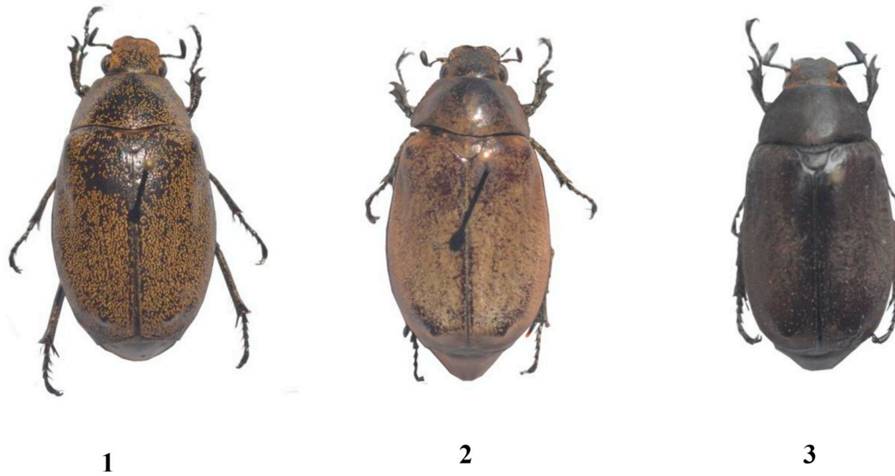


Fig. 1–3. *Leucopholis* spp. 1. *Leucopholis lepidophora*, 2. *Leucopholis burmeisteri*, 3. *Leucopholis coneophora*.

Leucopholis by morphological and genitalia characters. Prosternal process smooth and sub-rectangular, paramere arms are close together and lobe-like, endophallus membranous, broad and a pair of lateral basal sclerite present only at dorsal region.

2. *Leucopholis burmeisteri* Brenske (Fig. 2)

Material examined: Karnataka: 16♂ 10♀, Udupi, Bramhavara, 10.vi.2013, Coll. Naveen, N.L. & Adarsha, S.K.; 5♂ 4♀, Mangalore, Vitla, 01.vi.2018, Coll. Adarsha, S.K.

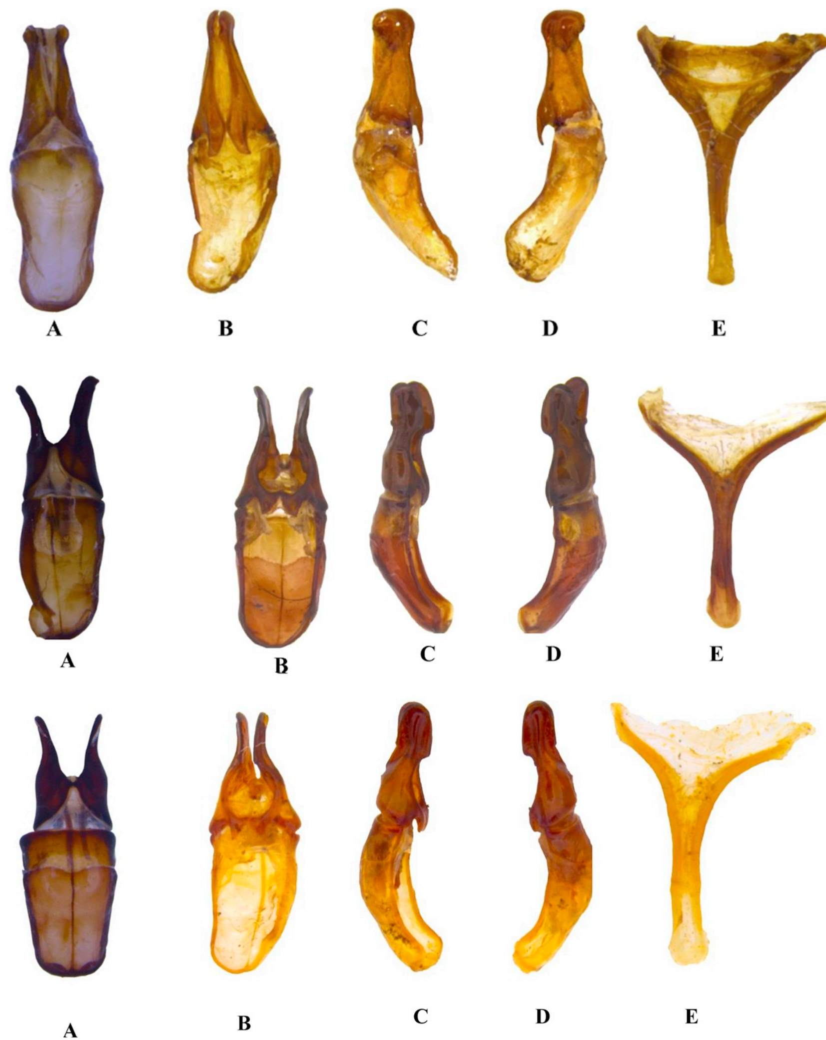


Fig. 4–6. Male genitalia structure, 4. *L. lepidophora*, 5. *L. burmeisteri*, 6. *L.coneophora*. A – dorsal view, B – Ventral view, C– right lateral view, D – left lateral view, E - Spiculum gastrale.

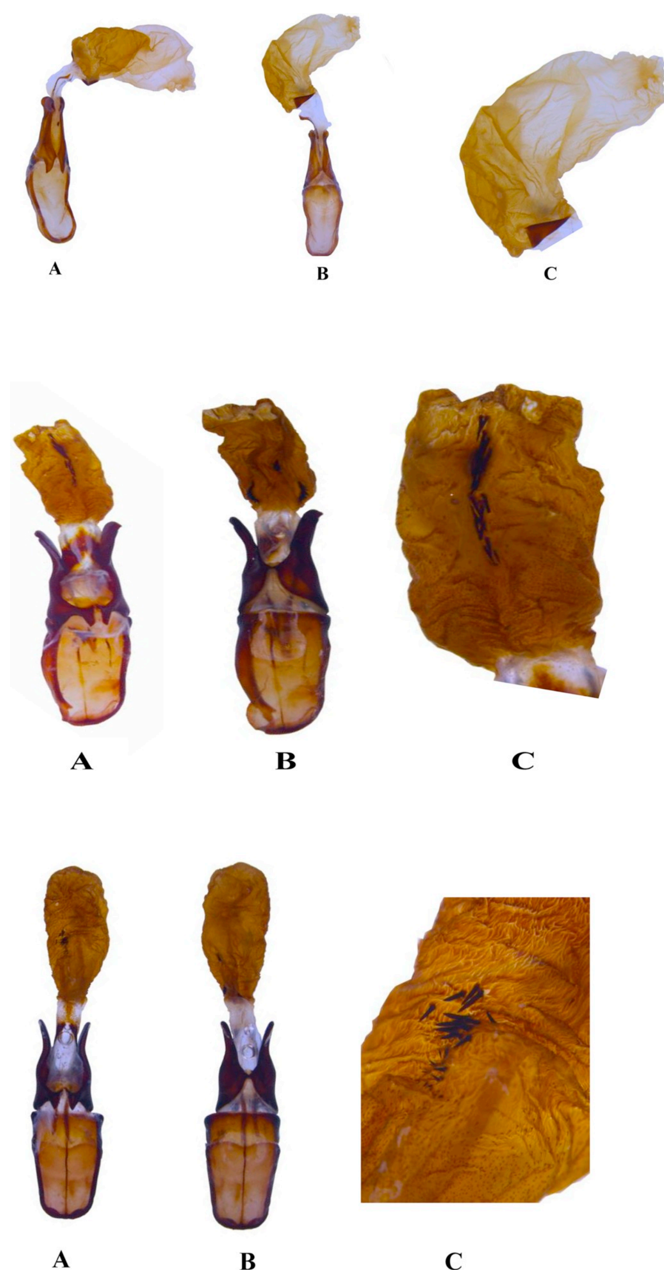


Fig. 7–9. Male genitalia with extracted endophallus, 7. *L. lepidophora*, 8. *L. burmeisteri*, 9. *L. coneophora*; A – ventral view, B – dorsal view, C – close view of endophallus.

Tegmen

Phallobase dorsal anterior margin connected to the paramere with membranous tissue and slightly lighter than paramere. Phallobase is larger than paramere, sub-circle, evenly sclerotized measuring a length of 7.02 mm and width 4.26 mm, broader anterior and slightly narrow at the base. Paramere ventral inner posterior margin is connected by sclerotized pointed arms. Both paramere arms are symmetrical, separated widely and lobe-like. Paramere measuring length of 6.39 mm and width of 4.74 mm, broader at the base and narrower at apical region. Paramere dorsal posterior margin inverted Y shaped and connected by the membranous sheath (Fig. 5A, B). The dorsal mid-posterior region of paramere is bell-shaped. The ventral region possess slight depression in the mid portion of paramere (Fig. 5C). Paramere possess lateral horizontal groove on mid posterior half of dorsum to the ventral and both sides of paramere bounded by an elevated ridge (Fig. 5D).

Enophallus

Endophallus short and membranous, extracted endophallus shorter than aedeagus. Endophallus fleshy and contains a single row of short chitinized spines running from ventral anterior to the mid-region (Fig. 8A & C). The extracted endophallus posses three batches of short chitinized spines at the dorsal posterior region. Temones long and connected to the base (Fig. 8B).

Spiculum gastrale

Y shaped sclerite, stem long measuring length of 4.34 mm and width of 0.64 mm. Arms are connected by membranous region measuring length of 3.72 mm. The posterior end of the stem is lobe-like (Fig. 5E).

Remarks

Externally similar to *L. coneophora* but can be distinguished by the endophallus which contains a single row of short chitinized spines running from ventral anterior to the mid-region.

3. *Leucopholis coneophora* Burmeister (Fig. 3)

Material examined: Kerala: 10♂ 5♀, Kasargod, 01.vi.2018, Coll. Adarsha, S.K.

Tegmen

Dorsal anterior margin of phallobase connected to the paramere with membranous tissue. Both paramere and phallobase are uniformly coloured. Phallobase is larger than paramaere, sub-circle, evenly sclerotized measuring a length of 7.43 mm and width 4.35 mm, broader anterior and slightly narrow at the base. Paramere ventral inner posterior margin is connected by sclerotized pointed arms. Both paramere arms are symmetrical, separated widely and lobe-like. Paramere measuring length of 6.82 mm and width of 4.99 mm, broader at the base and narrower at apical region. Dorsal posterior margin of paramere inverted Y shaped and connected by membranous integument (Fig. 6A, B).The dorsal mid-posterior region of paramere is bell-shaped. The ventral region possess slight depression in the mid-portion of paramere (Fig. 6C). Parameres possess lateral horizontal groove on mid posterior half of dorsum to the ventral and both sides of paramere bounded by an elevated ridge (Fig. 6D).

Enophallus

Endopallus short and membranous, extracted endophallus shorter than aedeagus. Endophallus fleshy and contains a mass of short chitinized spines in ventral mid anteriorregion (Fig. 9A & C). The extracted endophallus possesses three batches of short chitinized spines at the dorsal posterior region. Temones long and connected to the base (Fig. 9B).

Spiculum gastrale

Y shaped sclerite, stem long measuring length of 5.22 mm and width of 0.80 mm. Arms are lengthy (3.42 mm) and pointed towards the tip and connected by membranous structure (Fig. 6E).

Remarks

Leucopholis coneophora is very much similar to *L. burmeisteri* in all the morphometric characters. Only genital structure can be used to distinguish these two species. The endophallus contains a mass of short chitinized spines in ventral mid anterior region. Whereas, single row of short chitinized spines running from ventral anterior to the mid-region

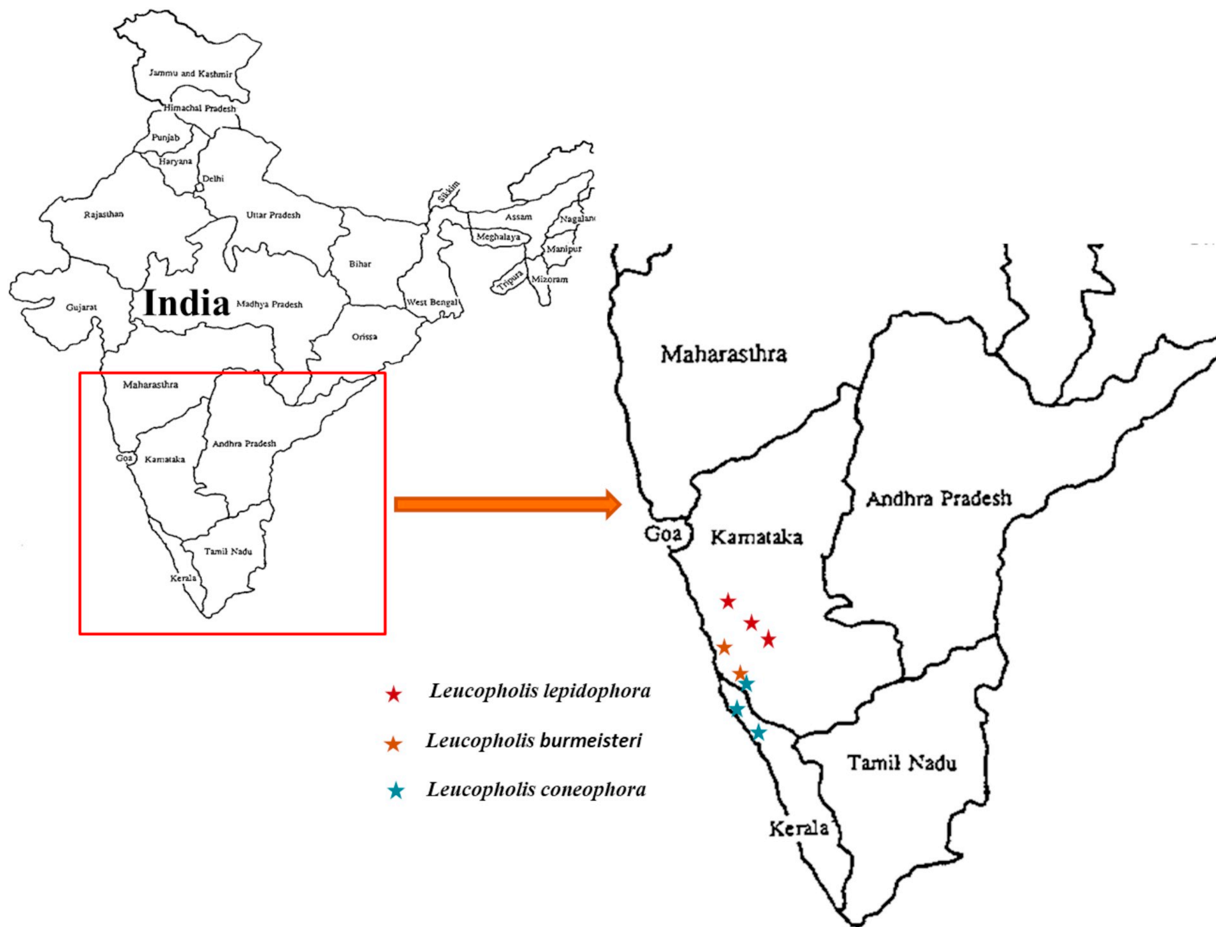
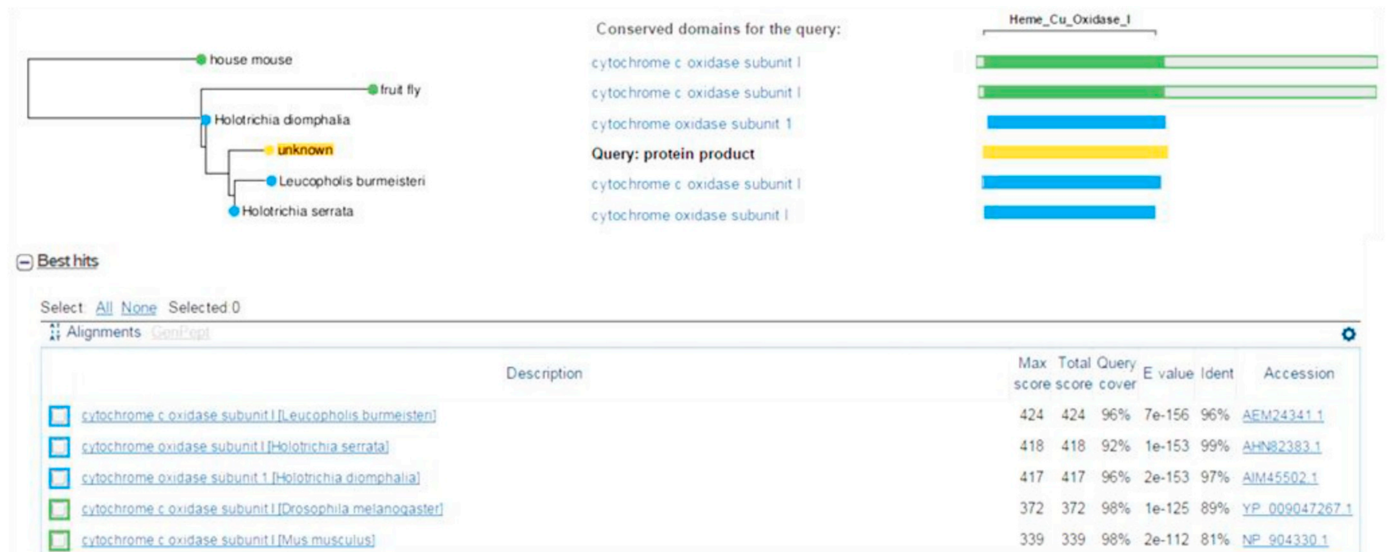


Fig. 10. The sampling of *Leucopholis* spp. for the description of male genitalia and molecular identification. *L. lepidiphora* from Shivamogga district, Karnataka, *L. burmeisteri* and *L. coneophora* from Coastal Karnataka (Udupi district) and Kerala (Kasaragod district).

of endophallus in *L. burmeisteri* (Fig. 8A & C).

Molecular identification and phylogenetic analysis of arecanut white grubs

The MtCOI *Leucopholis* plasmid species comparison of the triplicate sequences for the respective *Leucopholis* spp. did not show any mismatches and therefore no sequencing errors. Evidence of nuclear copies was not found in any of the analyzed sequences, with no indels, which was supported by the absence of stop codons and the basic composition was similar.



Description	Max score	Total score	Query cover	E value	Ident	Accession
cytochrome c oxidase subunit I [Leucopholis burmeisteri]	424	424	96%	7e-156	96%	AEM24341.1
cytochrome oxidase subunit I [Holotrichia serrata]	418	418	92%	1e-153	99%	AHN82383.1
cytochrome oxidase subunit 1 [Holotrichia diomphalia]	417	417	96%	2e-153	97%	AIM45502.1
cytochrome c oxidase subunit I [Drosophila melanogaster]	372	372	98%	1e-125	89%	YP_009047267.1
cytochrome c oxidase subunit I [Mus musculus]	339	339	98%	2e-112	81%	NP_904330.1

Fig. 11. Smart Blast searches a protein query against protein databases. The three best matches in the sequence database together with the two best matches from well-studied reference species, showing phylogenetic relationships based on multiple sequence alignment and conserved protein domains.

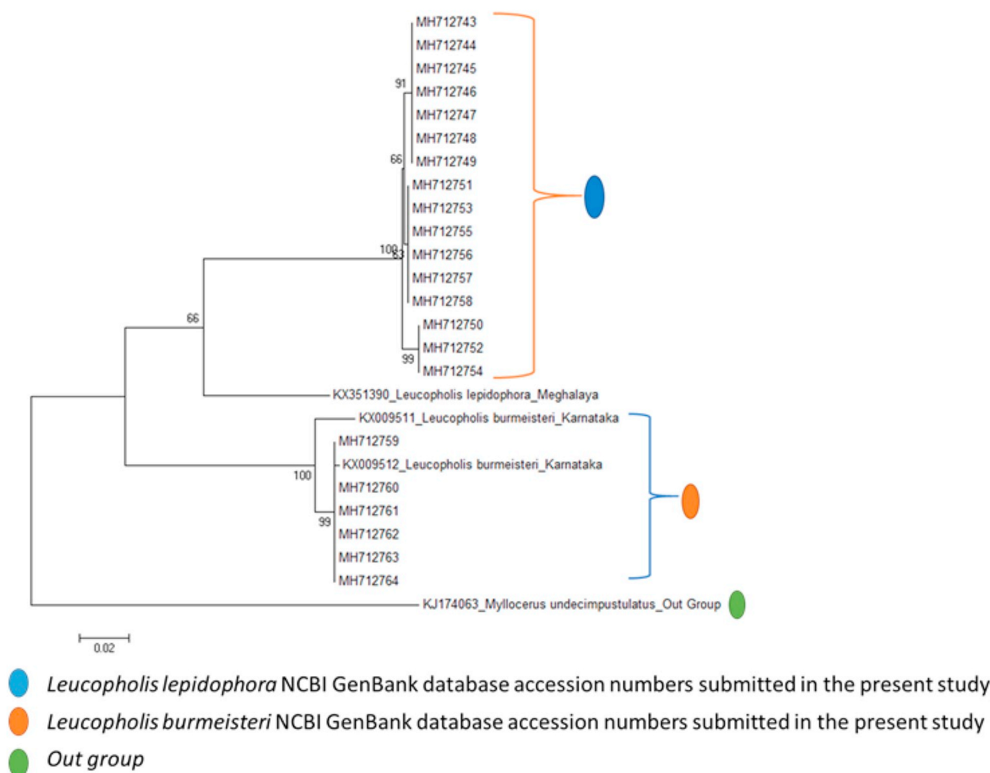


Fig. 12. The phylogenetic relationships of *Leucopholis* species samples, inferred from the 658 bp mitochondrial cytochrome oxidase I (COI) sequence using the Maximum Likelihood method based on the Tamura-Nei model. Bootstrap values (1000 replications) reproduced in < 60% replicates were collapsed and the bootstrap values are shown at the branch points. The GenBank accession numbers of the corresponding reference sequence are indicated. The analysis involved 205 nucleotide sequences. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor- Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. The nucleotide sequences were aligned using the MUSCLE and the evolutionary analyses were conducted in MEGA6.06.

Based on initial NCBI-BLASTn and BOLD searches *L. lepidophora* showed identity of 88% to KX351390/NCBI BLASTn, 88.07% identity in BOLD identification, *L. burmeisteri* sequences showed 100% identity to JN126323/NCBI BLASTn and 97.76% BOLD identification and *L. coneophora* showed 98% identity NCBI BLASTn, BOLD identification 100% matching to *L. burmeisteri*. As no sequence data available for *L. coneophora* at BOLD database. Further NCBI protein blast (BLASTp) search results showing the putative conserved domain of cytochrome oxidase subunit I. In addition, the results of smart blast searches of protein query against protein database showed that multiple sequence alignment and conserved protein domains indeed belong to *Leucopholis* spp. (Fig. 11).

The mitochondrial COI from all *Leucopholis* samples were successfully sequenced and sequences generated in this study were deposited to NCBI-GenBank. A total of 24 sequences were submitted to data base and accession numbers were obtained. These includes, MH712743, MH712744, MH712745, MH712746, MH712747, MH712748, MH712749, MH712750, MH712751, MH712752, MH712753, MH712754, MH712755, MH712756, MH712757, MH712758 (*L. lepidophora*), MH712759, MH712760, MH712761, MH712762, MH712763, MH712764 (*L. burmeisteri*), MK121983 and MK121984 (*L. coneophora*).

The nucleotide frequencies are Adenine 29.96% (A), Thymine 32.92% (T/U), Cytosine 20.87% (C), and Guanine 16.25% (G). From these results it is observed that across all sequences there was an excess of Thymine and Adenine over Cytosine and Guanine. The transition/transversion rate ratios are $k_1 = 2.44$ (purines) and $k_2 = 5.311$ (pyrimidines). The overall transition/transversion bias is $R = 1.946$.

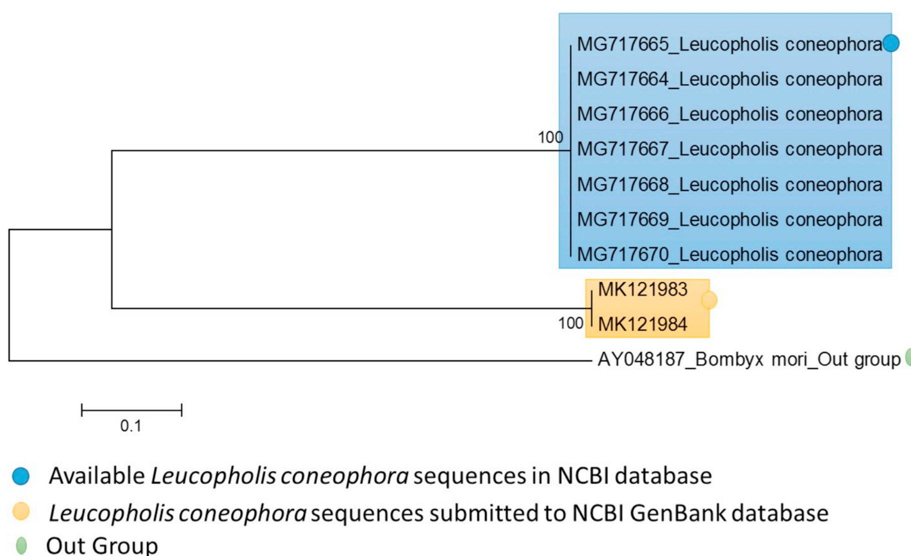


Fig. 13. Phylogenetic tree inferred using the Maximum Likelihood (ML) method of mtDNA CO1 region of *Leucopholis coneophora* sequences. (A) The tree is based on the Kimura 2-parameter method. (B) The tree is based on Tamura 3-parameter model with evolutionarily invariable (T92 + I). Both trees were resampled with 1000 bootstrap replicates. Bootstrap support values on the branches are given.

A Neighbor-Joining (NJ) and Maximum Likelihood (ML) Figs. 12 and 13 tree was produced from this study and GenBank. This indicates that the *Leucopholis* spp. to which the accession numbers were obtained in the present study are corroborated with previously documented from India. NJ and ML tree analysis showed that the individuals of the same species clustered together based on the MtCOI sequence similarity, regardless of their collection site and geographic location.

Discussion

In order to develop control tactics, rapid and accurate identification of *Leucopholis* spp. is crucial. Morphological identification of Melolonthinae: Scarabaeidae larvae or adult requires skilled taxonomists. In addition, the specimen examination is a long process using a microscopy to identify specific characteristics of the species (Gleeson et al., 2000). Identification will be uncertain if larvae/adults were damaged during the collection process. Many larval forms cannot be identified beyond the genus or even beyond the family level (Miller et al., 2005). The lack of adequate morphological taxonomic services makes the molecular approach an agreeable option.

To date, no research has been conducted on the systematics of the *Leucopholis* genus based on molecular data. In the present study, therefore, a phylogenetic analysis was carried out through molecular systematics comprising DNA analysis, PCR amplification and sequence variation. Three species are reported from Arecanut growing regions of Karnataka, *Leucopholis lepidophora* Blanchard, *L. coneophora* Burmeister and *L. burmeisteri* Brenske from kerala. The genus is characterized by the presence of scales on all parts of the body and a mesosternal spine and a prosternal process. *Leucopholis lepidophora* Blanchard is very distinct from the three species with the large subovate scales, black coloured body; the prosternal process is smooth. But the other two species, *L. coneophora* Burmeister and *L. burmeisteri* Brenske are very similar. These two species can only be distinguished by morphometrics.

The genomic DNA obtained with the presented methodology was of high quality. It was possible to extract DNA from the all the collected *Leucopholis* samples with the modified method. DNA concentration varied from 232.9 to 543.6 ng/μL. In most of the cases, the 260:280 absorbance ratios satisfied for pure DNA requirements and varied between 1.70 and 1.86. The efficacy of the method was indicated by amplifying and sequencing the MtCOI. The DNA samples were used for polymerase chain reaction (PCR) employing *viz.* LCO-1490 & HCO-2198 (Folmer et al., 1994) MtCOI primers. PCR for *Leucopholis* samples had produced the expected PCR amplicon size (~658 bp), which was confirmed on 1.5% agarose gel. The expected PCR amplicon (~658 bp) was eluted from the agarose gel using Nucleospin Extract II as per manufacture's protocol (Machery Nagal, Germany) subsequently cloned and sequenced. The morphological variation of the endophallus has revealed the information needed to solve problems and clarify the evolutionary relationships among the groups and also helps in cladistic analysis (Medina and Scholtz, 2005). *Leucopholis* samples were successfully sequenced based on cytochrome oxidase subunit I gene (COXI) sequences. Morphological and molecular analysis is very important step to develop integrated pest management strategies, proper species identification is required *viz.*, usage of female baited traps for mass trapping of male beetles (Kalleshwaraswamy et al., 2016), application of insecticides and biocontrol agents (Prabhu et al., 2011; Laznik and Trdan, 2014; Adarsha et al., 2015; Adarsha, 2017, Stanislav Trdan et al., 2019), application of insecticides with flooding (Adarsha et al., 2017 and Adarsha et al., 2018) and underground drainage and application of gravel soil (Adarsha, 2017).

Conclusion

There is a lack of taxonomic information on *Leucopholis* species in India. To overcome this aspect, *Leucopholis* species were identified based on morphological and molecular methods. Three species of *Leucopholis* can be identified based on male genitalia structures such as aedeagus and endophallus. *Leucopholis lepidophora* is distributed in hill regions of Western Ghats and morphological distinguished from other two species of *Leucopholis*. Whereas *L. coneophora* and *L. burmeisteri* are externally similar in all the cases, this can be distinguished based on male genitalia description. These are distributed in coastal belts of Karnataka and Kerala. *Leucopholis lepidophora* was found in high altitude regions compare to *L. coneophora* and *L. burmeisteri* present in lower altitude. Endophallus or internal sac shows major differences between the *Leucopholis* spp. Molecular and Phylogenetic analysis is an accurate method for identification of organisms at the species level; this may help in the formation of targeted pest management strategies such as cultural, biological, mechanical or chemical control. DNA barcoding is not replacing traditional morphological species identification; the combined use of morphological and molecular analysis can provide a very powerful tool for identifying pest at the species level. These morphological characters were very important for the understanding of the phylogenetic relationship among the species.

Conflct of interest

Authors declares no conflict of interest.

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