Complexity in *Dioryctria zimmermani* Species Group: Incongruence Between Species Limits and Molecular Diversity

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ABSTRACT *Dioryctria* (Zeller 1846) (Lepidoptera: Pyralidae: Phycitinae) moths, commonly known as coneworms, are a group of important coniferous pests. Interspecific overlap of molecular, morphological, and behavioral traits has made identification and delimitation of these species problematic, impeding their management and control. In particular, delimitation of members of the *Dioryctria zimmermani* species group, a diverse group of Nearctic species, is notoriously difficult. To clarify the species boundaries in this species group we examined two independent molecular markers (cytochrome *c* oxidase I and II and elongation factor 1α), larval host plant association, geographic distribution, and pheromone attraction in an integrated taxonomic framework. Congruence between these diagnostic traits and established species limits in the *zimmermani* group was variable. Some species showed well-supported congruence between established taxonomic limits and mitochondrial DNA gene tree topology, whereas other species showed little phylogenetic resolution, little correspondence with diagnostic traits, and incongruence with previously described species limits. Gene tree–species tree discordance may be caused by several evolutionary processes, such as imperfect taxonomy, incomplete lineage sorting, or introgression. Additional information, such as highly variable molecular markers, morphometrics, and larval host information, is needed to effectively evaluate and differentiate among these alternative hypotheses and fully resolve the species limits among *D. zimmermani* species group members.

KEY WORDS mitochondrial DNA, integrated taxonomy, *Dioryctria*, coneworm, species delimitation

Insects are known for their biological diversity, ecological importance, and economic impact (Scudder 2009), making accurate and timely taxonomic classification of insect species imperative (Wheeler 2009). Ideally, an integrative approach that incorporates many sources of evidence should be used to achieve this taxonomic goal. Integrative taxonomy combines morphological, molecular, behavioral, and ecological data to improve identification, discover new species, delimit species boundaries, and reconstruct phylogenetic relationships (Dayrat 2005, Will et al. 2005, Sperling and Roe 2009, Padial et al. 2010, Schlick-Steiner et al. 2010). Use of diverse data sources is invaluable in all aspects of insect taxonomy but is particularly important when examining closely related species (Roe and Sperling 2007, Sperling and Roe 2009, Roe et al. 2010). Evolutionary processes such as introgression and incomplete lineage sorting lead to fuzzy species boundaries, particularly between closely related species where insufficient evolutionary time has passed for diagnostic characters to become fully fixed. As such, incongruence may exist between species limits and diagnostic traits, which could be undetected when a single character set is examined (Rubinoff et al. 2006, Elias et al. 2007, Roe and Sperling 2007, Twewick 2007, Roe et al. 2010).

Dioryctria (Zeller 1846) (Lepidoptera: Pyralidae) is a large, distinct genus of phycitine moths that requires the use of integrative taxonomy for accurate species delimitation (Roe and Sperling 2007). Currently, there are 79 recognized *Dioryctria* species (Nuss et al. 2010) and at least several undescribed species (Du et al. 2005, Knölke 2007, Powell and Opler 2009). Although 12 species groups were erected to help clarify morphological variation within *Dioryctria* (Mutuura and Munroe 1972, 1974; Wang and Sung 1982; Neunzig 2003; Knölke 2007), accurate identification of species is still problematic (Roe and Sperling 2007, Roux-Morabito et al. 2008). Many species show interspecific overlap of molecular, morphological, or behavioral traits, thereby impeding species delimitation and identification (Roe et al. 2006, Roe and Sperling 2007, Roux-Morabito et al. 2008). Larvae of all *Dioryctria* species feed on conifers, many on or in the cones of economically important species (Pinaceae and Cupressaceae) (Neunzig 2003, Roux-Morabito et al. 2008, Whitehouse et al. 2011). As such, several *Dioryctria* species are considered economically important pests and require

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Table 1. Members of the *D. zimmermani* species group

Species	Wing color	Larval host		Pheromone[a]	Reference[b]
		Species	Tissue		
D. albovittella (Hulst)*	L	*P. monophylla* Torrey & Frémont, *P. cembroides* Zuccarini [*P. edulis* Engelmann][c]	Cones, shoots		Heinrich (1956), Cibrián-Tovar et al. (1986)
D. amatella (Hulst)*	D	*P. palustris* Miller, *Pinus* spp.	Cones, shoots cambium, flowers, rust cankers	Z11–16:Ac + C25-p	Heinrich (1956), Coulson and Franklin (1970), Hedlin et al. (1980), Meyer et al. (1986), Miller et al. (2010)
D. banksiella Mutuura, Munroe & Ross	D	*P. banksiana* Lambert	Rust cankers		Mutuura (1982), Mutuura et al (1969)
D. cambiicola (Dyar)*	D	*P. ponderosa* Douglas ex C. Lawson, *P. contorta* Douglas ex Loudon, *P. coulteri* D. Don	Cambium, rust cankers, shoots, cones		Heinrich (1956), Mutuura et al. (1969), Mutuura (1982)
D. contortella Mutuura, Munroe & Ross*	D	*P. contorta*	Cambium, rust cankers		Mutuura et al. (1969)
D. cuitecensis Neunzig	D	Unknown			
D. delectella (Hulst)	D	Unknown			
D. fordi Donahue & Neunzig*	L	[*P. sabiniana* Douglas ex D. Don][c]			Donahue and Neunzig (2002)
D. merkeli Mutuura & Munroe*	D	*P. elliottii* Engelmann, *P. palustris*	Flowers, shoots, cones	Z9–14:Ac + E9–14:Ac	Mutuura and Munroe (1979), Hanula et al. (1984), Meyer et al. (1984), 1986(??), Miller et al. (2010)
D. monticolella Mutuura, Munroe & Ross	D	*P. monticola* Douglas ex D. Don	Cambium		Mutuura et al. (1969)
D. mutuurai Neunzig	L	Unknown			
D. resinosella Mutuura*	D	*P. resinosa* Aiton	Shoots, cones	Z9–14:Ac + E9 14:Ac + Z9–14:OH + Z9–12:Ac	Mutuura (1982), Grant et al. (1993)
D. taedae Schaber & Wood	D	*P. taeda* L., *P. echinata* Miller	Cones, shoots		Schaber and Wood (1971)
D. taedivorella Neunzig & Leidy*	D	*P. taeda*	Cones		Neunzig and Leidy (1989)
D. tumicolella Mutuura, Munroe & Ross*	D	*P. ponderosa*	Rust cankers	Z9–16:Ac	Mutuura et al. (1969); G. Grant, unpublished
D. westerlandi Donahue & Neunzig	L	[*P. jeffreyi* Balfour][c]			Donahue and Neunzig (2002)
D. yatesi Mutuura & Munroe*	D	*P. pungens* Lambert	Cones		
D. zimmermani (Grote)*	D	*P. strobus* L., *P. resinosa*, *P. sylvestris* L., *P. mugo* Turra, *P. nigra* J. F. Arnold, *Pinus* spp.	Cambium, shoots	Z11–16:Ac	Heinrich (1956); Munroe (1959); Mutuura (1982); G. Grant, unpublished

Species examined in this study are indicated by an asterisk (*). Host plant information is summarized from Neunzig (2003) and Whitehouse et al. (2011), with additional host plant and pheromone references included.

[a] Z11–16:Ac, (*Z*)-11-hexadecenyl acetate; C25-p, (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-pentacospentaene; Z9–14:Ac, (*Z*)-9-tetradecenyl acetate; E9–14:Ac, (*E*)-9-tetradecenyl acetate; Z9–14:OH, (*Z*)-9-tetradecen-1-ol; Z9–16:Ac, (*Z*)-9-hexedecenyl acetate; Z11–16:Ac, (*Z*)-11-hexedecenyl acetate.

[b] Includes both pheromone and larval host literature.

[c] Hypothesized.

targeted management (Whitehouse et al. 2011, and references therein), necessitating accurate species identification.

Difficulties with species delimitation are common among members of the *zimmermani* species group and typify the taxonomic difficulties commonly found within *Dioryctria*. The *zimmermani* species group is one of the largest groups of *Dioryctria*, containing 18 described species (Table 1), all of which are exclusively Nearctic (Mutuura et al. 1969; Mutuura and Munroe 1979; Neunzig 1990, 2003). Species are characterized by distinctive genitalic structures and prominent forewing scale ridges (Fig. 1), and recent phylogenetic analyses support the monophyly of this group (Du et al. 2005, Roe et al. 2006, Knölke 2007). Although the majority of species have darkly colored forewings, several distinctive pale colored species occur in the western United States (Fig. 2; Table 1). The majority of species in the *zimmermani* group feed almost exclusively on *Pinus* (Munroe 1959, Neunzig 2003, Roe et al. 2006). Larvae feed internally on cambium, shoots, cones, wounds, and rust cankers, causing extensive economic damage, particularly in commercial pine seed orchards (Whitehouse et al. 2011), and

Fig. 1. *D. merkeli* habitus showing raised scale ridges that are found on all members of the *zimmermani* species group. (Online figure in color.)

host plant may be an important diagnostic character (Neunzig 2003). Pheromone lures, designed to improve management and control of *Dioryctria* pests, show distinct species differences (Table 1) (Meyer et al. 1986, Grant et al. 1993, Miller et al. 2010), although cross-species attraction does occur (Hanula et al. 1984).

Despite pheromone and host plant differences among species (Table 1), accurate species identification remains elusive and species limits in this group need further examination. Identification of species relies primarily on minor forewing differences, geographic distribution, and larval host plant associations (Neunzig 2003), although these traits show considerable interspecific overlap (Sopow et al. 1996, Roe et al. 2006), complicating species diagnostics. Furthermore, previous molecular work on *Dioryctria* has found low levels of molecular variation separating members of the *zimmermani* group, particularly among dark-scaled species (Richmond and Page 1995, Du et al. 2005).

The objectives of this study were to examine the genetic diversity found within species in the *zimmermani* species group and relate molecular variation to larval host plant association, geographic distribution, and pheromone attraction. Using this integrated taxonomic approach, we hope to clarify species boundaries within this difficult group.

Materials and Methods

Specimen Collection. Adult and larval specimens in the *D. zimmermani* group were sampled from sites across North America by using a variety of methods,

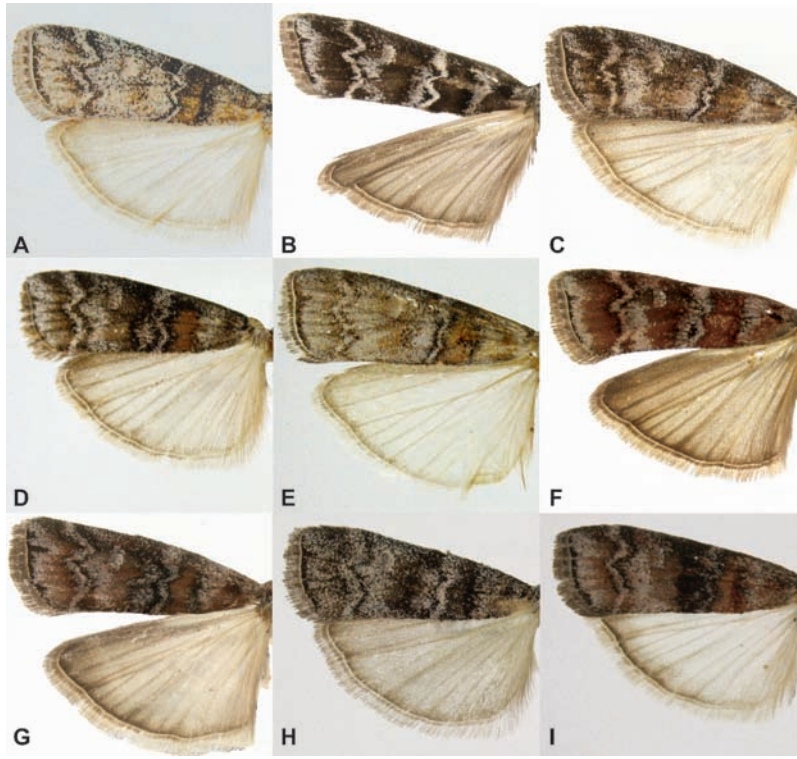

Fig. 2. Members of the *D. zimmermani* species group included in the study (missing *D. yatesi*). (A) *D. albovittella*, CO: 12 miles NW Fort Collins VIII-1971 R. Stevens reared *Pinus edulis* cones. (B) *D. amatella*, GA: Belleville 30-IX-1969 G. DeBarr reared *Pinus elliottii* second-year cones. (C) *D. cambiicola*, BC: Summerland 9-VIII-1967 reared *Pinus ponderosa* fresh pitch mass. (D) *D. contortella*, BC: Barriere 3-VII-1967 reared *Pinus contorta* blister rust, paratype. (E) *D. fordi*, CA: 3 miles NE Diablo 2,100 feet 4-X-1966 reared *Pinus sabiniana*. (F) *D. merkeli*, GA: Belleville reared *P. elliottii* second-year cones G. DeBarr, paratype. (G) *D. resinosella*, ME: TWP 30 Wash. Co. 2-VII-1980 G. S. Patterson, type series. (H) *D. tumicolella*, BC: Summerland 31-VII-1967 reared *P. ponderosa* old pitch mass. (I) *D. zimmermani*, ON: Gormley, Lk. Simcoe 10-VIII-1961 reared *Pinus sylvestris*. (Online figure in color.)

including light trapping, pheromone lures, and larval rearing (Table 2). Pheromone trapping was conducted in the southeastern United States as described by Miller et al. (2010). Identification of specimens was based on forewing morphology, host association, and geographic range, based on species descriptions in Neunzig (2003). All specimens are deposited in the Strickland Museum frozen tissue collection at the University of Alberta.

Molecular Methods. Total genomic DNA was extracted using a Qiagen DNeasy Blood & Tissue kit (QIAGEN, Valencia, CA) using manufacturer's instructions. Two independent molecular markers were sequenced from all samples. Mitochondrial (mtDNA) from the cytochrome *c* oxidase I and II gene regions (COI-COII) was obtained (Table 2) using primers described in Roe et al. (2006). For a subset of specimens, a 534-bp elongation factor 1α (EF1a) fragment was obtained using two overlapping sets of primers: E15f (5′ CGGACACGTCGACTCCGG 3′) to rcM44.9 (5′ CTTCATCAAATCYCTGTGTCC 3′) and M44–1 (5′ GCTGAGCGYGARCGTATCAC 3′) to E600rc (5′ TCCTTACGCTCAACATTCC 3′) (Cho et al. 1995, Reed and Sperling 1999). For specimens with DNA voucher numbers from AR28 to AR332, mtDNA polymerase chain reaction (PCR) amplification, purification, and cycle sequencing protocols are as in Roe et al. (2006). Protocols for all EF1a amplification and the remaining mtDNA sequences were as follows. PCR amplification was performed in 50 μl reactions using Takara *Taq* and supplied reagents (R001T, Takara, Otsu, Shiga, Japan). The reaction mix contained 0.25 μl of Takara *Taq* (5 U/μl), 5 μl of 10× PCR buffer, 4 μl of dNTP mixture (2.5 mM each), and 2 μl of extracted genomic DNA, 2 μl per primer (5 μM each). PCR products were purified with EXO-SAP (exonuclease I and shrimp alkaline phosphatase, 70073Z and 70092Y, USB Corp., Cleveland OH) according to manufacturer's instructions. Bidirectional sequencing of purified PCR products with ABI BigDye Terminator version 3.1 on an ABI 3730xl (Applied Biosystems, Foster City, CA) was performed at the DNA Sequencing and Analysis Facility in the University of Minnesota Biomedical Genomics Center. Sequence data were analyzed with Sequencher version 4.8 (Gene Codes Corp., Ann Arbor, MI). All sequence data were submitted to GenBank as follows: mtDNA, JN162706–JN162761; and EF1a, JN162704, JN162705.

Phylogenetic Analyses. Previously published *Dioryctria* sequences also were included in this study (Du et al. 2005, Roe et al. 2006): *D. cambiicola* (DQ295183, DQ296169, DQ296170), *D. fordi* (DQ295184, DQ296173, DQ296174), *D. taedivorella* (DQ247731), *D. tumicolella* (DQ247729), and *D. zimmermani* (DQ247730) (Table 2). All sequences were initially aligned in Sequencher version 4.8, followed with manual adjustments made by eye. Sequence fragment lengths were not equal and treated as missing data. Alignments of mtDNA and EF1a data sets were deposited in TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S11682).

Parsimony haplotype networks for mtDNA and EF1a data sets were calculated using TCS 1.21 (Clement et al. 2000). Haplotype networks were inferred using a statistical parsimony framework (Templeton 1998), with gaps treated as missing data and a connection limit of 95%. During network inference identical sequences were collapsed, leaving a unique haplotype set (Table 2).

Given the low EF1a variability, genetic diversity indices (nucleotide and haplotype diversity), uncorrected pairwise distances, and a maximum likelihood (ML) tree were calculated for only the mtDNA data set. Haplotype diversity and nucleotide diversity (Nei 1987) were calculated in DNAsp version 5.10.00 (Rozas et al. 2003). Uncorrected pairwise distances were estimated with PAUP* version 4.0b10. ML trees were calculated using only unique haplotypes under a maximum likelihood framework implemented in RaxML version 7.0.4 (Stamatakis 2006) by using the CIPRES portal version 1.0 (Cyberinfrastructure for Phylogenetic Research, http://www.phylo.org/portal/Home.do). Before ML analysis, two additional species were included as outgroup taxa: *D. okanaganella* Mutuura, Munroe & Ross (DQ295178, in the *D. ponderosae* group) and *D. penticronella* Mutuura, Munroe & Ross (DQ295180, in the *D. baumhoferi* group) (Roe et al. 2006). These species represent the two additional species groups characterized by raised forewing scales which have been shown to form a "raised scale" clade with the *zimmermani* group (Whitehouse et al. 2011).

Results

In total, 56 specimens were collected for this study through rearing, light, or pheromone trapping. When combined with previously published data, the total data set includes 66 specimens from 11 species (Table 2). Specimens were collected from across Canada and the United States, and represent half of the described species in the *zimmermani* group (Table 1). Identifications were based on previously published descriptions of forewing morphology, host plant associations, pheromone attraction, and geographic location (Neunzig 2003, and references therein).

Phylogenetic relationships and genetic diversity of the *zimmermani* group species were assessed with two independent loci, COI-COII (mtDNA) and EF1a (nuclear) (Figs. 3 and 4). mtDNA sequence length ranged from 450 bp of COI to the full 2.3 kb of COI-COII (Table 2). The *zimmermani* group formed a monophyletic clade, although the bootstrap support for this clade was low (Fig. 4). Morphologically, the *zimmermani* group can be circumscribed into two groups of species: dark-scaled and light-scaled species. The presence of a "dark-scaled" group is further supported in the ML tree, where it forms a well-supported monophyletic clade, whereas a "light-scaled" group was paraphyletic with respect to the "dark-scaled" clade (Fig. 4).

mtDNA gene tree topologies within the dark- and light-scaled groups contrasted sharply. For the light-scaled species, *D. fordi* and *D. albovittella*, mtDNA was

Table 2. Specimen collection data and haplotype information

Species	Locality	Latitude	Longitude	Date	Collector	Collecting information[a]	mtDNA	EF1a	DNA no.	GenBank
Dioryctria zimmermani gr.										
D. albovittella	USA, NV, White Pine Co., Baker	39.013	−114.123	14 June 2003	A. Cognato	*Pm cone*	33	E1	AR307	JN162706[b], JN162704
D. albovittella	USA, AZ, Coconino Co., Coconino N.F. near Happy Jack	34.743	−111.407	25 Aug. 2007	S. Shank		34		AR493	JN162707[c]
D. albovittella	USA, AZ, Yavapai Co., Mingus Mts.	34.698	−112.122	11 Aug. 2007	S. Shank		35		AR495	JN162708[c]
D. albovittella	USA, AZ, Yavapai Co., Mingus Mts.	34.698	−112.122	11 Aug. 2007	S. Shank		36		AR496	JN162709[c]
D. amatella	USA, SC, Berkeley Co, Francis Marion Ntnl Forest	33.167	−79.667	11 June 2002	A. Roe	MV-light	04		AR220	JN162722[c]
D. amatella	USA, GA, Baldwin Co., Milledgeville, Baldwin seed orchard	32.999	−83.209	28 Sept. 2006	D. Miller	Z11–16:Ac	04		AR379	JN162723[c]
D. amatella	USA, AL, Greene Co., Flatwood seed orchard	33.125	−87.867	Dec. 1995		*Pt cone*	05		AR331	JN162724[c]
D. amatella	USA, AL, Greene Co., Flatwood seed orchard	33.125	−87.867	Dec. 1995		*Pt cone*	06	E2	AR332	JN162725[b], JN162705
D. amatella	USA, GA, Baldwin Co., Milledgeville, Baldwin seed orchard	32.999	−83.209	28 Sept. 2006	D. Miller	Z11–16:Ac + C25-p	11		AR380	JN162710[c]
D. amatella	USA, GA, Baldwin Co., Milledgeville, Baldwin seed orchard	32.999	−83.209	28 Sept. 2006	D. Miller	Z11–16:Ac + C25-p	12		AR381	JN162711[c]
D. amatella	USA, GA, Baldwin Co., Milledgeville, Baldwin seed orchard	32.999	−83.209	28 Sept. 2006	D. Miller	Z11–16:Ac	13	E2	AR382	JN162712[b], JN162705
D. amatella	USA, GA, Baldwin Co., Milledgeville, Baldwin seed orchard	32.999	−83.209	4 June 2007	D. Miller	Z11–16:Ac + C25-p	14		AR474	JN162713[c]
D. amatella	USA, GA, Baldwin Co., Milledgeville, Baldwin seed orchard	32.999	−83.209	17 April 2007	D. Miller	Z11–16:Ac + C25-p	14		AR468	JN162714[c]
D. amatella	USA, GA, Baldwin Co., Milledgeville, Baldwin seed orchard	32.999	−83.209	4 June 2007	D. Miller	Z11–16:Ac + C25-p	19		AR465	JN162715[c]
D. amatella	USA, GA, Baldwin Co., Milledgeville, Baldwin seed orchard	32.999	−83.209	17 April 2007	D. Miller	Z11–16:Ac + C25-p	20		AR466	JN162716[c]
D. amatella	USA, GA, Baldwin Co., Milledgeville, Baldwin seed orchard	32.999	−83.209	17 April 2007	D. Miller	Z11–16:Ac + C25-p	21		AR467	JN162717[c]

Table 2. Continued

Species	Locality	Latitude	Longitude	Date	Collector	Collecting information[a]	mtDNA	EF1a	DNA no.	GenBank
D. amatella	USA, GA, Baldwin Co., Milledgeville, Baldwin seed orchard	32.999	−83.209	4 June 2007	D. Miller	Z11–16:Ac + C25-p	22		AR469	JN162718[c]
D. amatella	USA, GA, Baldwin Co., Milledgeville, Baldwin seed orchard	32.999	−83.209	4 June 2007	D. Miller	Z11–16:Ac + C25-p	23		AR471	JN162719[c]
D. amatella	USA, GA, Baldwin Co., Milledgeville, Baldwin seed orchard	32.999	−83.209	4 June 2007	D. Miller	Z11–16:Ac + C25-p	24		AR472	JN162720[c]
D. amatella	USA, GA, Baldwin Co., Milledgeville, Baldwin seed orchard	32.999	−83.209	4 June 2007	D. Miller	Z11–16:Ac + C25-p	25		AR473	JN162721[c]
D. cambiicola	CAN, BC, Prince George	53.917	−122.750	19 July 2001	A. Roe	UV-light	01	E2	AR78	DQ295183[b], JN162705
D. cambiicola	USA, OR, Jackson Co., Medford	42.327	−122.876		C. Masters	*Psm cambium*	01		AR89	DQ296169[d]
D. cambiicola	CAN, BC, CL62			21 July 2005	A. Roe	*Pp cambium*	01		AR390	JN162726[c]
D. cambiicola	CAN, BC, Prince George	53.917	−122.750	19 July 2001	A. Roe	UV-light	02		AR79	DQ296170[d]
D. cambiicola	CAN, BC, Prince George	53.917	−122.750	19 July 2001	A. Roe	UV-light	03		AR204	JN162727[d]
D. cambiicola	CAN, BC, CL62			21 July 2005	A. Roe	*Pp cambium*	03		AR389	JN162728[e]
D. contortella	CAN, BC, Prince George	53.917	−122.750	19 July 2001	A. Roe	*Pc cambium*	01	E2	AR387	JN162729[c], XXXXXX
D. contortella	CAN, BC, Pritchard	50.599	−119.892	5 Aug. 2003	A. Roe	MV-light	02	E2	AR322	JN162730[b], JN162705
D. fordi	USA, CA, Butte Co., Chico	39.728	−121.837	12 June 2001	A. Roe	UV-light	37	E1	AR157	DQ295184[b], JN162704
D. fordi	USA, CA, Butte Co., Chico	39.728	−121.837	4–6 Oct. 2002	A. Roe	MV-light	38		AR285	DQ296173[d]
D. fordi	USA, CA, Butte Co., Chico	39.728	−121.837	4–6 Oct. 2002	A. Roe	MV-light	38		AR288	DQ296173[d]
D. fordi	USA, CA, Butte Co., Chico	39.728	−121.837	4–6 Oct. 2002	A. Roe	MV-light	39		AR289	DQ296174[d]
D. fordi	USA, CA, Bakersfield, Kern Canyon	35.533	−118.619	13 July 2007	A. Roe	MV-light	40		AR434	JN162731[c]
D. merkeli	USA, GA, Baldwin Co., Milledgeville, Baldwin seed orchard	32.999	−83.209	28 Sept. 2006	D. Miller	Z9–14:Ac + C25-p	07	E2	AR373	JN162733[b], JN162705
D. merkeli	USA, GA, Baldwin Co., Milledgeville, Baldwin seed orchard	32.999	−83.209	28 Sept. 2006	D. Miller	Z9–14:Ac + C25-p	08		AR374	JN162734[c]
D. merkeli	USA, GA, Baldwin Co., Milledgeville, Baldwin seed orchard	32.999	−83.209	28 Sept. 2006	D. Miller	Z9–14:Ac	09	E2	AR375	JN162735[c], JN162705

Table 2. Continued

Species	Locality	Latitude	Longitude	Date	Collector	Collecting information[a]	mtDNA	EF1a	DNA no.	GenBank
D. merkeli	USA, GA, Baldwin Co., Milledgeville, Baldwin seed orchard	32.999	−83.209	28 Sept. 2006	D. Miller	Z9–14:Ac + C25-p	10		AR377	JN162732[c]
D. resinosella	USA, MI, Emmet Co., UMBS Stn Stream Lab	45.560	−84.674	2006	B. Scholtens	MV-light	14	E2	AR386	JN162736[b], JN162705
D. resinosella	USA, MI, Cheboygon Co., Wildwood Rd.	45.365	−84.652	15 July 2006	B. Scholtens		14		AR413	JN162737[c]
D. resinosella	USA, MN, Itasca Co., North Star Campgr.	47.557	−93.654	1 Aug. 2007	A. Roe	UV-light	14		AR480	JN162738[c]
D. resinosella	USA, MN, Itasca Co., North Star Campgr.	47.557	−93.654	1 Aug. 2007	A. Roe	UV-light	14		AR481	JN162739[c]
D. resinosella	USA, MN, Itasca Co., North Star Campgr.	47.557	−93.654	1 Aug. 2007	A. Roe	UV-light	14		AR482	JN162740[c]
D. resinosella	USA, MN, Itasca Co., North Star Campgr.	47.557	−93.654	1 Aug. 2007	A. Roe	UV-light	14		AR483	JN162741[c]
D. resinosella	USA, MN, Itasca Co., North Star Campgr.	47.557	−93.654	1 Aug. 2007	A. Roe	UV-light	14		AR484	JN162742[c]
D. resinosella	USA, MN, Itasca Co., North Star Campgr.	47.557	−93.654	1 Aug. 2007	A. Roe	UV-light	14		AR488	JN162743[c]
D. resinosella	USA, MN, Itasca Co., North Star Campgr.	47.557	−93.654	1 Aug. 2007	A. Roe	UV-light	14		AR489	JN162744[c]
D. resinosella	USA, MN, Chanhassen Co., MN Landscape Arboretum	44.863	−93.617	1 June 2007	A. Roe	*Pc cambium*	14		AR491	JN162745[c]
D. resinosella	USA, MN, Itasca Co., North Star Campgr.	47.557	−93.654	1 Aug. 2007	A. Roe	UV-light	23		AR486	JN162746[c]
D. resinosella	USA, MN, Itasca Co., North Star Campgr.	47.557	−93.654	1 Aug. 2007	A. Roe	UV-light	26		AR485	JN162747[c]
D. resinosella	USA, MN, Itasca Co., North Star Campgr.	47.557	−93.654	1 Aug. 2007	A. Roe	UV-light	27		AR487	JN162748[c]
D. resinosella	USA, MN, Itasca Co., North Star Campgr.	47.557	−93.654	1 Aug. 2007	A. Roe	UV-light	28		AR490	JN162749[c]
D. resinosella	USA, MN, Chanhassen Co., MN Landscape Arboretum	44.863	−93.617	1 June 2007	A. Roe	*Ps cambium*	29		AR492	JN162750[c]
D. taedivorella	USA, MD, Queen Anne's Co., Grasonville	38.958	−76.210	1986	D.C. Fergeson		32		Du119	DQ247731[b]
D. tumicolella	USA, NE			Aug. 1990	G. Grant	*Pp*	01		AR28	JN162751[d]
D. tumicolella	USA, KS, Crawford Co.	37.517	−94.850	2002	K.O. Bell		30		Du112	DQ247729[b]
D. yatesi	USA, GA, Rabun Co., Chattahoochee N.F. nr. Clayton	34.896	−83.362	1 Aug. 2004	J. Hanula	*Pu cone*	07		AR451	JN162753[c]
D. yatesi	USA, GA, Rabun Co., Chattahoochee N.F. nr. Clayton	34.896	−83.362	1 Aug. 2004	J. Hanula	*Pu cone*	07		AR454	JN162755[c]

Table 2. Continued

Species	Locality	Latitude	Longitude	Date	Collector	Collecting information[a]	mtDNA	EF1a	DNA no.	GenBank
D. yatesi	USA, GA, Rabun Co., Chattahoochee N.F. nr. Clayton	34.896	−83.362	1 Aug. 2004	J. Hanula	*Pu cone*	07		AR455	JN162757[c]
D. yatesi	USA, GA, Rabun Co., Chattahoochee N.F. nr. Clayton	34.896	−83.362	1 Aug. 2004	J. Hanula	*Pu cone*	07		AR456	JN162758[c]
D. yatesi	USA, GA, Rabun Co., Chattahoochee N.F. nr. Clayton	34.896	−83.362	1 Aug. 2004	J. Hanula	*Pu cone*	07		AR457	JN162759[c]
D. yatesi	USA, GA, Rabun Co., Chattahoochee N.F. nr. Clayton	34.896	−83.362	1 Aug. 2004	J. Hanula	*Pu cone*	07		AR459	JN162761[c]
D. yatesi	USA, GA, Rabun Co., Chattahoochee N.F. nr. Clayton	34.896	−83.362	1 Aug. 2004	J. Hanula	*Pu cone*	15		AR450	JN162752[c]
D. yatesi	USA, GA, Rabun Co., Chattahoochee N.F. nr. Clayton	34.896	−83.362	1 Aug. 2004	J. Hanula	*Pu cone*	16	E2	AR452	JN162754[b], JN162705
D. yatesi	USA, GA, Rabun Co., Chattahoochee N.F. nr. Clayton	34.896	−83.362	1 Aug. 2004	J. Hanula	*Pu cone*	17		AR453	JN162756[c]
D. yatesi	USA, GA, Rabun Co., Chattahoochee N.F. nr. Clayton	34.896	−83.362	1 Aug. 2004	J. Hanula	*Pu cone*	18		AR458	JN162760[c]
D. zimmermani	USA, MS, Hinds Co.	32.292	−90.417	1994	M.E. Poshore		31		Du118	DQ247730[b]
Outgroup										
Dioryctria ponderosae gr.										
D. okanaganella	USA, CA, Eldorado Co. Placerville	38.730	−120.799	13 June 2001	A. Roe				AR148	DQ295178[b]
Dioryctria baumhoferi gr.										
D. pentictonella	USA, CA, Butte Co., Chico	39.728	−121.837	3 Aug. 2000	C. Rudolf				AR15	DQ295180[b]

[a] Pheromone abbreviations as in Table 1; Host plant abbreviations as follows: *Pc, Pinus contorta; Pm, Pinus monophylla; Pp, Pinus ponderosa; Ps, Pinus strobus; Pt, Pinus taeda; Pu, Pinus pungens; Psm, Pseudotsuga menziesii.*

[b] Full 2.3-kb COI-COII sequence (TY-J-1460–C2-N-3782).

[c] Partial 800-bp COI-COII sequence fragment (C1-J-2183–TL2-N-3013).

[d] Partial 450-bp COI sequence fragment (C1-J-2183–C1-N-2659).

[e] Partial 1.5-kb COI-COII sequence fragment (TY-J-1460–C2-N-3389).

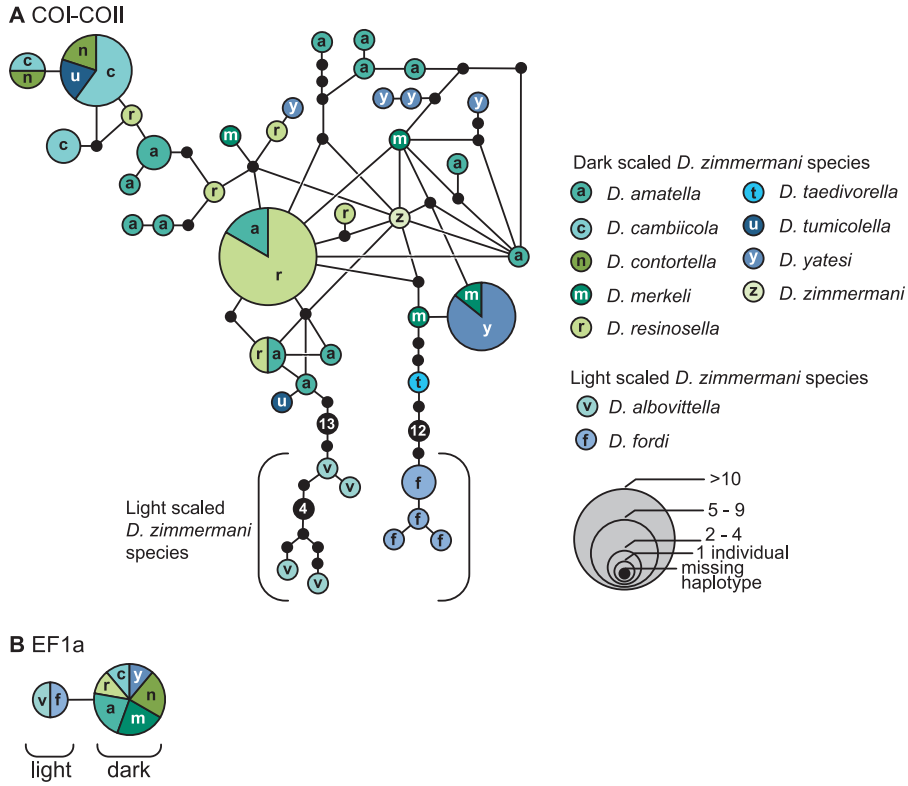

Fig. 3. Parsimony haplotype networks for two independent loci of 11 members of the *D. zimmermani* species group Shaded circles represent individual haplotypes, with circle size proportional to the number of specimens. Lines connecting haplotypes represent single mutational differences, with missing haplotypes represented by black circles. Numbers within black circles represent the number of missing haplotypes. (A) mDNA locus (COI-COII). (B) Nuclear locus (EF1a). (Online figure in color.)

congruent with previously described species limits (Figs. 3 and 4). These species had low levels of intraspecific variation and high, nonoverlapping levels of interspecific variation (Fig. 5), with no evidence of shared mtDNA haplotypes (Figs. 3 and 4). Both species formed strongly supported, monophyletic clades in the parsimony haplotype network and ML tree (Figs. 3 and 4). Haplotype diversity was also very high for both species, despite low levels of nucleotide diversity (Table 3).

In contrast to the light-scaled species, mtDNA diversity in the dark-scaled group was not congruent with previously described species limits, host plant association, pheromone attraction, or geographic location (Figs. 3A and 4). Often individuals from different host plants or pheromone blends were more closely related than individuals with similar ecological traits. All dark-scaled species had overlapping intra- and interspecific variation (Fig. 5), and several haplotypes were shared among species (Figs. 3A and 4). Five of the 32 dark-scaled haplotypes were shared between species, even when separated by large geographic distances (Fig. 4, e.g., mtDNA haplotype 14). There was little phylogenetic structuring among species (Figs. 3A and 4), and relationships among haplotypes were characterized by short internal branches with little to no bootstrap support (Fig. 4). For species with multiple individuals, nucleotide diversity was low, ranging from 0.00121 (*D. contortella*) to 0.0484 (*D. amatella*). Despite the low nucleotide diversity and shared haplotypes among species, overall haplotype diversity within species was high, above 0.900 in several species (Table 3), indicating that nearly all mtDNA sequences were unique.

The second locus, EF1a, was sequenced for a subset of individuals ($n = 11$), representing eight of the 11 species examined in this study (Table 2). In total, 584 bp were obtained which represented two unique haplotypes. A parsimony network (Fig. 3B) shows that these two haplotypes (E1 and E2) differ by a single mutation and coincide with the light-scaled and dark-scaled groups, which was congruent with the mtDNA results.

Discussion

Species limits among the dark-scaled members of the *zimmermani* group have always been considered problematic. Previous work on a *Dioryctria* species complex demonstrated that the examination of multiple molecular markers (COI and EF1a) and dense taxon sampling successfully clarified species limits

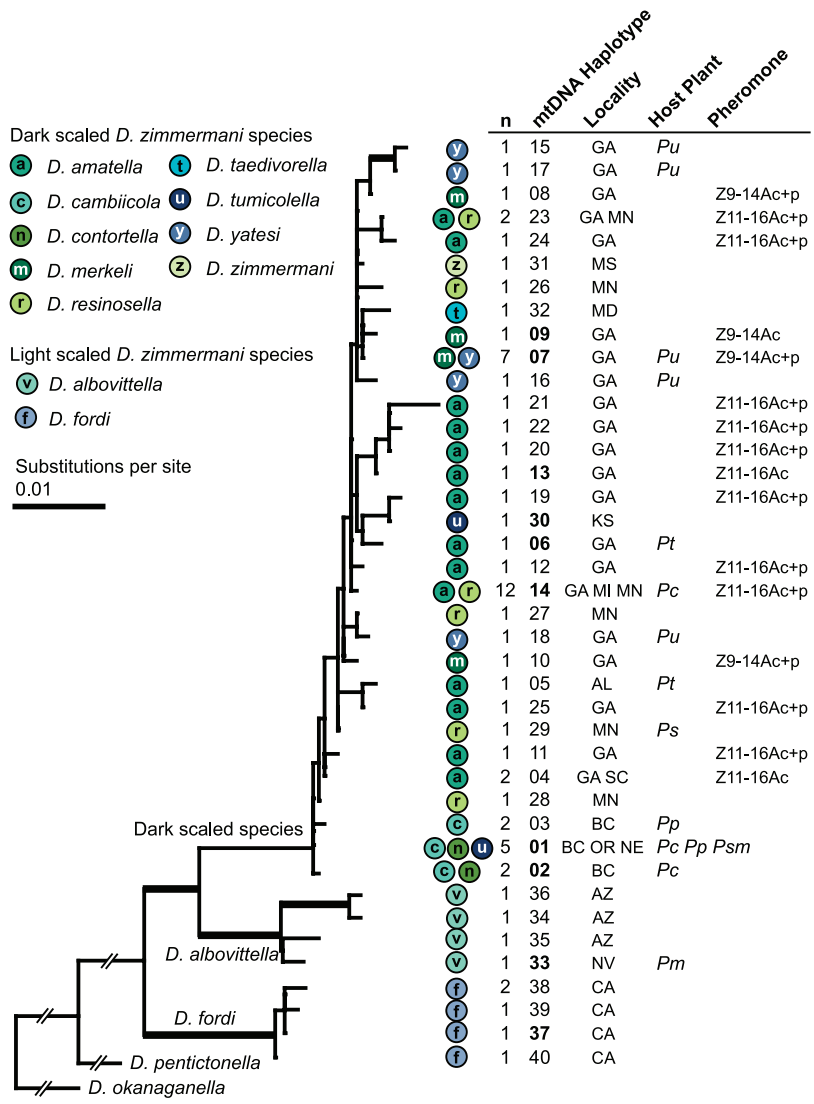

Fig. 4. Maximum likelihood phylogram (−ln −3380.800) of mtDNA (COI-COII) for the *D. zimmermani* species group. ML model information as follows: GTR+G; A = 0.299, C = 0.140, G = 0.135, T = 0.456; G = 0.0200; A-C = 0.0000170, A-G = 11.904, A-T = 2.474, C-G = 0.0000170, C-T = 29.935, G-T = 1.000. Thickened branches indicate clade support >70%. For each haplotype, sample size, haplotype number, sampling locality, host plant, and pheromone association are shown. Host plant abbreviations are as given in Table 2. Pheromone abbreviations are as given in Table 1. (Online figure in color.)

between two sympatric species (Roe and Sperling 2007). By applying a similar technique, we sought to clarify species limits, estimate the genetic diversity within species, and clarify the phylogenetic relationships among species within the *zimmermani* group.

Congruence of the mtDNA gene tree with established species limits in the *zimmermani* group was variable. Species limits and the mtDNA gene tree were clearly congruent for the light-scaled species. The two light-scaled species (*D. albovittella* and *D. fordi*) were characterized by high interspecific pairwise variation and low intraspecific variation (Fig. 5), and each species was well supported as monophyletic (Fig. 4). Gene tree congruence in the light-scaled species contrasts with the broad gene tree—species tree incongruence in the dark-scaled clade of the *zimmermani* group. The nine dark-scaled species showed little phylogenetic resolution (Fig. 4) and had overlapping interspecific pairwise variation (Fig. 5). The nuclear locus (EF1a) lacked species-level variation, despite diagnostic success in other *Dioryctria* species (Roe and Sperling 2007).

Discordance between molecular variation and species limits is not unusual. In a recent survey, Funk and Omland (2003) estimate that at >23% of taxa (26.5% of arthropods) show some species-level polyphyly (considered broadly to represent non-

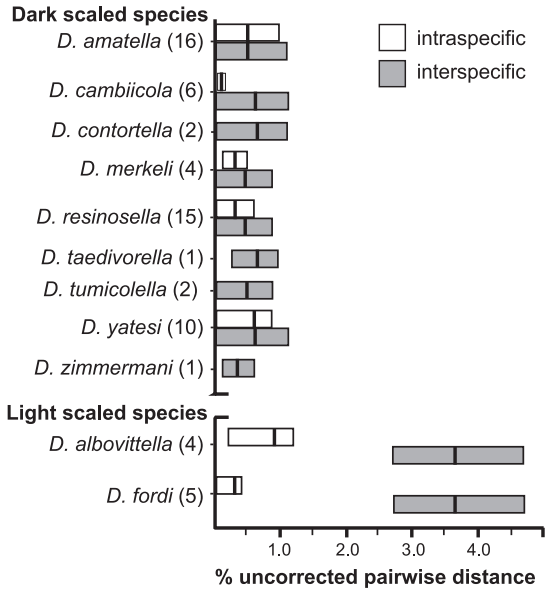

Fig. 5. Intra- and interspecific uncorrected pairwise mtDNA distances for 11 species in the *D. zimmermani* species group. Range (min.–max) of pairwise distances are shown with mean distance indicated by a black bar. Where species were represented by less than three sequences (*D. taedivorella*, *D. tumicollela*, *D. zimmermani*) only interspecific pairwise distance is shown.

monophyly). Several processes can lead to this phenomenon of gene tree—species tree incongruence.

First, it is possible that the currently recognized species limits are incorrect (i.e., imperfect taxonomy) and the mtDNA gene tree accurately represents the species tree. In the case of the dark-scaled species, the clade would be considered "overspilt" with all the taxa belonging to a single, widely distributed, highly polymorphic species, rather than multiple distinct species. Historically, these taxa have been separated based on minor forewing variation and larval host plant associations, although all authors acknowledge that complex species problems continue to exist within the group (Mutuura et al. 1969, Schaber and Wood 1971, Mutuura and Munroe 1979, Hedlin et al. 1980, Mutuura 1982, Sopow et al. 1996, Neunzig 2003). In fact, although Heinrich (1956) tentatively recognized *D. cambiicola* as a species, he postulated that it might actually represent a western race of *D. zimmermani*, rather than a distinct species, a sentiment later supported by Munroe (1959). Furthermore, many species have sympatric or parapatric distributions, as well as extensive overlap of diagnostic characters (Sopow et al. 1996), supporting the hypothesis of a single dark-scaled species.

Table 3. Genetic diversity estimates for members of the *D. zimmermani* species group

Species	*n*	H	Hd (±SD)	Pi (±SD)
Dark scaled				
D. amatella	16	14	0.983 (0.0280)	0.0484 (0.000510)
D. cambiicola	6	3	0.733 (0.155)	0.00323 (0.00133)
D. contortella	2	2	1.000 (0.500)	0.00121 (0.000610)
D. merkeli	4	4	1.000 (0.177)	0.00323 (0.000780)
D. resinosella	15	6	0.571 (0.149)	0.00162 (0.000530)
D. taedivorella	1	1	N.A.[a]	N.A.
D. tumicolella	2	2	1.000 (0.500)	0.00842 (0.00421)
D. yatesi	10	5	0.778 (0.137)	0.00365 (0.000900)
D. zimmermani	1	1	N.A.	N.A.
Light scaled				
D. albovittella	4	4	1.000 (0.177)	0.00908 (0.00209)
D. fordi	5	4	0.900 (0.161)	0.00295 (0.000670)

n, number of specimens; H, number of haplotypes; Hd, haplotype diversity; and Pi, nucleotide diversity.

[a] N.A., not applicable.

Widely distributed, highly polymorphic species are not unusual in *Dioryctria*. *Dioryctria abietivorella* (Grote), an important cone pest throughout North America, has broad larval host associations and a transcontinental distribution. This level of ecological and geographic variation would be comparable to the variation exhibited among the dark-scaled members of the *zimmermani* group. Morphological variability, particularly in forewing coloration, is also well known for other *Dioryctria* species (Roe et al. 2006, Roe and Sperling 2007). For example, *Dioryctria pentictonella* (Mutuura, Munroe, & Ross), another raised scale species, has highly plastic forewing coloration, ranging from nearly black to red to white, which all occur within a single season at a single collection locality (Roe et al. 2006). Again, forewing variability among the dark-scaled species is within the intraspecific range of variability previously documented in *D. pentictonella*.

The second possibility is that the current species limits in the dark-scaled clade are accurate and that the mtDNA gene tree fails to accurately reflect the evolutionary relationships among these species. Although many species show interspecific overlap of larval host associations, other species do not (Table 1). As well, distinct pheromone sex attractants have been described for several dark-scaled species, particularly for dark-scaled species in the southeastern United States (Miller et al. 2010). Although cross species attraction occurs (Hanula et al. 1984), recent work has shown that *Dioryctria* pheromones are complex (Millar et al. 2005, 2010) and pheromone races exist within *Dioryctria* species (Grant et al. 2009), although it is uncertain whether these races represent distinct species or show reduced inter-race gene flow.

If individuals in the dark-scaled clade represent a single species, we would expect to observe some phylogeographic structuring among the mtDNA haplotypes. Instead, haplotypes are shared across broad geographic ranges (e.g., mtDNA haplotype 14), with individuals collected in the same location more closely related to individuals from distant locations than to each other (Figs. 3A and 4). The lack of phylogeographic structuring and ecological variation among species suggests that more complex evolutionary processes may be responsible for the observed incongruence (Schmidt and Sperling 2008).

Gene tree–species tree discordance is a common issue when seeking to delimit species boundaries and

can be caused by several evolutionary processes (e.g., Maddison 1997, Funk and Omland 2003), such as incomplete lineage sorting or introgression. Incomplete lineage sorting results when gene lineages of closely related species have not had sufficient time to coalesce and achieve reciprocal monophyly. Generally, mtDNA is considered more robust to incomplete lineage sorting than nuclear genes (Hudson and Turelli 2003) but has been shown to fail among rapidly radiating clades (Funk and Omland 2003), particularly among groups experiencing ecological race formation (Dres and Mallet 2002, Scheffer and Hawthorne 2007). If dark-scaled *Dioryctria* species are undergoing rapid ecological divergence based on larval host association and pheromone attraction, then the species barriers separating these recently diverged species may be maintained by a small region of the genome (Matsubayashi et al. 2009), whereas other regions of the genome (e.g., mtDNA) will not have had sufficient time for purifying selection to produce reciprocally monophyletic clades (Funk and Omland 2003).

Conversely, interspecific hybridization and subsequent introgression is the movement of foreign genetic material into a conspecific genome. This process leads to reticulate evolutionary relationships and gene tree–species tree discordance, clouding genealogical species boundaries (Maddison 1997). Interspecific hybridization is surprisingly common (Mallet 2005), with hybridization rates ranging from 6 to 29% among species of Lepidoptera (Sperling 1990, Mallet et al. 2007). mtDNA introgression may occur without nuclear introgression (Ballard and Whitlock 2004, Petit and Excoffier 2009), particularly if mtDNA is impacted by direct or indirect selection (Ballard and Whitlock 2004, Hurst and Jiggins 2005). For hybridization to occur, species must be sympatric/parapatic, synchronic, and be capable of interbreeding (Schmidt and Sperling 2008). Dark-scaled *zimmermani* species have sympatric and parapatric distributions, overlapping flight times, and have shown evidence for cross-species pheromone attraction (Hanula et al. 1984, Whitehouse et al. 2011), all conditions necessary for hybridization to occur.

As stated previously in many studies, we must acknowledge that difficult species problems continue to exist in the *zimmermani* species group. Despite our dense taxon sampling and inclusion of multiple lines of evidence, we were unable to fully resolve species limits among dark *zimmermani* species group members. Many of the dark-scaled taxa are considered "good" species, with extensive information available on their behavioral and ecological differences, as well as their economic impacts (Whitehouse et al. 2011). Although mtDNA has been used extensively as a diagnostic marker in Lepidoptera (e.g., DNA barcoding; Hebert et al. 2003, 2004), and is successful in other species of *Dioryctria* (Roe and Sperling 2007), including light-scaled members of the *zimmermani* group, studies have shown that a single marker is prone to failure, particularly when differentiating closely related species (Roe and Sperling 2007, Schmidt and Sperling 2008, Roe et al. 2010). Given the economic importance of these dark-scaled species and in the interest of nomenclatural stability, we choose not to recommend any taxonomic changes to this group based on a single molecular marker.

Based on the currently available data, we are unable to differentiate among the alternative hypothesis for the cause of the gene tree–species tree discordance detected among the dark-scaled *zimmermani* species. Effective evaluation of these hypotheses requires data from multiple regions of the genome (Maddison 1997) and analytical means for resolving gene tree discordance (Degnan and Rosenberg 2009). Highly variable molecular markers, such as microsatellites or single-nucleotide polymorphisms from regions throughout the genome, in addition to behavioral, ecological, and morphological characters will be required to provide clarity to the dark-scaled *zimmermani* species complex.

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