Phylogenetic framework for *Dioryctria* (Lepidoptera: Pyralidae: Phycitinae) based on combined analysis of mitochondrial DNA and morphology

Yanli Du,[1] Amanda D. Roe, Felix A.H. Sperling[2]

Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

Abstract—Coneworms of the genus *Dioryctria* Zeller are important lepidopterous pests of conifer cones throughout the Holarctic region. Seventy-nine *Dioryctria* species are currently recognized and arranged into 11 species groups, but a globally unified classification of these species groups has not been attained. We surveyed 14 *Dioryctria* species belonging to 7 species groups recognized as being taxonomically problematic. Mitochondrial DNA sequences and morphological characters were used to resolve relationships among and within species groups and species. Sequences were obtained for 2.3 kb of the mitochondrial COI + COII genes and related to 52 morphological characters. Parsimony analyses of separate and combined data showed that (*i*) the five included Chinese species (*D. abietella* (Denis and Schiffermüller), *D. rubella* Hampson, *D.* nr. *rubella*, *D. magnifica* Munroe, and *D. yiai* Mutuura and Munroe) were distinct from the North American taxa, and their relationships were interspersed among Nearctic and European species; (*ii*) three of the four species groups represented by more than one species formed robust, well-supported clades (*abietella* group, *sylvestrella* group, and *zimmermani* group) for both mtDNA sequences and morphology; (*iii*) mtDNA and morphology gave conflicting interspecific and intergroup relationships for the *auranticella*, *schuetzeella*, *ponderosae*, and *baumhoferi* groups; (*iv*) all eight species for which more than one specimen was sampled were characterized by discrete clusters of mitochondrial DNA haplotypes, and mtDNA divergences among species in the same species group were generally less than those among species in different species groups; and (*v*) combining mtDNA data with morphological data increased support for most nodes in the phylogeny, with morphological characters providing support for species groups and mtDNA being essential for distinguishing species within species groups. This study demonstrates the value of a combined analysis of both mtDNA and morphological characters and establishes a phylogenetic framework for broader and more comprehensive studies of *Dioryctria* species.

Résumé—Les pyrales des cônes du genre *Dioryctria* Zeller sont des ravageurs importants des cônes de conifères dans toute la région holarctique. On reconnaît actuellement 79 espèces de *Dioryctria* regroupées en 11 groupes d'espèces, mais il n'existe pas de classification uniforme de ces groupes d'espèces à l'échelle globale. Nous avons étudié 14 espèces de *Dioryctria* appartenant à 7 groupes d'espèces reconnus comme posant des problèmes taxonomiques. Des séquences d'ADN mitochondrial et des caractères morphologiques nous ont servi à établir les relations entre les groupes d'espèces et les espèces et à l'intérieur de ces catégories. Nous avons obtenu des séquences de 2,3 kb des gènes mitochondriaux COI + COII et nous avons comparé 52 caractères morphologiques. Des analyses de parcimonie des données séparées et regroupées indiquent que (*i*) les cinq espèces chinoises étudiées (*D. abietella* (Denis et Schiffermüller), *D. rubella* Hampson, *D.* près de *rubella*, *D. magnifica* Munroe et *D. yiai* Mutuura et Munroe) sont distinctes des taxons nord-américains et leurs relations sont dispersées parmi des espèces européennes et néarctiques, (*ii*) trois des quatre groupes d'espèces étudiés et représentés par plus d'une espèce (groupes d'*abietella*, de *sylvestrella* et de *zimmermani*) forment des clades robustes bien définis tant par les séquences d'ADN que par la morphologie, (*iii*) les relations inter-groupes et intra-groupes établies à partir de l'ADNmt et de la morphologie sont souvent incompatibles entre elles chez les groupes d'*auranticella*, de *schuetzeella*, de *ponderosae* et de *baumhoferi*, (*iv*) les espèces

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[1]Present address: Department of Entomology, China Agricultural University, Haidian, Beijing, People's Republic of China, 100094

[2]Corresponding author (e-mail: felix.sperling@ualberta.ca).

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sont caractérisées par des regroupements distincts d'haplotypes d'ADN mitochondrial chez l'ensemble des huit espèces chez lesquelles plus d'un spécimen a été examiné et les divergences d'ADNmt entre les espèces dans un même groupe d'espèces sont généralement moindres qu'entre les groupes d'espèces eux-mêmes, (*v*) la combinaison des données d'ADNmt et des données morphologiques vient généralement raffermir la définition des principaux noyaux de la phylogénie; les données morphologiques appuient la définition des groupes d'espèces et les données d'ADNmt sont essentielles pour distinguer les espèces au sein des groupes d'espèces. Notre étude démontre l'avantage de combiner les caractéristiques de l'ADNmt et de la morphologie et elle fournit un cadre phylogénétique pour des études élargies et plus complètes des espèces de *Dioryctria*.

[Traduit par la Rédaction]

Introduction

The genus *Dioryctria* Zeller, 1846 (Lepidoptera: Pyralidae) is Holarctic in distribution, although a few species also occur in the northern tropics (Neunzig and Dow 1993; Neunzig 1996). Larvae feed on a wide range of conifer species (Hedlin *et al.* 1980), boring into cones, shoots, wounds, boles, and rust cankers, which may damage trees or significantly reduce seed production (Turgeon *et al.* 1994). The genus is composed of 79 currently recognized species in 11 species groups (Heinrich 1956; Mutuura and Munroe 1972, 1974; Wang and Sung 1982; Speidel 1996; Segerer and Pröse 1997; Neunzig 2003). Although 11 species groups have been described, no global phylogenetic framework for these species groups is available. Furthermore, many species within these groups are difficult to distinguish on the basis of morphology, and their identification often relies on host plant association or pheromone attraction.

Problems in species identification extend even across continents, and their resolution is particularly urgent in the face of recent increases in global trade. A phylogenetic framework for *Dioryctria* species could improve identification of species inadvertently transported as a result of global trade, thereby reducing the risk of invasive species being introduced (Normile 2004). For example, at ports of entry in the United States, 467 insect species accounted for 39% of total interceptions originating from China during 1996–1998 (Animal and Plant Health Inspection Service 2000). Statistics for the reverse direction are more difficult to obtain, but major outbreaks of at least one North American species, the red turpentine beetle (*Dendroctonus valens* LeConte, 1860; Coleoptera: Scolytidae), have recently occurred in China (Sun *et al.* 2003). Such occurrences are likely to increase in the future, especially if green wood continues to be used as a packing material for imported goods (Haack and Cavey 1997).

We examined representatives of 7 of the 11 previously defined species groups. The 4 remaining species groups contain only one (*mongolicella* and *erythropasa* groups) or two species (*taiella* and *pygmaella* groups) that are easily distinguished or rarely collected. China and western North America were well represented by the species sampled in our treatment. The 14 species included in our paper are placed in the following 7 previously defined species groups: (*i*) the *abietella* group (*D. abietella* (Denis and Schiffermüller, 1775) and *D. abietivorella* (Grote, 1878)) (Mutuura and Munroe 1972; Neunzig 2003); (*ii*) the *sylvestrella* group (*D. sylvestrella* (Ratzeburg, 1840), *D. rubella* Hampson, 1901, *D.* nr. *rubella*, and *D. magnifica* Munroe, 1958) (Mutuura and Munroe 1972; Wang and Sung 1985); (*iii*) the *auranticella* group (*D. auranticella* (Grote, 1883) and *D. yiai* Mutuura and Munroe, 1972) (Mutuura and Munroe 1972; Neunzig 2003); (*iv*) the *schuetzeella* group (*D. reniculelloides* Mutuura and Munroe, 1973) (Mutuura and Munroe 1972, 1973; Neunzig 2003); (*v*) the *ponderosae* group (*D. ponderosae* Dyar, 1914) (Mutuura *et al.* 1969*b*; Mutuura and Munroe 1972; Neunzig 2003); (*vi*) the *baumhoferi* group (*D. clarioralis* (Walker, 1863) (Mutuura *et al.* 1969*b*; Mutuura and Munroe 1972; Neunzig 2003); and (*vii*) the *zimmermani* group (*D. zimmermani* (Grote, 1877), *D. tumicolella* Mutuura, Munroe and Ross, 1969, and *D. taedivorella* Neunzig and Leidy, 1989) (Mutuura *et al.* 1969*a*; Mutuura and Munroe 1972; Neunzig 2003).

There is a substantial body of taxonomic research that examines *Dioryctria* classification based on morphological characters (Heinrich 1956; Munroe 1959; Roesler 1968; Mutuura *et al.* 1969*a*, 1969*b*; Schaber and Wood 1971; Mutuura and Munroe 1972, 1973, 1974, 1979;

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Mutuura 1982; Wang and Sung 1982, 1985; Blanchard and Knudson 1983; Yamanaka 1990; Neunzig 1990, 1996, 2003; Neunzig and Dow 1993; Sopow *et al.* 1996; Segerer and Pröse 1997; Speidel and Asselbergs 2000), although no formal phylogenetic analysis has been conducted. However, many problems in *Dioryctria* taxonomy remain, both in characterizing species groups and in distinguishing species within species groups. For example, although Wang and Sung (1985) gave a detailed revision of the *sylvestrella* group, the differences among the species are subtle, prompting the search for additional characters.

We used mitochondrial DNA (mtDNA) sequences to provide additional characters for elucidating relationships between species groups as well as among *Dioryctria* species within these groups. mtDNA was selected because for most animals it is maternally inherited, unlike nuclear genes, and thus the phylogenetic information in mtDNA sequences is less likely to be obscured by recombination (Moritz *et al.* 1987; Harrison 1989). Second, the utility of mtDNA sequence analyses in species-level studies has been demonstrated in several groups of Lepidoptera (Sperling 2003). Third, combined morphological and molecular data sets for Lepidoptera have produced excellent templates for testing hypotheses of the evolution of other phenotypic characters (*e.g.*, Brown *et al.* 1994). Many studies have demonstrated that a total evidence approach, in contrast to independent analysis of each data set, increases resolution and support for tree topology in phylogenetic reconstructions (Miller *et al.* 1997; Sperling *et al.* 1997; Damgaard *et al.* 2000; Giribet *et al.* 2000; Klompen *et al.* 2000; Normark 2000; Skevington and Yeates 2000; Kruse and Sperling 2002).

The economic importance of *Dioryctria* species, difficulties in species identification, and the increasing risk of transfer of species between continents as a result of global trade all lend urgency to the task of finding efficient characters for accurate identification of *Dioryctria* species. The objectives of this paper are to (*i*) evaluate the effectiveness of mtDNA as a source of additional characters for identifying species of *Dioryctria*, and (*ii*) elucidate the phylogeny of major species groups in *Dioryctria* using a combination of mtDNA and morphological characters. This study is the first published application of mtDNA sequence analysis to *Dioryctria* taxonomy, as well as the first explicitly phylogenetic study of the genus.

Materials and methods

Specimens

Specimens from the following collections were examined (Table 1).

CNC	Canadian National Collection of Insects, Arachnids, and Nematodes, Ottawa, Ontario, Canada
EMEC	Essig Museum of Entomology, University of California, Berkeley, California, United States of America
NFRC	Northern Forestry Research Center, Canadian Forest Service, Edmonton, Alberta, Canada
OSAC	Oregon State Arthropod Collection, Oregon State University, Corvallis, Oregon, United States of America
UASM	E.H. Strickland Entomological Museum, University of Alberta, Edmonton, Alberta, Canada
USNM	National Museum of Natural History, Washington, District of Columbia, United States of America
ZSM	Zoologische Staatssammlung, Munich, Germany

Taxonomic sampling

We scored morphological characters and sequenced mtDNA extending from $tRNA^{Tyr}$ through cytochrome-*c* oxidase subunit I (COI) + $tRNA^{Leu}$ + COII to $tRNA^{Lys}$ from 14 *Dioryctria* species (*D.* nr. *rubella* is missing 106 bp at the start of $tRNA^{Tyr}$–COI and *D. taedivorella* is missing 433 bp at the end of COII, but they were included in the analysis of the 2.3-kb fragment). We also sequenced 2 phycitine species as outgroups: *Oncocera* (*Laodamia*) *faecella* (Zeller, 1839) (Lepidoptera: Pyralidae) and *Ceroprepes ophthalmicella* (Christoph, 1881) (Lepidoptera: Pyralidae). According to Roesler's (1973) classification, these outgroups are included in the nominal subtribe and tribe of the subfamily Phycitinae together with *Dioryctria*. Specimens used in this study were provided by collaborators or were collected by the authors (Table 1). Specimens were identified to species based on previous morphological descriptions and comparisons with identified museum material.

DNA was extracted from two or three legs of dried specimens or from thoracic tissue of

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Table 1. List of specimens used for DNA sequencing or figures.

Species	Species group	Specimen	Locality	Collector and year	Preservation	Collection*	GenBank accession No.
Dioryctria abietella	*abietella*	Du64†	China: Mt. Baiyun, Henan Province	X. Wang, 2002	Alcohol	UASM	DQ247739
D. abietella	*abietella*	Du70‡	China: Mt. Baiyun, Henan Province	X. Wang, 2002	Alcohol	UASM	DQ247752
D. abietella	*abietella*	Du71‡	China: Mt. Baiyun, Henan Province	X. Wang, 2002	Alcohol	UASM	DQ247753
D. abietella	*abietella*	Du73‡	China: Mt. Baiyun, Henan Province	X. Wang, 2002	Alcohol	UASM	DQ247754
D. abietella	*abietella*	Du75‡	China: Mt. Baiyun, Henan Province	X. Wang, 2002	Alcohol	UASM	DQ247755
D. abietella	*abietella*	Du76‡	China: Mt. Baiyun, Henan Province	X. Wang, 2002	Alcohol	UASM	DQ247758
D. abietella	*abietella*	Du77‡	China: Mt. Baiyun, Henan Province	X. Wang, 2002	Alcohol	UASM	DQ247756
D. abietella	*abietella*	Du78‡	China: Mt. Baiyun, Henan Province	X. Wang, 2002	Alcohol	UASM	DQ247757
D. abietivorella	*abietella*	Du04†	USA: Chico, Butte Co., California	A. Roe, 2001	Frozen	UASM	DQ247740
D. abietivorella	*abietella*	Du05†	USA: Chico, Butte Co., California	A. Roe, 2001	Frozen	UASM	DQ247741
D. sylvestrella	*sylvestrella*	02087†	Germany: Landshut, Bavaria	H. Kolbeck, 2002	Dried	ZSM	DQ247745
D. sylvestrella	*sylvestrella*	Du130†	Germany: Parkstein-Hütten, Bavaria	A.H. Segerer, 2003	Dried	UASM	DQ247746
D. rubella	*sylvestrella*	Du08‡	China: Mt. Qipan, Weichang, Hebei Province	Y. Du, 2001	Dried	UASM	DQ247760
D. rubella	*sylvestrella*	Du14‡	China: Chengde, Hebei Province	Y. Du, 2001	Dried	UASM	DQ247761
D. rubella	*sylvestrella*	Du21†,§	China: Mt. Baxian, Tianjin	H. Li, 2001	Dried	UASM	DQ247743
D. rubella	*sylvestrella*	Du22‡	China: Mt. Qipan, Weichang, Hebei Province	?	Dried	UASM	DQ247762
D. nr. *rubella*	*sylvestrella*	Du07†,§	China: Mt. Fanjing, Guizhou Province	X. Wang, 2002	Dried	UASM	DQ247744
D. magnifica	*sylvestrella*	Du69†	China: Mt. Baiyun, Henan Province	X. Wang, 2002	Alcohol	UASM	DQ247742
D. magnifica	*sylvestrella*	Du74‡	China: Mt. Baiyun, Henan Province	X. Wang, 2002	Alcohol	UASM	DQ247759
D. auranticella	*auranticella*	Du02†,§	USA: Placerville, El Dorado Co., California	A. Roe, 2001	Frozen	UASM	DQ247736
D. auranticella	*auranticella*	Du03‡	USA: Chico, Butte Co., California	A. Roe, 2001	Frozen	UASM	DQ247751
D. yiai	*auranticella*	Du10‡	China: Mt. Baxian, Tianjin	H. Li, 2002	Dried	UASM	DQ247749
D. yiai	*auranticella*	Du12‡,§	China: Mt. Baxian, Tianjin	H. Li, 2002	Dried	UASM	DQ247747
D. yiai	*auranticella*	Du13†	China: Mt. Xiaowutai, Hebei Province	Y. Du, 2000	Dried	UASM	DQ247737
D. yiai	*auranticella*	Du15	China: Mt. Baishi, Hebei Province	H. Yu, 2000	Dried	UASM	DQ247750

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Table 1 (*concluded*).

Species	Species group	Specimen	Locality	Collector and year	Preservation	Collection*	GenBank accession No.
D. yiai	*auranticella*	Du16[§]	China: Xingcheng, Liaoning Province	R. Zhang, 1986	Dried	UASM	—
D. yiai	*auranticella*	Du17[†]	China: Mt. Baxian, Tianjin	H. Li, 2002	Dried	UASM	DQ247738
D. yiai	*auranticella*	Du90[‡,§]	China: Mt. Tianmu, Zhejiang Province	H. Li, 1999	Alcohol	UASM	DQ247748
D. reniculelloides	*schuetzeella*	Du01[†]	Canada: Fort McMurray, Alberta	A. Roe, 2001	Frozen	UASM	DQ247734
D. reniculelloides	*schuetzeella*	Du06[†]	Canada: Beaver Creek Campground, Alberta	A. Roe, 2001	Frozen	UASM	DQ247735
D. reniculelloides	*schuetzeella*	Du121[§]	Canada: Prince Albert, Saskatchewan	1953	Dried	NFRC	—
D. reniculelloides	*schuetzeella*	Du122[§]	Canada: Corner Lake, Manitoba	1956	Dried	NFRC	—
D. ponderosae	*ponderosae*	Du114[†,§]	USA: Sierra Diablo, Texas	D.C. Ferguson, 1973	Dried	USNM	DQ247733
D. clarioralis	*baumhoferi*	Du117[†]	USA: Tishomingo Co., Mississippi	R. Kergosien, 1994	Dried	USNM	DQ247732
D. zimmermani	*zimmermani*	—[§]	Canada: Vineland, Ontario	1942	Dried	OSAC	—
D. zimmermani	*zimmermani*	Du118[†,§]	USA: Hinds Co., Mississippi	M.E. Poshore, 1994	Dried	USNM	DQ247730
D. taedivorella	*zimmermani*	Du119[†,§]	USA: Grasonville, Maryland	D.C. Ferguson, 1986	Dried	USNM	DQ247731
D. tumicolella	*zimmermani*	Du112[†,§]	USA: Crawford Co., Kansas	K.O. Bell, 2002	Dried	USNM	DQ247729
Oncocera faecella	Outgroup	Du29[‡]	China: Mt. Manhan, Inner Mongolia	D. Zhang, 2002	Alcohol	UASM	DQ247764
O. faecella	Outgroup	Du30[‡]	China: Mt. Manhan, Inner Mongolia	D. Zhang, 2002	Alcohol	UASM	DQ247765
O. faecella	Outgroup	Du31[‡]	China: Mt. Manhan, Inner Mongolia	D. Zhang, 2002	Alcohol	UASM	DQ247766
O. faecella	Outgroup	Du33[†]	China, Mt. Manhan, Inner Mongolia	D. Zhang, 2002	Alcohol	UASM	DQ247727
O. faecella	Outgroup	Du35[‡]	China, Mt. Manhan, Inner Mongolia	D. Zhang, 2002	Alcohol	UASM	DQ247767
O. faecella	Outgroup	Du36[‡]	China, Mt. Manhan, Inner Mongolia	D. Zhang, 2002	Alcohol	UASM	DQ247763
Ceroprepes ophthalmicella	Outgroup	Du79[†]	China, Mt. Baiyun, Henan Province	X. Wang, 2002	Alcohol	UASM	DQ247728

*See Materials and methods for complete list of institutions.
[†]Specimen sequenced for 2.3 kb of COI–COII genes.
[‡]Specimen sequenced for only 394 bp of COI gene.
[§]Specimen used for genitalic figures.

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alcohol-preserved or live-frozen specimens. Vouchers of the remaining parts of each sample were kept pinned as standard museum specimens or were placed in gelatin capsules when too fragmented to pin directly. Vouchers were returned to the original collections listed in Table 1 or were deposited in UASM. All specimens were numbered and corresponding numbers were assigned to the extracted DNA samples, which are stored at –70 °C at the University of Alberta (Edmonton, Alberta).

Molecular techniques

Total genomic DNA was extracted using a QIAamp DNA Mini Kit following the manufacturer's instructions (QIAGEN, Valencia, California). Tissue was digested with proteinase K for a minimum of 3 h. Final elution volumes ranged from 200 µL to 75 µL depending on the preservation and age of the specimen. Decreased elution volumes were used to increase DNA concentrations from old or poorly preserved material. Polymerase chain reaction (PCR) amplifications were performed on a TGradient Thermocycler (Biometra, Göttingen, Germany) in a 50-µL reaction mix containing 1–4 µL of extracted DNA (dependent on template quality), 1–2 µL of each of two 5 pmol/µL heterologous primers, 1 µL of 10 mmol/L dNTPs (Roche Diagnostics, Indianapolis, Indiana), 5 µL of 10× PCR reaction buffer containing 15 mmol/L $MgCl_2$ (Promega Corporation, Madison, Wisconsin), 4 µL of 25 mmol/L $MgCl_2$ (Promega), and double-distilled water (Millipore Corp., Billerica, Massachusetts) to make up the remaining volume. After a "hot start" with a 2-min denaturation at 94 °C, the reaction was paused and 0.5 µL of Taq polymerase (approximately 5 U/µL) was added. Amplification parameters for the subsequent 35 cycles were as follows: 94 °C for 30 s (denaturation), 45 °C for 30 s (annealing), and 72 °C for 2 min (extension). The reaction was finished with a final 5-min extension at 72 °C. A single amplification was used for most fragments. Where the PCR product was weak, a second amplification using nested primers within the first PCR fragment was performed. Primers used in this study are listed in Table 2.

PCR products were visualized on a 1% agarose gel, stained with ethidium bromide, and sized against a ΦX174/*Hae*III DNA ladder (Promega) under UV light. PCR products were cleaned using a QIAquick PCR Purification Kit (QIAGEN). Cycle sequencing for both forward and reverse strands was performed using either a DYEnamic™ ET Terminator Cycle Sequencing Kit (Amersham, Buckinghamshire, England) or an ABI PRISM® BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California) according to the manufacturer's suggested thermal profile. DYEnamic™ ET sequencing reactions were 15 µL in total volume and each contained 4.0 µL of ET Premix, 1 µL of 5 pmol/µL primer used for PCR amplification, 1–3 µL of purified PCR product, and 7–9 µL of double-distilled water (Millipore Corp.). Parameters for cycle sequencing were as follows: 93 °C for 30 s; 27 cycles of 95 °C for 20 s, 45 °C for 15 s, and 60 °C for 1 min; and a final extension of 1 min at 60 °C. BigDye® sequencing reactions were 10 µL in total volume and each included 3.0 µL of 2.5× BigDye® Terminator Sequencing Buffer, 1.0 µL of BigDye® Terminator cycle sequencing mix, 0.5 µL of 5 pmol/µL primer used for PCR amplification, 2.0 µL of clean PCR product, and 3.5 µL of double-distilled water. Parameters for cycle sequencing were as follows: 96 °C for 1 min followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and extension for 6 min at 60 °C. The sequenced product was filtered through Sephadex G-50 columns (Amersham Biosciences Inc., Piscataway, New Jersey) and dried. This product was resuspended and visualized on an ABI PRISM® 377 automated sequencer (Applied Biosystems). The DNA sequence for each specimen was determined for both sense and antisense strands using the same primers used in the PCR amplification. Contig construction was performed using Sequencher 4.1 (Gene Codes Corp., Ann Arbor, Michigan). Sequences lacked insertions or deletions, so sequence alignments were done manually in PAUP* version 4.0 beta 10 (Swofford 2003).

Morphological techniques

Dissection methodology follows Li and Zheng (1996) and Winter (2000) except that after a 100% ethanol wash, all parts were either preserved in glass genitalia vials filled with glycerol or slide-mounted with Euparal mounting medium (BioQuip Products, Inc., Rancho Dominguez, California). Most morphological characters chosen for phylogenetic analysis had been used previously to describe species or delineate species groups (Heinrich 1956; Munroe 1959; Mutuura *et al.*1969*a*, 1969*b*; Schaber and Wood 1971; Mutuura and Munroe 1972, 1973, 1974; Wang and Sung 1982, 1985; Blanchard

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Table 2. List of primers used for PCR amplification and sequencing.

Region*	Source	Primer (5′–3′)
TY-J-1460a	Sperling *et al.* (1994)	TAC AAT TTA TCG CCT AAA CTT CAG CC
CI-J-1609	New	GAT GAT CAA ATT TAT AAT AC
C1-N-1687	Wells and Sperling (1999)	CAA TTT CCA AAT CCT CCA ATT AT
C1-J-1751e	New	GGA GCT CCA GAT ATA GCT TTC CC
C1-N-1840a	Sperling *et al.* (1995)	AGG AGG ATA AAC AGT TCA C/TCC
CI-N-1840a.1	New	RGG GGG RTA AAY WGT TCA WCC
CI-N-1840b.m	New	AGG GGG GTA GAC GGT TCA TCC
C1-J-2183a	Simon *et al.* (1994)	CAA CAT TTA TTT TGA TTT TTT GG
C1-N-2329	Simon *et al.* (1994)	ACT GTA AAT ATA TGA TGA GCT CA
C1-N-2329c	New	ACA GTA AAT ATA TGA TGA GCT CA
C1-J-2495a	New	CTT CTA TAC TTT GAA GAT TAG G
C1-J-2495y	New	CTT CTA TGT TAT GAA GTT TAG G
C1-J-2531	New	TTT ACT GTA GGA GGA TTA ACW GG
C1-N-2578f	New	TGA AAA TGA GCA ACA ACA TAA TA
C1-N-2659b	New	ACT AAT CCT GTG AAT AAA GG
C1-J-2792b	Wells and Sperling (1999)	ATA CCT CGG CGA TAC TCT GA
C1-J-2792c	New	ATA CCT CGA CGA TAT TCC GA
C1-J-2792d	New	ATA CCM CGA CGA TAY TCW GA
TL2-N-3013	Sperling *et al.* (1996)	TCC ATT ACA TAT AAT CTG CCA TAT TAG
TL2-J-3038b	Caterino *et al.* (2001)	CTA ATA TGG CAG ATT ATA TCT AAT GGA
C2-J-3120	New	GTT GTT CTA TTA AGG GTG AAG
C2-J-3138a	Sperling *et al.* (1995)	AGA GCC TCT CCT TTA ATA GAA CA
C2-N-3389b	New	TCA TAW CTT CAR TAT CAT TG
C2-J-3570a	New	GCA ACA GAT GTT ATT CAC TCT TG
C2-N-3661	Simon *et al.* (1994)	CCA CAA ATT TCT GAA CAT TGA CCA
TK-N-3775	Bogdanowicz *et al.* (1993)	GAG ACC ATT ACT TGC TTT CAG TCA TCT

*Nomenclature from Simon *et al.* (1994).

and Knudson 1983; Yamanaka 1990; Neunzig 1990, 1996, 2003; Neunzig and Dow 1993; Sopow *et al.* 1996; Segerer and Pröse 1997; Speidel and Asselbergs 2000). Additional morphological characters were chosen based on comparisons with outgroup taxa and variation observed between species. Using characters identified by previous authors allowed us to evaluate species descriptions and species-group delineations using molecular characters. Characters not previously used in *Dioryctria* classification were included to identify new informative morphological characters. For phylogenetic analysis, 52 morphological characters were scored for all included species and included 1 antennal, 1 abdominal, 2 palpal, 6 forewing, and 42 genitalic characters (Figs. 1–23; Appendix 1). All characters were coded as unordered and unweighted. All available specimens were examined when scoring characters, and multiple specimens were compared whenever possible. A. Roe independently confirmed character codings using identified material at CNC and USNM. We illustrate the genitalia of one male and one female in each *Dioryctria* species group, when possible. Additional illustrations of species in this treatment are in the following: Heinrich 1956; Munroe 1958; Mutuura 1958; Mutuura *et al.* 1969*a*, 1969*b*; Neunzig and Leidy 1989; Mutuura and Munroe 1972, 1973; Sopow *et al.* 1996; and Neunzig 2003.

Phylogenetic analyses

Phylogenetic reconstructions were obtained using unweighted parsimony in PAUP* 4.0b10 (Swofford 2003) and MacClade 4.05 OSX (Maddison and Maddison 2002). Variable nucleotide positions and morphological characters were treated as unordered characters with one state for each nucleotide or character. We used heuristic searches with 100 random-addition

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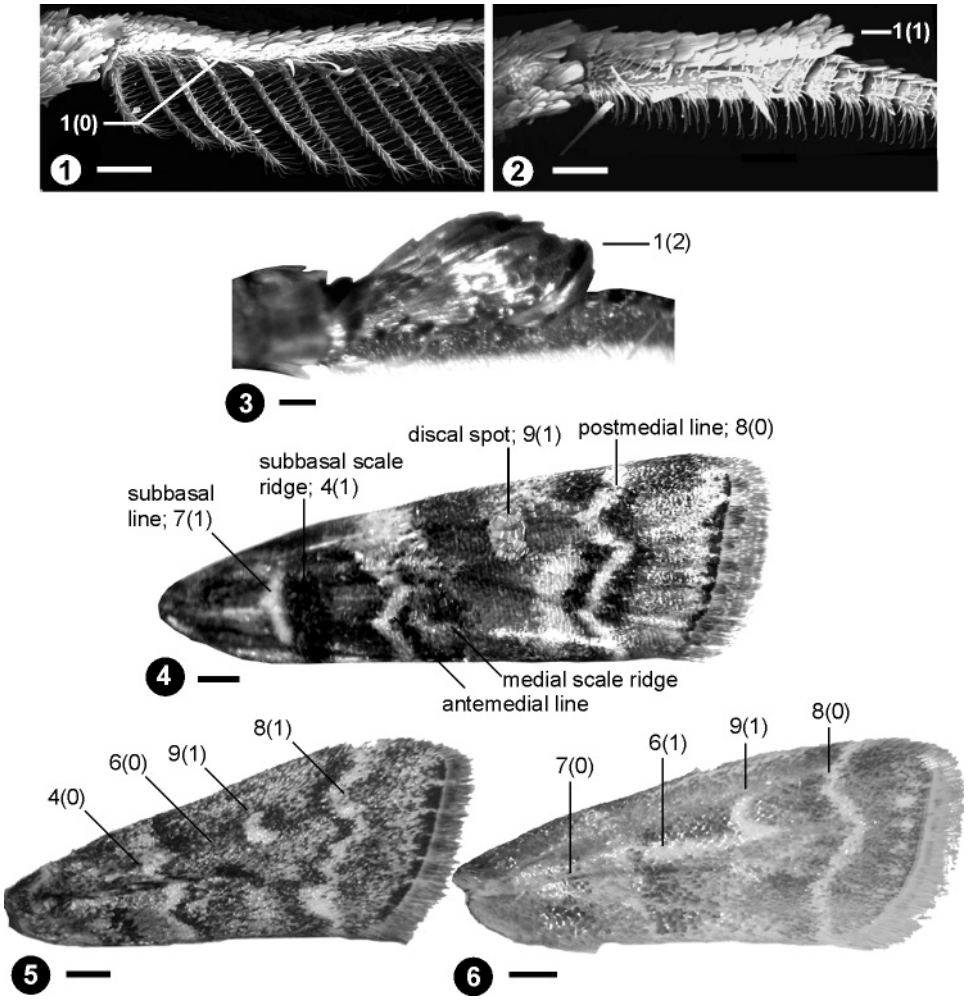
Figs. 1–6. 1–3, Male antennae of outgroup taxa and two *Dioryctria* species: 1, *Ceroprepes ophthalmicella* (China: Henan Province: Mt. Baiyun; from Y. Du, 2002); 2, *D. rubella* (China: Tianjin: Mt. Baxian; from Y. Du, 2002); 3, *D. yiai* (China: Liaoning Province: Xingcheng). 4–6, forewing structures of *Dioryctria* species: 4, *D. zimmermani* (Canada: Ontario: Vineland); 5, *D. reniculelloides* (Canada: Manitoba: Corner Lake); 6, *D. yiai* (China: Tianjin: Mt. Baxian). Scale bars = 0.1 mm (Figs. 1–3) and 1.0 mm (Figs. 4–6).



replicates, using tree bisection–reconnection (TBR) branch swapping. Sequence divergence was calculated using uncorrected pairwise distances.

Clade stability was estimated using both bootstrap percent and Bremer support values (Bremer 1994). Bootstrap values were generated in PAUP* from 100 replicates, each using a heuristic parsimony search. For combined analyses, we performed parsimony analyses using the same defaults as above. Partitioned Bremer support values (Baker and DeSalle 1997; Baker *et al.* 1998) were calculated in PAUP* by saving the most parsimonious tree found in a heuristic search constrained to not include one of the nodes on the simultaneous analysis tree, which was performed using TreeRot 2.0 (Sorenson 1999).

Results

mtDNA phylogeny

We sequenced a 2308-bp mtDNA region (including the COI + $tRNA^{Leu}$ + COII genes) for a total of 20 specimens of 14 *Dioryctria* species and 2 outgroups. For *D.* nr. *rubella*, 2202 bp of mtDNA was obtained (start of COI gene was missing), and for *D. taedivorella* we obtained 1927 bp of mtDNA (end of COII gene was

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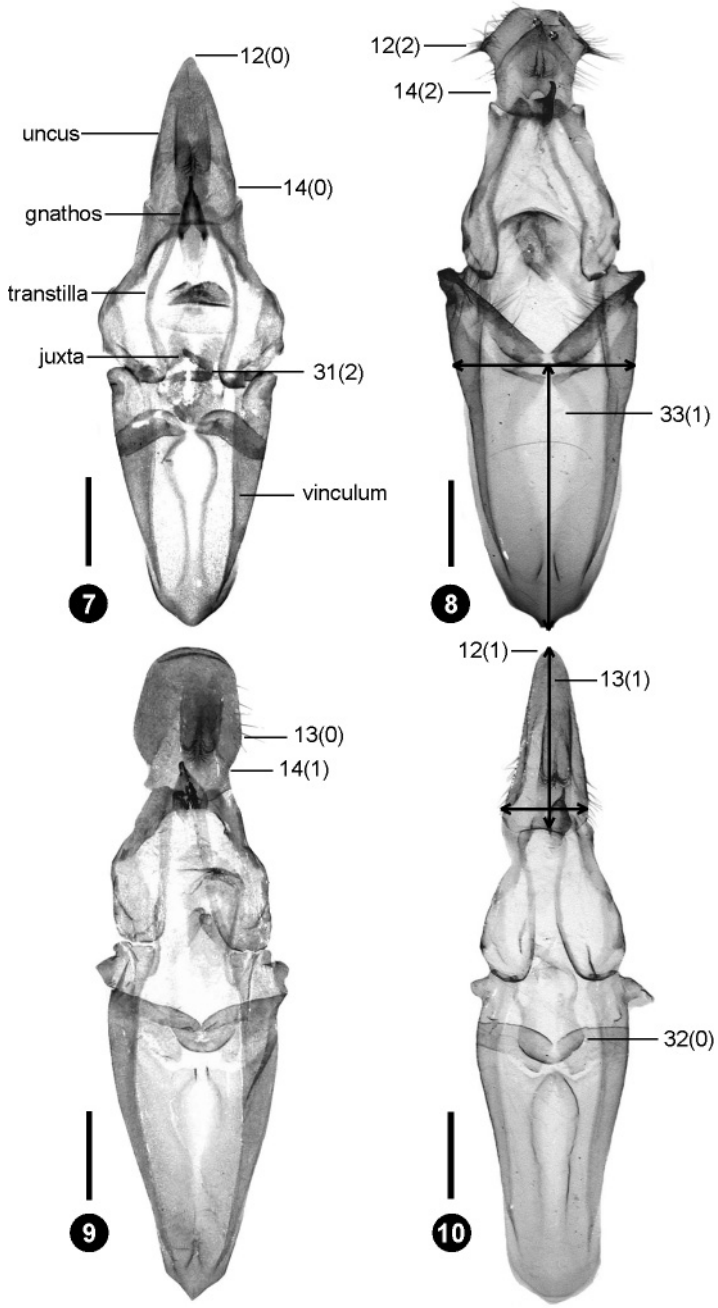
Figs. 7–10. Male *Dioryctria* genitalia with valva and aedeagus removed: 7, *D. yiai* (China: Zhejiang: Mt. Tjanmu); 8, *D. taedivorella* (USA: Maryland: Grasonville); 9, *D. rubella* (China: Tianjin: Mt. Baxian); 10, *D. reniculelloides* (Canada: Manitoba: Corner Lake). Scale bars = 0.5 mm.



missing). The full 2.3-kb fragment was thus obtained for 18 of the 20 specimens, including 2 specimens from each of 4 *Dioryctria* species (Table 1). In total, 1729 nucleotide sites were constant in this data set, and 401 were parsimony-informative characters.

The two most parsimonious (MP) trees (length = 1112, CI = 0.638, RI = 0.699) were

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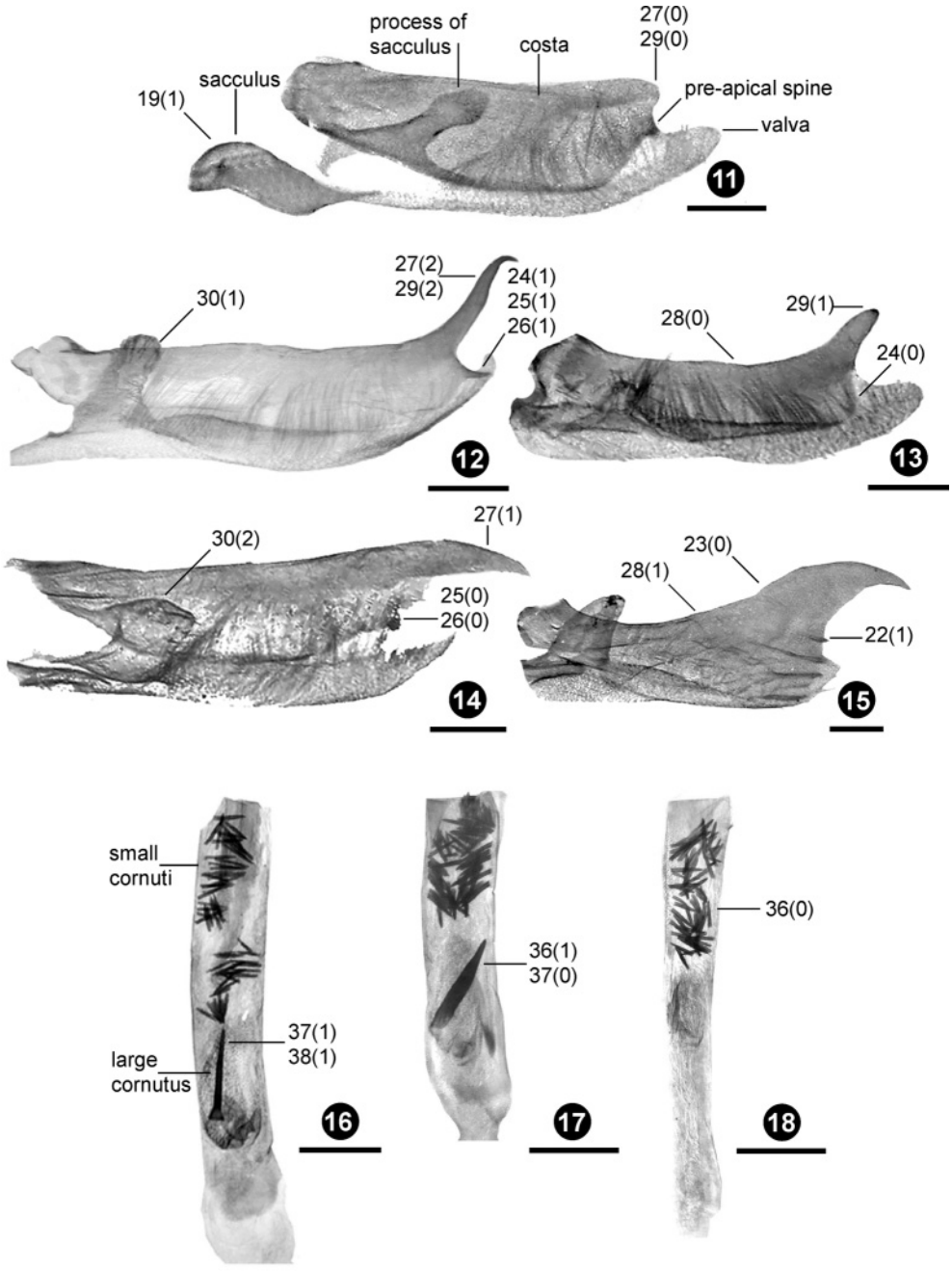
Figs. 11–18. 11–15, right valva of male *Dioryctria* spp.: 11, *D. yiai* (China: Zhejiang: Mt. Tjanmu); 12, *D. tumicolella* (USA: Kansas: Crawford Co.); 13, *D. ponderosae* (USA: Texas: Sierra Diablo); 14, *D. reniculelloides* (Canada: Manitoba: Corner Lake); 15, *D. rubella* (China: Tianjin: Mt. Baxian). 16–18, aedeagus of male *Dioryctria* spp.: 16, *D. taedivorella* (USA: Maryland: Grasonville); 17, *D. ponderosae* (USA: Texas: Sierra Diablo); 18, *D. reniculelloides* (Canada: Manitoba: Corner Lake). Scale bars = 0.25 mm (Figs. 11–15) and 0.5 mm (Figs. 16–18).



obtained by a heuristic search of the 2.3-kb mtDNA data set. Differences between these two trees involve changes in placement of the *abietella* and *schuetzeella* + *auranticella* groups. Figure 24 shows the bootstrap consensus tree with Bremer support values added to

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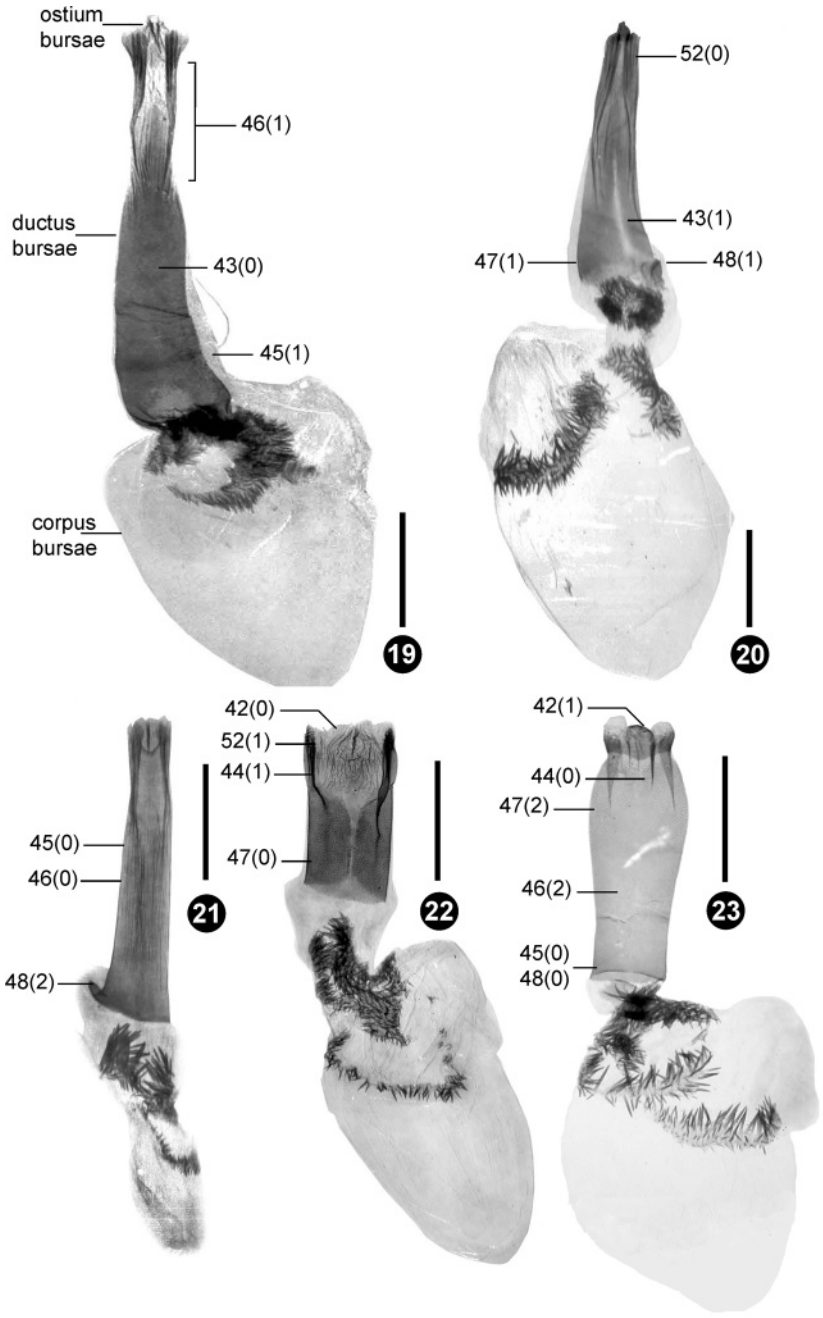
Figs. 19–23. Female *Dioryctria* genitalia: 19, *D. zimmermani* (USA: Mississippi: Hinds Co.); 20, *D. abietella* (China: Henan Province, Mt. Baiyun); 21, *D. reniculelloides* (Canada: Saskatchewan: Prince Albert); 22, *D. yiai* (China: Hebei Province: Mt. Baishi); 23, *D. clarioralis* (USA: Mississippi: Tishomingo Co.). Scale bars = 1.0 mm.



show an alternative measure of support for nodes (Fig. 24).

Within species, each of the four pairs of sequences (*D. abietivorella*, *D. reniculelloides*, *D. sylvestrella*, and *D. yiai*) were strongly supported as monophyletic (Fig. 24). Three of the four species groups that were represented by multiple species also formed strongly supported monophyletic clades (the *abietella*, *sylvestrella*, and *zimmermani* groups). Relationships within

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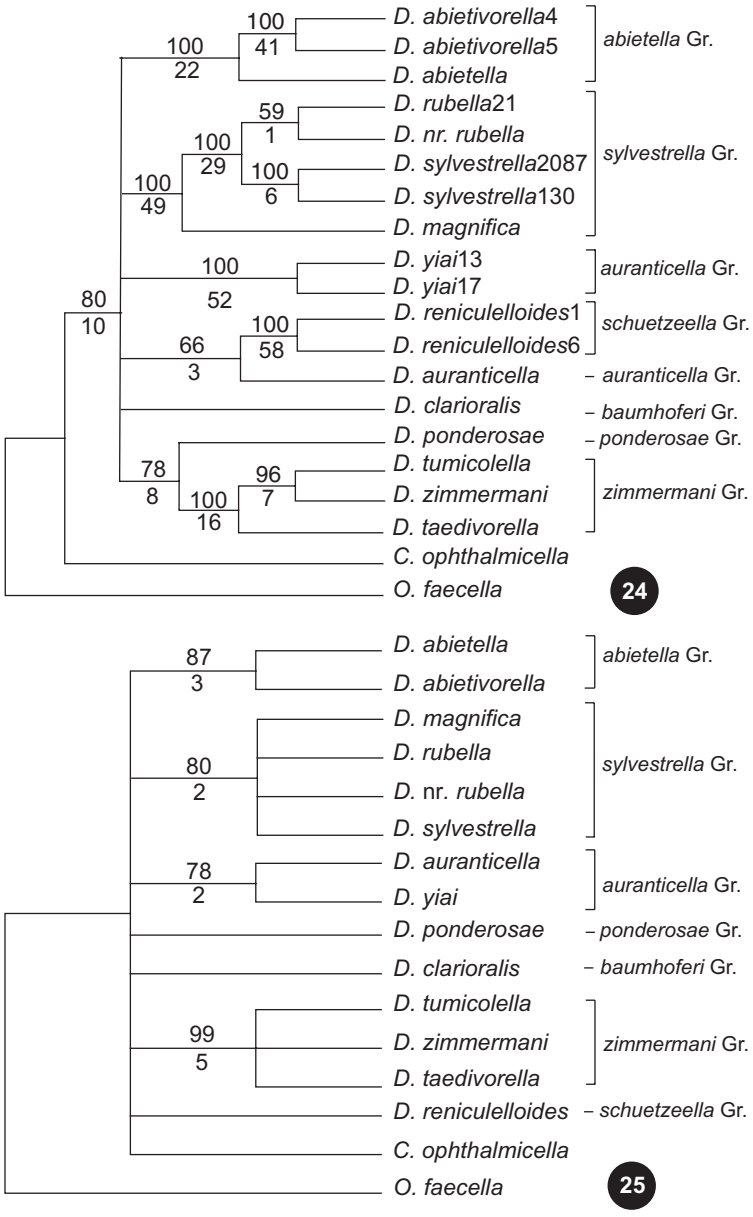
Figs. 24–25. 24, a bootstrap consensus tree based on the two most parsimonious trees from the parsimony analysis of 2.3 kb of mtDNA (COI + $tRNA^{Leu}$ + COII genes) in 20 specimens representing 14 *Dioryctria* species and 2 outgroup species (length = 1112, CI = 0.638, RI = 0.699). 25, a bootstrap consensus tree based on the four most parsimonius trees from parsimony analysis of 52 morphological characters in 14 *Dioryctria* species and 2 outgroup species (length = 133, CI = 0.622, RI = 0.659). Species-group classifications are based on Mutuura and Munroe (1972) and Neunzig (2003). Bootstrap support is shown above branches and Bremer support values are below branches of the tree.



these groups were generally strongly supported. Within the *sylvestrella* group, *D. rubella* and *D.* nr. *rubella* were sister species, though this relationship was poorly supported. The two *D. sylvestrella* specimens were strongly supported as sister species to the *D. rubella* + *D.* nr. *rubella* clade, and *D. magnifica* was strongly supported as sister species to this larger clade. Within the *zimmermani* group, *D. tumicolella* and *D. zimmermani* were strongly supported as

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sister species, and *D. taedivorella* was strongly supported as sister species to this clade. The fourth species group, the *auranticella* group (*D. auranticella* and *D. yiai*), was paraphyletic with respect to the *schuetzeella* group (*D. reniculelloides*), though this relationship was weakly supported. Higher-level relationships were generally poorly supported by the mtDNA phylogeny. Placement of the *abietella* group, *auranticella* + *schuetzeella* group, *sylvestrella* group, and *baumhoferi* group was variable in the MP trees. The one exception was the *ponderosae* group (*D. ponderosae*), which formed a moderately well-supported sister group relationship to the *zimmermani* group.

Genetic divergences

In addition to the 20 specimens sequenced over all or most of the 2.3-kb region of the COI–COII genes, 16 additional *Dioryctria* specimens and 5 outgroup specimens were sequenced over a 394-bp fragment of the COI gene. This short fragment was located downstream of the middle of the COI gene and was amplified with primers C1-J-2183a and C1-N-2578f (Table 2). Phylogenetic analyses of all forty-one 394-bp sequences, including those contained in longer 2.3-kb sequences, resolved 20 *Dioryctria* haplotypes belonging to 14 species and 4 haplotypes belonging to 2 outgroup species. Trees produced from this analysis (not shown) were congruent with those in the previous analysis (Fig. 24); species groups were similarly resolved and well supported and relationships between species groups were poorly supported.

Average divergences within *Dioryctria* species and between species of the same group were tabulated based on uncorrected pairwise distances (Table 3). Eight species had two or more specimens sequenced over the 394-bp fragment of the COI gene. Average within-species variation for the 394-bp fragment ranged from 0.0% to 0.5% in *Dioryctria* and was 0.8% in one outgroup species. Within-species variation for the 2.3-kb fragment ranged from 0.1% to 0.3% for the four species with multiple specimens sequenced over the region (Table 3). Although pairwise comparisons of specimens indicated that within-species variation was usually less than 0.4%, two exceptions were found. First, a specimen of *D. yiai* was 0.7% diverged from other *D. yiai* specimens in the analysis. This individual was geographically isolated from the other *D. yiai* specimens that were sampled from similar localities or geographical areas (Table 1), which may explain the observed sequence divergence. A second exception was a specimen initially identified as *D. rubella*. In relation to other *D. rubella* specimens, it had an average divergence of 0.9% over the 394-bp fragment and 1.2% over the 2.3-kb fragment. Several unique morphological characters were also found upon reexamination of the voucher specimen and additional museum material. Thus, the specimen is treated here as a separate but undescribed taxon (*D.* nr. *rubella*) (Y. Du, unpublished data).

We examined variation within the species groups defined by Mutuura and Munroe (1972) and reevaluated by Neunzig (2003) (Tables 3, 4). In general, the 2.3-kb fragments had slightly higher sequence divergence than the 394-bp fragments and, comparatively, these two fragments showed similar trends. Within the *zimmermani* group, there was less than 0.7% divergence between *D. tumicolella* and *D. zimmermani* in both fragments (Table 4). Divergences between these two species and *D. taedivorella* were greater, though generally less than those separating species in other species groups (Table 4). Three members of the *sylvestrella* group (*D. sylvestrella*, *D. rubella*, and *D.* nr. *rubella*) were separated by only 1.2%–1.4% sequence divergence over the 2.3-kb fragment. In comparison, *D. magnifica* had greater sequence divergence from other members of the group (2.3-kb fragment, 3.8%–4.1%). Members of the *abietella* and *auranticella* species groups had divergences of 3.8%–5.6% between their respective species. The *baumhoferi*, *ponderosae*, and *schuetzeella* groups were each represented by a single species, so within-group divergence could not be evaluated.

Average sequence divergence between different species groups (Table 4) ranged from 3.3% to 8.4% (394-bp fragment) and from 5.1% to 9.2% (2.3-kb fragment). Raw divergences support the paraphyletic relationship of the *schuetzeella* and *auranticella* groups. *Dioryctria auranticella* was less diverged from *D. reniculelloides* (394-bp fragment, 4.3%; 2.3-kb fragment, 5.6%) than from haplotypes of *D. yiai* (394-bp fragment, 4.9%; 2.3-kb fragment, 5.6%).

Morphological phylogeny

Although not all known species of *Dioryctria* were included in this analysis, our phylogenetic

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Table 3. Mitochondrial DNA variation within species groups and species where sequences for more than one species or specimen were available.

	Within species (no. of specimens)			Within group (no. of species)	
Species	2.3 kb	394 bp	Species group*	2.3 kb	394 bp
D. abietella	—	0.1 (8)	*abietella*	3.8 (2)	4.4 (2)
D. abietivorella	0.1 (2)	0.2 (2)			
D. sylvestrella	0.1 (2)	0.0 (2)	*sylvestrella*	2.7 (4)	2.2 (4)
D. magnifica	—	0.0 (2)			
D. rubella	—	0.3 (4)			
D. auranticella	—	0.0 (2)	*auranticella*	5.6 (2)	4.9 (2)
D. yiai	0.3 (2)	0.5 (6)			
D. reniculelloides	0.1 (2)	0.0 (2)	*schuetzeella*		
	—	—	*zimmermani*	1.0 (3)	0.5 (3)
O. faecella	—	0.8 (6)	Outgroups		

Note: Sequence divergence is measured as uncorrected average pairwise distances. Numbers in parentheses refer to the number of sequences available for examination.

*Species groups based on Mutuura and Munroe (1972) and Neunzig (2003).

framework provides a useful hypothesis-testing exercise for relating the characters we found supporting various clades to the characters previously used in the classifications of species groups (Mutuura and Munroe 1972, 1974; Wang and Sung 1982; Neunzig 2003).

A heuristic parsimony search in PAUP* using 52 morphological characters for 14 *Dioryctria* species and 2 outgroup species resulted in four MP trees of 133 steps (CI = 0.622, RI = 0.659). Differences among the MP trees lie in the relationships between species groups and the placement of *C. ophthalmicella* in the phylogeny. Figure 25 shows the bootstrap consensus tree with Bremer support values added to show an alternative measure of support for nodes (Fig. 25). Seven major lineages were identified within *Dioryctria*, and all species groups with more than one species (*zimmermani*, *abietella*, *sylvestrella*, and *auranticella* groups) were strongly or moderately well supported as monophyletic.

Within the strongly monophyletic *zimmermani* group, there are few morphological differences among *D. zimmermani*, *D. tumicolella*, and *D. taedivorella*. As in previous classifications (Mutuura *et al.* 1969*a*; Mutuura and Munroe 1972; Neunzig 2003), males of this clade are characterized by an uncus with a strongly broadened midpoint forming a triangular prominence and a large, prominent preapical spine on the terminal edge of the costa (Fig. 8: 14(2); Fig. 12: 25(1)). Females have a long, curved ductus bursae (Fig. 19: 45(1)). Fine longitudinal wrinkles restricted to the posterior half of the corpus bursae were identified by our analysis as another character defining the group (Fig. 19: 46(1)).

The single species representing the *ponderosae* group (*D. ponderosae*) formed a distinct lineage in the morphology-based phylogeny (Fig. 25). As in previous classifications (Mutuura *et al.* 1969*b*; Mutuura and Munroe 1972; Neunzig 2003), males of this lineage are characterized by an elongate costal apex with a blunt, hooked tip and lack a preapical spine on the costal edge (Fig. 13: 24(0), 29(1)). Females are characterized by a ductus bursae with heavy longitudinal fluting.

The single species representing the *baumhoferi* group (*D. clarioralis*) formed a distinct lineage in the morphology-based phylogeny. As in the classifications of Mutuura *et al.* (1969*b*), Mutuura and Munroe (1972), and Neunzig (2003), males of this lineage are characterized by an uncus narrowing to an acute apex and a straight, elongate costal apex lacking a hooked tip, and the female ductus bursae lack longitudinal wrinkles. Although previous descriptions of the *baumhoferi* group mention this character (Mutuura *et al.* 1969*b*; Mutuura and Munroe 1972; Neunzig 2003) and we have confirmed the presence of the character in four other species in the group (*D. baumhoferi* Heinrich, 1956, *D. substracta* Heinrich, 1956, *D. pentictonella* Mutuura, Munroe and Ross, 1969,

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Table 4. Mitochondrial DNA sequence divergence (%) among *Dioryctria* and outgroup species.

Group	Species	*abt*	*abv*	*syl*	*rub*	nr. *r*	*mag*	*aur*	*yia*	*ren*	*pon*	*cla*	*zim*	*tae*	*tum*	*O.fa*	*C.op*
abietella	*abt*	—	4.4	8.4	7.7	8.4	8.1	6.6	6.1	7.6	5.1	6.1	7.6	7.1	7.4	8.2	9.1
	abv	3.8	—	7.0	6.5	6.5	7.0	5.5	6.0	6.2	4.7	6.0	6.7	6.7	6.5	8.1	9.3
sylvestrella	*syl*	8.4	8.7	—	0.9	1.0	3.6	6.3	7.2	5.8	5.6	6.9	6.3	6.3	6.6	8.5	10.2
	rub	8.3	8.7	1.3	—	0.9	3.5	5.7	6.8	5.3	4.8	6.1	5.8	5.8	6.1	7.7	9.2
	nr. *r*	8.1	8.4	1.4	1.2	—	3.0	6.1	7.2	5.8	5.6	6.9	6.3	6.3	6.7	8.5	9.9
	mag	7.3	7.6	3.8	4.1	4.1	—	6.6	7.7	5.3	6.4	7.9	7.4	7.4	7.1	9.3	11.4
auranticella	*aur*	6.1	6.0	7.9	8.0	8.1	7.1	—	4.9	4.3	3.3	4.6	5.3	5.3	5.1	6.9	8.4
	yia	6.4	6.8	8.1	8.0	8.1	7.3	5.6	—	5.7	3.4	5.7	5.7	5.7	5.7	7.9	8.5
schuetzeella	*ren*	6.4	6.6	8.4	8.5	8.5	7.8	5.6	5.9	—	4.3	6.1	5.3	5.3	5.6	7.7	9.6
ponderosae	*pon*	6.4	6.9	8.9	8.8	8.7	8.1	6.9	6.7	7.2	—	3.6	4.1	4.1	4.7	5.8	5.6
baumhoferi	*cla*	7.4	7.8	9.1	9.2	8.9	8.7	7.7	8.0	8.3	8.2	—	6.6	6.1	6.6	6.1	7.4
zimmermani	*zim*	6.1	6.5	7.4	7.6	7.3	7.2	6.3	5.9	6.5	5.1	6.6	—	0.5	0.3	8.5	7.6
	tae	6.3	6.4	7.5	7.7	7.4	7.3	5.9	5.7	5.1	5.5	6.7	1.5	—	0.8	8.5	8.1
	tum	6.1	6.7	7.8	7.9	7.6	7.3	6.4	6.1	6.7	5.1	6.6	0.7	1.8	—	8.5	7.6
Outgroups	*O.fa*	7.3	7.7	8.9	8.8	8.8	8.1	6.9	7.0	7.4	7.8	8.9	7.2	7.4	7.5	—	10.1
	C.op	9.1	9.7	11.3	11.3	11.3	10.3	8.8	9.1	9.8	8.8	10.5	9.4	9.3	9.5	9.1	—

Note: Uncorrected average pairwise distances are shown for sequences from 2.3 kb of the cytochrome oxidase I + II genes (COI + COII; below diagonal) and 394 bp of the COI gene (above diagonal). Shaded regions outline the extent of variation within species groups. Species groups are based on the classification outlined in Mutuura and Munroe (1972). Species abbreviations are as follows: *abt*, *D. abietella*; *abv*, *D. abietivorella*; *syl*, *D. sylvestrella*; *rub*, *D. rubella*; nr. *r*, *D.* nr. *rubella*; *mag*, *D. magnifica*; *aur*, *D. auranticella*; *yia*, *D. yiai*; *ren*, *D. reniculelloides*; *pon*, *D. ponderosae*; *cla*, *D. clarioralis*; *zim*, *D. zimmermani*; *tae*, *D. taedivorella*; *tum*, *D. tumicolella*; *O.fa*, *Oncocera faecella*; *C.op*, *Ceroprepes ophthalmicella*.

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and *D. vancouverella* Mutuura, Munroe and Ross, 1969), all male specimens of *D. clarioralis* examined in this study lacked a preapical tooth on the costal edge of the valve. The presence of minute microsculpture on the surface of the ductus bursae and a posterior expansion of the ductus bursae were identified by our analysis as additional characters defining the lineage (Fig. 23: 47(2)).

Members of the *abietella* group formed a clade strongly supported by bootstrap and Bremer support values. The clade is composed of two species, *D. abietella* and *D. abietivorella*, which can be separated by genitalic differences, in accordance with previous studies (Munroe 1959). As in previous classifications (Mutuura and Munroe 1972; Neunzig 2003), males are characterized by an uncus with a slight marginal expansion, though less prominent than in *D. rubella* (Fig. 9: 14(1)), and a large preapical spine on the costal edge. Females are characterized by a curved ductus bursae with a lateral lobe on the right side and a central longitudinal membranous region (Fig. 20: 43(1)).

Members of the *sylvestrella* group formed a clade strongly supported by bootstrap and Bremer support values. This clade is represented by *D. sylvestrella*, *D. rubella*, *D.* nr. *rubella*, and *D. magnifica*, which are separated by genitalic differences, and is distinct from other clades in the phylogeny. As in previous classifications (Mutuura and Munroe 1972), males are characterized by a broadly expanded uncus with a margin forming a rounded edge (Fig. 9: 14(1)) and a costa with three or more distinct terminal longitudinal ridges (Fig. 15: 22(1)). Females are characterized by a long, straight ductus bursae with full-length longitudinal wrinkles and lack an anterior lateral process (Fig. 23: 48(0)).

The *schuetzeella* group, represented by a single species (*D. reniculelloides*), formed a distinct lineage in the morphology-based phylogeny. As in previous classifications (Mutuura and Munroe 1972, 1973; Neunzig 2003), males of this lineage are characterized by an uncus with a narrow, rounded apex (Fig. 10: 12(1)) and the absence of a large cornutus in the aedeagus (Fig. 18: 36(0)). Females are characterized by a lateral prominence on the left side of the ductus bursae (Fig. 21: 48(2)).

Members of the *auranticella* group formed the final clade, which was moderately well supported by bootstrap and Bremer support values. The clade is composed of two species, *D. auranticella* and *D. yiai*, which are diagnosable by genitalic differences. As in previous classifications (Mutuura and Munroe 1972; Neunzig 2003), males of this clade are characterized by distally expanded maxillary palps and a valve with a broad costal apex lacking elongation (Fig. 11: 27(0), 29(0)). Females are characterized by a ductus bursae with a central membranous region and transverse medial wrinkles. A strongly infolded posterior margin of the ductus bursae was identified by this analysis as an additional character defining the group (Fig. 22: 52(1)).

Relationships between species groups were poorly supported (Fig. 25), though the species groups themselves formed distinct, well-supported lineages where more than one species per group was examined. Although the *baumhoferi*, *ponderosae*, and *zimmermani* groups share three character states (forewing with raised scale ridges (Fig. 4: 4(1)), male aedeagus with a large cornutus (Fig. 17: 36(1)), and a valve with a costa with an elongate apex (Fig. 12: 27(2); Fig. 14: 27(1)), no phylogenetic relationship was found between them. As well, a monophyletic genus was not supported by bootstrap and Bremer support values, although members of the ingroup do share the following synapomorphies: forewing with distinct transverse bands and a single discal spot (primarily white with the exception of *D. clarioralis*); males with a scale tuft at the base of the antenna, genitalia distinctly elongated, a broadly sclerotized costa, weakly to moderately sclerotized juxta, and a narrowed sacculus (Figs. 2–15).

Comparisons among data sets

Compared with the morphology-based phylogeny, the mtDNA topology generally showed increased resolution, especially for relationships within species groups. There were also several relationships that differed between the morphological and mtDNA phylogenies (Figs. 24, 25): (*i*) the *auranticella* group was monophyletic in the morphological data set, whereas *D. reniculelloides* was nested within the *auranticella* species group in the mtDNA phylogeny; (*ii*) relationships in the *zimmermani* group were unresolved in the morphology-based phylogeny, whereas *D. zimmermani* was strongly supported as sister taxon to *D. tumicolella* in the mtDNA phylogeny; (*iii*) the relationship of *D. ponderosae* to other taxa was unresolved in the morphological analysis, whereas this species was sister to the *zimmermani* group in the mtDNA phylogeny; (*iv*) relationships within the *sylvestrella* clade were unresolved with morphological data,

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whereas well-supported relationships between species within the species group were resolved in the mtDNA phylogeny; and (*v*) almost every node, especially internal nodes, had greater support in the mtDNA phylogeny.

Combined analysis

An unweighted parsimony analysis of the combined morphological and mtDNA data sets resulted in a single MP tree (Fig. 26; length = 1260, CI = 0.634, RI = 0.698). The monophyly of the genus *Dioryctria* had increased support in the combined analyses in comparison with either separate analysis. Generally, relationships among species groups lacked resolution in the combined analysis, as was seen in each separate analysis. Furthermore, the combined analysis showed decreased support for a higher-level grouping that was resolved in the mtDNA phylogeny (Figs. 24, 26). In the mtDNA phylogeny, *D. reniculelloides* was placed as sister taxon to *D. auranticella*, making the *auranticella* group paraphyletic (Fig. 24); the combined analysis placed *D. reniculelloides* as sister to the *auranticella* group, though this relationship had less than 50% bootstrap support (Fig. 26).

Though the combined analysis failed to resolve the majority of higher-level relationships, there was one exception. The placement of *D. ponderosae* as sister to the *zimmermani* group was moderately supported in the mtDNA phylogeny but was well supported in the combined analysis, despite lack of support in the morphological phylogeny. In the combined analysis, the *zimmermani* group + *D. ponderosae* clade was defined by the following morphological characters: a raised ridge of scales on the forewing; a basally constricted uncus; the presence of a large cornutus; and a hooked tip on the elongate costal apex of the valve.

Bootstrap and Bremer support values improved for the species-group clades. The *zimmermani*, *abietella*, and *sylvestrella* clades that were resolved in both separate analyses were also found in the combined analysis with similar or higher support values (Fig. 26). For the *auranticella* group, combined analysis and morphology favored monophyly for the group, in contrast to the mtDNA phylogeny.

Relationships within species groups also improved in the combined analysis (Fig. 26). For species within the *zimmermani* and *sylvestrella* groups, the combined analysis was congruent with the mtDNA phylogeny and relationships had roughly equal or higher support values (Figs. 24, 26). This contrasts with the morphology-based phylogeny, which lacked resolution for species within these groups (Fig. 25). Species in the *abietella* and *auranticella* groups were resolved in all three analyses, with higher support in the combined analysis.

Discussion

For the species included in this paper, mtDNA and combined mtDNA–morphological analyses confirmed the monophyly of the genus *Dioryctria*. Morphological analyses did not produce a well-supported monophyletic ingroup, although several distinct genitalic and forewing characters support the genus. Robust species groupings were obtained in both separate and combined analyses for the *zimmermani*, *abietella*, and *sylvestrella* groups, while relationships in the *auranticella* group and the *schuetzeella* group varied between analyses.

A partitioned Bremer support (PBS) analysis can help to evaluate the relative strength of nodes in a combined analysis and can assess the contribution of each data partition to a final topology (Fig. 26) (Baker and DeSalle 1997). This method has been widely employed in simultaneous analyses of multiple data sets (DeSalle and Brower 1997; Baker *et al.* 1998, 2001; O'Grady *et al.* 1998; Remsen and DeSalle 1998; Gatesy *et al.* 1999; Caterino *et al.* 2001). Positive PBS values show that the majority of nodes at the species-group level are supported by both data sets (Fig. 26), although there are conflicts between the mtDNA and morphological data sets for the *auranticella* group and *D. reniculelloides*. Based on PBS values, the topology of the combined data set is more congruent with the mtDNA data partition than with the morphological data set, though some nodes do receive support from morphology in the face of mtDNA conflict. This incongruence may result from (*i*) large numbers of molecular characters overwhelming smaller morphological data subsets, even though the bias may not be severe (DeSalle and Brower 1997), or (*ii*) differences between rates of evolution in morphological and molecular characters, allowing morphological evidence to provide substantial support for internal nodes that are supported by few molecular synapomorphies (Brown *et al.* 1994; Sperling *et al.* 1997). As seen in separate analyses, higher-

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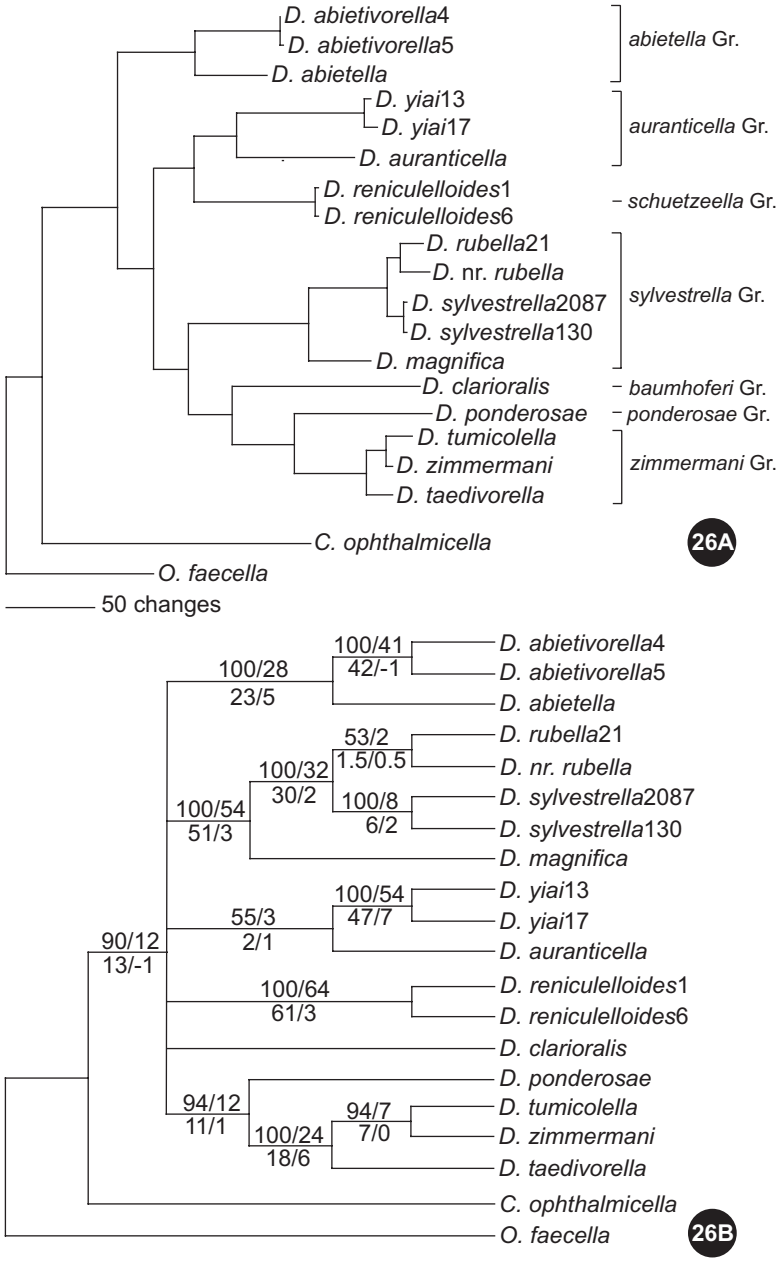
Fig. 26. A single most-parsimonious phylogram (A) and bootstrap consensus (B) obtained from combined analysis of morphology and mtDNA data of 20 specimens representing 14 *Dioryctria* species and 2 outgroup species (length = 1255, CI = 0.635, RI = 0.699). Species-group classifications are based on Mutuura and Munroe (1972) and Neunzig (2003). Numbers above each branch indicate bootstrap and Bremer support values, while numbers below are partitioned Bremer support values for mtDNA and morphology, respectively.



level relationships between species groups at the base of *Dioryctria* are poorly supported in both data sets, leading to low PBS values, suggesting that additional characters and taxa are needed to improve resolution of the species-group relationships. Other gene regions are particularly promising avenues, since many nuclear genes have much slower rates of evolution

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in comparison with mtDNA and may provide support for higher-level species-group relationships (Mallarino *et al.* 2005).

mtDNA and classification

Mitochondrial DNA sequence divergences among species in the same group (0.3%–5.6%) were generally less than those among species in different groups (3.3%–9.2%), although some overlap was apparent (Table 4). The exceptions to this trend were easily distinguished using morphological characters. Discrete clusters of mtDNA haplotypes characterized all species and three species groups (*zimmermani*, *abietella*, and *sylvestrella*), although not all species were resolved in the morphology-based phylogeny.

Conversely, relationships among *D. auranticella*, *D. yiai*, and *D. reniculelloides* in the combined and mtDNA phylogenies conflicted with previous classifications of their groups (Figs. 24–26) (Mutuura and Munroe 1972). Genitalia of species in the *auranticella* group lack an elongate costal apex (Fig. 11: 27(0)) and have a central membranous longitudinal region in the ductus bursae (Fig. 20: 43(1)), whereas *D. reniculelloides* have an elongate costal apex (Fig. 14: 27(1)) and a fully sclerotized ductus bursae with a prominence on the left side (Fig. 21: 48(2)). Based on the genitalic similarities between *D. auranticella* and *D. yiai*, it was surprising that mtDNA sequence data showed *D. auranticella* to be sister to *D. reniculelloides*. Increased taxon sampling and an examination of additional molecular markers may clarify this relationship. If these relationships continue to conflict with previously described classifications, changes to the definition of the *auranticella* species group will be necessary to reflect this relationship.

Based on mtDNA and combined analyses, *D. zimmermani*, *D. tumicolella*, and *D. taedivorella* were resolved as distinct species. Though supported, these three species were separated by less than 1.8% sequence divergence, less than the divergences observed among species in other species groups (Table 4). Moreover, morphological characters failed to resolve these three species as distinct. Additional characters and material, for both morphological and molecular characters, will be necessary to fully evaluate the distinctness of these lineages.

From both separate and combined analyses we confirmed that (*i*) the *zimmermani*, *abietella*, and *sylvestrella* groups form natural groups; and (*ii*) the *zimmermani* and *ponderosae* groups form a clade defined by both molecular data and the following morphological characters: multiple regions of raised scales on the forewing, a basally constricted uncus, the presence of a large cornutus, and a hooked tip on the elongate costal apex of the valve. With the limited number of species and geographical haplotypes included in this analysis, our results cannot be used to determine whether the *schuetzeella*, *ponderosae*, *baumhoferi*, and *auranticella* groups are monophyletic.

The morphological data provided more consistent support for deeper clades than the mtDNA data, whereas mtDNA characters strongly supported species groups as well as internal nodes. Neither data set provided strong support for the higher-level phylogeny among species groups. Thus, morphological characters were valuable in analyzing *Dioryctria* relationships, which is of particular importance considering that such characters can be collected easily and often nondestructively from museum material and, if necessary, from good illustrations. At the same time, mitochondrial DNA sequences are excellent for species-level studies in Lepidoptera and can provide a wealth of variation that may be particularly useful in determining species boundaries in taxa where morphological differences are subtle (Sperling and Hickey 1994; Caterino and Sperling 1999; Wahlberg and Zimmermann 2000; Kruse and Sperling 2001, 2002; Wahlberg *et al.* 2003). Thus, mtDNA data may compensate for insufficient information in morphological characters for identifying closely related or superficially similar species. Nonetheless, morphology has provided and will continue to provide important and necessary characters for understanding taxonomy, species limits, and phylogeny.

Combined data sets that include molecular and morphological data for insects have produced trees with improved resolution and support compared with trees produced from any single data set alone (Miller *et al.* 1997; Sperling *et al.* 1997; Remsen and DeSalle 1998; Damgaard *et al.* 2000; Skevington and Yeates 2000). This study provides additional evidence for this pattern, as nearly all nodes in the combined topology received stronger support,

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from both bootstrap and Bremer support values, than those in either separate analysis. For example, the monophyly of *Dioryctria* was supported by a bootstrap value of 90% and a Bremer support value of 12 in the combined tree, which is better than the support provided by either mtDNA or the morphological data alone. Together, these two types of data have allowed us to identify several major nodes in the first study of the phylogeny of *Dioryctria* on a global scale. We expect that examination of the remaining species in the genus will further refine our understanding of both deeper lineages and more recent divergences within *Dioryctria* and improve our ability to identify these important species.

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Appendix 1

Table A1. Morphological character matrix of 14 *Dioryctria* species and 2 outgroup taxa.

	Character
	000000000111111111122222222223333333334444444444555
Species	123456789012345678901234567890123456789012345678901234567890123456789012
O. faecella	200000000111111201111000991012000002002120100020010 1
C. ophthalmicella	000110100001001110000909999990010010990009999990009 9
D. tumicolella	110100102112020201122001112021201111112221001110110 0
D. zimmermani	100100102112020201122001112021201111112221001110110 0
D. taedivorella	100100102112020201122001112021201111112221001110110 0
D. clarioralis	100100101110101201131000991020101111012121000210111 0
D. ponderosae	100100102112010201111000992011201001002221000000110 0
*D. reniculelloides*1	100000112111101201112001001022201010992220000012010 0
*D. reniculelloides*6	100000112111101201112001001022201010992220000012010 0
D. auranticella	111031002111101201111000990001201011002120110000010 1
*D. yiai*13	201020102110101201111001100101201001012020110100010 1
*D. yiai*17	201020102110101201111001100101201001012020110100010 1
D. abietella	100000112111111201112001011122201011112121101111110 0
*D. abietivorella*4	100000112111110201112001111122201011112121101011110 0
*D. abietivorella*5	100000112111110201112001111122201011112121101011110 0
D. magnifica	100010102112111201112100991122201011112221000010010 0
*D. rubella*21	100010102112110201112100991122201011112221000010010 0
D. nr. *rubella*	100010102113110201112110991122201011112221000010010 0
*D. sylvestrella*2087	100010102112110201110100991122201111112221000010010 0
*D. sylvestrella*130	100010102112102011010099112220111111222100001001 00

Note: Character names and states are given in Table A2.

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Table A2. Morphological characters used in the phylogenetic analysis of 14 *Dioryctria* species and 2 outgroup taxa.

Character No.	Character	States
	Antennal morphology	
1	Scale tuft at base of male antenna	0, lacking (Fig. 1)
		1, weak (Fig. 2)
		2, strong (Fig. 3)
	Palp morphology	
2	3rd segment of labial palp*	0, less than 0.5× length of 2nd segment
		1, greater than 0.5× length of 2nd segment
3	Male maxillary palp	0, narrows distally
		1, broadens distally
	Forewing morphology	
4	Forewing scale structure	0, lacking raised scales
		1, raised scales present (Fig. 4)
5	Forewing ground colour	0, brownish grey, dusted with white scales
		1, brownish grey, dusted with brown scales
		2, maroon, dusted with yellow scales
		3, orange, dusted with yellow scales
6	Forewing with white longitudinal streak	0, absent (Fig. 5)
		1, present, beginning before antemedial line and ending on discal cell (Fig. 6)
7	Subbasal line of forewing	0, absent (Fig. 6)
		1, present (Figs. 4, 5)
8	Post-medial line of forewing	0, weakly dentate, with two dentations at most (Figs. 4, 6)
		1, strongly dentate, with at least four dentations (Fig. 5)
9	Discal spots at end of discal cell	0, two separate black spots
		1, absent
		2, white spot or bar on discocellular vein (Figs. 4–6)
	Abdominal morphology	
10	Ventral scale tufts of 8th abdominal segment	0, one pair
		1, two or more pairs
	Male genitalic morphology	
11	Length of male genitalia†	0, not elongate, less than 0.6× width
		1, elongate, greater than 0.8× width
12	Shape of terminal apex of uncus	0, acute (Fig. 7)
		1, narrowly rounded (Fig. 10)
		2, broadly rounded, truncate, or slightly concave (Figs. 8, 9)
13	Uncus length‡	0, approximately 1× width (Figs. 8, 9)
		1, at least 1.4× width (Figs. 7, 10)
14	Uncus margin	0, narrowing to apex (Figs. 7, 10)
		1, broadly expanded, forming rounded edge (Fig. 9)

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Table A2 (*continued*).

Character No.	Character	States
		2, strongly expanded at mid-point, forming triangular prominence (Fig. 8)
15	Lateral uncus margin	0, concave
		1, incurved
16	Gnathos length	0, longer than uncus
		1, ranges from 1× to 0.5× length of uncus
		2, less than 0.5× length of uncus (Figs. 7–10)
17	Gnathos shape	0, lacking forked tip (Figs. 7–10)
		1, forked tip
18	Transtilla lobes	0, connected by sclerotized posterior arch
		1, separated (Figs. 7–10)
19	Sacculus shape	0, broad, as wide as valva base
		1, distinctly narrower than base of valve (Fig. 11)
20	Costa shape	0, thin, bar-like
		1, broad, length less than 6× width
		2, length 6–8× width
		3, length greater than 8× width
21	Costa length	0, equal to setiferous region of valva
		1, shorter than setiferous region of valva
		2, longer than setiferous region of valva
22	Terminal ridges of costa	0, absent (Figs. 11–14)
		1, present (Fig. 15)
		9[§], if character 20 was scored 0
23	Upper costal margin	0, smooth (Fig. 15)
		1, with one or more toothed prominences
24	Preapical spine on costa	0, absent (Figs. 13, 15)
		1, present (Figs. 11, 12, 14)
		9, if character 20 was scored 0
25	Size of preapical spine	0, short and obscure (Fig. 14)
		1, large and distinct (Figs. 11, 12)
		9, if characters 20 or 24 were scored 0
26	Shape of preapical spine	0, blunt (Figs. 11, 14)
		1, acute, thorn-like (Fig. 12)
		9, if characters 20 or 24 were scored 0
27	Shape of costal apex	0, lacking elongate apex (Fig. 11)
		1, straight elongate apex (Figs. 14, 15)
		2, hooked elongate apex (Figs. 12, 13)
		9, if character 20 was scored 0
28	Costal margins of sclerotized region	0, nearly parallel prior to apex (Figs. 12–14)
		1, clearly not parallel (Figs. 11, 15)
		9, if character 20 was scored 0
29	Shape of apex tip	0, broad (Fig. 11)
		1, narrowed and blunt (Fig. 13)
		2, narrowed and acute (Figs. 12, 14, 15)
		9, if character 20 was scored 0

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Table A2 (*continued*).

Character No.	Character	States
30	Shape of sacculus	0, reduced
		1, long and thin (Figs. 11–13)
		2, large and broad (Figs. 14, 15)
31	Juxta sclerotization	0, well sclerotized
		1, moderately sclerotized
		2, weakly sclerotized (Fig. 7)
32	Juxta shape	0, U-shaped (Fig. 10)
		1, V-shaped
33	Vinculum width‖	0, equal to length of vinculum
		1, distinctly greater than length of vinculum (Fig. 8)
34	Vinculum length	0, equal or slightly greater than 0.5× total length¶
		1, distinctly greater than 0.5× total length
35	Aedeagus length**	0, less than 4.5× width (Fig. 17)
		1, greater than 6× width (Figs. 16, 18)
36	Cornuti composition	0, only small spines (Fig. 18)
		1, cluster of small spines and a large cornutus (Figs. 16, 17)
		2, cluster of small spines and two large cornuti
37	Large cornutus length	0, less than 7× medial width (Fig. 17)
		1, greater than 10× medial width (Fig. 16)
		9, if character 36 was scored 0
38	Shape of large cornutus	0, curved
		1, straight (Fig. 12)
		9, if character 36 was scored 0
	Female genitalic morphology	
39	Antrum	0, strongly sclerotized
		1, membranous
40	Ductus bursa length	0, less than 2× width (Fig. 22)
		1, 2–4× width (Fig. 20)
		2, greater than 5× width (Figs. 19, 21, 23)
41	Ductus bursa sclerotization	0, absent, membranous
		1, heavily sclerotized (Figs. 19–23)
42	Posterior medial lobe on ductus bursa	0, absent (Figs. 21, 22)
		1, present (Figs. 19, 20, 23)
		9, if character 41 was scored 0
43	Longitudinal membranous region of ductus bursa	0, absent (Figs. 19, 21, 23)
		1, present (Figs. 20, 22)
		9, if character 41 was scored 0
44	Medial partially transverse wrinkles in posterior region of ductus bursa	0, absent (Figs. 19–21, 23)
		1, present (Fig. 22)
		9, if character 41 was scored 0
45	Ductus bursa shape	0, straight (Figs. 21–23)
		1, curved (Figs. 19, 20)

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Table A2 (*concluded*).

Character No.	Character	States
		9, if character 41 was scored 0
46	Longitudinal wrinkles of ductus bursa	0, extensive through bursa
		1, restricted to posterior half of bursa
		2, nearly absent, bursa primarily smooth
		9, if character 41 was scored 0
47	Ductus bursa width	0, equal throughout length (Fig. 22)
		1, anterior expanded (Figs. 19–21)
		2, posterior expanded (Fig. 23)
		9, if character 41 was scored 0
48	Lateral prominence of ductus bursa	0, absent (Figs. 19, 22, 23)
		1, present on right of bursa (Fig. 20)
		2, present on left of bursa (Fig. 21)
49	Anterior sclerite bearing spines of ductus bursa	0, absent (Figs. 21, 22)
		1, present (Figs. 19, 20, 23)
50	Signum	0, scobinate folds
		1, spines
51	Ductus bursa surface	0, smooth, lacking sculpture
		1, minute spicules along entire length
		9, if character 41 was scored 0
52	Infolded ductus bursa margin	0, absent (Figs. 19–21, 23)
		1, present (Fig. 22)
		9, if character 41 was scored 0

*Length measured with scales present.
[†]Measured from apex of uncus to anterior tip of vinculum and apices of extended valva.
[‡]Measurements shown in Fig. 10.
[§]A score of 9 represents missing data.
[‖]Measurements shown in Figure 8.
[¶]Total length measured from apex of uncus to anterior tip of vinculum.
**Measured length of sclerotized region.

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