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Morpho-molecular identification of the bark beetle *Hylesinus macmahoni* (Stebbing, 1909) (Coleoptera: Curculionidae: Scolytinae) infesting *Olea europaea* subsp. *cuspidata* (Wall. & G.Don) Cif., along with a brief biological synopsis

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Abstract

In Jammu and Kashmir's Govindpora (Ramban) and Salamabad (Baramulla), horticultural nurseries with a majority of *Olea europaea* subsp. *cuspidata* (Wall. & G.Don) Cif. plantings were examined for possible bark beetle attacks. These olive tree plantations are infested with a variety of insect pests, but the bark beetle *Hylesinus macmahoni* (Stebbing, 1909) did significant harm to the olive trees that carry fruit. The beetle attacks and consumes the phloem tissues of the plant during its development, causing the afflicted trees to become weak and eventually die. At both sites, infestations of *H. macmahoni* were discovered. The cytochrome oxidase subunit I (COI) gene was used to validate the species' diagnosis, and its morphological characteristics were re-described. The species has a polygynous matting system (one male and two females), producing two full and a third partial generation annually in Kashmir.

Key words

bark beetle, diagnosis, *COX-1* gene, phylogenetic assessment, biology

Introduction

The olive tree, *Olea europaea* subsp. *cuspidata* (Wall. & G.Don) Cif. is a species of small, evergreen tree that belongs to the family Oleaceae and is native to the coastal areas of the Eastern Mediterranean region, from Lebanon and the maritime parts of Asia Minor to northern Iran at the southern end of the Caspian Sea (Haber and Mifsud 2007). Its fruit is of major agricultural importance in the Mediterranean region and other Mediterranean-type climates as a source of olive oil and table olives. The olive trees are mainly grown in two semi-temperate regions of Jammu and Kashmir viz. Govindpora (Ramban) and Salamabad (Baramulla) (Shant 1999). Many diseases and insect pests are known to damage olive trees (Borg 1932; Mifsud 1997; Porta-Puglia and Mifsud 2005; Lozano et al. 2009). Bark beetles utilise a wide variety of hosts including living, damaged or dying trees, cut logs, cones, needles and roots (Wood 1982). The bark beetle *Phloeotribus scarabae-*

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oides (Bernard, 1788) is a common insect pest of olive trees throughout the Mediterranean coast and in a number of other countries (Arambourg 1984). The Scolytine beetle *Hylesinus macmahoni* (Stebbing, 1909) has been reported on *Olea europaea* subsp. *cuspidata* earlier from Ramban area of Jammu and Kashmir (Schedl 1957). However, due to inaccurate taxonomic information in the relevant groups, Gupta et al. (2023) recently misidentified the beetle as *P. scarabaeoides* besides providing other misleading taxonomic information.

The objective of the present study was to investigate bark beetles (if any) on olive trees grown mainly in the horticultural nurseries at Govindpora (Ramban) and Salamabad (Baramulla) areas of Jammu and Kashmir. We also provide species diagnosis using DNA data. A brief biology of the species is also given.

Material and methods

Collection

During the survey of various host trees in 2018–2019, the bark beetle *H. macmahoni* was collected from the olive nurseries located in Salamabad (34° 05.186′ N and 074° 01.765′ E) and Govindpora (33° 07.053′ N and 075° 18.967′ E) regions of Jammu and Kashmir (Fig. 1). The host trees showed positive signs of *Hylesinus* infestation (emergence holes and frass coming out of the active gallery systems) on both the live trees as well as the pruned logs. The infested branches were examined, and the adult forms were collected after debarking the galleries. The specimens were then preserved in ethanol (96%) for subsequent taxonomic treatment.

Identification

For identification, we follow the nomenclature used by Wood and Bright (1992) as well as later taxonomic and systematic adjustments (e.g. Maiti and Saha 2009; Knížek 2011; Bright 2021). Then, the specimens were morphologically examined under a Leica M205A Stereozoom microscope (Leica Microsystems GmbH, Wetzlar, Germany). The images were taken with a Leica DFC295 camera attached to the microscope and having LAS Montage Multifocus Software (version 4.10). Measurements of the specimens were also taken using Leica Automontage Software. The specimens are deposited in the Museum, Department of Zoology, Uni-

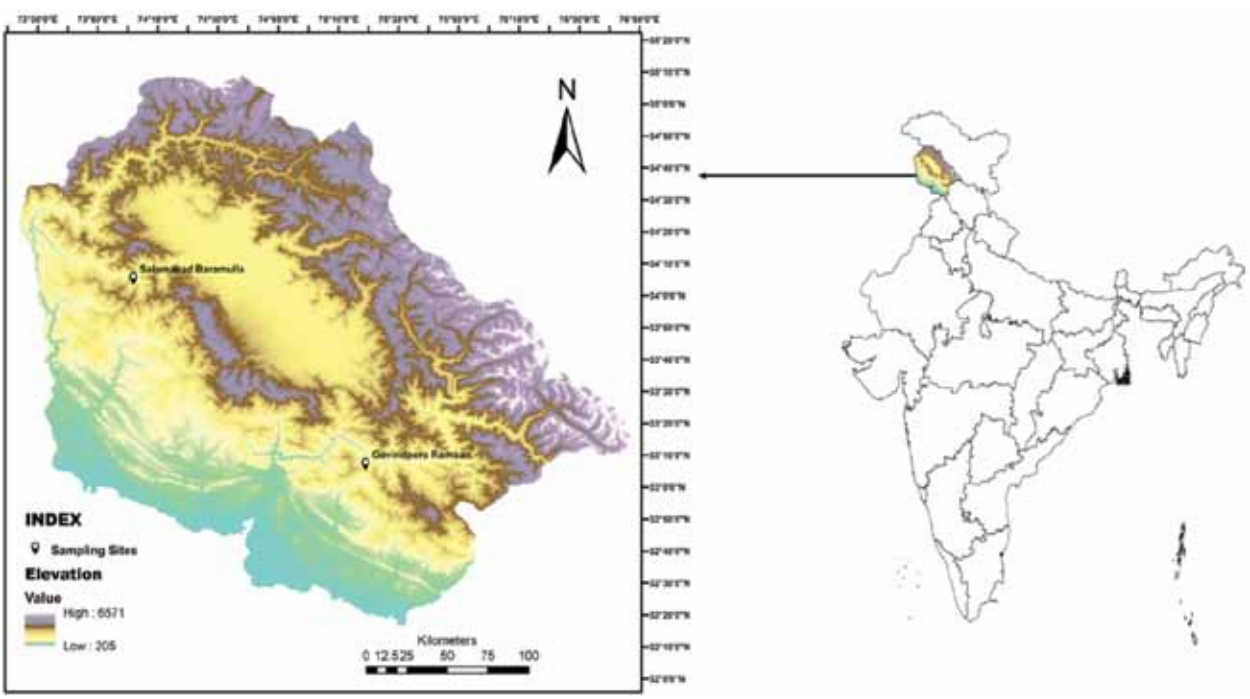


Figure 1. Sampling sites of *Hylesinus macmahoni* in the Pir Panjal range of Jammu and Kashmir (ArcGIS 10.2; http://resources.arcgis.com/en/help/ main/10.2/ index.html)

versity of Kashmir, Srinagar and some specimens are deposited in Milos Knížek Collection, Prague.

Polymerase Chain Reaction and Sequencing

The samples for DNA extraction were preserved in extra pure (96%) ethanol and stored at -20°C. Adult specimens were carefully crushed and DNA was extracted using Nucleospin Insect DNA kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. The amplification of the DNA was carried out in 25µl reactions containing 5µl Q5 reaction buffer, 0.5µl deoxynucleoside triphosphates (dNTPs), 0.5µl of each primer: LCO-1490 5'-GGTCAACAAATCATAAAGATATTGG-3' (forward) and HCO-2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (reverse) (Folmer et al. 1994), 3µl of template DNA, 0.25µl Q5 high-fidelity DNA polymerase, 5µl Q5 high GC enhancer and 10.25µl of nuclease-free water. The amplifications were carried out with an initial denaturation step of one cycle of 2 min at 95°C, 35 cycles for 20 sec at 95°C, 40 sec at 52°C, 2 min at 72°C and a final elongation cycle of 10 min at 72°C. The amplified products were separated on 2% agarose stained with ethidium bromide and visualised using a UV trans-illuminator. The sequence reactions were performed in both directions on a thermal cycler using Applied Biosystems BigDye Terminator V3.1 Cycle sequencing kit. The sequencing products were loaded on Applied Biosystems 3130 Genetic Analyser at GeneOmbio Technologies Pvt. Ltd. (Pune, India). The sequences of the haplotypes derived from this study were deposited in the Gene Bank with accession numbers: MW715433 *H. macmahoni* HT-1 and MW715434 *H. macmahoni* HT-2.

Data Analysis

The DNA sequence alignment (for cytochrome oxidase subunit I [COI] genes) was performed by ClustalX (Thompson et al. 1997) using default setting. Distance analysis and construction of trees were performed by the Neighbor-Joining (NJ) algorithm (Saitu and Nei 1987) as it is implemented in the MEGA-11 (Tamura et al. 2021). For comparison, the GenBank entries of desired Scolytine species including outgroup were taken in the analyses.

Laboratory Rearing

To study the biological parameters, the branches infested with *H. macmahoni* were cut into logs of specific size range (30–40 cm length and 8–10 cm in diameter). The logs were then brought to the rearing laboratory and placed in rearing boxes of similar design with dimensions of 70 × 30 × 35 cm and 40 × 30 × 30 cm, made up of glass fitted with a muslin cloth on their top end. To maintain the culture, some fresh branches (30–40 cm long and 8–10 cm in diameter) were placed with the naturally infested branches inside the rearing boxes as soon as the adults started emerging. The fresh branches at their cut ends were sealed with wax. The mating system, gallery pattern and the number of generations were recorded.

Results

Morphological identification

H. macmahoni (Stebbing, 1909)

= *macmahoni* Stebbing, 1909 (*Sphaerotrypes*)
= *alternans* Schedl, 1959
= *fraxinoides* Schedl, 1959 (*Leperisinus*)

Description (Figs 2–4)

Frons somewhat flat and feebly impressed above epistomal margin; surface finely reticulate, with close punctures of irregular shape and size and dense stout setae, except on median smooth, impunctate area; vertex convex, finely reticulate and with minute punctures; eyes elongate and entire; antennal scape long, funicle with seven segments, club conical with three distinct sutures; pronotum 1.5 times as wide as long, widest at base, lateral sides rounded and gradually incurved from the base, with distinct antero-lateral constriction; anterior margin sub-rounded; surface rugosely punctate, punctures more prominent, shallow and irregular towards the basal half; asperities of different sizes on the lateral margins more prominent anteriorly; scale-like setae on the pronotal surface, somewhat longer laterally and recumbent towards the basal margin; elytra two times as long as pronotum, lateral sides sub parallel on basal two-fifths, gradually narrowing posteriorly with broadly rounded apex; basal margin each with 12–13 transverse crenulations; striae impressed and narrow with somewhat indistinct punctures; interstriae much

wider than striae; interstrial vestiture with three to four rows of scale-like setae, scales blunt at the apex; pale scales form a wide band from the postero-lateral part of elytra to scutellum, with a further pale area on elytral declivity; rows of tuberculations visible on interstriae more prominent towards the elytral base; declivity convex, moderately steep with second interstriae somewhat depressed; colour blackish brown; body length 2.9 mm. Males are distinct from females in having broadly impressed frons.

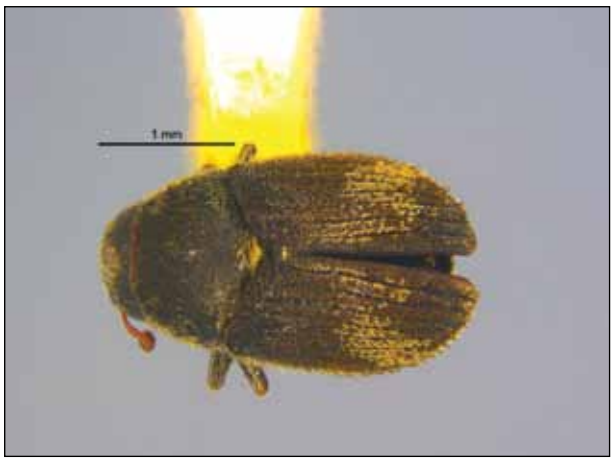


Figure 2. *Hylesinus macmahoni* ♂ (dorsal view)



Figure 3. *Hylesinus macmahoni* ♂ (lateral view)

Remarks: *H. macmahoni* is very similar to *H. tupolevi* Stark, 1936 in the elytral colouration, but the setae on the elytra in *H. tupolevi* are rather sharply pointed and in *H. macmahoni,* the setae are blunt. The species is also very similar to *H. cingulatus* Blandford 1894*,* but differs from it again mainly in the elytral setae, which are very short, somewhat rounded and blunt in *H. cingulatus*.



Figure 4. *Hylesinus macmahoni* ♂ (frontal view)

Specimens examined

Syntypes in F.R.I. Dehradun

New records: India: Kashmir, Baramulla, Salamabad (34° 05.186′ N, 074° 01.765′ E, 4415 ft.), A.A. Buhroo, 30.09.2017 (KUIC). Jammu, Ramban, Govindpora (33° 07.053′ N, 075° 18.967′ E, 5420 ft.), A.A. Buhroo and R.M. Zubair, 10.05.2018 (KUIC)

Distribution

India: Jammu and Kashmir

Hosts

Fraxinus excelsior, F. floribunda, Olea europaea subsp. *cuspidata*

Phylogenetic assessment

Phylogenetic analysis of *H. macmahoni, H. varius* (Fabricius, 1775)*, H. crenatus* (Fabricius, 1787)*, H. fraxini* Panzer, 1799 and *H. wachtli orni* Fuchs, 1906, along with the out-group *Hylurgus ligniperda* (Fabricius, 1787) resulted in the formation of clades having bootstrap values greater than 90% (Fig. 5). *H. macmahoni* was separately monophyletic within the group with well-supported sister clades, and its haplotypes were displayed in a single clade with high bootstrap values (100%). The nucleotide

difference between the haplotypes of *H. macmahoni* was 2.2% (Table 1). The interspecific nucleotide divergence was evident and well above the set criteria (6.1%) for *Ips* sister species (Cognato and Sun 2007). It ranged 13.1%–13.8% between *H. macmahoni* and *H. varius*, 12.3%–14.6% between *H. macmahoni* and *H. wachtli* and 14.7%–16% between *H. macmahoni* and *H. crenatus*. The earlier systematic adjustments (Knížek 2011, Bright 2021) have put *H. fraxini* under the synonymy of *H. varius*. However, the consensus tree also showed that *H. varius* and *H. fraxini* formed one clade with 100% bootstrap values, which also supports their synonymy being morphologically similar species.

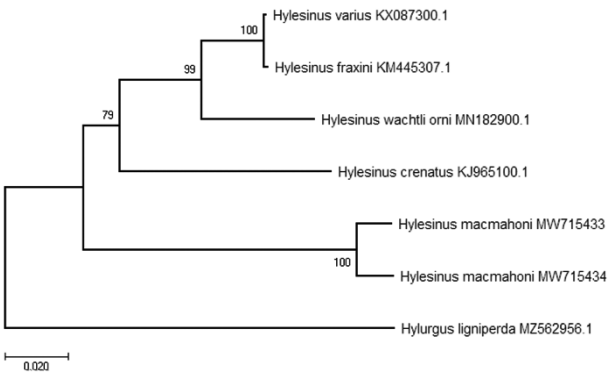


Figure 5. A phylogenetic tree obtained from NJ for the COI genes of *H. macmahoni* and related species. Numbers at the branches represent the bootstrap values.

Table 1. Interspecific nucleotide differences of *H. macmahoni* and its related species

Variable	*H. macmahoni* HT-1	*H. macmahoni* HT-2	*H. varius*	*H. wachtli orni*	*H. crenatus*
H. macmahoni HT-1	x	2.2	13.1	14.6	16
H. macmahoni HT-2	2.2	x	13.8	12.3	14.7

Gallery patterns

H. macmahoni excavates a characteristic transverse mother gallery in the phloem and sapwood under the bark. Because of polygamous mating system, the mother galleries are mostly bi-armed, unequal in length and run opposite to each other on the two sides of nuptial chamber (Fig. 6). The adjacent mother galleries run parallel to one another and are evenly spaced. The length of the mother gallery ranged from 2.7 to 8 cm with a mean of 5.61 (±1.87 standard deviation [SD]) cm (Tab. 2). The larval mines were around 50–55 in number radiating from both margins of the mother gallery. They were wavy and completely separated, running up and down, with the middle ones being straight while the outer ones radiated straight at first and then bent outwards, away from the mother gallery. The larval mines measured 4.4–7.0 cm in length with a mean of 5.25 (±0.74 SD) cm, with increasing dimensions as they moved away. The adults emerged through the exit holes, forming a specific pattern on the bark surface and leaving a characteristic impression in the inner bark specific to a species.

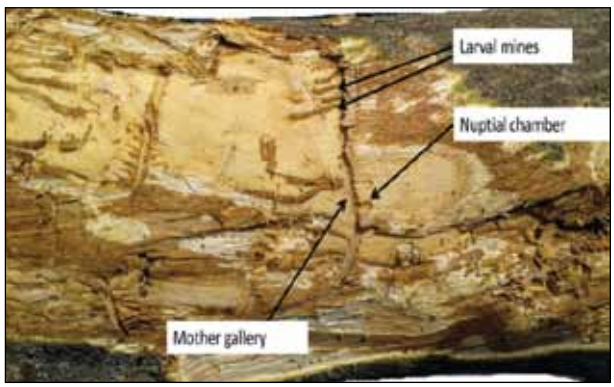


Figure 6. Debarked log of *Olea europaea* subsp. *cuspidata* showing the gallery pattern

Table 2. Measurement of galleries of *H. macmahoni*

Variable	n	Minimum (cm)	Maximum (cm)	Mean (cm)	SD (cm)
Maternal gallery	10	2.7	8	5.61	1.87
Larval gallery	10	4.4	7	5.25	0.74

n – number of observations.

Biology

The female locates a suitable host tree and then bores into the bark, excavating a small depression called as nuptial chamber meant for mating. This was evident by the presence of females in most of the newly constructed galleries containing solitary individuals. The female then girdles out transverse single- or double-armed mother gallery (Fig. 6). Single-armed galleries were few, and majority of the galleries were bi-armed with unequal length of the arms; probably, these gal-

lery systems are initially the work of a single female to which the secondarily arriving male later attracts the second female, resulting in two-armed gallery with equal or unequal arm lengths. About 55 (±5 SD, $n = 10$) eggs were deposited at regular intervals in small individual niches along the whole length on both sides of the mother gallery. After hatching out from the eggs, the larvae ate out distinct wavy and completely separated mines, running up and down, the middle ones straight while the outer ones radiated straight at first and then bent outwards. The larval mines ended up in pupal chambers, where adults matured and finally emerged straight through the exit holes, forming a specific pattern on the bark surface.

During the field surveys and laboratory observations, it was found (Fig. 7) that this species overwintered in larval and pupal stages inside the galleries from the last week of October onwards. The overwintering generation resumed its activity in spring of the following year from the first week of March onwards. The first swarming adults appeared from the first week of April. The adults again searched fresh and suitable trees to continue the life cycle. The second swarming of adults emerged in the later part of June. Emergence of adults was again seen in September of the year. This showed that there were two complete and a third partial generation per year in Jammu and Kashmir.

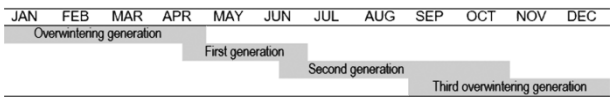


Figure 7. Generations of *H. macmahoni* in Kashmir

Discussion

Biogeographically, the Himalayas constitutes a transition zone between the Palaearctic and the Indo-Malayan realms, and an intermixing of species from both the realms is evident in its faunal composition (Corbett and Hill 1992). Most species of the genus *Hylesinus* are distributed in the Holarctic realm, but in the Palaearctic, they occupy the range of *Fraxinus* up to India and northern Africa (Knížek 2011; Petrov 2011). The genus includes 37 recent species, of which at least 12 are known from the Palaearctic. All Palaearctic species breed in the genera *Fraxinus* and *Olea* of Oleaceae trees, but exceptionally can attack branches of *Fagus, Quercus, Tilia* and *Syringa*. The current research found that olive trees in Jammu and Kashmir, particularly in the areas of Govindpora and Salamabad, were attacked by *H. macmahoni*. This species infested both cultivated and wild varieties of its host *Olea europaea* subsp. *cuspidata* in the areas visited. Thus, the fascinating biodiversity of this region is the consequence of complex interplay between biogeographic, altitudinal and topographic factors. The morphology of the species is re-described supplemented by digital photography and DNA data.

In polygamous species, males usually initiate gallery construction in the form of a nuptial chamber (e.g. *Ips* spp.) (Wermelinger 2004); however, in *H. macmahoni*, the galley construction was pioneered by females, similar to that of *H. fraxini* (Lukášová and Zimová 2015). Sometimes, only one mating pair was seen in the gallery, usually with only one arm. The emergence and girdling by the adults on the branches of olive trees was seen in April of the year, and the species completed two full and a third partial generation annually in Kashmir.

Much remains to be done to increase the knowledge of Scolytine biodiversity in the Himalayan region, particularly with respect to their association with host plants. The unique topography of the region indicates that species diversity could be higher and needs to be explored in future.

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