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Phylogeny of *Dendroctonus* bark beetles (Coleoptera: Curculionidae: Scolytinae) inferred from morphological and molecular data

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Abstract. Bark beetles in the genus *Dendroctonus* may attack and kill several species of coniferous trees, some of them causing major economic losses in temperate forests throughout North and Central America. For this reason, they have been widely studied. However, various aspects of the taxonomy and evolutionary history of the group remain contentious. The genus has been subdivided in species groups according to morphological, biological, karyological or molecular attributes, but the evolutionary affinities among species and species groups within the genus remain uncertain. In this study, phylogenetic relationships among *Dendroctonus* species were reassessed through parsimony-based cladistic analysis of morphological and DNA sequence data. Phylogenetic inference was based on 36 morphological characters and on mitochondrial DNA sequences of the cytochrome oxidase I (COI) gene. Analyses were carried out for each dataset, as well as for the combined data analysed simultaneously, under equal and implied weights. According to the combined analysis, the genus *Dendroctonus* is a monophyletic group defined by at least three synapomorphic characters and there are four main lineages of varied composition and diversity within the genus. Within these lineages, several monophyletic groups match, to some extent, species groups defined by previous authors, but certain groups proposed by those authors are polyphyletic or paraphyletic.

Introduction

The bark beetle genus *Dendroctonus* Erichson (Coleoptera: Curculionidae: Scolytinae) comprises 20 recognized species (Wood, 1982; Armendáriz-Toledano *et al.*, 2015). Most species are distributed in North and Central America, but two native and one introduced species are present in Eurasia (Wood & Bright, 1992; Bright, 2014). Several of them are among the most destructive agents of coniferous forests (Fig. 1). Despite their ecological and economic significance, some relevant issues of the evolutionary history of the group remain controversial, particularly those concerning the phylogenetic relationships among its members, the delimitation of species and species groups, the geographical origin of the genus and its evolutionary trends.

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The genus has been subdivided into species groups (or complexes) according to morphological, biological, karyological or molecular affinities among its members (Wood, 1963, 1982; Lanier, 1981; Bentz & Stock, 1986; Stock *et al.*, 1987; Kelley & Farrell, 1998; Cognato, 2011; Reeve *et al.*, 2012). However, these groupings, derived from different data sources and different methods of analysis for such data, differ with regard to the precise delimitation of species groups and, particularly, the relative phylogenetic position of each group within the genus.

In his revision of the genus, Wood (1963) recognized 14 species and placed them in five groups based on morphological and biological affinities, ordering these groups according to their level of 'specialization'. Wood's proposal does not specify the relationships between groups, and he points out that 'evolutionary relationships among the groups is [*sic*] uncertain' (Wood, 1963: 24). However, level of 'specialization' was defined considering the degree of morphological and behavioural divergence of each species with respect to allied genera. Hence,

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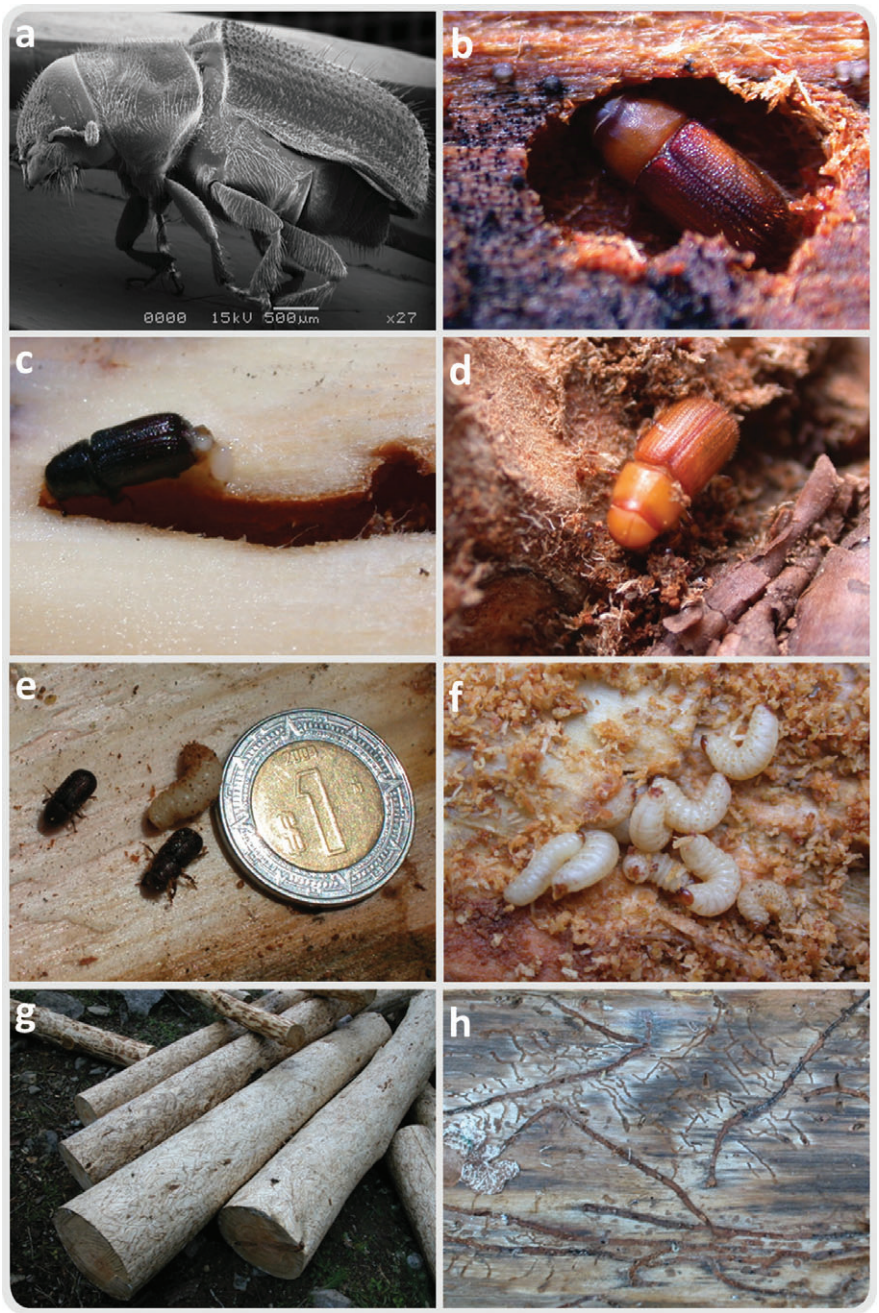

Fig. 1. *Dendroctonus* bark beetles and their damage to host trees: (a, b) *D. pseudotsugae* adults, collected from Douglas-fir (*Pseudotsuga menziesii*) in Durango, Mexico. (c) *D. adjunctus* adult on Hartweg's pine (*Pinus hartwegii*) in Jalisco, Mexico. (d) *D. rhizophagus* adult on Apache pine (*P. engelmannii*) in Chihuahua, Mexico. (e) *D. valens* adults and larva on Jeffrey pine (*Pinus jeffreyi*) in Baja California, Mexico. (f) *D. valens* larvae on lodgepole pine (*Pinus contorta*) in Idaho, U.S.A. (photograph by Malcolm Furniss, used with permission). (g) Trunks with bark removed from felled Gregg's pine (*Pinus greggii*) trees infested with *D. frontalis* in Queretaro, Mexico, showing the gallery systems constructed by the beetles. (h) Close-up of *D. frontalis* galleries under the bark of a *P. greggii* tree.

the proposed ordering of the groups implies an evolutionary sequence in which the less specialized (or more 'primitive') group is the *Dendroctonus frontalis* Zimmermann group, while the most specialized or 'modern' is the group comprising *Dendroctonus pseudotsugae* Hopkins and *Dendroctonus simplex* LeConte. In a subsequent taxonomic update, Wood (1982) recognized 19 valid species but maintained the division of the genus in five groups, in which he placed 18 of the species (not including *Dendroctonus armandi* Tsai & Li, an endemic species from China). Recently, a new species from southern

Mexico and Central America has been recognized and subsequently described (Sullivan *et al.*, 2012; Armendáriz-Toledano *et al.*, 2014, 2015), being very similar to the species in the *D. frontalis* group.

Lanier (1981) re-evaluated the original classification proposed by Wood (1963) based on the analysis of the karyotype of 14 *Dendroctonus* species. He presented an arrangement of the genus in six species groups, according to similarities and differences in features such as chromosome number and configuration of sex chromosomes in males (i.e. neo-XY or Xy_p). The main disagreement with respect to Wood's (1963) proposal refers to the ordering of the groups and the evolutionary implications of such an ordering scheme. The criterion used by this author to define the sequential arrangement of his groupings was comparison with the karyotype of *Hylurgops pinifex* (Fitch), a bark beetle belonging to the tribe Hylastini (*sensu* Wood, 1982), considered by him as 'primitive'. Lanier (1981) ordered his groups following a sequence in which members of his group I [*Dendroctonus rufipennis* (Kirby) and allied species] correspond to the most 'primitive' species in the genus – followed by species in the group II (*D. pseudotsugae* and *D. simplex*) – while species in the group VI (*D. frontalis* and closely related species) represent 'the most advanced' ones. This ordering is nearly the opposite of that postulated by Wood (1963). Another significant disagreement with Wood's (1963) proposal relates to the placement of *Dendroctonus adjunctus* Blandford, a species included in the *Dendroctonus ponderosae* Hopkins group (along with *D. ponderosae* and *Dendroctonus jeffreyi* Hopkins) but placed by Lanier (1981) in a group of its own (his group V) as he considered it to be 'chromosomally intermediate' between the *D. ponderosae* group (his group IV) and the *D. frontalis* group (his group VI).

Bentz & Stock (1986) used allozyme data to estimate genetic relationships among ten *Dendroctonus* species [representing the species groups recognized by Wood (1963) and Lanier (1981)] using phenetic methods. The patterns of relationship obtained by these authors include some groupings coincident with previous proposals (i.e. the clusters *D. simplex*–*D. pseudotsugae* and *Dendroctonus terebrans* (Olivier)–*Dendroctonus valens* LeConte) but also have notable differences. The authors conclude that '*D. adjunctus*, *D. approximatus*, and *D. rufipennis* are the most primitive species, and that *D. simplex*, *D. pseudotsugae*, *D. frontalis*, *D. terebrans*, and *D. valens* are among the more evolutionarily advanced species in the genus' (Bentz & Stock, 1986: 533).

Later, Stock *et al.* (1987) reanalysed these data but incorporated *Dendroctonus micans* (Kugelann), a Eurasian species, to determine its relationship with the ten American species included in the original study. The phenogram derived from their cluster analysis shows mostly the same groups obtained by Bentz & Stock (1986), although *D. micans* grouped with *D. terebrans* and *D. valens* (Fig. 2a), a result that disagrees with previous hypotheses by Wood (1963) and Lanier (1981).

By using *Dendroctonus* beetles as a model system for studying the evolution of specialization in host use in phytophagous insects, Kelley & Farrell (1998) inferred a phylogeny of the genus through parsimony analysis of nucleotide sequences of a fragment of the mitochondrial cytochrome oxidase I gene (COI) from 18 of the species then recognized by Wood (1963) (*Dendroctonus parallelocollis* Chapuis was not considered). The most parsimonious tree found in this study supports the monophyly of many of the groups proposed by Wood (1963, 1982) and Lanier (1981), although *D. adjunctus* appears as sister to the clade formed by *Dendroctonus approximatus* Dietz plus *Dendroctonus brevicomis* LeConte (Fig. 2b). Consequently, the *D. frontalis* group (*sensu* Wood, 1963) or group VI (*sensu* Lanier, 1981) was paraphyletic, and the *D. ponderosae* group (*sensu* Wood 1963), was polyphyletic. This cladogram also showed a division of the genus into two major clades (aside from *D. armandi*, which appeared as sister to all remaining species), each one comprising several species groups of Wood (1963) and Lanier (1981).

Cognato (2011) conducted a parsimony analysis of eight *Dendroctonus* species to assess the phylogenetic placement of *D. frontalis* within the genus. This analysis was based on combined DNA sequence fragments from five genes (18S ribosomal subunit, 28S ribosomal subunit, elongation factor 1α, enolase and COI). Despite the limited taxon sampling – most groups of Wood (1963) and Lanier (1981) are represented by only one species, relationships among the included taxa are largely consistent with the results of Kelley & Farrell (1998) and nearly all of them are highly supported, except the sister-group relation of *Dendroctonus murrayanae* Hopkins and *D. pseudotsugae*. This node was statistically equivocal in the tree, as was the case for the clade joining their corresponding species groups in the most parsimonious tree of Kelley & Farrell (1998). The main disagreement with the latter authors involves the position of *D. terebrans*, which appears as sister to the rest of *Dendroctonus* species in Cognato (2011) (Fig. 2c).

Aiming to trace the evolution of a number of ecological and life-history traits correlated with the ability to kill healthy trees in *Dendroctonus* beetles, Reeve *et al.* (2012) also used DNA sequences of a fragment of the COI gene from 17 species (*Dendroctonus mesoamericanus* Armendáriz-Toledano & Sullivan, *D. parallelocollis* and *Dendroctonus vitei* Wood were not included) to estimate the phylogeny of this group. They used the phylogeny as a framework for reconstructing the ancestral states of the attributes of interest at various nodes on the tree, employing maximum likelihood and Bayesian methods. The maximum likelihood tree presented by these authors showed some remarkable differences with previous proposals (Fig. 2d): *D. armandi* was not sister to the rest of the genus, but instead appeared as sister to the *D. simplex*–*D. pseudotsugae* clade; the clade comprising *D. terebrans*, *Dendroctonus rhizophagus* Thomas & Bright and *D. valens* showed a sister-group relationship to the group consisting of *D. rufipennis*, *D. micans*, *D. murrayanae* and *Dendroctonus punctatus* LeConte, not to the *D. ponderosae* and *D. frontalis* groups; relationships among *D. micans*, *D. murrayanae*, *D. punctatus* and *D. rufipennis* also exhibited a different pattern, as well as relationships within the *D. frontalis* group (particularly regarding the non-sister grouping of *D. frontalis* and *Dendroctonus mexicanus* Hopkins).

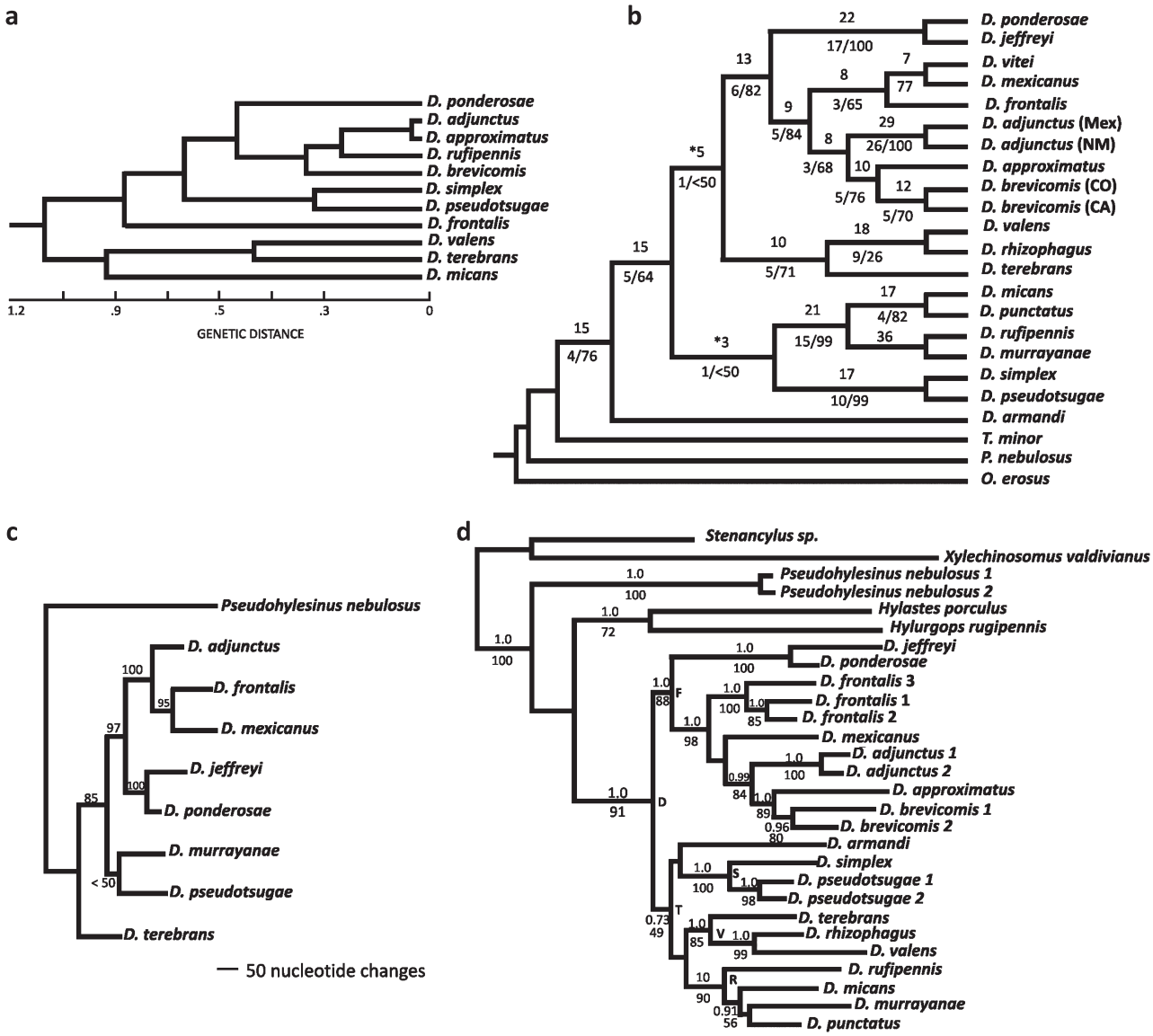

Fig. 2. Previous phylogenetic hypotheses for the genus *Dendroctonus*: (a) phenogram presented by Bentz & Stock (1986), illustrating relationships among 11 species based on allozyme data. (b) Single most parsimonious tree presented by Kelley & Farrell (1998), illustrating relationships among 18 species based on parsimony analysis of DNA sequences of a fragment of the cytochrome oxidase I (COI) gene. Numbers of synapomorphies are presented above the branches; Bremer support and bootstrap values (calculated without *D. vitei*) are presented below the branches; asterisks indicate clades with bootstrap support lower than 50. (c) Most parsimonious tree presented by Cognato (2011), illustrating relationships among eight species based on parsimony analysis of combined DNA sequence fragments from five genes (18S, 28S, EF-1α, enolase and COI). Bootstrap values are shown at the base of the branches; branch lengths indicate the number of character state changes. (d) Best-known likelihood tree presented by Reeve *et al.* (2012), illustrating relationships among 17 species based on maximum likelihood (ML) analysis of DNA sequences of a fragment of the COI gene. Values at the base of each clade correspond to posterior probabilities (from a parallel Bayesian analysis) and ML bootstrap support values; letters identify clades used by the authors in their ancestral state reconstructions.

In the present study, phylogenetic relationships of all *Dendroctonus* species were reassessed through a cladistic analysis, incorporating data from morphology of adult and immature stages as well as other data sources such as biological features and COI mitochondrial DNA sequences, analysed simultaneously under parsimony in an attempt to increase the resolution and explanatory power of available data.

Materials and methods

The examined material includes specimens of the 20 species in the genus *Dendroctonus* (Wood, 1982; Armendáriz-Toledano *et al.*, 2015). The species *Pseudohylesinus variegatus* (Blandford), *Tomicus piniperda* (Linnaeus) and *Hylurgus ligniperda* (Fabricius) were used as outgroups. The

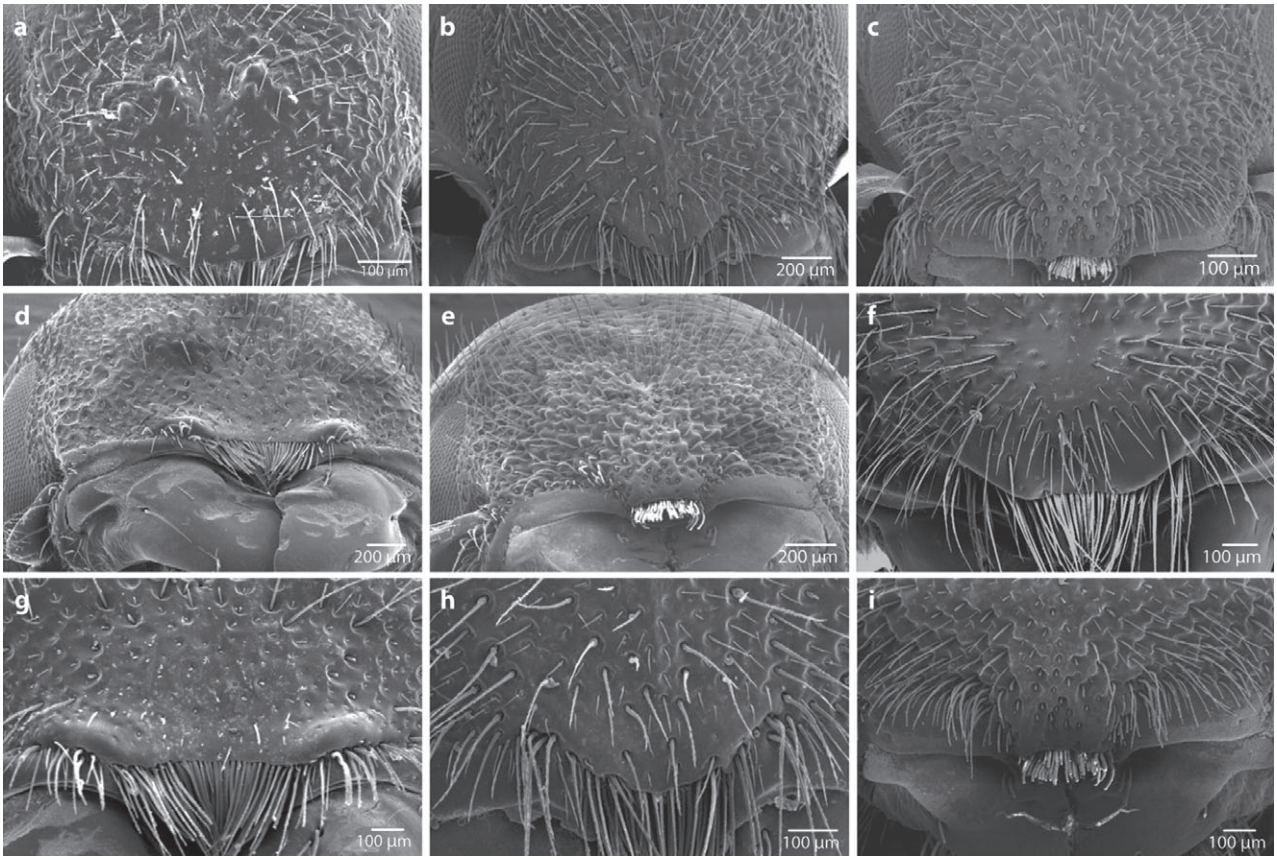

Fig. 3. Head (all genuses are *Dendroctonus*). (a–c) Frontal region: (a) *D. frontalis* (male); (b) *D. rufipennis* (female); (c) *D. pseudotsugae* (female). (d, e) Epistomal region: (d) *D. valens*; (e) *D. pseudotsugae*. (f–i) Epistomal process: (f) *D. adjunctus*; (g) *D. valens*; (h) *D. rufipennis*; (i) *D. pseudotsugae*.

collections from which specimens were examined are the following:

CEAM	Colegio de Postgraduados, Montecillo, Mexico
CNC	Canadian National Collection of Insects, Ottawa, ON, Canada
CNIN	Colección Nacional de Insectos, Instituto de Biología, UNAM, Mexico City, Mexico
ENCB	Escuela Nacional de Ciencias Biológicas, IPN, Mexico City, Mexico
MCMC	Museo de Historia Natural de la Ciudad de México, Mexico City, Mexico
NFRC	Northern Forestry Centre, Canadian Forest Service, Edmonton, AB, Canada
OSAC	Oregon State Arthropod Collection, Oregon State University, Corvallis, OR, USA
UACC	División de Ciencias Forestales, Universidad Autónoma Chapingo, Chapingo, Mexico
UVGC	Universidad del Valle de Guatemala, Guatemala City, Guatemala
WFBM	W. F. Barr Entomological Museum, University of Idaho, Moscow, ID, USA
WFIC	Western Forest Insect Collection, USDA Forest Service, Corvallis, OR, USA

Different methods of analysis were used to examine the morphological variation in the genus and recognize potentially useful characters (primary hypotheses of homology). First, the examination of the characters in all available specimens was done under a stereoscopic microscope (usually a Nikon SMZ800), at magnifications ranging from 10× to 80×. A subset of these specimens was dehydrated, critical point-dried, mounted on aluminium stubs and gold-coated for examination with a JEOL JSM-5800LV scanning electron microscope (at an acceleration voltage of 15 kV; JEOL, Tokyo, Japan) for further refinement of initial observations and illustration of informative features. Genitalia of male specimens were also dissected, slide-mounted and examined under a Nikon Eclipse E100 compound light microscope.

In addition, larval specimens from most *Dendroctonus* species were obtained and examined for inclusion of characters from immature stages in the analysis. Specimens from these species were dissected and mounted on microscopic slides for morphological study, following the protocol described by Thomas (1957); a subset of these specimens was also processed for examination with scanning electron microscopy. Other characters such as karyotype and ecological-behavioural features (particularly patterns of gallery construction in host trees) were also examined. Coding of these characters was based on direct observations and field work whenever possible, although for some species first-hand access to this information was not

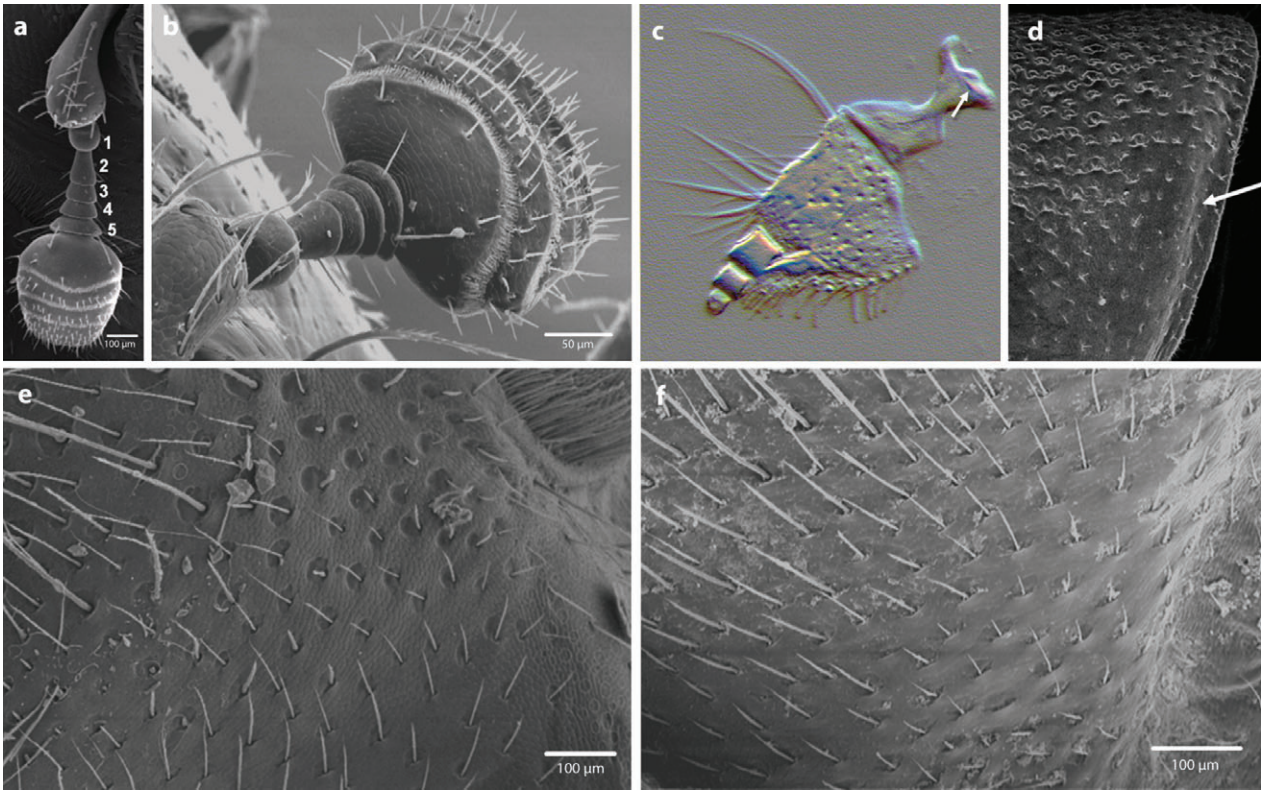

Fig. 4. (a, b) Antennae: (a) *D. valens*; (b) *D. mexicanus*. Maxilla: (c) *D. ponderosae*. Prothorax (lateral view): (d) *D. frontalis* (female). (e, f) Episternal area of prothorax: (e) *D. micans*; (f) *D. terebrans*. (All genuses are *Dendroctonus*.)

available and, in such cases, illustrations and descriptions from pertinent literature were used.

Variations between the different species in the genus (interspecific variation) and within each species (intraspecific variation) were evaluated for each character considered. Historically, scolytine taxonomy has suffered from ambiguously defined characters and states (e.g. abundant use of relative terms) and overlap among character states (Hulcr *et al.*, 2007). Therefore, when a character was recognized as potentially useful for phylogenetic analysis, character states were defined and delimitated as discrete, mutually exclusive, alternative states. Primary hypotheses of homology were established among states of the character based on the criterion of topographic similarity (*sensu* Remane, 1952, as cited by Wiley, 1981). Terminology used for description of characters and their states is based on Hopkins (1909, 1915), Wood (1963, 1982), Bright (1976) and Duncan (1987) for external morphology of adults, Thomas (1957, 1965) for larval morphology, and Hopkins (1915) and Cerezke (1964) for morphology of male genitalia. Examination of intraspecific and interspecific variation resulted in a matrix of 36 characters that represent potential homologies for phylogenetic analysis (Table S1). Twenty-six were characters from adult morphology (including male genitalia) and five from larval morphology, plus two chromosomal and three behavioural features (relative to patterns of gallery construction inside host tree tissues). Twenty-seven characters were coded as binary and nine as multistate. All characters were considered as unordered and with equal weights. Table S2 provides supplementary descriptive information for some characters that need further explanation due to their history of previous ambiguous or equivocal definitions and uses in scolytine literature. The selected characters and their states are included in the Appendix and illustrated in Figs 3–7.

The data matrix was created and edited using WINCLADA version 1.00.08 (Nixon, 2002). Parsimony-based phylogenetic analyses were carried out using the software TNT version 1.1 (Goloboff *et al.*, 2008b). A heuristic search strategy, with tree–bisection–reconnection branch swapping, was employed in order to find the most parsimonious trees (MPTs), starting with 1000 random addition sequences and holding ten trees during each replication. Trees were viewed and summarized in WINCLADA. Character optimization was also visualized in WINCLADA. In the case of characters with ambiguous optimization, both accelerated (ACCTRAN) and delayed (DELTRAN) transformations of character states were used to explore equally parsimonious reconstructions, as recommended by Agnarsson & Miller (2008). Bremer support (Bremer, 1988, 1994) and jackknife values (Farris *et al.*, 1996) were calculated in TNT to evaluate clade support. Bremer support values were obtained using the following command sequence – sub10; h500; find*; bsupport; – while jackknife values were estimated after 1000 replications of resampling and expressed as GC frequency differences (Goloboff *et al.*, 2003).

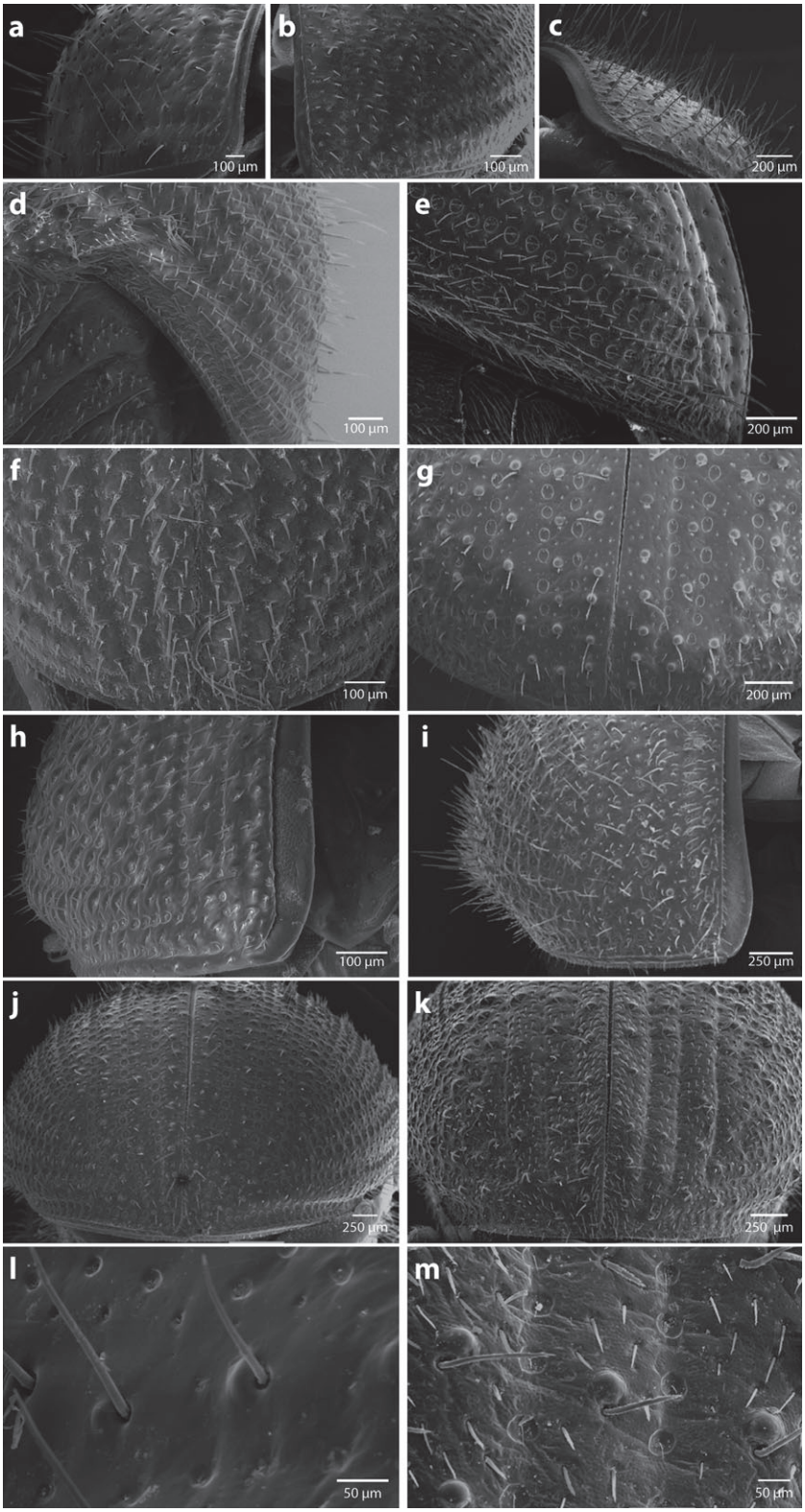

Fig. 5. Elytra (all genuses are *Dendroctonus*). (a–c) Vestiture: (a) *D. adjunctus*; (b) *D. brevicomis*; (c) *D. murrayanae*. (d, e) Elytral declivity (lateral view): (d) *D. vitei*; (e) *D. simplex*. (f–k) Elytral declivity (posterior view): (f) *D. mexicanus*; (g) *D. pseudotsugae*; (h) *D. mesoamericanus*; (i) *D. rufipennis*; (j) *D. valens*; (k) *D. jeffreyi*. (l, m) Microsculpture of interstriae: (l) *D. adjunctus*; (m) *D. jeffreyi*.

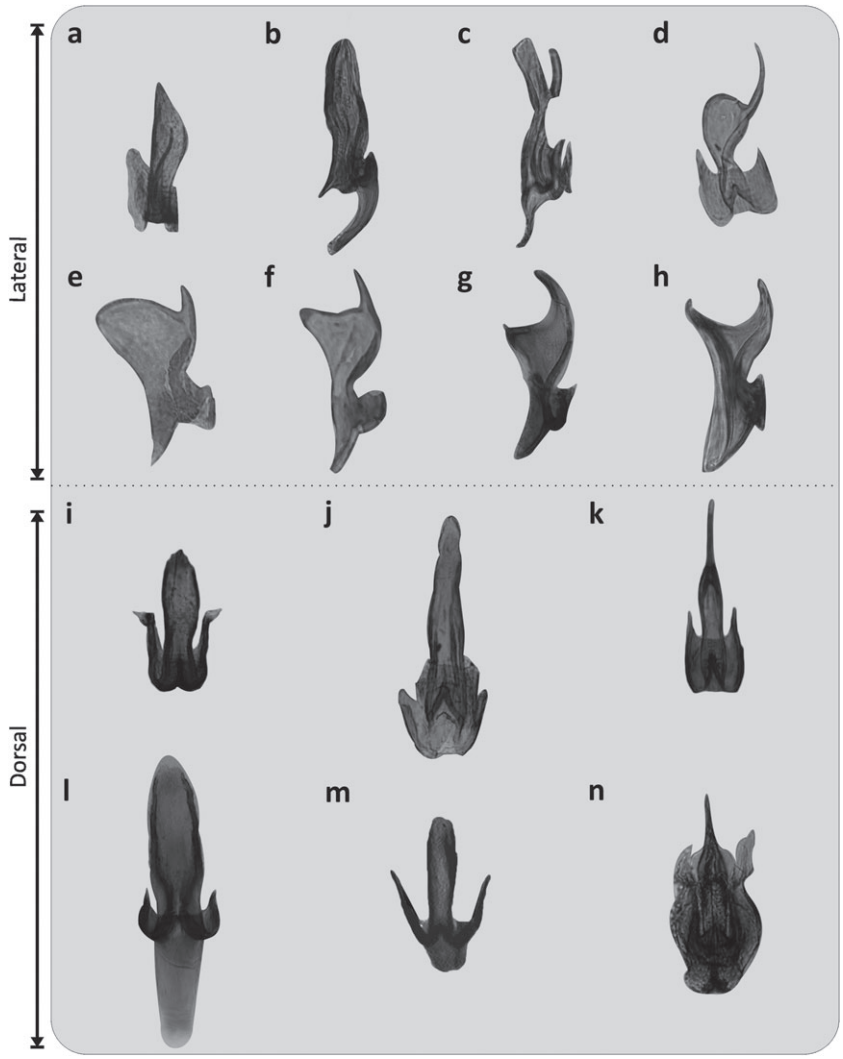

Fig. 6. Seminal rod (all genuses are *Dendroctonus*, unless noted otherwise). (a–h) Lateral view: (a) *D. approximatus*; (b) *D. rhizophagus*; (c) *Tomicus piniperda*; (d) *D. adjunctus*; (e) *D. frontalis*; (f) *D. mesoamericanus*; (g) *D. vitei*; (h) *D. mexicanus*. (i–n) Dorsal view: (i) *D. approximatus*; (j) *D. brevicomis*; (k) *D. adjunctus*; (l) *D. valens*; (m) *D. rufipennis*; (n) *D. pseudotsugae*.

Furthermore, additional searches were performed with implied weighting, which weights characters according to their levels of homoplasy (Goloboff, 1993). It has been demonstrated that properly down-weighting characters according to their homoplasy produces more strongly supported groups and more stable results in analyses of morphological datasets (Goloboff *et al.*, 2008a). Different values of the constant of concavity *k*, which determines how strongly homoplasious characters are down-weighted, were used in analyses under implied weights, to compare the results obtained with each value and thus evaluate stability of results through different analytical conditions (Goloboff *et al.*, 2008a). The *k*-values ranged from 1, which represents a stronger down-weighting of the homoplasious characters, to 10, which represents a milder down-weighting.

Cytochrome oxidase I nucleotide sequences for *Dendroctonus* species and the three outgroups were downloaded from GenBank (Table S1). When two or more different COI sequences were available for a single species, the most complete sequence was selected. In the case of *Pseudohylesinus*, the COI sequence available corresponds to *P. nebulosus* (LeConte) (closely related to *P. variegatus*, used in the morphological analysis). In addition, more complete COI sequences were obtained from *D. parallelocollis*, *D. vitei* and *D. mesoamericanus*. DNA was extracted using the Qiagen DNeasy tissue kit (Qiagen, Valencia, CA, U.S.A.) following the manufacturer's instructions. COI amplification was carried out using primer pairs C1-J-2183 (modified as 5′ CAACACTTATTTTGATTTTTTGG 3′) and TL2-N-3014, as well as TY-J-1460 and C1-N-2329 (modified as 5′ ACTGTGAATATATGATGGGCTCA 3′) (Simon *et al.*, 1994). Each polymerase chain reaction (PCR) was conducted in a final volume of 25 µL, containing 50 ng of DNA, 0.5 µM of each primer, 200 µM of each dNTP (dATP, dCTP, dGTP and dTTP), 3.5 mM of $MgCl_2$, 1× buffer, and 1 unit of Taq DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.).

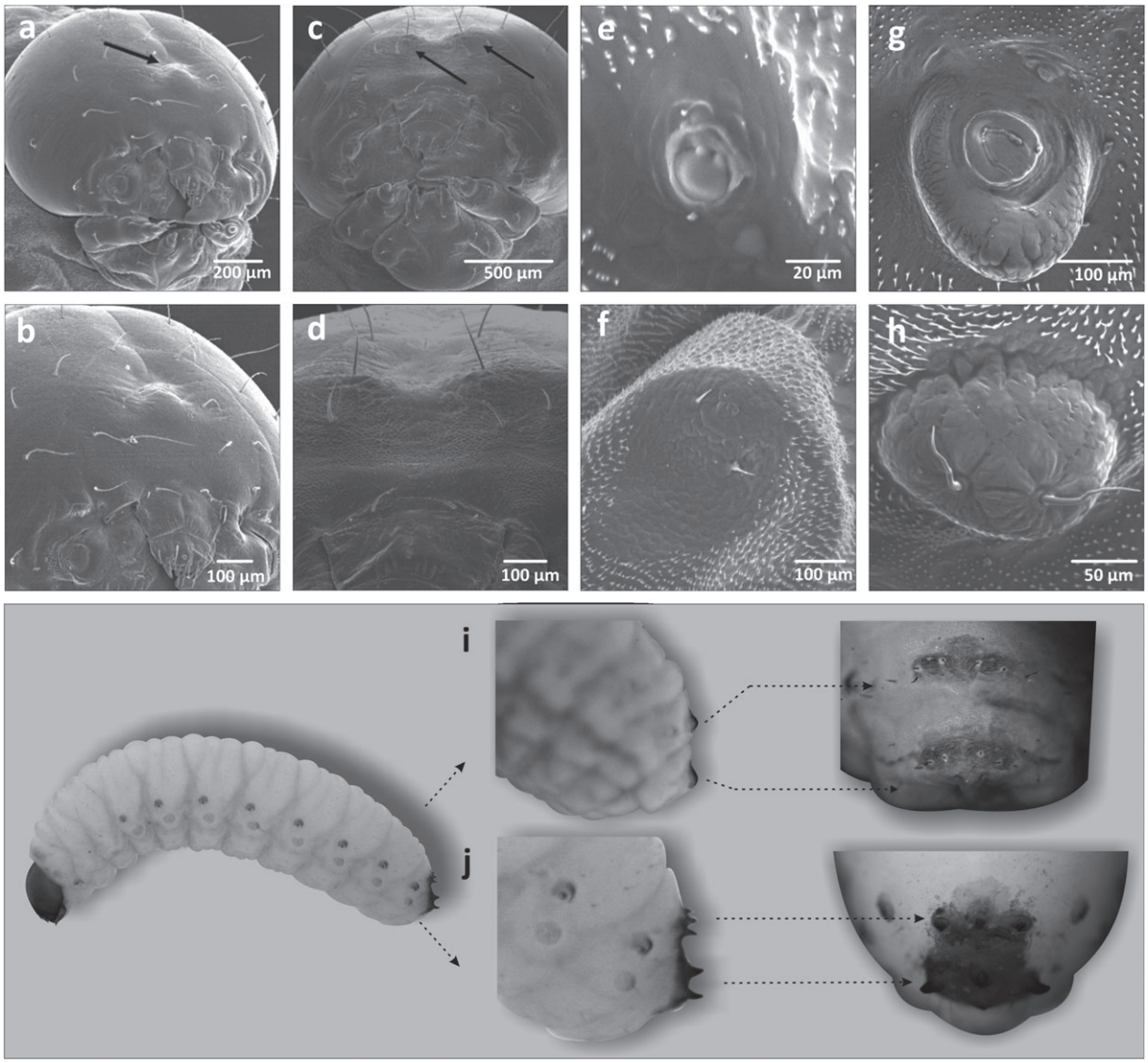

Fig. 7. Larvae (all genuses are *Dendroctonus*). (a–d) Head capsule: (a, b) *D. adjunctus*; (c, d) *D. jeffreyi*. (e–h) Abdominal pleura: (e, f) *D. frontalis*; (g, h) *D. valens*. (i, j) Abdominal terguites 8 and 9: (i) *D. rufipennis*; (j) *D. terebrans*.

PCR was performed on a Biometra thermal cycler (Biometra, Göttingen, Germany) under the following conditions: samples were preheated for 5 min at 95°C, followed by 35 amplification cycles of 60 s at 95°C, 60 s at 50°C, and 90 s at 72°C, and a final extension of 10 min at 72°C. PCR products were purified using GFX PCR DNA and Gel Band Purification kit (GE Healthcare Life Sciences, Buckinghamshire, UK). Purified PCR products were sequenced using a CEQ 8000 Genetic Analysis System automated sequencer (Beckman Coulter, Fullerton, CA, U.S.A.). Sequences were edited with the software BIOEDIT version 7.0.9 (Hall, 1999), and aligned with the aid of the program CLUSTALX version 2.0.12 (Larkin *et al.*, 2007). Parsimony analysis of aligned sequences was performed, with the same software and search strategy used for the morphological dataset, and the same nodal support values were calculated as well.

Finally, the combined matrix of morphological characters and COI sequences was simultaneously analysed in order to maximize the explanatory power of available evidence. This analysis comprised the 22 aforementioned taxa (including the composite terminal *Pseudohylesinus variegatus*/*nebulosus*). The combined matrix file is available as supporting information (File S1). The same software and heuristic search strategy described for previous analysis was used to find the MPTs from this combined matrix. Likewise, jackknife and Bremer support values were calculated for each node in the MPTs found using the same procedure described earlier, although in this case command sequence used for calculation of Bremer support was: sub40; h500; find*; bsupport;. Besides, the combined matrix was analysed under implied weights using the same *k*-values as in morphological analysis.

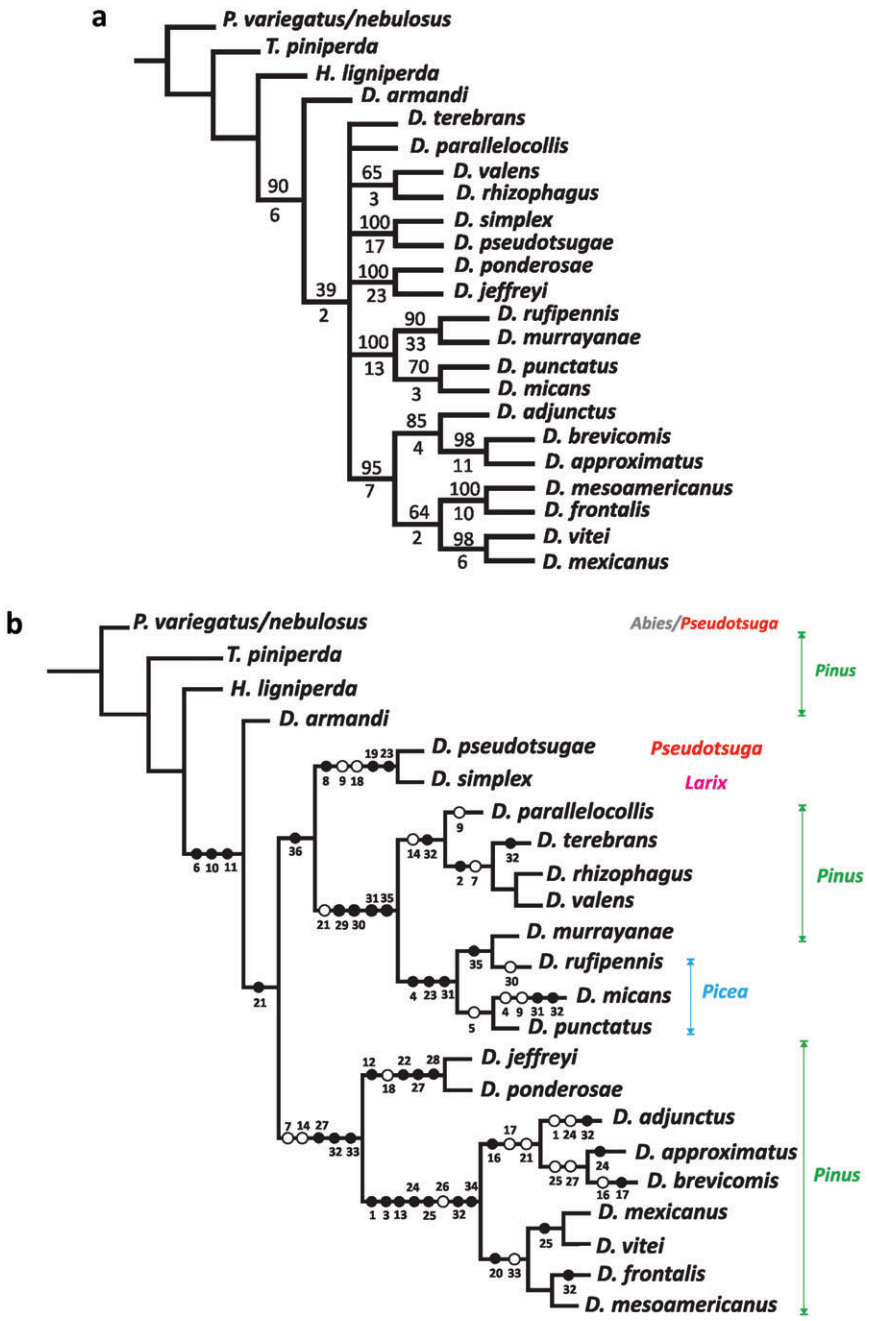

Fig. 8. Phylogenetic reconstructions from the combined analysis of morphological and DNA data: (a) strict consensus of five most parsimonious trees (MPTs) (length, $L = 1948$, consistency index = 0.48, retention index = 0.44); values at the base of each clade correspond to jackknife nodal support (above) and Bremer support (below). (b) Single cladogram obtained under implied weighting of morphological and DNA data (for k-values of 3–10), showing character optimization under accelerated transformation (ACCTRAN); black circles indicate synapomorphic characters, and white circles indicate homoplasic changes. The preferred host tree genus is indicated at the right of each species/clade.

Results

Phylogenetic analysis: morphology

Fifteen MPTs were found [length (L) = 69, consistency index (CI) = 0.75, retention index (RI) = 0.87]. The consensus topology (Figure S1a) shows four main clades, the major of them subdivided in several monophyletic groups, with the sequence ((*D. jeffreyi*, *D. ponderosae*) (*D. brevicomis* (*D. adjunctus*, *D. approximatus*) (*D. frontalis*, *D. mexicanus*, *D. vitei*, *D. mesoamericanus*))). The 15 individual MPTs differ in five essential points: (i) the position of *D. parallelocollis*; (ii) the

position of the (*D. rhizophagus*, *D. terebrans*, *D. valens*) clade; (iii) the position of the (*D. pseudotsugae*, *D. simplex*) clade; (iv) the sister group of *D. brevicomis*; and (v) internal relationships among members of the clades (*D. rhizophagus*, *D. terebrans*, *D. valens*), (*D. micans*, *D. murrayanae*, *D. punctatus*, *D. rufipennis*), and (*D. frontalis*, *D. mexicanus*, *D. vitei*, *D. mesoamericanus*).

The majority-rule tree (Figure S1b) is better resolved and sustains the sister-group relationship between the clades ((*D. murrayanae*, *D. rufipennis*) (*D. micans*, *D. punctatus*)) and (*D. rhizophagus*, *D. terebrans*, *D. valens*), as well as the position of *D. parallelocollis* as the sister taxon of the latter group.

Parsimony analysis under implied weights revealed three optimal trees with the most *k*-values tested ($k = 2–10$), and in all these cases the strict consensus is identical to the majority-rule tree from the unweighted analysis (Figure S1c).

Phylogenetic analysis: nucleotide sequences

Parsimony analysis of aligned COI sequences revealed three MPTs ($L = 1874$, CI = 0.47, RI = 0.41). These trees mainly disagree in the sister group relation of *D. terebrans*, as well as in the position of the clades (*D. parallelocollis*, *D. rhizophagus*, *D. valens*), (*D. pseudotsugae*, *D. simplex*), and ((*D. murrayanae*, *D. rufipennis*) (*D. micans*, *D. punctatus*)). Consensus of these trees is shown in Figure S2a, and the majority-rule tree is presented in Figure S2b. Relevant differences with respect to morphological analysis include the sister group relationships of *D. approximatus* and *D. parallelocollis*, as well as a stronger signal for the position of the *D. valens* group as sister to the *D. frontalis* group instead of the *D. rufipennis* group.

Phylogenetic analysis: combined matrix (morphology + COI)

Simultaneous analysis of morphological and DNA data retrieved five MPTs ($L = 1948$, CI = 0.48, RI = 0.44). The consensus tree (Fig. 8a) reveals that these MPTs differ in the position of *D. terebrans* and *D. parallelocollis*, and in the relationships among the major clades, particularly the clades (*D. terebrans* (*D. parallelocollis*, *D. rhizophagus*, *D. valens*)) and ((*D. murrayanae*, *D. rufipennis*) (*D. micans*, *D. punctatus*)).

Analysis under implied weighting produced a single MPT, with the same topology under concavity constant values from 3 to 10. The topology of this tree (Fig. 8b) is more resolved than either consensus tree obtained in separate analyses of individual partitions. Some groupings overlap with the morphological analysis (e.g. the sister-group relationship of the *D. valens* group and the *D. rufipennis* group) and some with the analysis of COI sequences (e.g. the sister-group relationship of *D. brevicomis* and *D. approximatus*). Four well-supported and clearly recognizable main clades were present in majority-rule trees for each analysis performed (either with equal or with implied weights), and three of them invariably appeared in all most parsimonious trees recovered from all analyses.

Discussion

Three synapomorphic characters support the monophyly of *Dendroctonus*: a well-developed epistomal process, antennal funicle with five antennomeres, and a flattened antennal club. Within the genus, several monophyletic groups are recognized. The first of these clades includes only the sister species *D. pseudotsugae* and *D. simplex*, equivalent to the *D. pseudotsugae* group of Wood (1963, 1982) and group II of Lanier (1981). This group is characterized by: (i) a very distinctive epistomal process, with its lateral margins almost perpendicular to its apical rim; (ii) interstria 2 on elytral declivity markedly narrower than interstria 3; and (iii) a pronounced sexual dimorphism in sculpture of interstriae on elytral declivity, with tubercles in declivital interstriae of females but not in those of males. The epistomal process is also atypically flat (convergent in *D. micans* and *D. parallelocollis*). These two species also share a conspicuous elevation of the first interstria on elytral declivity, although this feature has evolved convergently in the common ancestor of *D. jeffreyi* and *D. ponderosae*. Remarkably, these species also have very distinct host preferences, as neither of them attack *Pinus* trees: *D. pseudotsugae* feeds on Douglas-fir (*Pseudotsuga*), while *D. simplex* feeds on larch (*Larix*) (Fig. 8b).

The second clade consists of the species *D. murrayanae*, *D. rufipennis*, *D. micans* and *D. punctatus*, comprising the *D. rufipennis* group of Wood (1963, 1982) or group I of Lanier (1981). It is diagnosed by: (i) the reduction of tubercles in declivital interstriae of males, with respect to those in females; (ii) the presence of a median carina in the frontal region of the head in most members of this group (except *D. micans*); and (iii) larvae with two posterior sclerotized dorsal plates, on the eighth and ninth abdominal segments (although in *D. micans* there is only one plate on segment 9). *Dendroctonus micans* diverged from its sister species in aforementioned characters as well as in other attributes, such as the completely flat epistomal process. This morphological differentiation is correlated with the atypical geographic range of the species, which is Northern Europe and Asia (Wood, 1963). However, this species is very similar to *D. punctatus* in almost every other respect, and both share the diagnostic feature of a smooth frontal surface, with punctures but lacking granules and crenulations (while in *D. murrayanae* the frontal sculpture is quite diminished but still present). Furniss (1996) hypothesized that the ancestor of *D. micans* possibly migrated from refugial spruce forests in Alaska to Siberia through Beringia, which may have occurred during the Wisconsinan glaciation (0.08–0.01 Ma). As evidence in support of this theory, there are 24 genera of scolytine beetles shared between Eurasia and North America, and among them there are 12 cases of pairs of sibling species distributed in each of the two continents (Wood, 1982), including *D. micans* and *D. punctatus*. Interestingly, all these species breed in spruce (*Picea*) trees, which is a group of conifers with circumpolar distribution in the northern hemisphere. An additional unifying feature for the species in this group is their preference, unusual within the genus, for non-*Pinus* hosts, feeding mostly on *Picea* (with the exception of *D. murrayanae*, the only pine-feeding species of the clade) (Fig. 8b). Furthermore, the oldest known fossil

of the genus presumably belongs to this clade. It is a bark engraving made by a bark beetle, discovered in fossil wood of *Larix altoborealis* found in a location of the Canadian Arctic (Labandeira *et al.*, 2001). Dating of this fossil wood indicates a middle Eocene age, and after a thorough analysis of the details of gallery morphology, Labandeira *et al.* (2001) conclude that it was engraved by beetles from a species probably akin to *D. rufipennis*.

A third monophyletic group present in most MPTs recovered is composed of *D. rhizophagus*, *D. terebrans* and *D. valens*. This clade is roughly equivalent to the *D. valens* group of Wood (1963, 1982) and to group II of Lanier (1981), with the difference that Wood included *D. parallelocollis* in the group, despite the fact that the latter species lacks one of the defining synapomorphies of the clade (i.e. a rounded median protuberance in the frontal region of head in females). In fact, *D. parallelocollis* represents a peculiar case; this species displays a unique combination of character states and a set of putative autapomorphies, which makes it a very distinctive taxon, but at the same time obscures its phylogenetic position within the genus. This situation is reflected in the fact that *D. parallelocollis* is located in different positions in the MPTs found after the various analyses. However, *D. parallelocollis* appears as the sister taxon of the rest of the species forming this group in 12 of the 15 MPTs found in the morphological analysis and in the analysis with implied weights of the combined matrix of morphology and COI sequences. Another unifying feature of these species is the prominent sclerotized dorsal plate of the larvae, conspicuously armed with spines and covering both abdominal tergites 8 and 9. Larvae of *D. parallelocollis* were not available for study, so the shared presence of this character in such species could not be tested. Lanier (1981) did not study the karyology of either this species or *D. rhizophagus*, but Zúñiga *et al.* (1998) typified the karyotype of both and found that their chromosome numbers are similar to those of *D. valens* and thus placed them together in group II.

A particularly interesting result of this work is the suggested sister-group relationship between the *D. valens* group and the *D. rufipennis* group, although this grouping is not as strongly supported as the previously discussed clades. It does not appear in all MPTs retrieved either by the morphological data or by the COI sequences alone, but it does appear in nine of the 15 MPTs found in the morphology-based analysis, and it is also recovered in the implied weighting analysis of both morphology-only and simultaneous analysis of combined morphology and COI data. There are morphological characters supporting this relationship, being larval morphology and behaviour particularly informative. Putative larval synapomorphies for this clade include the presence of sclerotized tubercles around the spiracular openings, as well as prominent and heavily sclerotized and pigmented dorsopleural lobes. In addition, the two groups share a communal feeding of larvae, at least in the earliest instars (Thomas & Bright, 1970; Wood, 1982; Furniss, 1995).

A fourth, more diverse, clade is characterized by the presence of true mycangia in adults (although of varied nature and location), ornamentations of different kinds on the frontal sclerite of the head capsule of larvae, construction of winding parental galleries and egg deposition in individual niches, and a neo-XY configuration of sex chromosomes in males (although with posterior reversions for most of these characters in several species of the clade). The concept of the mycangium has been frequently used in a very general and unspecific sense, applying the term to any structure which serves for temporary storage and transport of symbiotic fungi, independently of its form, structure, location or origin (Livingston & Berryman, 1972; Whitney, 1982; Furniss *et al.*, 1987; Six, 2003). According to this broad definition, a mycangium could be a saccular structure, a groove, a pit or even a group of setae, thus tending to be a highly homoplasious feature, independently originated in multiple lineages (Grebennikov & Leschen, 2010). Therefore, a more restricted definition was adopted here, considering a true mycangium only a saccular structure with a strict and documented morphological specialization for transport and storage of symbiotic fungi (Batra, 1963; Whitney & Farris, 1970; Barras & Perry, 1971; Happ *et al.*, 1971; Paine & Birch, 1983; Harrington, 2005). The presence of mycangial structures is a derived trait within the genus, and the clades in which this feature has appeared comprise almost half of the taxa in the ingroup and include some of the most widely distributed and most generalist-feeding species. This attribute consequently represents a key innovation in the evolutionary history of the genus.

Within this fourth clade, two well-defined subgroups can be recognized. One includes the sister species *D. jeffreyi* and *D. ponderosae*, and is strongly supported in all analyses and defined by four synapomorphic features, i.e. maxillary mycangia and microsculpture of interstriae on elytral declivity distinctively rugose (adult), and a pair of tubercles on the frontal area of head and a tubercle on the base of the mandible (larva). This grouping is comparable to the *D. ponderosae* group of Wood (1963, 1982), although he includes *D. adjunctus* as well, and to group IV of Lanier (1981).

The other subgroup of this clade comprises *D. adjunctus*, *D. approximatus*, *D. brevicomis*, *D. frontalis*, *D. mesoamericanus*, *D. mexicanus* and *D. vitei*. It is diagnosable by the presence of a median groove in the frontal region of the head, which in males is flanked by prominent tubercles (secondarily lost in *D. adjunctus*), thoracic mycangia, and construction of typically sinuate parental galleries in their host trees. This clade includes the *D. frontalis* group of Wood (1963, 1982) plus *D. adjunctus*, and groups V and VI of Lanier (1981). However, relationships among members of this group show conflict between analyses of different datasets, particularly regarding relationships among *D. adjunctus*, *D. approximatus* and *D. brevicomis*. Conversely, all datasets agree on the monophyly of a cluster comprising the pairs of sister species *D. mexicanus*–*D. vitei* and *D. frontalis*–*D. mesoamericanus*. This latter taxon is remarkable, as it is morphologically very similar to the other three species and particularly to *D. frontalis*, but at the same time it presents a unique combination of characters, different from any other *Dendroctonus* species, including *D. frontalis*. This contributes to the lack of resolution within this clade in the morphological analysis (because this species shares several character states exclusively with *D. frontalis* but also with *D. mexicanus* and *D. vitei*), while on the other hand this evidence supports the idea

that *D. mesoamericanus* is a separate taxon, distinct from the rest of the species in the genus. This conclusion agrees with recent additional evidence from chemical ecology, karyology, morphometric data and DNA sequences (Sullivan *et al.*, 2012; Armendáriz-Toledano *et al.*, 2014).

The Chinese species *Dendroctonus armandi* appears to be the sister species of the rest of the genus in both DNA and combined analyses. The present geographic range of this species is restricted, and closely linked to that of its main host, *Pinus armandii* (the Chinese White Pine), a pine species mainly distributed in central China (Yin *et al.*, 1984; Zhongqi, 1989).

Many of the monophyletic groups that can be recognized match, to some extent, the species groups previously defined by Wood (1963, 1982) and Lanier (1981), but our analysis shows that some of their groups are polyphyletic (e.g. the *D. valens* and *D. ponderosae* groups *sensu* Wood, 1963). Similarly, results of this study clearly support the inclusion of *D. adjunctus* within the *D. frontalis* group *sensu lato* (cf. Lanier, 1981; Wood, 1982). Wood's (1963) groups were mainly defined based on plesiomorphic characters and/or combinations of characters. Ideally, taxonomic groups should be defined on the basis of synapomorphic characters, but in practice, many of them are actually diagnosed only by particular combinations of homoplasies, as shown by several recent studies that have re-examined traditional diagnostic characters within a phylogenetic framework for selected genera of Scolytinae (Hulcr *et al.*, 2007; Smith & Cognato, 2010, 2014; Cognato *et al.*, 2015). Various morphological attributes have been proposed to define species groups in *Dendroctonus*, but until now, it has been untested whether these characters represent synapomorphies.

Conclusion

The simultaneous analysis of the combined matrix reflects an influence of both morphological and DNA sequence data, despite morphological data representing less than 10% of informative characters in the combined matrix. The most relevant contribution of molecular data seems to be reflected in the basal position within the genus of *D. armandi* and the definition of *D. approximatus* as sister taxon to *D. brevicomis* (while morphology alone places the former as sister to *D. adjunctus*). On the other hand, the phylogenetic signal of morphological data seems evident in the implied weights analysis of the combined matrix, resolving the inclusion of *D. terebrans* as member of the *D. valens* group, as well as the place of *D. parallelocollis* as sister taxon to this clade. The main source of conflict between morphological and COI data precisely concerns not only internal relationships within this latter clade but also the position of the whole clade within the genus. Consensus trees from separate analyses reflect that neither morphology nor COI sequences alone can unambiguously resolve this issue, although majority-rule trees indicate that morphological data favour the location of this group as the sister taxon of clade ((*D. murrayanae*, *D. rufipennis*) (*D. micans*, *D. punctatus*)), in opposition to a sister-group relationship with ((*D. jeffreyi*, *D. ponderosae*) (*D. brevicomis* (*D. adjunctus*, *D. approximatus*) (*D. frontalis*, *D. mexicanus*, *D. vitei*, *D. mesoamericanus*))) suggested by molecular data. This conflict is not solved in the combined analysis with equal weights, where both alternatives are present in the five MPTs obtained, but, remarkably, simultaneous analysis with implied weights retrieves the sister-group relationship between the *D. valens* group and the *D. rufipennis* group as supported by morphology alone (in addition to the fact that there are at least three putative synapomorphies sustaining this relationship). It is worth mentioning that this result contradicts the most parsimonious tree presented by Kelley & Farrell (1998), in which the *D. valens* group appears as sister to the *D. ponderosae* and *D. frontalis* groups, yet agrees with the likelihood tree obtained by Reeve *et al.* (2012). Despite these points of contention, there are several recurring clades recovered from both morphological and molecular datasets, although almost all of them are more strongly supported in combined analyses of all available evidence, which generated the most robust and precise phylogenetic hypothesis.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/syen.12149

Figure S1. Phylogenetic reconstructions from the analysis based on morphological characters: (a) strict consensus of 15 most parsimonious trees (MPTs) ($L = 69$, CI = 0.75, RI = 0.87) – values at the base of each clade correspond to jackknife nodal support (above) and Bremer support (below); (b) majority-rule tree of 15 MPTs – values at the base of each clade correspond to its frequency among the MPTs; and (c) strict consensus of three trees obtained under implied weighting of morphological characters (for *k*-values of 2–10), showing character optimization under accelerated transformation (ACCTRAN) – black circles indicate synapomorphic characters, and white circles indicate homoplasic changes.

Figure S2. Phylogenetic reconstructions from the analysis based on COI sequences: (a) strict consensus of three most parsimonious trees (MPTs) ($L = 1874$, CI = 0.47, RI = 0.41) – values at the base of each clade correspond to jackknife nodal support (above) and Bremer support (below); and (b) majority-rule tree of three MPTs – values at the base of each clade correspond to its frequency among the MPTs.

Table S1. Data matrix used in the morphological analysis and GenBank accession numbers of the COI sequences used in the molecular analysis.

Table S2. Supplementary descriptive notes on selected characters from the morphological analysis with previous ambiguous/equivocal use in scolytine taxonomy.

File S1. TNT file of combined matrix of morphological characters and COI sequences.

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APPENDIX: Character state descriptions.

Adult morphology

1. Frontal region of head in males: (0) without frontal tubercles; (1) with a pair of prominent frontal tubercles (Fig. 3a).
2. Frontal region of head in females: (0) without protuberances; (1) with a rounded median frontal protuberance.
3. Median groove in the frontal region of head: (0) absent; (1) present (Fig. 3a).
4. Median carina in the frontal region of head: (0) absent; (1) present (Fig. 3b).
5. Sculpture on surface of frons: (0) only with punctures (granules and crenulations absent); (1) with punctures, plus granules and/or crenulations, conspicuous and abundantly distributed (Fig. 3c).
6. Epistomal area of head: (0) sculptured, but never with a well-developed epistomal process; (1) with a clearly defined epistomal process (*sensu* Hopkins, 1909), covering the median epistomal area (Fig. 3d–i).
7. Relative width of epistomal process: (0) broad (50% or more of interocular distance) (Fig. 3d); (1) narrow (always less than 40% of interocular distance) (Fig. 3e).
8. Lateral margins of epistomal process: (0) oblique (up to 60° with respect to horizontal axis, in frontal view) (Fig. 3f–h); (1) almost perpendicular to horizontal axis (> 80° in frontal view) (Fig. 3i).
9. Lateral margins of epistomal process (males): (0) flat (not elevated) (Fig. 3e, i); (1) transversely elevated (i.e. separated from basal surface of epistoma by a distance greater than that separating the median section of the process) (Fig. 3d, g). In some species, elevation of the lateral margins of the epistomal process is much more subtle in females, so examination of male specimens is usually required for proper evaluation of this feature.
10. Number of antennomeres in antennal funicle: (0) seven; (1) six; (2) five (Fig. 4a).

11. Shape of antennal club: (0) conical; (1) compressed (flat) (Fig. 4b).
12. Maxillary mycangium (at the base of the cardo): (0) absent; (1) present (Fig. 4c).
13. Thoracic mycangium (females): (0) absent; (1) present (Fig. 4d).
14. Episternal area of prothorax: (0) punctured (Fig. 4e); (1) granulate (Fig. 4f).
15. Type of elytral vestiture: (0) scales and setae; (1) exclusively setae (Fig. 5a–c).
16. Colour of setae on elytral declivity: (0) yellowish setae; (1) dark setae.
17. Relative length of setae on elytral declivity: (0) evenly long (i.e. length of all setae on declivity are as long as or longer than the average width of an interstria) (Fig. 5a); (1) evenly short (i.e. length of all setae on declivity are much shorter than the average width of an interstria) (Fig. 5b); (2) two or more sizes mixed (Fig. 5c).
18. Elevation of interstria 1 on elytral declivity: (0) interstria not elevated (its posterior margin at the same level as the remainder of the interstriae, or very weakly elevated) (Fig. 5d); (1) interstria conspicuously elevated (Fig. 5e).
19. Relative width of interstria 2 on elytral declivity: (0) as wide as interstria 3 (Fig. 5f); (1) markedly narrower than interstria 3 (Fig. 5g).
20. Interstria 2 on elytral declivity: (0) straight through its whole extension (thus, not constricted apically) (Fig. 5h); (1) strongly constricted apically (due to curvature of stria 2 towards elytral suture) (Fig. 5i).
21. Strial lines on elytral declivity: (0) weakly impressed (i.e. striae shallow) (Fig. 5g, j); (1) strongly impressed (i.e. striae deeply marked) (Fig. 5f, k).
22. Microsculpture on surface of interstriae on elytral declivity: (0) surface smooth (Fig. 5l); (1) surface finely rugose (Fig. 5m).
23. Sexual dimorphism in sculpture of interstriae on elytral declivity: (0) absent (granules or tubercles present in males and females, of the same size in both sexes); (1) tubercles present in declivital interstriae of both sexes, but much smaller in males; (2) tubercles only in declivital interstriae of females, absent in males.
24. Granulation on interstriae of elytral declivity (females): (0) uniseriate (tubercles forming a single row in each interstria) (Fig. 5g); (1) non-uniseriate (tubercles or granules scattered throughout the width of interstriae) (Fig. 5f); (2) uniseriate in interstria 2 and scattered in contiguous interstriae.
25. Distal end of seminal rod of the male genitalia: (0) entire (Fig. 6a, b); (1) bifurcated on both the dorsoventral and lateral axes (Fig. 6c); (2) bifurcate on the dorsoventral axis, with a rounded dorsal lobe (Fig. 6d–f); (3) bifurcate on the dorsoventral axis, with a pointed dorsal lobe (Fig. 6g, h).
26. Valve of seminal rod: (0) without posterior plate (or lobe) (Fig. 6i–k); (1) extending posteriorly into a plate (visible as a lobe in dorsal view) (Fig. 6l–n).

Larval morphology

27. Frontal area of head capsule: (0) without protuberances; (1) with a prominent central tubercle (Fig. 7a, b); (2) with a pair of tubercles (Fig. 7c, d).
28. Mandibular surface: (0) without tubercles; (1) with a tubercle on its outer face (contiguous to dorsal articular condyle).
29. Spiracular openings: (0) simple (without adjacent spiracular tubercles) (Fig. 7e); (1) surrounded by sclerotized spiracular tubercles (Fig. 7g).
30. Dorsopleural lobes beneath spiracles: (0) reduced, visible just as flat, unpigmented circular areas bearing a pair of small setae (Fig. 7f); (1) very prominent, heavily sclerotized and pigmented, with a pair of evident setae arising from each of them (Fig. 7h).
31. Sclerotized dorsal plates on abdominal segments 8 and 9: (0) absent; (1) one simple plate, covering tergite 9; (2) two separate, simple plates (one on each segment) (Fig. 7i); (3) a single, prominent plate covering both abdominal tergites 8 and 9, armed with spines (Fig. 7j).

Karyotype

32. Chromosome number: (0) $2n = 30$; (1) $2n = 28$; (2) $2n = 26$; (3) $2n = 24$; (4) $2n = 22$; (5) $2n = 16$; (6) $2n = 14$; (7) $2n = 12$.
33. Meiotic configuration of sex chromosomes in males (i.e. sex determination system): (0) Xy_p, (1) neo-XY.

Coding of chromosomal characters was based mostly on Lanier (1981); character states for *D. parallelocollis* and *D. rhizophagus* were coded based on Zúñiga *et al.* (1998), and coding for *D. micans* was based on Zúñiga *et al.* (2002).

Gallery construction behaviour

34. Shape of parental galleries: (0) mostly straight; (1) sinuate.
35. Larval galleries: (0) individual; (1) communal; (2) communal at first, individual at later instars.
36. Pattern of egg deposition: (0) in individual niches; (1) in collective niches.