Identification of *Dioryctria* (Lepidoptera: Pyralidae) in a Seed Orchard at Chico, California

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ABSTRACT Species of *Dioryctria* Zeller (Lepidoptera: Pyralidae) are important pests of conifers, particularly in seed orchards, and accurate species identification is needed for effective monitoring and control. Variable forewing morphology and lack of species-specific genitalic features hinder identification, prompting the search for additional diagnostic characters. Mitochondrial DNA (mtDNA) sequences from the cytochrome *c* oxidase I and II genes (COI and COII) were obtained from specimens collected at lights, pheromone traps, and host plants in the Pacific Northwest, focusing on a U.S. Forest Service seed orchard in Chico, CA. A 475-bp fragment of COI was used to identify eight distinct genetic lineages from 180 *Dioryctria* specimens, and these were identified as eight described species. Comparisons among mtDNA variation, adult morphology, larval host association, and pheromone attraction were used to assign individuals to species groups and to identify diagnostic characters for species identification. A 2.3-kb fragment of COI-COII was sequenced for 14 specimens to increase resolution of phylogenetic relationships. Species groups were well resolved using both the 475-bp and "DNA barcode" subsets of the 2.3-kb sequences, with the 475-bp fragment generally showing lower divergences. The *zimmermani* and *ponderosae* species groups were sister groups and had similar male genitalic morphology and larval feeding habits. The *pentictonella* group was sister to the *zimmermani* + *ponderosae* group clade, and all species have raised scales and a *Pinus* sp. larval host (where known). Combining molecular characters with morphological and behavioral characters improved identification of *Dioryctria* species and supported previous species group relationships.

KEY WORDS *Dioryctria*, mtDNA, cytochrome oxidase *c*, morphology, DNA barcoding

Dioryctria Zeller (Lepidoptera: Pyralidae) is a Holarctic genus currently composed of 70 species, with 40 species described from North America, north of Mexico (Heinrich 1956; Munroe 1959; Mutuura et al. 1969a,b; Schaber and Wood 1971, Coulson et al. 1972, Mutuura and Munroe 1972, 1973, 1979; Mutuura 1982, Blanchard and Knudson 1983, Neunzig and Leidy 1989, Neunzig 2003, Donahue and Neunzig 2005) and at least six additional species from Mexico (Cibrián-Tovar et al. 1986, Neunzig 1990). The distribution of the genus matches that of its coniferous hosts, with species ranging from subtropical to subarctic coniferous forests (Neunzig 2003).

The majority of larvae feed internally on coniferous trees, attacking regions with rapid growth (meristematic tissue) such as cones, stems, cambium, wounds, and blister rust galls [e.g., *Endocronartium harkenssii* (Moore) Y. Hiratsuka. and *Cronartium coleosporioides* Arthur], although several species feed externally on foliage and buds (Neunzig 2003). Damage often leads to substantial economic loss, particularly in tree farms, plantations, shelterbelts, and seed orchards (Lyons 1957, Hedlin et al. 1980, Blake et al. 1989, Mosseler et al. 1992). Cone feeding destroys seeds, reducing seed production, especially during years of low seed set (Hedlin et al. 1980, Schowalter et al. 1985). Stem, trunk, and shoot mining results in branch breakage and bud loss, causing tree deformation during heavy infestations (Hainze and Benjamin 1984).

Dioryctria infestations at the USDA Forest Service Genetic Resource Center (GRC) in Chico, CA, illustrate the importance of these pests. The GRC supports 54.9 ha (122.8 acres) of grafted breeding stock and produces seedlings for three breeding zones of ponderosa pine, *Pinus ponderosa* Douglas ex Lawson & C. Lawson; two breeding zones of sugar pine, *Pinus lambertiana* Douglas; and five breeding zones of Douglas-fir, *Pseudotsuga menziesii* (Mirbel) Franco that aid reforestation efforts throughout northern California. Twenty years of heavy *Dioryctria* infestations have drastically reduced the number of viable seeds harvested from this orchard, hindering its ability to produce seedlings (G. Norcross, personal communication). Traditional methods of managing insect infestations, such as insecticide sprays and injections, have resulted in inconsistent control, so alternative methods such as pheromone monitoring and mating disruption are being pursued.

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Development of pheromone monitoring and control methods requires accurate species identification. As a genus, adult *Dioryctria* is one of the most easily identified groups in the subfamily Phycitinae (Heinrich 1956, Neunzig 2003). Seven species groups delineated by genitalic and forewing characters were originally erected to improve the taxonomy and aid identification of this difficult group (Mutuura and Munroe 1972). Two additional North American species groups were delineated (Neunzig 2003); however, identification and taxonomy of many *Dioryctria* species within and between groups remains problematic. Previous taxonomic work in North America has relied upon minor genitalic variation, slight differences in forewing pattern, geographic range, and larval host associations. Wing pattern differences can be polymorphic, compounding the problem. Also, many species are sympatric and occur on the same larval host. Thus, it is difficult to rely on these characters for species identification (Sopow et al. 1996), and additional characters are needed to reliably identify *Dioryctria* spp.

Nucleotide sequence data, particularly mitochondrial DNA (mtDNA), have been useful in resolving difficult species problems by providing a suite of additional characters (Simon et al. 1994, Caterino et al. 2000, Sperling 2003a). mtDNA is maternally inherited and essentially haploid. Mutations can accumulate rapidly, allowing the separation of closely related species. mtDNA is also robust to degradation, permitting the use of pheromone-trapped material and some museum specimens. The cytochrome *c* oxidase gene regions I and II (COI, COII) have been shown to be particularly useful for resolving species problems across a range of lepidopteran families (Caterino et al. 2000, Sperling 2003a). The use of COI for identifying species has been popularized by DNA barcoding (Hebert et al. 2003), although the use of this technique as the sole method for identifying species has been questioned (Lipscomb et al. 2003, Sperling 2003b, Will and Rubinoff 2004). Previous molecular work on *Dioryctria* has used isozymes, cuticular hydrocarbons, and nucleotide sequence data to examine genetic variation within and between *Dioryctria* species (Richmond 1995, Richmond and Page 1995, Knölke et al. 2005, Du et al. 2005) but has not focused on the full suite of species likely to be encountered in any one region.

This study is intended to provide a foundation for molecular identification and to clarify and confirm morphological, larval host association and pheromone characters that may be used for identification in the field. There were four primary objectives of this study. First, we used a 475-bp region of COI to identify distinct mtDNA lineages of *Dioryctria* from the Genetic Resource Center in Chico, CA, including additional specimens from northern California, Oregon, and British Columbia. Second, we associated these genetic lineages with previously described species by using adult morphology, locality, larval host association, and pheromone attraction. Third, we sequenced 2.3 kb from each major genetic lineage and obtained a well supported preliminary phylogeny for species and species groups identified in the region. Fourth, we used subsets of the 2.3-kb sequences to compare divergences between the 475-bp fragment used in this study and the 658 bp DNA barcoding region of Hebert et al. (2003) to examine the information content of these regions in *Dioryctria* spp. The overall aim of this project was to use mtDNA sequence data to identify *Dioryctria* spp., evaluate boundaries, and provide a preliminary assessment of the phylogenetic relationships in the genus.

Materials and Methods

Collection Sites and Species. In total, 180 *Dioryctria* specimens were collected from northern California, Oreogn, and British Columbia (Table 1). Collecting was focused on the U.S. Forest Service conifer seed orchard at GRC in Chico, CA, and 146 specimens were collected at this locality. An additional 34 specimens were collected from northern California, western Oregon, and British Columbia were included to expand species sampling and provide an assessment of geographic variation. Samples were collected to represent the range of pheromone attraction, larval host plant association, and morphology that was considered likely to be seen in *Dioryctria* species throughout the region, especially from northern California and Oregon.

All six species groups previously recorded in northern California were represented in the study. Eight species of *Dioryctria* were sampled in this survey, with four recorded at Chico and six in northern California (Table 1): *Dioryctria abietivorella* (Grote) (*abietella* group); *D. auranticella* (Grote) and *Dioryctria rossi* Munroe (*auranticella* group); *Dioryctria pentictonella* Mutuura, Munroe & Ross (*baumhoferi* group); *Dioryctria okanaganella* Mutuura, Munroe & Ross (*ponderosae* group); *Dioryctria pseudotsugella* Munroe (*schuetzeella* group); and *Dioryctria cambicola* (Dyar) and *Dioryctria fordi* Donahue & Neunzig (*zimmermani* group). *Dioryctria* specimens were identified based on wing characters, genitalic morphology, and geographic range by using keys and other published materials (Heinrich 1956; Munroe 1959; Mutuura et al. 1969a,b; Mutuura and Munroe 1972, 1973; Neunzig 2003; Donahue and Neunzig 2005). Four additional species of *Dioryctria* have been recorded from northern California (Neunzig 2003), but they were not included because they are generally rare in the region and no fresh material could be obtained for this study. *Dioryctria muricativorella* Neunzig, *Dioryctria mutuurai* Neunzig, and *Dioryctria westerlandi* Donahue & Neunzig have been described recently from California, but they have highly restricted geographic ranges, and few specimens are known. *Dioryctria ponderosae* Dyar also was not collected during this study, although this species is recorded at low frequency at various sites in the study region (Neunzig 2003). Two additional species in the Phycitini, *Oncocera faecella* (Zeller) and *Ceroprepes*

Table 1. Locality and collection information for *Dioryctria* specimens used in mtDNA surveys in northern California, Oregon, and British Columbia

Locality data	Collection[a]	Date[b]	No.	Collector, yr	Haplotype	GenBank accession no.	
						475 bp	2.3 kb
abietella Group							
D. abietivorella							
USA: CA: Butte Co., Chico	Pheromone: I, II, V	June–Aug.	11	C. Rudolf, G. Grant (2000, 2001)	AB1	DQ296154	DQ295185
USA: CA: Butte Co., Chico	Pheromone trap	Aug.–Sept.	3	C. Rudolf, G. Grant(1998)	AB1		
USA: CA: Butte Co., Chico	Cone: Pp, Pb, Pl, Df		37	C. Rudolf (1995, 2000–2001)	AB1		
USA: CA: Butte Co., Chico	MV-light	June	8	A. Roe (2001)	AB1		
USA: CA: Butte Co., Chico	MV-light	June	1	A. Roe (2001)	AB4		DQ247740
USA: CA: Butte Co., Chico	MV-light	June	1	A. Roe (2001)	AB5		DQ247741
USA: CA: Placer Co., Foresthill	Cone: Pp		3		AB1		
USA: OR: Lane Co., Cottage Grove, Dorena Tree Center	Cone: Pm, Lo		3	J. Berdeen (2001)	AB1		
USA: OR: Clackamas Co., Colton, Horning Tree Center	Cone: Df		1	B. Willhite (2001)	AB1		
USA: CA: Butte Co., Chico	Cone: Pb, Pl, Df		6	C. Rudolf (2000–2001)	AB2	DQ296156	
USA: CA: Butte Co., Chico	MV-light	June	1	A. Roe (2001)	AB2		
USA: CA: Butte Co., Chico	Cone: Df		1	C. Rudolf (2001)	AB3	DQ296155	
auranticella Group							
D. auranticella							
USA: CA: El Dorado Co., Placerville	MV-light	June	2	A. Roe (2001)	OS1	DQ296157	DQ295176
USA: CA: El Dorado Co., Placerville	MV-light	June	1	A. Roe (2001)	OS4		DQ247736
USA: CA: Butte Co., Chico	MV-light	June	1	A. Roe (2001)	OS2		
USA: CA: Butte Co., Chico	Pheromone: II	June	1	C. Rudolf, G. Grant (2001)	OS2		
USA: CA: El Dorado Co., Placerville	MV-light	June	2	A. Roe (2001)	OS2	DQ296158	
D. rossi							
CAN: BC: 35 km E Summerland	MV-light	Aug.	2	A. Roe (2003)	OS3	DQ296159	DQ295177
schuetzeella Group							
D. pseudotsugella							
USA: OR: Benton Co., Corvalis	UV-light	July	2	J. Adams (2001)	RE1	DQ296160	DQ295186
USA: OR: Benton Co., Corvalis	UV-light	July	1	J. Adams (2001)	RE2	DQ296161	
USA: OR: Benton Co., Corvalis	UV-light	July	1	J. Adams (2001)	RE3	DQ296162	
CAN: BC: 8 km E Adams Lake	MV-light	Aug.	1	A. Roe (2003)	RE4	DQ296163	
CAN: BC: 10 km SW Pritchard	MV-light	Aug.	1	A. Roe (2003)	RE5	DQ296164	
baumhoferi Group							
D. pentictonella							
USA: CA: Butte Co., Chico	Pheromone: V, III, IV	May–Sept.	46	C. Rudolf, G. Grant (2000, 2001)	RS1a	DQ296165	DQ295180
USA: CA: Butte Co., Chico	Pheromone trap	April–Sept.	6	C. Rudolf, G. Grant (1998)	RS1a		
USA: CA: Siskiyou Co., Ball Mt.	Cone: Pa		2	J. Stein (1994)	RS1a		
USA: CA: Butte Co., Chico	Cone: Pb		1	C. Rudolf (1997)	RS1a		
USA: CA: El Dorado Co. Placerville	MV-light	Aug.	1	A. Roe (2001)	RS1a		
CAN: BC: 35 km E Summerland	MV-light	Aug.	1	A. Roe (2003)	RS1a		
USA: CA: Butte Co., Chico	Pheromone: V, III	May–June	14	C. Rudolf, G. Grant (2000, 2001)	RS1b	DQ296166	
USA: CA: Butte Co., Chico	Pheromone trap	May, July	2	C. Rudolf, G. Grant (1998)	RS1b		
USA: CA: Alameda Co. Berkeley	UV-light	June	1	F.A.H. Sperling (1998)	RS1b		
USA: CA: Butte Co., Chico	Pheromone trap	May	1	C. Rudolf, G. Grant (1998)	RS1c		DQ295181
USA: CA: El Dorado Co., Placerville	MV-light	June	1	A. Roe (2001)	RS1d		DQ295182
USA: CA: Butte Co., Chico	Pheromone: III	Oct.	1	C. Rudolf, G. Grant (2000)	RS1e	DQ296167	
USA: CA: Siskiyou Co., Ball Mt.	UV-light	Sept.	1	C. Frank	RS1f	DQ296168	
zimmermani Group							
D. cambiicola							
USA: OR: Medford	Bark: Df		1	J. Berdeen (2001)	RS2a	DQ296169	
CAN: BC: Prince George Tree Improvement Station	Bark: Pc		2	A. Roe (2001)	RS2b	DQ296170	DQ295183
D. fordi							
USA: CA: Butte Co., Chico	MV-light	June	1	A. Roe (2001)	RS2c		DQ295184
USA: CA: Butte Co., Chico	MV-light	Oct.	2	A. Roe (2002)	RS2h	DQ296173	
USA: CA: Butte Co., Chico	MV-light	Oct.	1	A. Roe (2002)	RS2g	DQ296174	
ponderosae Group							
D. okanaganella							
USA: CA: El Dorado Co., Placerville	MV-light	June	1	A. Roe (2001)	RS2d	DQ296171	
USA: CA: El Dorado Co., Placerville	MV-light	June	1	A. Roe (2001)	RS2e		DQ295179
USA: CA: El Dorado Co. Blodgett Research Stn. 15 mi E Georgetown	MV-light	Aug.	1	A. Roe (2002)	RS2e	DQ296172	
USA: CA: El Dorado Co., Placerville	MV-light	June	1	A. Roe (2001)	RS2f		DQ295178
Outgroups							
Oncocera faecella							
China: Inner Mongolia: Mt. Manhan	Light	Aug.	1	D. Zhang (2002)	O. fae		DQ247727
Ceroprepes ophthalmicella							
China: Henan Province: Mt. Baiyun	Light	July	1	X. Wang (2002)	*C. oph.*		DQ247728

[a] Host records from material reared to adult or extracted as larvae. Host abbreviations: Pb, Afghan pine [*Pinus brutia* ssp. *eldarica* (Medw.) Nahal]; Pl, sugar pine (*Pinus lambertiana*); Pp, ponderosa pine (*Pinus ponderosa*); Pc, lodgepole pine (*Pinus contorta* Douglas ex Loudon); Pa, whitebark pine (*Pinus albicaulis* Engelmann); Pm, western white pine (*Pinus monticola* Douglas ex D. Don); Df, Douglas-fir (*Pseudotsuga menzesii*); Lo, western larch (*Larix occidentalis* Nuttall).

[b] Date is not given for reared material due to unreliability of emergence times in artificial conditions.

Table 2. Mitochondrial DNA primers used in surveying *Dioryctria* spp. over 2.3 kb of COI-COII

Primer name	Direction and location (3′ end)[a]	Sequence (5′–3′)
K698	TY-J-1460	TAC AAT TTA TCG CCT AAA CTT CAG CC
RonV	C1-J-1751	GGA GCT CCA GAT ATA GCT TTC CC
K699	C1-N-1840	AGG AGG ATA AAC AGT TCA (C/T)CC
K808	C1-N-1840	TGG AGG GTA TAC TGT TCA ACC
Jerry[b]	C1-J-2183	CAA CAT TTA TTT TGA TTT TTT GG
JerryV[b]	C1-J-2183	CAA CAT TTA TTT TGA TTC TTT GG
Nancy	C1-N-2191	CCC GGT AAA ATT AAA ATA TAA ACT TC
K525	C1-N-2329	ACT GTA AAT ATA TGA TGA GCT CA
Brian	C1-J-2495	CTT CTA TAC TTT GAA GAT TAG G
MilaIII[b]	C1-N-2659	ACT AAT CCT GTG AAT AAA GG
George	C1-J-2792	ATA CCT CGA CGT TAT TCA GA
GeorgeIII	C1-J-2792	ATA CCT CGG CGA TAC TCT GA
GeorgeV	C1-J-2792	ATA CCT CGA CGA TAT TCC GA
PatII	TL2-N-3013	TCC ATT ACA TAT AAT CTG CCA TAT TAG
Pierre	C2-J-3138	AGA GCC TCT CCT TTA ATA GAA CA
Marilyn	C2-N-3389	TCA TAA GTT CA(A/G) TAT CAT TG
MarilynII	C2-N-3389	TCA TA(T/A) CTT CA(A/G) TAT CAT TG
MarilynIII	C2-N-3389	TCA TAT CTT CAG TAT CAC TG
Preston	C2-J-3570	GCA ACA GAT GTT ATT CAC TCT TG
Eva	C2-N-3782	GAG ACC ATT ACT TGC TTT CAG TCA TCT

[a] Following Simon et al. 1994: J/N, majority/minority (equivalent to sense/antisense for COI-COII).
[b] Primer combination used for the 475-bp fragment.

ophthalmicella (Christoph) (Lepidoptera: Pyralidae: Phycitinae), were included as outgroup taxa.

Collection Methods. Specimens examined in this study were provided by collaborators or collected by the authors (Table 1). Larvae were extracted or reared from cones, cambial tissue, and pitch masses from eight conifer species, with the majority of material obtained from the GRC. Live larvae and reared adults were preserved in 96–100% ethanol. Adults were sampled using both light and pheromone-baited traps. Live light-trapped specimens were frozen at −20 or −70°C or were placed in 96–100% ethanol.

Dioryctria specimens were collected by pheromone trapping at GRC in 1998, 2000, and 2001. These lures differed either in chemical composition or concentration. Pheromone lures were developed based on previously described lures for *Dioryctria disclusa* Heinrich (Meyer et al. 1982), *Dioryctria abietella* (Denis & Schiffermüller) (Löfstedt et al. 1983), *Dioryctria clarioralis* (Walker) (Meyer et al. 1984), *Dioryctria amatella* (Hulst) (Meyer et al. 1986), *Dioryctria reniculelloides* Mutuura & Munroe (Grant et al. 1987), and *Dioryctria resinosella* Mutuura (Grant et al. 1993). Pheromone trapping with undefined blends obtained a small number of specimens in 1998, but because these lures could not be confidently associated with chemical blends they were not considered in the pheromone analysis. Pherocon 1CP pheromone traps baited with the lures (Trécé Inc., Salinas, CA) were placed in a replicated, randomized block pattern in tree tops throughout the orchard to maximize trap catch (Grant et al. 1987). Traps were checked biweekly, and specimens were removed, scored for wing pattern, and frozen at −20°C.

Molecular Techniques. Genomic DNA was extracted from thoracic muscle or legs of specimens using a QIAamp DNA mini kit (250) (QIAGEN, Valencia, CA) and visualized on 0.8% agarose gels (Invitrogen, Carlsbad, CA). mtDNA was amplified using a polymerase chain reaction (PCR) with pairs of heterologous primers (Simon et al. 1994) on either a Whatman Biometra TGradient or TPersonal Thermocycler (Whatman Biometra, Göttingen, Germany) with *Taq* polymerase (University of Alberta, Edmonton, Alberta, Canada) added in a hot start at the end of an initial denaturation cycle at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 45°C for 30 s, 72°C for 2 min, and a final extension at 72°C for 5 min. A 475-bp fragment in the COI region was obtained using either Jerry or JerryV and MilaIII (Table 2) for 180 specimens in the study region. From the specimens examined for 475 bp, 14 specimens representing eight distinct genetic lineages in the region were sequenced over the full 2.3 kb of COI-COII. A complete list of primers used to obtain the 2.3-kb fragment is shown in Table 2. PCR products were cleaned using QIAquick PCR purification kit (250) (QIAGEN) and cycle sequenced on either a Whatman Biometra TGradient or TPersonal Thermocycler using Amersham Bioscience DYEnamic ET Dye Terminator kit (GE Healthcare, Little Chalfont, Buckinghamshire, England) according to the following profile: initial denaturation at 93.0°C for 30 s, 28 cycles of 95°C for 20 s, 45°C for 15 s, 60°C for 1 min, with a final extension at 60°C for 30 s. The sequenced product was purified by filtration through Sephadex columns (GE Healthcare) and dried. This product was resuspended in formamide and sequenced on an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA). All fragments were sequenced in both directions and manually aligned to the sequence of *Drosophila yakuba* Burla (Clary and Wolstenholme 1985).

Phylogenetic Analyses of DNA Sequence. Initially, the 475-bp COI fragments from all 180 individuals were compared and identical mtDNA haplotypes were combined. In total, 28 unique haplotypes of

Dioryctria were found among all specimens sampled (Table 1). Sequences were aligned by eye and nucleotides were treated as unordered, unweighted characters. Phylogenetic analysis using maximum parsimony (MP) was conducted with PAUP 4.0*b10 (Altivec) (Swofford 2002). MP analysis was performed using heuristic searches with the following parameters: 100 random addition replicates, stepwise addition, and tree bisection-reconnection (TBR). Branch support was calculated using bootstrap and Bremer support values. Bootstrap values were obtained with 100 bootstrap replicates by using heuristic search methods as described above. Bremer support was calculated from a strict consensus MP tree by using AutoDecay 5.03 (Eriksson 2002). Analysis of the 2.3 kb of sequence data for the 14 available specimens was identical to that described for the 475-bp sequence data.

Morphological Techniques. A wing phenotype classification was developed with the intent of linking forewing pattern to genetic variation, seasonality or pheromone lure attraction. Specimens were initially scored by C. Rudolf (USDA–Forest Service Pacific Southwest Research Station) as wing pattern types that were identified and described by J.D.S. Specimens were subsequently characterized using a simplified wing phenotype character system developed by A.D.R., in which forewing pattern variability was scored for three characters: presence of raised scales, primary forewing color and color of subbasal area. Colors were standardized against the Munsell Soil Color Charts 1994 revised edition (Munsell Color, New Windsor, NY) and listed after each description. Character 1, presence of raised scales, was scored as present (R) or absent (N). Raised scales, when present, occurred as a patch in the basal area, as a subbasal ridge, a medial ridge and on the discocellular spot. Character 2, primary forewing color, was scored as black to dark gray (B) (2.5/N to 6/N), white to pale gray (W) (7/N to 8/N), orange (O) (7.5YR 7/8), or brown (Br) (10YR 6/2 to 5/2). Wings that looked white or pale gray often had white tipped scales with dark interiors, lightening the overall appearance of the wing. Individuals with this coloration were scored (W) to reflect the pale appearance of the wing. Character 3, color of subbasal area, was scored as black to dark gray (B) (2.5/N to 6/N), white to pale gray (W) (8/N to 7/N), or tan to dark red (R) (5YR 4/6–7/8 to 10R 3/6–5/6 to 2.5YR 3/6–6/6). Each specimen was then assigned a wing phenotype code that reflected all three characters. For example, a forewing with raised scales that is primarily black and has a red subbasal area would be RBR. Any larvae or specimens with damaged forewings were listed as unscorable (UNS). All specimens were scored by A.D.R. and were confirmed by an independent observer.

Genitalia dissection methods were adapted from techniques outlined by Sopow et al. (1996) and Winter (2000). The abdomen was removed and placed in 5 ml of 10% KOH solution and boiled in a beaker of water for 10 min or until the abdomen was softened. The abdomen was placed in a 30% ethanol solution and scales were removed using a No. 0 insect pin and a soft hair paintbrush. The genitalia were extracted using a pair of fine forceps and a No. 0 insect pin and disconnected from adjoining membranes. A glass capillary tube pulled to 5 μm in diameter was attached to microtubing and a fine syringe and was used to evert male vesicae. This apparatus was filled with 30% ethanol solution and then slowly injected into the aedeagus causing the vesicae to evert. Vesicae contained many cornuti that hindered the process, so a hooked No. 0 insect pin was used to help eversion. After examination, genitalia were placed in glycerin in genitalia microvials and pinned with the specimen voucher.

Images of wings and genitalia were taken with a Nikon COOLPIX 990 digital camera mounted on a dissecting microscope. Multiple images were taken of each specimen and compiled in AutoMontage (Syncroscopy, Frederick, MD). Wings, head capsule, and remaining structures were preserved in gelatin capsules for morphological comparisons and future identifications. Vouchers and images are deposited in the E.H. Strickland Entomological Museum, University of Alberta.

Results

Analysis of 475-bp COI Fragment. In the 475-bp fragment of COI examined in 180 specimens, 101 positions were variable and 77 were parsimony informative, with an AT bias of 68.8% and a transition/transversion ratio of 3.23. Six of the 28 haplotypes were found in at least two populations throughout the study range, some of which were separated by large geographic distances (Table 1). Twenty-two haplotypes were restricted to single localities, and 19 of these haplotypes were unique and found in a single individual.

A heuristic maximum parsimony search found 178 most parsimonious trees that were 182 steps in length. Multiple most-parsimonious trees resulted from rearrangements between similar haplotypes and between lineages. Eight major genetic lineages were obtained from specimens in the study region. Each of the eight lineages contained more than one specimen and was identified based on relatively long basal branch lengths, high bootstrap values (93–100%), and Bremer support values (3–14) (Fig. 1A and B). Some lineages (lineages 1 and 8) contained two to three specimens, whereas lineages 6 and 7 each contained more than 70 specimens. (Table 1; Fig. 1).

Sequence divergences within and between lineages for the 475-bp sequences were measured using uncorrected pairwise distances. Divergences within lineages ranged from 0.0 to 1.7%. Divergences between lineages ranged from 3.6% (lineages 1 and 2) to 8.0% (lineages 2–6). Divergences between species groups ranged from 4.0% (*baumhoferi* Gr. to *auranticella* Gr.) to 8.0% (*abietella* Gr. to *zimmermani* Gr.). Divergences between outgroup taxa and the ingroup ranged from 6.7% (RS1 g to *C. ophthalmicella;* RS1 g to

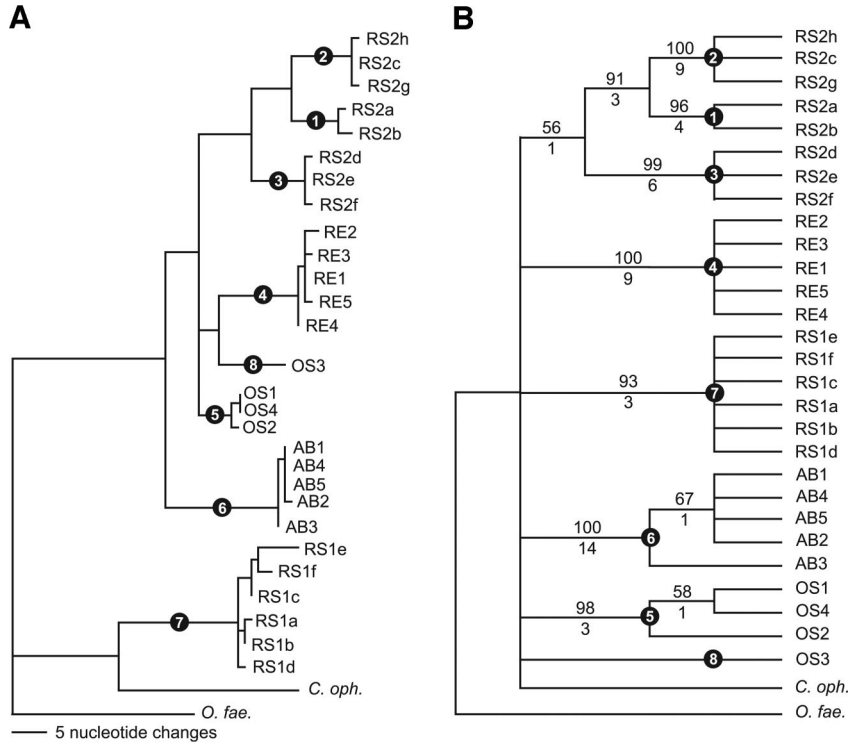

Fig. 1. Maximum parsimony analysis of 475-bp COI. (A) Phylogram of one of 178 most-parsimonious trees (length = 182 steps; CI = 0.692; RI = 0.862) showing phyletic branch lengths. Lineages labeled 1–8 are discussed in text. (B) Strict consensus of 178 most-parsimonious trees, with bootstrap values >50% shown above branches and Bremer support values below branches.

O. faecella; RS1b to *O. faecella*) to 9.9% (RE5 to *C. ophthalmicella;* AB2 to *C. ophthalmicella*).

Support for relationships between lineages was variable. Lineages 1 + 2 formed a well supported sister group relationship, but relationships between the remaining lineages were poorly resolved (Fig. 1B). Monophyly of *Dioryctria* was not obtained in any of the most-parsimonious trees. The outgroup taxon *C. ophthalmicella* grouped with lineage 8 in all trees, although this relationship was not supported by bootstrap or Bremer support values.

Morphological Identifications. Forewings of all voucher specimens used for mtDNA sequencing were examined by A.D.R. for structural characters (raised scales), color, and wing pattern. In total, 146 specimens were scored for wing phenotype, whereas the remaining 34 individuals were unscorable (UNS), because of damage from sticky traps or specimens were collected as larvae. Forewings were assigned a three-letter phenotype code based on the presence or absence of raised scales, primary forewing color, and color of the subbasal area (Table 3). Specimens initially formed two distinct groups: specimens lacking raised scales (N) and those with raised scales (R). For specimens lacking raised scales, three main groups of primary forewing color were present: black, orange, or brown. These three groups corresponded to lineage 6 (mainly NBB), lineage 5 (NOR), lineage 8 (NOR), and lineage 4 (NBrR). For specimens with raised scales, forewings were either black or white. Wing phenotype codes could not distinguish between the raised scale lineages (lineages 1, 2, 3, and 7) because of variability in forewing pattern in lineage 7 (*D. pentictonella*).

Genitalic morphology and additional wing characters linked the eight lineages to eight previously described species from six species groups described from North America (Heinrich 1956; Mutuura et al. 1969a,b; Mutuura and Munroe 1972, 1973; Neunzig 2003).

Lineage 1 (three specimens) was identified as *D. cambiicola* (*zimmermani* group). These individuals could not be fully scored for wing phenotype because of forewing damage.

Lineage 2 (four specimens) was identified as *D. fordi* (*zimmermani* group). Specimens in this lineage all had a wing phenotype code RWR, indicating the presence of raised scales (R) with a primarily white forewing (W) and red subbasal area (R) (Table 3).

Lineage 3 (four specimens) was identified as *D. okanaganella* (*ponderosae* group). Three specimens were scored for wing phenotype. Each specimen had raised scales (R) and a primarily black forewing (B), but variation in the color of the subbasal area (black [B] or red [R]) produced two phenotypes for this species (RBB and RBR) (Table 3). The phenotypes

Table 3. Wing phenotypes for *Dioryctria* specimens used in mtDNA survey

Species	Haplotype	Wing phenotype[a] (no.)
D. abietivorella	AB1	NBB (31), NBR (9), NBW (3), NWB (1), UNS (22)
	AB2	NBB (4), UNS (3)
	AB3	NBB (1)
	AB4	NBB (1)
	AB5	NBB (1)
D. auranticella	OS1	NOR (2)
	OS2	NOR (2), UNS (2)
	OS4	NOR (1)
D. rossi	OS3	NOR (2)
D. pseudotsugella	RE1	NBrR (2)
	RE2	UNS (1)
	RE3	NBrR (1)
	RE6	NBrR (1)
	RE7	NBrR (1)
D. pentictonella	RS1a	RWR (15), RBB (14), RBR (14), RWB (5), RWW (4), RBW (1), UNS (4)
	RS1b	RWR (6), RBR (4), RBB (1), RWB (3), RWW (2)
	RS1c	RBR (1)
	RS1d	RWR (1)
	RS1e	RWR (1)
	RS1f	UNS (1)
D. cambiicola	RS2a	UNS (1)
	RS2b	UNS (2)
D. fordi	RS2c	RWR (1)
	RS2g	RWR (1)
	RS2h	RWR (2)
D. okanaganella	RS2d	RBB (1)
	RS2e	RBB (1), UNS (1)
	RS2f	RBR (1)

[a] Code for wing phenotypes. First position: raised scales: N, absent; R, present. Second position: primary forewing color: B, black to dark grey; W, white to pale grey; O, orange; Br, brown to tan; third position: color of subbasal area; B, black to dark grey; W, white to pale grey; R, tan to dark red.

scored for this species overlapped with those scored for lineage 7.

Lineage 4 (six specimens) was identified as *D. pseudotsugella* (*schuetzeella* group). The identification of *D. pseudotsugella* was based primarily on geographic range, rather than morphology, because of overlap of diagnostic characters with *D. reniculelloides*, a morphologically similar species. Although they are similar, mtDNA sequence data suggest that two sister taxa are distinct species (A.D.R., unpublished data). Five specimens were scored for wing phenotype. All scored specimens had a phenotype code NBrR, indicating the absence of raised scales (N), a primarily brown forewing (Br), and a tan-to-orange subbasal area (R) (Table 3).

Lineage 5 (seven specimens) was identified as *D. auranticella* (*auranticella* group). Five specimens were scored for wing phenotype. All scored specimens had a phenotype code NOR, indicating the absence of raised scales (N), a primarily orange forewing (O), and a reddish orange subbasal area (R). The wing phenotypes scored for this lineage are identical to those scored for lineage 8.

Lineage 6 (76 specimens) was identified as *D. abietivorella* (*abietella* group) and the majority were reared from hosts at the GRC. In total, 51 specimens were scored for wing phenotype. The majority of scored specimens had phenotype NBB, indicating the absence of raised scales (N), a primarily black forewing (B), and a black subbasal area (B). Nine additional specimens had a phenotype code NBR, indicating the presence of a tan-to-pale yellow patch in the subbasal area. Three specimens had a pale white subbasal area (NBW), and a single specimen had a primarily pale gray forewing (NWB). Specimens could be separated from all other species at the GRC based on the previously described wing phenotypes.

Lineage 7 (78 specimens) was identified as *D. pentictonella* (*baumhoferi* group). The majority of specimens were males collected at pheromone traps at the GRC in Chico, CA, and equal numbers of males and females were reared or collected at lights. In total, 73 specimens were scored for wing phenotype. There was considerable overlap between the wing phenotypes found in *D. pentictonella* and those in other raised scale lineages. Six phenotypes were found in specimens of *D. pentictonella*, with RBB, RBR, and RWR the most common. All specimens had raised scales, but the primary color of the forewing ranged from solid black to nearly white, and the color of the subbasal area was black, white, or red. Based on collection dates for all adult material in the study, *D. pentictonella* exhibits three distinct flight periods (16 April–16 June; 1 July–August 16; and 1 September–16 October) (Fig. 2). *D. pentictonella* wing phenotypes were grouped by these three periods to determine if morphological variation was seasonal (Fig. 3). The three most common phenotypes (RBB, RBR, and RWR) were present in all three flight periods, whereas two phenotypes (RWW and RWB) were present in two periods. A single specimen with phenotype RBW occurred during a single flight period.

Lineage 8 (two specimens) was identified as *D. rossi* (*auranticella* group) and contained two specimens. All specimens had a phenotype code NOR, indicating the absence of raised scales (N), a primarily orange forewing (O), and a reddish orange subbasal area (R). The wing phenotypes scored for this lineage are identical to those scored for lineage 5.

Larval Host Plant Associations. In total, 57 specimens in this study were reared or extracted as larvae from host plant material. Specimens were reared from cones, cambium, or blister rust tissue on the following conifers: Douglas-fir; ponderosa pine; sugar pine; lodgepole pine, *Pinus contorta* Douglas ex Loudon; whitebark pine, *Pinus albicaulis* Engelmann; western white pine, *Pinus monticola* Dougl. ex D. Don; Afghan pine; *Pinus brutia* ssp. *eldarica* (Medw.); and western larch, *Larix occidentalis* Nuttall.

There were 33 individuals reared from Douglas-fir cones, and every specimen was identified as *D. abietivorella* (lineage 6; Table 1). In total, 21 specimens were reared from cones of other conifer species, 18 of which also were identified as *D. abietivorella.* One specimen reared from an Afghan pine cone and two specimens reared from whitebark pine cones were identified as *D. pentictonella* (lineage 7). Three

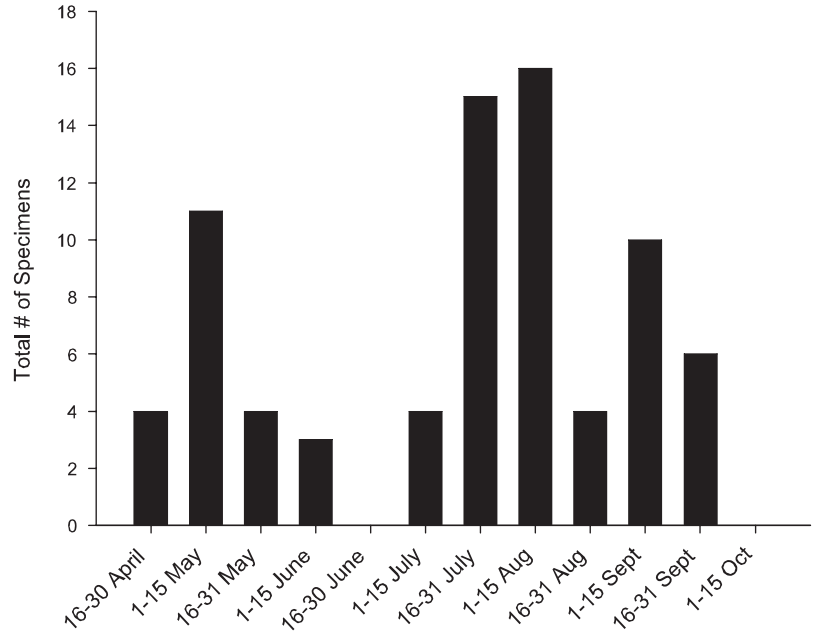

Fig. 2. Flight period for *D. pentictonella* based on all pheromone and light trapped material included in study, including 1998 pheromone trapped material.

specimens were reared from cambial tissue or blister rust tissue in Douglas-fir and lodgepole pine and were identified as *D. cambiicola* (lineage 1).

Pheromone Analysis. Specimens were trapped by five pheromone blends: I, II, III, IV, and V in 2000 and 2001 (Table 4). In total, 73 pheromone trapped specimens were sequenced and represented the range of flight period, lure attraction, and morphological variation occurring at the GRC. Three species of *Dioryctria* were trapped at pheromone lures: *D. pentictonella*, *D. abietivorella*, and *D. auranticella* (Table 4). The numbers of individuals captured varied substantially by lure (Table 4; Fig. 4). Lure I trapped nine specimens from 1 June to 31 August. All

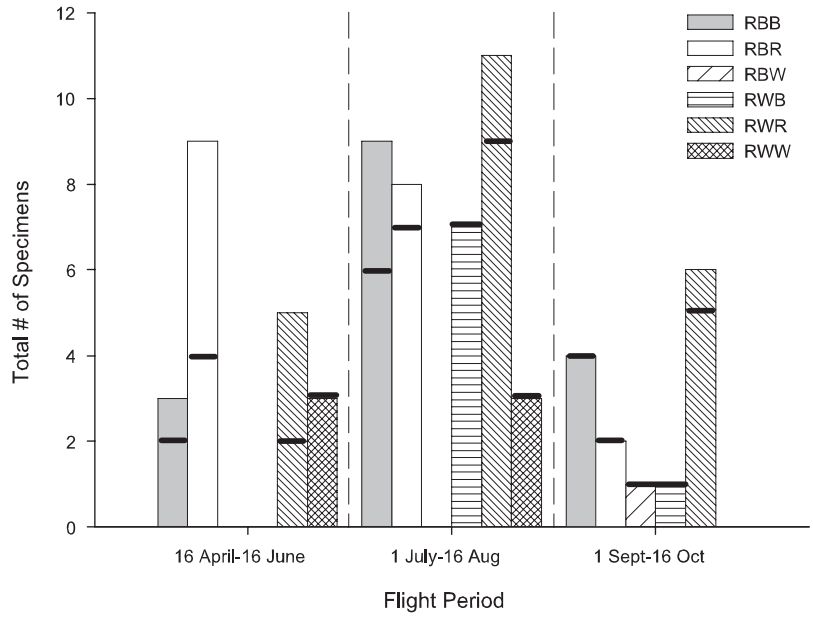

Fig. 3. Wing phenotypes collected during three flight periods of *D. pentictonella*. Dark horizontal bars separate number of specimens collected by pheromone traps (below) from other methods (above). Wing phenotype coding is discussed in text.

Table 4. ***Dioryctria*** **specimens collected at five pheromone blends, characterized for mtDNA haplotypes and wing phenotypes (all from the Genetic Resource Center, Chico, CA, 2000–2001)**

Species	Haplotype	No.	Blend	Components[a] (dose [μg])	Wing phenotype (no.)
D. abietivorella	AB1	9	I	Z (100) + E (1)	NBR (4), NBB (2), NWB (1), UNS (2)
		1	II	T (100)	UNS
		1	V	T (100) + D (5)	NBR
D. auranticella	OS1	1	II	T (100)	NOR
D. pentictonella	RS1a	42	V	T (100) + D (5)	RWR (12), RBB (10), RBR (10), RWB (5), RWW (3), RBW (1), UNS (1)
		3	III	T (10) + D (0.5)	RBB, RBR, RWR
		1	IV	T (1) + D (0.05)	RWW
D. pentictonella	RS1b	12	V	T (100) + D (5)	RBR (4), RWB (3), RWR (2), RWW (2), RBB (1)
		2	III	T (10) + D (0.5)	RBB, RWR
D. pentictonella	RS1e	1	III	T (10) + D (0.5)	RWR

[a] Chemical names of *Dioryctria* pheromone blend components. Z, (Z,*E*)-9,11-tetradecadienyl acetate; E, (Z,*E*)-9,12-tetradecadienyl acetate; T, (Z)-9-tetradecenyl acetate; D, (Z)-7-dodecenyl acetate.

nine individuals trapped by Llre I were identified as *D. abietivorella*, similar to the material reared from cones. Lure II trapped one specimen of *D. auranticella* and one specimen of *D. abietivorella.* Lure III trapped seven specimens throughout the season, and all were identified as *D. pentictonella.* Lure IV had the same chemical composition as lure III but with a lower dosage (Table 4) and trapped a single specimen of *D. pentictonella.* Lure V had the same chemical composition as the previous two lures, but with a higher dosage, and trapped 55 individuals, although the number of specimens trapped throughout the season varied (Fig. 4). All but a single specimen was identified as *D. pentictonella.* The other specimen caught by lure V was identified as *D. abietivorella.*

Phylogenetic Analysis of 2.3-kb Fragment. Based on the eight genetic lineages recovered from the 475-bp fragment, 14 representative specimens were sequenced across the full length of COI and COII genes. Of the 2,307 bp examined, 418 were variable and 309 were parsimony-informative, with an AT bias of 71.3% and a transition/transversion ratio of 2.66.

A heuristic maximum parsimony search found a single most-parsimonious tree 840 steps in length. A phylogram with bootstrap and Bremer support values is shown (Fig. 5). Eight distinct lineages were resolved by the 2.3-kb sequences, like the 475-bp sequences. Single specimens were sequenced for most lineages, but where multiple specimens were used (lineages 3, 5, 6, and 7), the lineages were well supported.

Sequence divergences among the 2.3-kb sequences were measured using uncorrected-pairwise distances. Divergences within lineages with more than one specimen ranged from 0.0 to 0.44%. Sequence divergence within species groups ranged from 2.2% (*zimmermani* Gr.) to 5.4% (*auranticella* Gr.). Divergences between species groups were also variable, ranging from 4.5% (*ponderosae* Gr. to *zimmermani* Gr.) to 7.5% (*abietivorella* Gr. to *ponderosae* Gr.). Divergence between outgroup and ingroup taxa ranged from 6.7% (*D. auranticella* to *O. faecella*) to 9.9% (*D. okanaganella* to *C. ophthalmicella*).

With the 2.3-kb sequences, species group and higher level relationships were more resolved and showed increased support, compared with the 475-bp sequences (Figs. 1 and 5). Well supported nodes (100% bootstrap values) from the 2.3-kb tree included the *zimmermani* Gr. (node G), *ponderosae* Gr. (lineage 3), *abietella* Gr. (lineage 6), and *baumhoferi* Gr. (lineage 7). The *auranticella* Gr. was paraphyletic,

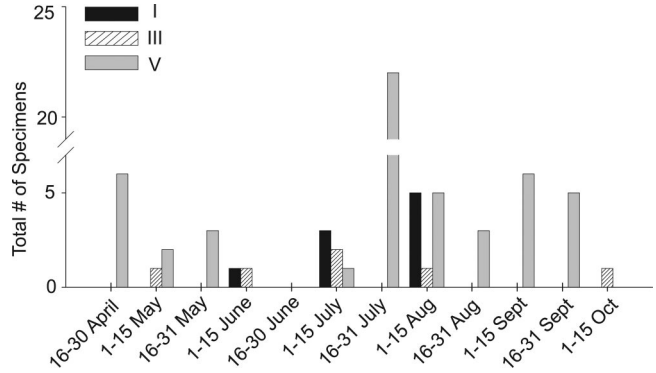

Fig. 4. Flight times for pheromone trapped material sequenced for mtDNA from 2000 and 2001. Lures with catch totals of less than three specimens are shown.

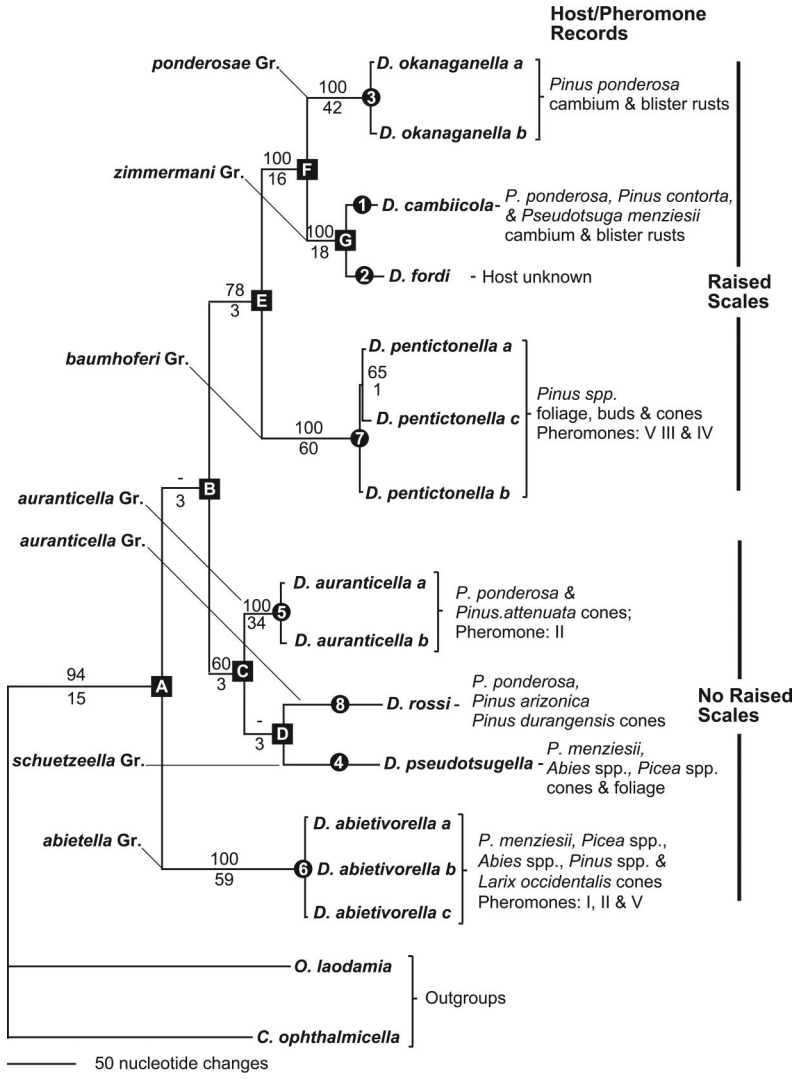

Fig. 5. Phylogram of single most parsimonious tree for 2.3 kb COI-COII (length = 840; CI = 0.714; RI = 0.746). Lineages labeled 1–8 and nodes A–G are discussed in text. Known host associations and pheromone attraction are indicated for each species. Bootstrap values >50% are shown above branches and Bremer support values below branches.

with *D. rossi* grouping with *D. pseudotsugella*, although this relationship was poorly supported (node D). *Dioryctria auranticella* + (*D. rossi* + *D. pseudotsugella*) formed a poorly supported clade (node C). The *abietella* Gr. (*D. abietivorella*) was sister to the remaining species groups, although this relationship was poorly supported (node B). The *baumhoferi* Gr. was sister group to the *zimmermani* Gr. + *ponderosae* Gr. clade and this relationship was moderately supported (node E). The sister group relationship between *zimmermani* Gr. and *ponderosae* Gr. was well supported (node F). Monophyly of *Dioryctria* was resolved and well supported (node A).

Information Content of Sequence Fragments. For specimens with the full 2.3 kb of COI + COII, uncorrected pairwise sequence divergences were compared between the 475-bp fragment used in this study and the 658-bp DNA barcoding region of Hebert et al. (2003). Divergences based on the full 2.3 kb of COI + COII were used as a reference. A wide degree of variability in sequence divergence between and within species groups was apparent between the two fragments (Fig. 6). Sequence divergences between species groups were generally higher in the DNA barcoding fragment and lower in the 475-bp fragment than the full 2.3-kb sequence, although exceptions did occur. In two cases, the 475-bp fragment had slightly higher sequence divergence than both the barcoding and 2.3-kb fragments. Additionally, divergences as high as 1.7% were found in the larger data set of 475-bp sequences (RS1e to RS1d). Haplotype RS1e of *D. pentictonella* was collected at the same locality as many other *D. pentictonella* specimens, but it occurred later in the season than most other haplotypes (Table 1).

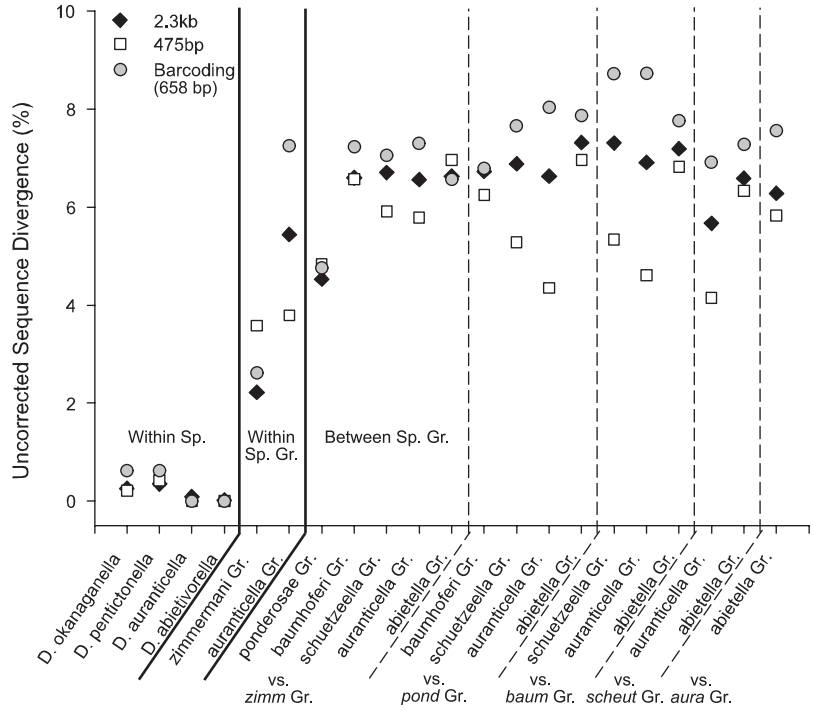

Fig. 6. Comparison of uncorrected sequence divergences within species, within species groups and between species groups for *Dioryctria* specimens sequenced across the full 2.3 kb of COI +COII. Abbreviations of between species groups comparisons are as follows: *zimm* Gr., *zimmermani* Gr; *pond* Gr., *ponderosae* Gr.; *baum* Gr., *baumhoferi* Gr.; *scheut* Gr., *scheutzeella* Gr.; and *aura* Gr., *auranticella* Gr.

Although sequence divergence was relatively high, haplotype RS1e was not considered a distinct lineage because it was only found in a single specimen, and the haplotype was located terminally among other RS1 haplotypes (Fig. 1). Tree topologies of the two subsets (data not shown) were compared with the 2.3-kb tree (Fig. 5). Basal relationships in the 475 bp and DNA barcoding subsets were both poorly supported and lacked resolution. However, trees from both subsets resolved all the well supported nodes found previously with the full set of 475-bp sequences and with the 14 sequences of 2.3 kb.

Discussion

mtDNA Lineages. DNA sequence from the 475-bp fragment of COI provided enough phylogenetic information to successfully delineate eight *Dioryctria* species in six species groups. By using a short fragment, we were able to survey a large numbers of individuals, and sequence variation was examined across a range of morphological characters, pheromone lure associations, and geographic locations. Previous phylogenetic studies among Lepidoptera have demonstrated the effectiveness of using mtDNA lineages for identifying distinct species (Landry et al. 1999, Kruse and Sperling 2001) and delineating species boundaries (Sperling et al. 1999, Caterino et al. 2000, Sperling 2003).

Preliminary tests demonstrated that known *Dioryctria* species were correctly delineated using the 475-bp fragment, so it was used to survey all specimens collected from the study region. This same region also has been used to investigate species problems in *Choristoneura* tortricids (Sperling and Hickey 1994). However, a different 658-bp region at the start of COI, known as the "DNA barcoding" region (Hebert et al. 2003), has more recently been used with increased frequency to identify closely related species or to associate specimens such as different sexes or immatures (Paquin and Hedin 2004, Simmons and Scheffer 2004, Hebert et al. 2005). Because the full COI gene was sequenced in a subset of *Dioryctria* specimens to improve resolution for the phylogeny, these sequences fortuitously allowed a comparison of the utility of these two regions for identifying *Dioryctria* species.

When using distance data to separate closely related species, it is particularly important to use the most informative region available. The variability seen in this data set suggests that the region of greatest divergence varies between taxa, which means that reliance on a single region within a gene could be misleading, although the 475-bp region was most divergent in the *zimmermani* Gr., contrary to the general trend where the DNA barcoding region showed the greatest differences. This variability also was seen be-

tween species groups, particularly between the *abietella* Gr. and *auranticella* Gr.

Such variation in divergence could be because of variation in the mutation rate or because of the level of constraint between these two fragments. Both of these processes could lead to differences in their observed substitution rate and their susceptibility to the effects of saturation because of multiple substitutions. Corrections, such as Kimura-2-parameter model (K2P) or LogDet can help mitigate the effects of multiple substitutions, although these corrections do not, in practice, fully eliminate the effects of saturation (Felsenstein 2004). The neighbor-joining method, favored by the DNA barcoding advocates (Hebert et al. 2003), is a distance method and may be significantly affected by saturation. A K2P correction of the pairwise distances in the current data set did not reduce the variability seen in pairwise comparisons (A.D.R., unpublished data). Substitution rate heterogeneity, secondary structure, mutation hot spots, or even recombination could explain the divergence variability observed in our data set (Lunt et al. 1996, Hagelberg 2003, Howell et al. 2003, Doan et al. 2004, Ho et al. 2005). Variability between pairwise sequence divergence within these sequence fragments not only raises concerns regarding the use of a single region of mtDNA for predicting the presence of distinct species but also contradicts any assumption of neutral or nearly neutral molecular evolution and provokes closer examination of the processes affecting mitochondrial DNA evolution.

Short fragments of COI (400–800 bp) have commonly been used to identify closely related species, particularly in Lepidoptera (Caterino et al. 2000). Although short fragments are used extensively and have been popularized for DNA barcoding (Hebert et al. 2003), such reliance on short COI fragments has been questioned (Wahlberg et al. 2003). Short fragments may have low numbers of phylogenetically informative characters, reducing their utility for separating closely related species. This effect is compounded when only a single specimen is used to define a lineage. Thus, all major lineages recognized in this study contained at least three specimens, and, when possible, specimens from multiple populations (Table 1). These problems can be further reduced if longer DNA fragments are included in the analysis (Mitchell et al. 2000, Wahlberg and Nylin 2003, Wahlberg et al. 2003).

Although the 475-bp fragment resolved the eight species included in this study, mtDNA phylogenies represent a single genetic tree and therefore caution must be exercised when relying on mtDNA for delineating species. Cases where species trees and mtDNA gene trees are incongruent have been well documented, and they are especially common between the most closely related species (Avise and Ball 1990, Avise 1991, Nichols 2001, Funk and Omland 2003, Ballard and Whitlock 2004). To be confident that species delineated by the mtDNA data constitute unique biological entities (e.g., species), additional characters such as morphology, geographic range, and larval host plant should be examined.

Morphological Identification. Although morphological characters were generally sufficient to identify the lineages to species, the highly variable forewing pattern of some species was problematic. Specimens of *D. pentictonella* showed a wide range of forewing variation, ranging from pale, nearly white phenotypes to dark red and black phenotypes. Many of these phenotypes resembled other species in the region (e.g., *D. fordi* or *D. okanaganella*), particularly *D. ponderosae.* Although *D. ponderosae* was not collected during this study, it occurs in species lists for California (Furniss and Carolin 1977; http://elib.cs.berkeley.edu/eme/calmoth.html); however, caution must be exercised when relying on these identifications, because of the similarity between this species and some wing phenotypes of *D. pentictonella.* Genitalic characters definitively separate these two species, because they are in separate species groups, but these characters are often not examined.

The phenotype RBR was similar to the original description for *D. pentictonella* (Mutuura et al. 1969b) and was found throughout the year, but several other wing phenotypes were also common (RBB and RWR; Table 3; Fig. 3). The wing phenotypes were compared with genetic variation, seasonality, and pheromone lure attraction, but none of these factors were able to account for the forewing pattern variation observed in the population (Tables 3 and 4; Fig. 3). Similar forewing variability was seen among the smaller number of *D. pentictonella* females. Forewing pattern in *D. pentictonella* thus seems to be highly variable and plastic, even within a single population. Such phenotypic plasticity is quite common in Lepidoptera, particularly with respect to forewing color patterns. An extreme example of phenotypic forewing variation occurs in *Acleris cristana* (Denis & Schiffermüler) (Lepidoptera: Tortricidae) where 119 forms were described for a single species in Britain (Manley 1973). Understanding the morphological variability seen in *D. pentictonella* will require a detailed examination of the environmental and genetic factors influencing the development of wing pattern in this species.

Reared Material and Pheromone Attraction. mtDNA and morphological comparisons were essential for identifying *Dioryctria* species reared from host material or captured with pheromone traps. A single specimen of *D. auranticella* was captured in a pheromone trap (Table 4), and although considered a cone pest, no specimens were reared from ponderosa pine cones at the GRC ,even though it has previously been recorded on this host (Mutuura and Munroe 1972, Hedlin et al. 1980, Neunzig 2003). *D. auranticella* does not usually occur in large populations and often affects only a small percentage of cones in a given area (Hedlin et al. 1980), which may explain the lack of reared material. *D. cambiicola* specimens were reared from wounds on Douglas-fir and blister rust tissue on lodgepole pine. Although described from several species of pine, *D. cambiicola* has not been previously recorded on Douglas-fir.

The majority of specimens reared from cones were identified as *D. abietivorella,* and, based on these

numbers, likely caused the majority of cone damage at the GRC. *D. abietivorella* larvae are generalist feeders and have been reared from a variety of larval hosts (Table 1). Although large numbers of specimens were present in cones, relatively low numbers of specimens were captured in pheromone traps (Table 4), suggesting that the pheromone lures used in the study are only weakly attractive to *D. abietivorella.* Recent work on pheromone blends of *D. abietivorella* has found that (3Z,6Z,9Z,12Z,15Z)-pentacosapentaene is a key component and is attractive as a 5:1 ratio with (9Z,11*E*)-14:Ac (Millar et al. 2005) .

D. pentictonella, however, was captured in higher numbers by pheromone traps, and three distinct flight periods were observed (Fig. 2). In contrast to the number of specimens captured in pheromone traps, only three specimens were reared from host material. *D. pentictonella* is primarily a foliage feeder, although it has occasionally been recorded on cones (Neunzig 2003). Collecting effort was targeted at species feeding within cones, rather than on foliage, which may explain the discrepancy between the numbers of adults captured by pheromone traps and those reared from host plants.

Multiple collecting methods were essential for sampling species in the study region, and mtDNA sequences allowed clear associations to be made among specimens collected by different methods, independently of morphological characters. Examination of reared material helped to identify the species causing the majority of cone damage at the GRC but provided incomplete sampling of *Dioryctria* diversity in the area. Pheromone trapping provided insight into the attractiveness of different pheromone lures deployed to sample *Dioryctria* populations in the area but was limited by the range of the blends deployed. Recent work also has shown that pheromone trapping may not accurately sample local populations, particularly in populations at the edges of ranges, and as a result may inaccurately evaluate genetic diversity and gene flow (Salvato et al. 2005). Most of the species, but not *D. pentictonella* at Chico, were sampled by UV or MV light trap (Table 1). Reliance on pheromone trapping, rearing, or light trapping alone therefore would not have documented the diversity of species in the area. Collecting with a variety of methods across a broad temporal and geographic range provides the most accurate estimation of population structure and representation of species in the region.

A combination of mitochondrial, morphological, and behavioral characters was needed to simplify identification of *Dioryctria* species in the study region. By sequencing mtDNA from specimens reared from cones, *D. abietivorella* was identified as the primary seed pest at the Genetic Resource Center in Chico, CA. This finding, in combination with clarifications of diagnostic morphological characters that identify this species, provides significant assistance for pest management. Likewise, the combination of these data demonstrated that *D. pentictonella* has a wide range of intraspecific forewing polymorphism. This species was trapped in large numbers by pheromone lures but was not responsible for the majority of cone damage at the GRC. Using mtDNA to clarify morphologically difficult groups and improve pest identifications has proven very effective (Sperling et al. 1995, Kerdelhué et al. 2002, Scheffer et al. 2004, Simmons and Scheffer 2004). Identification of mtDNA lineages can allow detection and testing of morphological characters that more conveniently diagnose pest species and are correlated with identification on the basis of other characters. Contamination of molecular samples is always a concern, so having a suit of morphological characters to validate molecular identifications is essential. Morphology can more easily be examined in the field and does not require laboratory facilities for identification of specimens. This speeds up identifications and improves the ability of forest managers to manage outbreaks, making morphological characters an important component of diagnostic keys and an essential complement to molecular studies.

Phylogenetic Relationships. Morphological identifications identified and assigned mtDNA lineages to eight *Dioryctria* species in six previously described species groups (Table 1). These species groups were originally described based on genitalic variation and forewing differences (Mutuura and Munroe 1972). Deeper phylogenetic relationships between lineages, particularly between species groups, were only poorly resolved by the 475-bp sequence data (Fig. 1). Consequently, we increased character sampling by sequencing the entire COI-COII region (2.3 kb) to resolve some of these relationships (Fig. 5).

Several interesting patterns were revealed when the phylogenetic relationships of the six *Dioryctria* species groups were examined in light of other characteristics (Fig. 5). For example, the *zimmermani* Gr. and *ponderosae* Gr., which were well supported as sister groups (node F) in the molecular data, also were supported by several morphological and ecological synapomorphies. Larvae in this clade feed in the cambium of host plants, forming pitch masses in wounds or blister rusts (with the possible exception of *D. fordi* whose host is undetermined). Males have a constricted uncus and a valve with a hooked apical projection. Females have longitudinal wrinkles on the ductus bursa, though the size and depth of those wrinkles are variable.

The *baumhoferi* Gr., which had a moderately supported sister group relationship with the *zimmermani* Gr. + *ponderosae* Gr. clade (node E; Fig. 5), was supported by two additional synapomorphies. All three species groups have raised scales in several regions of the forewing and form a monophyletic "raised-scale" group. The majority of species also specialize on pines, although exceptions do occur (e.g., *D. cambiicola;* Table 1). The species groups in this clade are the most speciose in North America north of Mexico, with 30 of 40 described species classified under these three groups. Species of this clade seem to specialize on only one or two *Pinus* species, rather than feeding on a wide range of host plants like *D. abietivorella* (Hedlin et al. 1980, Neunzig 2003). During the Tertiary, *Pinus* experienced a diversifica-

tion throughout North America as a result of climatic change (Millar 1998). *Dioryctria* in the "raised-scale" clade may have radiated onto the pines during or sometime after their diversification leading to the present species diversity. No appropriate phycitine fossils are available to calibrate divergence rates for *Dioryctria*, and there is noticeable divergence rate heterogeneity among different species groups, so any hypothesis of evolution between *Dioryctria* and their larval hosts remains speculative.

The *schuetzeella* Gr. and *auranticella* Gr. formed a poorly supported clade (node C). This clade lacked raised scales, as did *D. abietivorella* and the outgroups, making it a pleisiomorphic character. Species in this clade also lacked a constricted uncus and a prominent preapical spine, characters shared by members of the *baumhoferi* Gr. Larval host associations differ between the members of these two clades, with the *schuetzeella* Gr. feeding in cones, foliage, and cambium in a wide range of conifer species (Mutuura and Munroe 1973, Neunzig 2003), whereas species in the *auranticella* Gr. feed primarily in cones on *Pinus* species (Munroe 1959, Mutuura and Munroe 1972, Neunzig 2003).

The *abietella* Gr. was sister group to the rest of the *Dioryctria* species groups in the analysis of 2.3-kb sequences, but this relationship was poorly supported. *D. abietivorella* was the only representative of the species group and lacked raised scales like the previous two species groups. *D. abietivorella* feeds primarily on cones, like members of the *auranticella* Gr., although it will switch to foliage during times of low cone production (Trudel et al. 1999). This species is a generalist and feeds on a wide range of conifer hosts, unlike those in the "raised-scale" clade.

Increased character sampling, by examining the full COI-COII sequence, provided enough phylogenetically informative characters to develop a preliminary phylogeny for species and species groups of *Dioryctria*, but some parts of the phylogeny were still poorly supported. *Dioryctria* is a diverse genus and sampling of additional species is needed throughout North America, particularly from the speciose *zimmermani* Gr. and *baumhoferi* groups. Additional characters, such as from nuclear gene sequences or morphological analyses, will be needed to fully elucidate the relationships within this genus and to improve the resolution of the deeper clades in the phylogeny.

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