Systematics of the *Dioryctria abietella* Species Group (Lepidoptera: Pyralidae) Based on Mitochondrial DNA

G. ROUX-MORABITO,[1,2] N. E. GILLETTE,[3] A. ROQUES,[1] L. DORMONT,[4] J. STEIN,[5] AND F.A.H. SPERLING[6]

ABSTRACT   Coneworms of the genus *Dioryctria* Zeller include several serious pests of conifer seeds that are notoriously difficult to distinguish as species. We surveyed mitochondrial DNA variation within the *abietella* species group by sequencing 451 bp of cytochrome oxidase subunit 1 (COI) and 572 bp of cytochrome oxidase subunit 2 (COII) genes from 64 individuals of six major species in the group. In addition to examining phylogenetic relationships within European members of the group, the study focused on the two most damaging species, *D. abietivorella* Grote from North America and *D. abietella* Denis & Schiffermüller from Europe and Asia, which have been considered taxonomically synonymous in the past. To detect different levels of divergence, we extensively sampled in seed orchards and natural forests for *D. abietella* on different hosts. Maximum parsimony and maximum likelihood analyses confirmed the monophyly of the *abietella* species group and its separation into three clades. The grouping of North American species (clade A) received strong support in both analyses, whereas relationships between clade A and the two European clades were weakly supported. *Dioryctria simplicella* Heinemann could not be unambiguously separated from *D. abietella* populations. The diverse haplotypes observed in the network analysis conducted with eight populations of polyphagous *D. abietella* suggested the presence of two distinct lineages in France.

KEY WORDS   *Dioryctria*, mitochondrial DNA, COI, COII, seed orchard

The greater diversity of phytophagous insect clades compared with their nonphytophagous sister groups has lead biologists to postulate that host plants strongly influence the diversification and speciation of herbivorous insects (Kelley et al. 1999). Two patterns of association between phytophagous insects and their host plants exist, with some species using a diversity of host taxa (polyphagy), and others being restricted to one particular plant species (monophagy). According to numerous studies (Bush 1975, Mitter and Futuyma 1979, Diehl and Bush 1984, Tauber and Tauber 1989, Dres and Mallet 2002, Rundle and Nosil 2005), changes in host preference can be critical to the formation of new species. Such genetic differentiation has been associated with host use in several polyphagous species, which frequently consist of locally specialized populations, races, or even sibling species complexes (Menken 1996). Lepidoptera include nu-

merous examples of species complexes in which there are evolutionarily significant entities that may or may not represent species (Sperling 2003, Wahlberg et al. 2003). Because morphological characters of such related species or subspecies are very hard to distinguish, their autecology and host plants are often used for taxonomic identification, but the use of such labile characters raises doubts about the validity of the taxonomic status of these taxa.

Coneworms of the genus *Dioryctria* Zeller comprise several species groups within which numerous species have been identified mainly on the basis of their larval host plant, but also by forewing morphology and geography (Neunzig 2003). These coneworms are serious pests of conifer seed cones in the Holarctic region (Turgeon et al. 1994), where >70 species have been recorded (Du et al. 2005, Roe et al. 2006). Most of them are associated with the Pinaceae, especially with *Pinus* L., *Picea* A. Dietrich, *Abies* Miller, *Larix* Miller, and *Pseudotsuga* Carrière species (Hedlin et al. 1980, Roques 1983, Cibrián-Tovar et al. 1986, Neunzig 1990, Turgeon and de Groot 1992), with a few being observed on Taxodiaceae (Merkel 1984). Because these species may drastically limit crops of genetically superior seeds in seed orchards, their biology has been studied extensively during the past 40 yr (Lyons 1957, Zocchi 1961, Neunzig et al. 1964, Charles and Roques 1977, Grant et al. 1993, Millar et al. 2005).

[1] INRA, Centre d'Orléans, Unité de Zoologie Forestière, BP20619 Ardon, 45166 Olivet cedx, France.

[2] Corresponding author, e-mail: geraldine.roux-morabito@orleans.inra.fr.

[3] Pacific Southwest Research Station, USDA–Forest Service, Albany, CA 94710.

[4] Centre d'Ecologie Fonctionnelle et Evolutive, CNRS UMR 5175, 1919 Route de Mende, 34293 Montpellier Cedex 5, France.

[5] Forest Health Technology Enterprise Team, USDA–Forest Service, Morgantown, WV 26505.

[6] Department of Biological Sciences, University of Alberta, Edmonton, AB T6G 2E9, Canada.

However, a paucity of clear morphological characters for separating *Dioryctria* taxa creates taxonomic uncertainties that hinder the analysis of their host relationships (Chatelain and Goyer 1980, Hedlin et al. 1980, Sopow et al. 1996). Using a combination of external characters and adult genitalia, Mutuura and Munroe (1972) defined seven species groups but could not definitively place all the studied species into groups (Du et al. 2005). Among these groups, the *abietella* species group comprises 13 species and is defined by the absence of raised scales on the forewings, a feather-like maxillary palpus in the male, and a narrow valva in the male genitalia. According to Mutuura and Munroe (1972, 1973) and Neunzig (2003), this group includes the widespread Palaearctic species *D. abietella* Denis & Schiffermüller, and species from Europe (*D. pineae* Staudinger, *D. mendacella* Staudinger, *D. simplicella* Heinemann = *D. mutatella* Fuchs, Fazekas 2002), North Africa (*D. alloi* Barbey, *D. peyerimhoffi* Joannis), Asia (*D. stenopterella* Amsel, *D. assamensis* Mutuura, *D. raoi* Mutuura), North America (*D. abietivorella* Grote, *D. ebeli* Mutuura & Munroe, *D. pinicolella* Amsel), and Central America (*D. sysstratiotes* Dyar).

Dioryctria abietella and *D. abietivorella* are undoubtedly the most important lepidopteran pests of conifer cones in Europe and North America (Roques 1983, Hedlin et al. 1980). They both have a wide host range for larval development. So far, *D. abietella* has been recorded from Western Europe and Scandinavia to the Russian Far East and northern China on a broad range of hosts, including cones of pine (*Pinus* spp.), spruce (*Picea* spp.), larch (*Larix* spp.), fir (*Abies* spp.), and Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franko], and more rarely twigs, buds, and adelgid-induced galls (Roques 1983). *D. abietivorella* has been reported from Alaska to central Mexico, and from California to Newfoundland, on >20 different hosts, including pine, spruce, and Douglas-fir (Lyons 1957, Hedlin et al. 1980, Turgeon and de Groot 1992). Because these two species are generally similar in genital characters, they have variously been considered under the names *D. abietella*, *D. assamensis*, and *D. raoi* (Munroe 1959, Byun et al. 1998).

More recently, there has been similar confusion among European *Dioryctria* species such as *D. abietella*, *D. simplicella*, and even species outside the *abietella* group such as *D. schuetzeella* Fuchs (Charles and Roques 1977, Roques 1983). For example, *D. resiniphila* Segerer and Pröse was recently identified in cones of *Abies cephalonica* Loudon (Segerer and Pröse 1997) in Greece, but in the past these coneworms had been identified as *D. abietella.* Species-specific treatments such as semiochemicals (DeBarr et al. 2000) or pathogens (Verma et al. 1996, Perez et al. 1999, Glynn and Weslien 2004) are frequently required for the control of such cryptic pests, which are not amenable to control by using pesticides without resorting to highly toxic organophosphate and carbamate insecticides (Bhandari et al. 2003) or multiple injections of systemic insecticides (Grossman et al. 2002). The success of such species-specific treatments relies upon correct pest species diagnoses. Thus, it is important to focus on clarifying the status of taxonomically intractable taxa like *Dioryctria* species groups, so that effective control measures can be tailored to each species.

In taxonomic groups where morphological identification is difficult, molecular characters, particularly mtDNA, can help in assessing species boundaries (Sperling and Hickey 1994, Brower 1999, Caterino et al. 2000, Templeton 2001, Kerdelhué and Rasplus 2002, Hebert et al. 2003a, Wahlberg et al. 2003, Roe and Sperling 2007b). Several properties make mtDNA a good marker of species limits (Avise 1991, Sperling 2003, Wahlberg et al. 2003). It is a nonrecombining, maternally inherited genome, with a smaller effective population size that leads to shorter coalescence times (Moore 1995). Moreover, gene trees may be more likely to reflect species trees when using mitochondrial markers (Avise 2000, Sperling 2003). Thus, the rapid evolution of mtDNA sequences has often been used in intraspecific studies (Avise 2000) as well as in investigating relationships of closely related species in Lepidoptera (Bogdanowicz et al. 1993; Brower and DeSalle 1994; Brower 1999; Brown et al. 1994; Sperling and Hickey 1994; Sperling et al. 1995; Landry et al. 1999; Kruse and Sperling 2001; Sperling 2003; Wahlberg et al. 2003; Du et al. 2005; Roe and Sperling 2007a,b). So far, no molecular study has been performed across the widespread and economically important European *D. abietella* coneworm group; and genetic and biochemical analyses of *Dioryctria* have so far been primarily limited to North American and Chinese species (Richmond and Page 1995, Du et al. 2005, Roe et al. 2006).

Here, we review species delimitations and phylogenetic relationships within the *Dioryctria abietella* species group based on mtDNA sequences. We give special emphasis to the taxonomic status of the two most damaging *Dioryctria* species of this group, namely, European *D. abietella* and North American *D. abietivorella.*

Materials and Methods

Coneworm Collections. A total of 67 specimens were selected for this study, 61 within the *Dioryctria abietella* species group (29 specimens identified as *D. abietella*, nine *D. mendacella*, two *D. pineae*, eight *D. simplicella*, 11 *D. abietivorella*, and two *D. ebeli*) and six additional specimens representative of three other *Dioryctria* species groups [two specimens of the pine stem borer, *D. sylvestrella* Rartzeburg belonging to the *sylvestrella* group; two specimens of *D. amatella* (Hulst) belonging to the *zimmermani* group; one specimen of *D. pseudotsugella* Munroe; and one of *D. reniculelloides* Mutuura & Munroe belonging to the *schuetzeella* group]. The list of corresponding larval hosts and locations is presented in Table 1. To ascertain insect–host relationships, only insects reared from identified host cones were considered. We carried out most of the sampling, but some specimens were provided by collaborators. In addition, three

Table 1. Collection and sequence data for specimens used in this study

Identification[a]	Abbreviation	Haplotype[b] (no. of specimens)	Host tree species	Locality[c]	GenBank accession no.	
					451bp COI	572bp COII
D. abietella	abieTUE	abt1 (3)[d]	Picea abies	France, Alps, Tueda, Meribel, 1,750 m (mixed forest of Swiss stone pine and spruce) (nat)	EU407723	EU407749
D. abietella	cembTUE	abt1 (3),[d] abt2 (1)	Pinus cembra	France, Alps, Tueda, Meribel, 1,750 m (mixed forest of Swiss stone pine and spruce) (nat)	EU407723 EU407724	EU407749 EU407750
D. abietella	cembBOS	abt1 (4)	Pinus cembra	Italy, Alps, Bosco Aleve, Casteldelfino, 1,600–1,800 m (large forest of Swiss stone pine) (nat)	EU407723	EU407749
D. abietella	abieLAT	abt1 (1), abt5 (1), abt6 (1), abt9 (1)	Picea abies	France, Latronquire seed orchard, Lot (art)	EU407723 EU407727 EU407728 EU407731	EU407749 EU407753 EU407754 EU407757
D. abietella	menzLAT	abt1 (1), abt3 (1), abt8 (1), abt12 (1)	Pseudotsuga menziessii	France, Latronquire seed orchard, Lot (art)	EU407723 EU407725 EU407730 EU407734	EU407749 EU407751 EU407756 EU407760
D. abietella	deciLAT	abt1 (1), abt7 (1)	Larix decidua	France, Latronquire seed orchard, Lot (art)	EU407723 EU407729	EU407749 EU407755
D. abietella	smitBAR	abt4 (1), abt10 (2), abt12 (2)	Picea smithiana	France, Les Barres Arboretum, Loiret (art)	EU407726 EU407732 EU407734	EU407752 EU407758 EU407760
D. abietella	koraBAR	abt10 (1), abt11 (1)	Pinus koraiensis	France, Les Barres Arboretum, Loiret (art)	EU407732 EU407733	EU407758 EU407759
D. abietella (Du et al. 2005)	Du64Ch	abt13 (1)	Unknown	China, Henan Province, Mt. Baiyun	DQ247739	DQ247739
D. simplicella	SylvFON	sim1 (2)	Pinus sylvestris	France, Fontainebleau, Yvelines	EU407741	EU407767
D. simplicella	SylvJPOL	sim2 (2)	Pinus sylvestris	Poland Jedinia 300 m	EU407742	EU407768
D. simplicella	SylvZPOL	sim2 (4)	Pinus sylvestris L.	Poland Zawady 220 m	EU407742	EU407768
D. mendacella	HalTUN	mend1 (1), mend2 (1), mend3 (1), mend4 (1), mend5 (1)	Pinus halepensis	Tunisia, Jebel Bel Oulid	EU407735 EU407736 EU407737 EU407738 EU407739	EU407761 EU407762 EU407763 EU407764 EU407765
D. mendacella	HalTUN2	mend5 (1)	Pinus halepensis	Tunisia, Kasserine Ft. Oum Jeddour	EU407739	EU407765
D. mendacella	HalFR	mend5 (2)	Pinus halepensis	France, Venelles, 13	EU407739	EU407765
D. mendacella	PinTUN	mend5 (1)	Pinus pinea	Tunisia (Sejnane Ft. MTHibeus)	EU407739	EU407765
D. pineae	HalGR	pin1 (2)	Pinus halepensis	Greece, Thessaloniki	EU407740	EU407766
D. abietivorella	LambLG	abv1 (3)[e]	Pinus lambertiana (Douglas)	USA, Oregon, La Grande	EU407720	EU407746
D. abietivorella	MenzCHI	abv1 (2)	Pseudotsuga menziessii	USA, California, Butte Co., Chico	EU407720	EU407746
D. abietivorella	StrobSM	abv2 (1), abv3 (1)	Pinus strobus (L.)	Canada, Ontario, Sault Ste Marie	EU407721 EU407722	EU407747 EU407748
D. abietivorella	LambGP	abv1 (2)	Pinus lambertiana	USA, Oregon, Grants Pass	EU407720	EU407746
D. abietivorella (Roe et al. 2006)	Du04CHI	abv4 (1)	Unknown	USA, California, Butte Co., Chico	DQ247740	DQ247740
D. abietivorella (Roe et al. 2006)	Du05CHI	abv5 (1)	Unknown	USA, California, Butte Co., Chico	DQ247741	DQ247741

Table 1. Continued

Identification[a]	Abbreviation	Haplotype[b] (no. of specimens)	Host tree species	Locality[c]	GenBank accession no.	
					451bp COI	572bp COII
D. ebeli	TaedaWSO	ebe1 (2)	*Pinus taeda* (L.)	USA, North Carolina, Weyerhaeser seed orchard	EU400415	EU400416
D. sylvestrella	PstLAN	sylv1 (2)	*Pinus pinaster* (Aiton)	France (Landes)	EU407743	EU407769
D. amatella	TaedaWSO	ama1 (2)	*Pinus taeda*	USA, North Carolina, Weyerhaeser seed orchard	EU407745	EU407771
D. pseudotsugella	pseudotsug	psg1 (1)[e]	*Pinus* sp.	USA, Nevada, Clark Co, McWilliams Campgr. (3,000 m Mt Charleston)	EU407744	EU407770
D. reniculelloides	renicul	ren1 (1), FS.b-371	*Picea glauca* (Moench)	Canada, Alberta, Manning Hawk Hills	EU477758	
Oncocera faecella (Du et al. 2005)	Du29	oncocera (1)		China, Inner Mongolia, Mt Manhan	DQ247727	
Ceroprepes ophthalmicella (Du et al. 2005)	Du79	ceroprepes(1)		China, Henan Province, Mt Baiyun	DQ247728	

All specimens were sequenced over 451 bp of COI (primers no. 4-jerry and 7-mila) and 572 bp of COII (primers no. 10-pierre and 12-eva), except where indicated.
[a] Based on morphological characters or larval host plant.
[b] Haplotypes as in Figs. 1–3.
[c] Natural forest (nat) and artificial plantations (art) as in Table 4 (AMOVA analysis).
[d] One specimen sequenced over 1,975 bp of COI, tRNA leu, and COII.
[e] One specimen sequenced over 2,272 bp of COI, tRNA leu, and COII.

published sequences were incorporated to relate our study to previous publications (Table 1). These included one *D. abietella* from China (Du et al. 2005) and two *D. abietivorella* from Chico, CA (Roe et al. 2006).

Sampling of *D. abietella* was designed to survey different potential levels of divergence. Populations were defined according to the larval host tree and collecting localities. Thus, sympatric populations of *D. abietella* developing simultaneously on different hosts were sampled on three locations in France (Fig. 1): 1) in a natural forest with mixed *Pinus cembra* (L.) and *Picea abies* (L.) in the northern Alps; 2) in a seed orchard that included the native species *Picea abies* and *Larix decidua* (Miller), and the exotic species *P. menziesii* at Latronquière (south central France); and 3) in an arboretum that included, among other species, the exotic species *Picea smithiana* (Wall.) and *Pinus koraiensis* (L.) (Les Barres, north central France). No natural forests surrounded the Latronquière seed orchard.

Dioryctria larvae were extracted from damaged cones, and either reared until adult emergence or killed immediately in 95% ethanol and kept at −80°C until DNA extraction. Adults were identified using morphological descriptions from Zocchi (1961) and Mutuura and Munroe (1972, 1973), or on the basis of the larval host (Charles and Roques 1977) when morphological identification was uncertain and compared with adult DNA from the same species (four larval specimens were identified as *D. mendacella* and five larvae identified as *D. simplicella*).

DNA Protocols. The methods used for DNA extraction, amplification using the polymerase chain reaction (PCR), and sequencing follow Sperling et al. (1994). Genomic DNA was extracted from the thorax of both larvae and adults. The remaining body parts, including head, legs, wings, and abdomen were stored at −80° and retained as vouchers at the University of Orleans. Specimens were vacuum-dried to remove ethanol before extraction. DNA was purified using a phenol/chloroform-based extraction and eluted in 200 μl of LTE buffer.

One microliter of extracted DNA was used as template for amplification of mtDNA fragments by PCR following methods primarily developed for spruce budworm, *Choristoneura fumiferana* (Clemens) (Sperling and Hickey 1994). Using Promega *Taq*, 30 cycles of amplification were performed as follows in 50-μl reaction volumes: denaturation step at 94°C for 1 min, annealing step at 45°C for 1 min, and extension step at 72°C for 1 min 30 s. An initial cycle used a 3-min denaturation at 95°C and a final cycle had an extension step of 72°C for 5 min.

Overlapping sections of a 2,272-bp region of one individual of each of *Dioryctria abietivorella* and *D. reniculelloides*, and 1,975-bp for two specimens of *D. abietella* that are, respectively, homologous to bases 1,457–3,729 and 1,754–3,729 in *Drosophila yakuba* Burla (Clary and Wolstenholme 1985) were PCR amplified using heterologous primers (list in Table 2). This region includes the gene coding for the cyto-
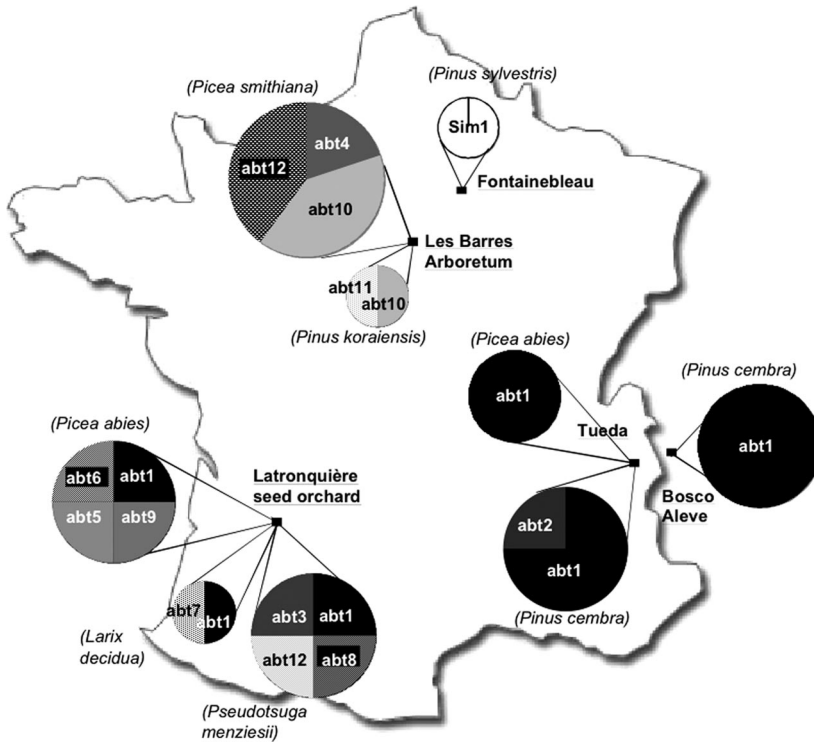
Fig. 1. Sampling sites and haplotype distributions for 12 mtDNA haplotypes detected in European populations of *D. abietella* (abt1 to abt12), plus the French haplotype of *D. simplicella* (sim1). Each haplotype is defined as in Table 1.

chrome oxidase subunit 1 (COI), extends through the tRNA leucine gene, and ends in the cytochrome oxidase subunit 2 gene (COII). An additional 60 samples, representing one to five specimens per population, were sequenced over 451 bp of COI (primers 4, 7) and 572 bp of COII (primers 10, 12). Both strands of the PCR product were sequenced for all samples. Fragments were sequenced directly using Big Dye Terminator (Applied Biosystems, Foster City, CA) and detected with an ABI 377 automatic sequencer (Applied Biosystems).

Data Analysis. DNA contigs were constructed using Sequence Navigator (Applied Biosystems) and aligned

manually with published sequences of *D. abietella* from China and *D. abietivorella* from the United States (Du et al. 2005, Roe et al. 2006). Sequences of two species in the Phycitini, *Oncocera faecela* Zeller and *Ceroprepes ophthalmicella* Christoph (Du et al. 2005), were used as outgroups to root *Dioryctria*.

Maximum parsimony (MP) and maximum likelihood (ML) phylogenetic analysis were performed with PAUP*4b10 (Swofford 2002). For maximum parsimony analysis, a heuristic search was implemented with the tree bisection-reconnection (TBR) branch-swapping option. Variable nucleotide positions were treated as unordered characters with one state for

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

Location[a]	No.	Reference	Sequence (5′–3′)
TY-J-1460a	0	Sperling et al. (1994)	TACAATTTATCGCCTAAACTTCAGCC
C1-J-1709	1	Stump et al. (2003)	ATAATTGGAGGATTTGGAAATTG
C1-J-1751a	2	Bogdanowicz (1993)	GGATCACCTGATATAGCATTCCC
C1-N-1945	3	Stump et al. (2003)	ATTGTAGTAATAAAATTAATTGCTCC
C1-J-2183a	4	Simon et al. (1994)	CAACATTTATTTTGATTTTTTGG
C1-N-2191	5	Bogdanowicz (1993)	CCCGGTAAAATTAAAATATAAACTTC
C1-J-2441	6	New	ACAGGWATTAAAATTTTTAGTTGATTAGC
C1-N-2659d	7	New	GTTAGTCCTGTAAATAGAGG
C1-J-2792b	8	Wells and Sperling (1999)	ATACCTCGGCGATACTCTGA
C1-N-2800	9	Sperling et al. (1994)	CATTTCAAGYTGTGTAAGCATC
C2-J-3138a	10	Sperling et al. (1995)	AGAGCCTCTCCTTTAATAGAACA
C2-N-3389b	11	Du et al. (2005)	TCATAWCTTCARTATCATTG
TK-N-3775	12	Bogdanowicz et al. (1993)	GAGACCATTACTTGCTTTCAGTCATCT

[a] Following Simon et al. (1994).

Table 3. Comparison of uncorrected sequence divergences (mean pairwise divergences) within lineage (within *D. abietella*), between lineages (within *D. abietella* species group) and between species groups for *Dioryctria* haplotypes defined from 451 bp of COI, 572 bp of COII, and across both COI+COII

	COI	COII	COI+COII
Within lineage	0–0.011 (0.002)	0–0.035 (0.018)	0–0.023 (0.010)
Between lineages	0,011–0.049 (0.027)	0.014–0.044 (0.029)	0.011–0.045 (0.027)
Between species groups	0.051–0.086 (0.068)	0.049–0.096 (0.074)	0.057–0.086 (0.069)

each nucleotide base. The relative level of support for each phylogenetic grouping was assessed with the bootstrap method (Felsenstein 1985). For analysis, MODELTEST version 3.07 (Posada and Crandall 1998) was used to determine the model of evolution across the COI and the COII genes. To test for homogeneity of our data set, we used a partition homogeneity test (ILD) for detecting incongruence caused by differences in evolutionary constraints and/or tree topologies (Farris et al. 1994). We performed the ILD test in PAUP by using heuristic searches with TBR branch swapping, and 100 random taxon addition replicates.

Sequence divergences were calculated using uncorrected pairwise distances with PAUP. A statistical parsimony network was constructed with *D. abietella* haplotypes by using TCS version 1.21 (Clement et al. 2000).

Genetic structure within and among European *D. abietella* populations was examined by analysis of molecular variance (AMOVA) (Excoffier et al. 1992) as implemented in ARLEQUIN version 3.0. Populations were grouped by geographical location (region) or by host species or by host origin (see Fig. 1 and Table 4 for details). Levels of significance were determined through 1,000 random permutation replicates.

Results

Sequence Selection. Sequence was obtained for the full 2,272-bp region (including the COI+tRNAleu+COII genes) in one *D. abietivorella* specimen (GenBank accession no. EU407773). For two specimens of *D. abietella* a fragment of 1,975 bp (start of COI missing) was obtained (GenBank no. EU407772). Overall sequence divergence between these two species was estimated at 3.7% (73 substitutions, 51 in COI and 22 in COII), based on the 1,975-bp region. Ten of the variable sites showed transversions (eight in COI and two in COII). There was variation in amino acids at four locations in COI and COII. No insertion or deletion of sequence was observed between the two species. On the basis of variation observed in these longer sequences, as well as the relative effectiveness of different primer combinations, two shorter regions were chosen to survey mitochondrial sequence variation for the remainder of the study.

Thirteen haplotypes were found among the 29 *D. abietella* specimens, five haplotypes among the 11 *D. abietivorella* specimens, five haplotypes among the nine *D. mendacella* specimens, and two haplotypes among the eight *D. simplicella* specimens. Diver-

gences within and between lineages (species) and species groups of *Dioryctria* were compared between the 451-bp COI versus the 572-bp COII fragments and across the two combined gene fragments (Table 3 and *Appendix* for complete data of pairwise divergences). For all data sets (COI, COII, COI+COII), maximum divergence between lineages did not overlap minimum divergence between species group, and uncorrected pairwise distances were comparable with divergences already reported in the genus *Dioryctria* by Roe and Sperling (2007a,b) and Du et al. (2005). By contrast, intraspecific divergence in *D. abietella* (within lineages) exceeded the interspecific divergence between sister pairs of the *abietella* species group for two data sets (maximum 0.035 in *D. abietella* versus minimum 0.014 in *abietella* species group and maximum 0.023 versus minimum 0.011 for COII and COI+COII, respectively). Moreover, when sequence divergence was compared within *D. abietella*, mean pairwise distances differed substantially between the two genes (0.002 in COI versus 0.018 in COII), the number of variable nucleotide sites being five times less in COI (four transitions with 1-0-3 changes at first, second, and third codon positions, respectively) than in COII (18 transitions and two transversions with 2-1-17 changes at first, second, and third codon positions, respectively). There were three amino acid replacements within the COII gene, including one valine versus isoleucine (bp 506), one phenylalanine versus leucine (bp 518), and one phenylalanine versus serine (bp 952). No ambiguous site (double peak) was detected. When compared between species belonging to the *abietella* species group, mean pairwise distances were similar (0.027 in COI versus 0.029 in COII). Mean pairwise comparisons between the two species *D. abietivorella* and *D. abietella* were higher in the COI fragment gene (0.041) versus in the COII gene (0.034). To minimize the great variability detected in intraspecific divergence rate between the COI fragment gene and the COII gene and to minimize the stochastic variation across taxa, we used the combined sequence because this gave a better average of overall divergence rates across COI and COII.

The ILD test between full-length sequences of COI and COII genes revealed no significant conflict ($P = 0.13$). Modeltest was applied to determine the most appropriate model of sequence evolution. The general time reversible model with the following base composition (A = 0.33290; C = 0.10870; G = 0.12960; T = 0.42880), rate of invariable sites (0.6068), and gamma distribution (0.6041) (GTR+I+G, Tavaré 1986) was the substitution model selected for the combined COI+COII data set.

Phylogenetic Reconstruction. One sequence for each combination of haplotype, host and locality was retained for phylogenetic reconstruction. The consensus of three most parsimonious (MP) trees (CI = 0.572; RI = 0.863; excluding uninformative characters) and the ML tree, using 451 bp in COI and 572 bp in COII, are shown in Fig. 2. The monophyly of the *abietella* species group relative to species in the three other Dioryctria groups is strongly supported by MP analysis but weakly by ML analysis (100 and 56% bootstrap values, respectively). The monophyly of the genus *Dioryctria* relative to the two outgroup species in other Phycitini genera was supported by 79% in MP and 72% in ML analysis.

When the MP and ML phylogenetic reconstructions were compared, there were some differences at the ingroup level. The most conspicuous difference was the position of the North American species relative to European species. The *D. abietella* species group was separated into three major clades (A, B, and C). Clade A contained all specimens from North America, *D. abietivorella* and *D. ebeli.* According to both MP and ML analyses this first clade was clearly separated from European and Chinese specimens (100 and 96% bootstrap support). Clades B and C were weakly supported by both analyses (bootstrap values comprised between 52 and 75%). Clade B mainly consisted of *D. abietella* haplotypes plus the *D. simplicella* haplotypes. The abt12 haplotype, corresponding to three *D. abietella* specimens collected at Latronquière on Douglas-fir and at the Les Barres Arboretum on *Picea smithiana* (Wall.) Boiss., formed a clade with specimens of *D. simplicella* collected in Fontainebleau and in Poland on *Pinus sylvestris* L. The pairwise distance between abt12 and *D. simplicella* was 1.2%, whereas distances between abt12 and other *D. abietella* haplotypes ranged between 1.6 and 3.8% for haplotypes from the same localities. Clade C grouped together specimens of *D. mendacella* and *D. pineae*, all of which were collected on the Mediterranean pines *P. halepensis* Mill. and *P. pinea* L. This clade had a basal trichotomy of three haplotype lineages that were separated by 1.4–1.5% from each other. One of the lineages (pin1) represented *D. pineae*, and the two others (mend1+2+3 and mend4+5) were identified as *D. mendacella*, indicating uncertainty in the phylogenetic relationships of these two species.

The European pine stem borer, *D. sylvestrella* (*sylvestrella* species group), was clearly separated from the *abietella* group and clustered with specimens of *D. amatella* (*zimmermani* species group). The specimen of the North American species *D. pseudotsugella* Munroe formed a well-supported monophyletic group with the specimen of *D. reniculelloides*, supporting the placement of both species in a separate species group.

***D. abietella* Haplotype Network.** Thirteen mitochondrial haplotypes were detected among the 29 *D. abietella* specimens analyzed. Most of the haplotype diversity was distributed in artificial stands in France (Fig. 1; Table 1). Of the 11 specimens from natural stands in the French and Italian Alps, 10 had the abt1 haplotype, regardless of host and locality. This major haplotype (abt1) was also present in an artificial stand in the Latronquière seed orchard, on three different hosts (*P. abies*, *P. menziesii*, and *L. decidua*). Seven other unique haplotypes (abt3, abt5–9, and abt11) were found in this locality, plus one divergent haplotype (abt12) shared with the *P. smithiana* population from the Les Barres Arboretum. Three remaining haplotypes were also found in Les Barres (abt4 on *P. smithiana*, abt11 on *Pinus koraiensis* s Sieb. et Zucc, and abt10 on both species). Haplotypes from China (abt13) clustered with the major haplotype abt1.

The host plant association and phylogenetic relationships of all haplotypes in *D. abietella* are summarized in a network (Fig. 3), based on COI and COII sequences. This network revealed two haplotype groups separated by seven mutational steps. One group comprises all 11 individuals from natural forests in the Alps (abt1, abt2) plus the Chinese sequence, separated by five mutational steps from three individuals from the Latronquière seed orchard and the Les Barres Arboretum (abt3, abt4). The second group, more polymorphic, comprises only individuals from artificial stands (arboretum and seed orchard, abt5, 6, 8–11), with the exception of one haplotype (abt7) displayed by one individual on *L. decidua* from the Latronquière seed orchard. The three individuals that had the abt12 haplotype were well separated from the other *Dioryctria* haplotypes and were not be included in the haplotype network produced by TCS.

Genetic Structure of *D. abietella* Populations. The results of the AMOVA analyses performed in *D. abietella* are presented in Table 4. When populations were grouped by geographic region (Alps, central France, and southwestern France) (Fig. 1), genetic variation was partitioned half between regions (51.08%) and half within each population (48.92%), this result being significant. When populations were grouped by host plant, 58% of the genetic variation was found within populations, 40% between populations within hosts and 2% between hosts. Only genetic variation within populations was significant. When populations were grouped by type of stand, 55.6% of the genetic variation was significantly found between groups, i.e., natural forest versus artificial stands, variation within population being also significant.

Discussion

Sequence Divergence in COI versus COII. Although mtDNA genes have long dominated the field of molecular systematics, gene choice, and fragment length are crucial when inferring phylogenetic relationship between species. The COI+COII gene region has frequently been sequenced in Lepidoptera (Sperling 2003) and a recent review (Roe and Sperling 2007a) examined patterns of evolution of these two mitochondrial genes and ramifications for delineating species boundaries in Lepidoptera and Diptera. They demonstrated that DNA substitution patterns can vary between independent lineages and change as taxa become increasingly diverged (also see Galtier et al. 2006). Nevertheless, studies of intraspecific patterns
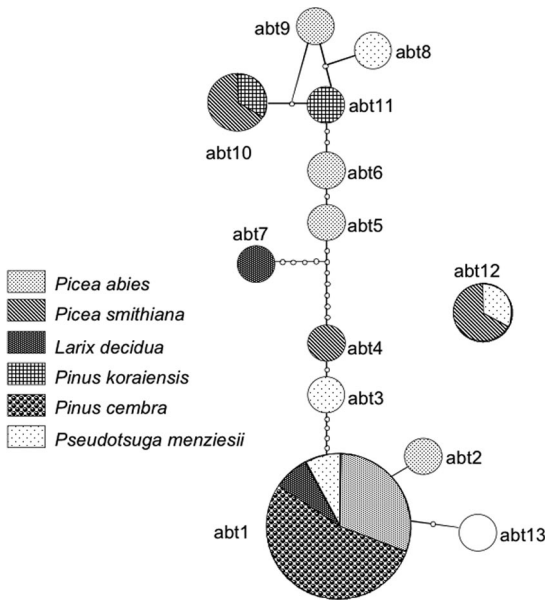
Fig. 2. Phylograms of the consensus tree for parsimony analysis (MP) and ML of haplotypes representing *Dioryctria* species and two outgroup species, for a combined data set COI (451 bp) + COII (572 bp). Bootstrap support values of >50% are shown above branches (500 and 100 replicates for MP and ML analysis, respectively).

Fig. 3. Haplotype network for 13 mtDNA haplotypes detected in populations of *D. abietella*. Each line between circles represents one mutational change. Small empty circles represent inferred, undetected interior haplotypes. Haplotype frequencies are approximated by the area of the circle. Each haplotype is defined as in Table 1, with different pattern codes for host tree species.

of divergence are performed with a limited number of conspecific populations (Wahlberg et al. 2003, Roe and Sperling 2007a). Better sampling throughout the geographic range of the species should maximize sampling of mtDNA haplotype diversity and consequently minimize the effect of localized stochastic mutational anomalies.

Our study provides an opportunity to evaluate the variability of divergence rates between COI and COII in a species group with sequences available at different taxonomic levels (populations, sister species, and species groups). The high variability observed in intraspecific divergence rate (five times more in COII than in

COI within *D. abietella*) was unexpected and contrasts with the interspecific divergence rates in *Dioryctria* demonstrated by Roe and Sperling (2007a). We ruled out technical artifacts due to DNA contamination or PCR-sequencing errors. However, extreme sequence divergence in COII could reflect the presence of nuclear copies of mitochondrial DNA (numts) that had contaminated sequences of *D. abietella.* Nevertheless, there were neither ambiguous polymorphic sites, nor unexpected stop codons in any of the sequences analyzed, nor elevated numbers of amino acid changes. Numts have been more commonly found in plants than in animals and few studies have been reported in insects (Bensasson et al. 2001, Keller et al. 2007). The presence of numts was inferred but not proven in tropical Lepidoptera by Hajibabaei et al. (2006). Similar divergence variability between COI and COII also was observed within the longhorn beetle *Monochamus galloprovincialis* (Olivier), in which numerous numts have been detected (Koutroumpa et al. 2008).

Roe and Sperling (2007a) found that maximum intraspecific diversity in Lepidoptera, including *D. pentictonella* Mutuura, Monroe & Ross, was usually found in COI. Although such patterns of sequence variation may be due to random stochastic variability, they also may suggest that the common assumption of neutral molecular evolution in mtDNA is not justified. Nonrandom regional variation has previously been shown to occur in mtDNA (Broughton and Reneau 2006, Galtier et al. 2006), and several factors such as functional constraints, mutation hot spots, or adaptive substitutions could explain heterogeneous evolutionary rates observed in *Dioryctria* (Lunt et al. 1996, Stoneking 2000, Innan and Nordborg 2002).

Our study highlights the importance of considering other genes than COI, such as COII, as well as independent markers such as nuclear markers, when studying phylogenetic relationships between closely related species, especially if they display high genetic diversity or low interspecific divergence. In this context, it is reasonable to ask whether the COII gene is optimally informative by itself for reconstructing phylogenetic relationships of closely related species. Short fragments have commonly been used to identify

Table 4. Analysis of molecular variance (AMOVA) among European populations of *D. abietella*, with grouping by geographic region, by host species, and by origin of host

	Source of variation	Variance components	% of variation
Grouping by region[a]	Among groups	3,30165Va	51.08**
	Among populations within groups	0Vb	0
	Within populations	3,16250Vc	48.92*
Grouping by host[b]	Among groups	0,10220Va	1.85NS
	Among populations within groups	2,20925Vb	40.04NS
	Within populations	3,20596Vc	58.11**
Grouping by origin of host[c]	Among groups	4,04406Va	55.6*
	Among populations within groups	0,06708Vb	0.92*
	Within populations	3,16250Vc	43.48**

*, $P < 0.05$; **, $P < 0.01$; NS, nonsignificant.

[a] Three regions: 1, Alps (French and Italian); 2, central France (Les Barres Arboretum, Fontainebleau); and 3, southwestern France (Latronquire seed orchard).

[b] As in Table 1.

[c] Two groups: 1, natural forest; and 2, artificial plantations (see Table 1).

sister species, especially for DNA barcoding (Hebert et al. 2003a). However, such reliance on short fragments has been controversial (Wahlberg et al. 2003, Roe et al. 2006), especially when only a single specimen is used to define a lineage. Roe and Sperling (2007a), argued that it may be advantageous to focus on regions that give accurate and consistent estimates of divergences relative to longer mtDNA regions. They identified a 600-bp fragment as the best indicator of total COI-COII divergence (mean percent divergence of 100.7% relative to total COI-COII divergence) for sister species in Lepidoptera and Diptera. The COI gene fragment used in our study partially spanned this region and showed overall interspecific and intraspecific pairwise divergences similar to other Lepidoptera (Wahlberg et al. 2003, Blum et al. 2003, Du et al. 2005) (0–0.011). In contrast, the COII gene showed lower minimum interspecific divergence (0.14) and higher maximum intraspecific divergence (0.35) compared with distances previously recorded in *Dioryctria* species (Du et al. 2005, Roe et al. 2006).

When separate data sets (COI or COII) for phylogenetic reconstructions of *Dioryctria* species group were compared, COI gave a more accurate indication of species boundaries than COII (data not shown), all haplotypes being grouped according to their respective taxon relationship in COI, whereas the COII gene separated *D. abietella* into two clades. Incongruences between species trees and mtDNA trees have often been reported in closely related taxa (Avise 1991, Funk and Omland 2003, Ballard and Whitlock 2004). Thus, reliance on a single DNA region can be misleading, in part due to underestimates or overestimates of sequence divergence between taxa, particularly between sister pairs.

Intraspecific Variability of the Polyphagous European *D. abietella*. Although short fragments of COI have commonly been used to identify closely related species in Lepidoptera (Caterino et al. 2000) and for DNA barcoding (Hebert et al. 2003b), the combination of more than one DNA region (or longer DNA fragments) to identify closely related species or to distinguish populations is strongly supported (Wahlberg et al. 2003, Roe et al. 2006). In addition to the fact that most *D. abietella* populations in our study contained at least three specimens, the combination of two mtDNA fragments showing contrasting evolutionary rates, targeting a region of maximum divergence (COII gene that was more divergent than COI in this species), should improved resolution considerably in intraspecific analysis.

Analysis of molecular variance within *D. abietella* did not show any clear genetic differentiation among hosts (Table 4), even if most variation in the plains haplotypes was restricted to single host species (see Fig. 1 and Table 1). *D. abietella* is unusual among European members of its group in that it is relatively polyphagous, feeding on host plant species from a number of unrelated coniferous genera. According to numerous studies (Johnson et al. 1996, Funk and Omland 2003, Rundle and Nosil 2005), divergent habitat preferences are more likely to cause prezygotic iso-

lation when mating occurs in or near the preferred habitat, for example, between herbivorous insect populations that mate on the plant on which they feed. But according to Emelianov et al. 2001, mating behavior in many plant-feeding insects does not depend directly on host cues. Hence, in most Lepidoptera, the host plant is not required for mating, and females call for males using long-range pheromones. Because of its polyphagy, *D. abietella* populations are less likely to experience disruptive selection after shifts to novel host plants (Mopper and Strauss 1998, Berlocher and Feder 2002, Funk and Omland 2003). Furthermore, our sampling was performed on numerous exotic conifer trees that may not represent of the natural host range of the species. Additional studies of the ecology and genetics of this species are needed to investigate further insect-host relationships.

Results of the AMOVA revealed the presence of significant population structure, showing that 51% of the variation was due to the subdivision of populations by geographic origin (Table 4). This result was mainly due to the strong divergence of populations from the plains, fixed for a number of unique singleton haplotypes, compared with populations from the Alps that displayed the widespread haplotype abt1 and only one singleton (abt2). Furthermore, when populations are grouped by origin of stand (native forest versus artificial plantation), the percentage of variance accounted for is higher (56%), which is more indicative of geographic distribution of the stand than of the type of stand (i.e., native forests in Alps versus artificial plantations in plains localities, and Latronquière seed orchard versus the Les Barres Arboretum).

Distinct selection pressures could play a role in the difference between genetic diversity in populations from the Alps and those in the plains. For most insects that are specific to cones, annual fluctuations in resource availability are a major driving force governing their population dynamics (Turgeon et al. 1994). In the natural forests of the Alps, it is likely that coneworm populations have evolved together with the hosts and adapted to masting, i.e., substantial annual fluctuations in cone abundance. In contrast, the plain populations, and especially those developing within the Latronquière seed orchard, faced only limited fluctuations in annual cone crop because the orchard trees were submitted to treatments to promote annual flowering. So, diet breadth might be an important parameter in the observed genetic patterns, as can be observed in other forest insects (Kerdelhué et al. 2002), and the low genetic variability observed in the Alps could be due to more episodic cone production than populations from the plains. However, we cannot rule out the hypothesis that past climatic oscillations during the Quaternary period may have affected the patterns of genetic diversity of *D. abietella* (Hewitt 1996). The low genetic diversity in present-day populations from the natural forest in the Alps could be attributable to a single mountain refugium, whereas the high genetic diversity in introduced areas could result from multiple origins from different refugial sources during the ice ages or, more likely, from move-

ments of insects due to transportation of cones into these areas. It would be useful to conduct a more comprehensive phylogeographic study on this Palearctic species.

The unexpected discovery of two distinct groups of *D. abietella* haplotypes, which was particularly evident in the haplotype network (Fig. 3), may indicate the presence of at least two diverged lineages within *D. abietella.* The widespread abt1 haplotype found mainly in the Alps and in some plains localities is very close to the Chinese haplotype, and it is therefore most likely to represent the specimens originally described as *D. abietella* from the vicinity of Vienna (Wienergegend) in Austria (Denis and Schiffermüller 1776). The second haplotype group seemed to be much more difficult to define without further exploration. However, even species placed in different species groups may be difficult to distinguish. For example, damage from the trunk borer *D. splendidella* Schaeffer (Jactel et al. 1994) has long been confused with that of *D. abietella* in France or *D. schuetzeella*, because it is also known to attack cones of *P. abies* in Europe (Schwenke 1982). Nonetheless, we did not observe specimens that were morphologically diagnosable to *D. splendidella* or *D. schuetzeella* in our surveys, nor did we find any haplotypes similar to the *D. schuetzeella* mtDNA (Knölke et al. 2005). The recently identified *D. resiniphila* (Segerer and Pröse 1997) also will need to be considered in further analyses.

Molecular Systematics of the *abietella* Species Group. Our results from mtDNA sequence variation among species are congruent with the findings of Du et al. (2005) and Roe et al. (2006), with *D. abietivorella* and *D. abietella* supported as a separate species. The results confirm the original diagnosis of Munroe (1959) who separated *D. abietella* from *D. abietivorella* using external and internal morphological criteria, including larger size, darker hind wings, more conspicuous pale markings, more transverse dark lines on forewings, and a different configuration of the male valva in *D. abietella.* Numerous other studies have advocated the use of mtDNA sequences as a valuable marker for identifying closely related species, especially where morphological differences are subtle, in some cases confirming and in others refuting previous interpretations (Sperling and Hickey 1994, Sperling et al. 1995, Caterino and Sperling 1999, Cognato et al. 1999, Kerdelhué et al. 2002, Kruse and Sperling 2001, Wahlberg et al. 2003, Damgaard and Cognato 2006).

The topology of the phylogenetic reconstruction shown in Fig. 2 reveals the monophyly of the *abietella* group and its separation from members of the three other *Dioryctria* groups (*sylvestrella, schuetzella,* and *zimmermani*) as defined by Mutuura and Munroe (1972) and Neunzig (2003). When considering the mitochondrial data set, within-species group genetic distances (1.1–4.5%) were always lower than between-species group pairwise distances (5.7–8.6%). A recent study of the same genus showed similar results but more overlap, with sequence divergence ranging from 0.3 to 5.6% among species within groups and 3.3 to 9.2% among species in different groups (Du et al.

2005). Low rates of overlap in mtDNA genetic distances within and between species have been found in other Lepidoptera. In Neotropical Nymphalidae, for example, distances ranged from 3 to 5% between sister species and from 5 to 8% between species in the three separate phyletic lineages defined in the genus *Anartia* (Blum et al. 2003).

Both MP and ML analyses strongly supported clade A, which comprises the two North American species (*D. abietivorella* and *D. ebeli*). mtDNA genetic distances between *D. abietivorella* and *D. abietella* ranged between 3.3 and 4.2% (mean sequence divergence of 0.037, whatever the fragment length considered, i.e., 1975, 451, or 572bp). This result was similar to divergence observed within Tortricidae for the *Argyrotaenia franciscana* species group (Landry et al. 1999), although these pairwise divergences were higher than these found between most closely related species or within species complexes in Lepidoptera. For example, within the spruce budworm species complex, divergences ranged from 2.7 to 2.9% between *C. fumiferana* and the other members of the group, divergences between these other members were all <1% (Sperling and Hickey 1994). Sequence divergence within the *Archips argyrospila* complex ranged from 1.47 to 2.53% between populations of *A. argyrospila* (Walker) and *A. goyerana* Kruse (Kruse and Sperling 2001). Less than 1% divergence was observed among three species of ermine moths (Yponomeutidae) (Sperling et al. 1995). In contrast, some swallowtail butterfly species groups showed higher sequence divergences, ranging from 2.6 to 5.4% in the *Papilio machaon* group, from 1.3 to 3.7% in the *P. glaucus* group, and from 7.3 to 9.4% in the *P. dardanus* group (Caterino and Sperling 1999).

The high variability of sequence divergence between closely related species of Lepidoptera implies that it is not a good predictor of whether two unknown populations constitute reproductively isolated species (Landry et al. 1999, Sperling 2003, Cognato 2006). Nevertheless, sequence divergence observed within the *D. abietella* species group most likely reflects relatively recent separation of the mitochondrial lineages, during the Quaternary ice ages, according to the mtDNA clock of Brower (1994). This pattern has been observed in other sibling species of forest insects (Emelianov et al. 1995, Boato and Battisti 1996, Cognato et al. 1999, Stauffer et al. 1999, Nice and Shapiro 2001). Although *D. abietella* and *D. abietivorella* have very close morphological characters and similar degrees of polyphagy and broad geographic ranges, they differ strongly in their distributions, the first species being Palearctic and the second species Nearctic. According to Hewitt (1996), most species are confined to continents and closely related species often occupy different parts of a continent.

European *Dioryctria* species of the *abietella* group fall into two major groups, one groups comprises species that develop on Mediterranean pinecones (*D. mendacella* and *D. pinae*), and the other group comprises species that develop on cones of more northern conifers (*D. abietella* and *D. simplicella*). The delim-

itation between *D. mendacella* and *D. pineae* is not clear. The fact that specimens of *D. mendacella* collected on the same host as *D. pineae* fell into two distinct haplotype lineages could indicate a complex of three cryptic species instead of two, because the two currently recognized species can be found in sympatry on the same hosts [*Pinus pinaster* (Aiton), *P. halepensis* Miller, and *P. pinea*, Roques 1983; and *Pinus brutia* Tenore, Karanicola 1998]. Nevertheless, further investigation with more extensive sampling of their ecology and genetic variation, especially with nuclear markers, is needed to clarify this result.

The clade represented by *D. abietella* and *D. simplicella* seemed even more problematic. The separation between the two species was not well supported, and three *D. abietella* specimens collected on *P. menziesii* and *P. smithiana* and showing the abt12 haplotype clustered together with specimens of *D. simplicella* collected on *P. sylvestris.* These three specimens were collected in different localities from *D. simplicella*, and, to date, *D. simplicella* has not been found on *P. menziesii* or on *P. smithiana.* This species is recorded from cones as well as shoots of diverse coniferous species including *P. sylvestris* (Charles and Roques 1977). The morphological characters of adults were clear and no differences in genitalia were noted between these specimens, whereas the genitalia of *D. simplicella* differ greatly from those of *D. abietella* (Zocchi 1961). Nevertheless, it seems plausible that abt12 specimens were *simplicella* specimens. Further sampling is needed to clarify the incongruence between morphological and molecular data.

For the North American species, the identity of the two specimens labeled as *D. ebeli* is open to question, because the *D. ebeli* mtDNA haplotype showed only 0.1–0.6% divergence from *D. abietivorella* haplotypes. Other *Dioryctria* species also display low sequence divergence [<1.8% separate *D. zimmermani* (Grote), *D. tumicolella* Mutuura, Monroe & Ross, and *D. taedivorella* Neunzig & Leidy], but morphological characters were not effective in confirming the distinctness of these lineages (Du et al. 2005). As for the European species of the *abietella* group, additional sampling is needed to resolve the specific status of *D. ebeli.*

Our study confirmed that nucleotide diversity within and between taxa was quite variable across both COI and COII genes. Because divergences are low between sister species of *Dioryctria*, it is crucial to target regions with maximum divergence to ensure the greatest probability of consistently delimiting species boundaries by sequencing regions with the most informative nucleotide variation (Roe and Sperling 2007a). Mitochondrial DNA data may compensate for insufficient information from morphological characters, especially at the species and species group levels, but it also shows that currently recognized taxonomic relationships, based on morphological similarities and host plant origin, need to be reevaluated in the European *D. abietella* species complex. Because an integrative approach is essential to testing species delimitations (Roe et al. 2007b), multiple independent

markers such as independent molecular loci (nuclear markers), morphology, larval host plant and geographic range also should be considered to be confident of species delineations.

Acknowledgments

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