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Mitochondrial phylogeny of pine cone beetles (Scolytinae, *Conophthorus*) and their affiliation with geographic area and host

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Abstract

Pine cone beetles (*Conophthorus* spp.) feed and kill immature cones of *Pinus* species, thereby reducing seed production and seriously impairing reforestation of forest ecosystems. Population variation of *Conophthorus* reproductive behavior has hampered the development of semiochemical control of these pests. This difficulty is compounded by a lack of taxonomic knowledge and species diagnostic characters. Researchers and managers rely, in part, on host associations and geographic locality for species identifications and these have arguable taxonomic utility. However, host use and/or geographic separation may influence *Conophthorus* lineage diversification. To improve *Conophthorus* taxonomy and understand the association of host and geography with lineage diversification, a phylogeny of 43 individuals, including all valid species and a robust sample of *C. ponderosae* from different hosts, is reconstructed using 785 nucleotides of the 3′-end of the mitochondrial cytochrome oxidase I gene. Thirty trees were recovered in a parsimony analysis and the strict consensus was well resolved and supported by branch support measures. *Conophthorus* was monophyletic but mitochondrial polyphyly was uncovered for several species. The data also suggested an underestimation of species diversity. Phylogenetically related *Conophthorus* lineages were significantly associated with geographic proximity but not with host, as indicated by comparisons of character optimized geographic distributions and host associations against randomized distributions of these attributes on the parsimony tree. These results suggest that geographic separation better explains the mode of *Conophthorus* lineage diversification than does host specialization. Based on these results, researchers and managers of *Conophthorus* should consider populations as potentially different evolutionary entities until species boundaries are delineated via a robust phylogenetic revision of *Conophthorus*.

Keywords: Scolytidae; Molecular systematics; Forest pest; Host use; Evolution

1. Introduction

Pine cone beetles (*Conophthorus* spp.) seriously impair reforestation of forest ecosystems that have been subjected to harvest, wildfires, or pest epidemics (Cibrián-Tovar et al., 1986; Hedlin et al., 1980; Rappaport, 1995). Thirteen species are recognized in the genus and all attack North American pines (*Pinus* spp.) in commercial and natural pine plantations (Wood and Bright, 1992). Relationships among species and populations remain unknown, and such phylogenetic information is potentially useful in understanding the evolution

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of the host associations of the species as well as in designing more effective control programs.

Pine cone beetles do not kill their host trees but oviposition in young cones destroys crops of many economically and ecologically important pines such as *P. ponderosa*, *P. monticola*, and *P. lambertiana* (Kinzer et al., 1970). Many haploxylon (soft) pines are crucial to wildlife species (Graham, 1990; Keane and Arno, 1993; Kendall and Arno, 1990; Kinloch and Scheuner, 1990; Lanner, 1993), but populations of these pines are declining because of their susceptibility to white pine blister rust (*Cronartium ribicola*). These pines depend completely on artificial regeneration for their maintenance in western forest ecosystems and genetic resource programs are in place to develop reliable sources of disease-resistant seed (Zobel, 1971). However, pine cone beetles can greatly reduce the harvestable seed crop. For example, Keene (1958) reported losses of at least 90% of sugar pine seed, and damage to *P. rudis* and *P. hartwegii* in Mexico fluctuates between 40 and 87% of the total cone crop (Cibrián-Tovar et al., 1986). Elucidation of behavioral chemicals has great promise for monitoring and control of these insects (Birgersson et al., 1995; Pierce et al., 1995; Rappaport et al., 2000). However, different populations of *C. ponderosae* beetle respond to different chemicals, which gives reason to question the taxomomic boundaries of this species (Rappaport et al., 2000). The ability to implement semiochemical monitoring and control methods is hampered by this and other systematic problems throughout the genus *Conophthorus*.

Conophthorus species are morphologically similar and the taxonomic characters that distinguish them are poorly defined. Many species were described based on host utilization (Hopkins, 1915). Species identification solely on the basis of host use is considered suspect and has resulted in many species synonymies (Wood, 1977; Wood and Bright, 1992) (Table 1). However, chemical and genetic data suggest that these synonymies are questionable and host use polymorphism may promote lineage diversification. Differences in cuticular hydrocarbon profiles suggest a degree of genetic isolation between *C. ponderosae* from different hosts (Page et al., 1990). Other factors, such as cone attack behavior, body size, and host selection behavior in areas of host sympatry, support the hypothesis that *C. ponderosae* may represent more than one species. This is not surprising given that cryptic species are common

Table 1
Recognized *Conophthorus* species, synonymies, and biological data (Cibrián-Tovar et al., 1986; Wood and Bright, 1992)

Species	Synonymies	Distribution	*Pinus* spp hosts
apachecae Hopkins	None	MEX: Chihuahua, Durango, USA: SE AZ	*engelmannii*
conicolens Wood	None	MEX: Colima, Distro Federal, Durango, Estado Mexico, Hidalgo, Jalisco, Michoacan, Morelos, Puebla, Tlaxcala	*douglasiana*, *leiophylla*
coniperda (Schwarz)		CAN: Nova Scotia, Ontario, Quebec USA: ME, MD, MA, MI, MN, NH, NY, NC, PA, VA, WV, WI	*strobus*
	taedae Hopkins	USA: VA	*taeda*
	clunicus Hopkins	USA: no other data given	Not recorded
echinatae Wood	None	USA: MO	*echinata*
edulis Hopkins		USA: AZ, CO, NM, TX, UT	*edulis*, *discolor*
	cembroides Wood	MEX: Baja California Sur, Chihuahua, Coahuila, Durango, Guanajuato, Hidalgo, Nuevo Leon, Queretaro, Puebla, San Luis Potosi, Sonora, Zactecas, USA: SE AZ	*cembroides*
mexicanus Wood	None	MEX: Hidalgo, Puebla, Veracruz	*patula*
michoacanae Wood	None	MEX: Michoacan	*michoacana*
monophyllae Hopkins	None	MEX: Baja California Norte, USA: CA, ID, NV, UT	*monophylla*
ponderosae Hopkins		MEX: Chihuahua, Coahuila, Distro Federal, Durango, Estado Mexico, Hidalgo, Jalisco, Michoacan, Morelos, Nuevo Leon, Puebla, Sonora, Tlaxcala, Veracruz, USA: OR	*aristata*, *arizonica*, *ayacahuite*, *cooperi*, *douglasiana*, *durangensis*, *jeffreyi*, *leiophylla*, *montezumae*, *ponderosa*, *pringlei*, *pseudostrobus*, *rudis*, *strobiformis*, *washoensis*
	contortae Hopkins	USA: OR	*contorta*
	flexilis Hopkins	USA: CO	*flexilis*
	lambertianae Hopkins	USA: CA	*lambertiana*
	monticolae Hopkins	CAN: British Columbia, USA: ID	*monticola*
	scopulorum Hopkins	USA: AZ, CO, AZ	*ponderosa*
radiatae Hopkins	None	USA: CA	*radiata*
resinosae Hopkins		CAN: Nova Scotia, USA: MN. NH, NJ, NY, WI	*resinosa*
	virginianae Hopkins	USA: WV	*virginiana*
	banksianae McPherson	CAN: Ont., Que., USA: MI	*banksiana*
teocotum Wood	None	MEX: Estado Mexico, Michoacan	*teocote*
terminalis Flores & Bright	None	MEX: Nuevo Leon	*cembroides*

among bark and pine cone beetles (de Groot et al., 1992; Lanier and Wood, 1968). Recent progress has been made in resolving taxonomic ambiguities in eastern North American *Conophthorus* species (de Groot and Ennis, 1990; de Groot, 1991; de Groot and Borden, 1991; de Groot et al., 1992). This work, which was based on ethology, cytogenetics, allozyme electrophoresis, and behavioral chemistry, resulted in the synonymy of *C. resinosae* and *C. banksianae*, but supported the validity of *C. coniperda*.

While these studies provided diagnostic characters, they did not deal with *Conophthorus* phylogeny. It is now widely recognized that a phylogeny may provide an evolutionary basis for the delimitation of taxa, given the criterion of monophyly (Hennig, 1966), and is useful for evaluating ecological hypotheses (Brooks and McLennan, 1991). Phylogenetic analysis of DNA sequences can provide taxonomic resolution for taxa that are morphologically indistinguishable (Cognato and Sperling, 2000; Scheffer and Lewis, 2001; Simon et al., 1994). Mitochondrial DNA (mtDNA) sequence data are particularly useful for species-level phylogenetic reconstruction (Caterino et al., 2000). Coalescence times for maternally inherited genes are generally faster than for nuclear genes and thus mtDNA is more likely to resolve hierarchical relationships for closely related species, and to reflect recent speciation associated with host use or geographic separation (Avise, 2000). In addition, mtDNA has already been demonstrated to provide phylogenetic information at the population and species levels for bark beetles (Cognato et al., 1999, 2003; Cognato and Sperling, 2000; Jordal et al., 2004; Kelley and Farrell, 1998; Stauffer et al., 1999).

This study tests whether *Conophthorus* species taxa (mainly *C. ponderosae*) are evolutionary entities (in this case, mtDNA lineages) via cladistic analysis of mtDNA sequences (Hey et al., 2003). Haplotypes that represent species taxa should be monophyletic for each taxon, if they are congruent with evolutionary entities. This means of species delimitation is similar to the cladistic haplotype aggregation method except that our initial species taxa hypotheses are based on published species taxonomy instead of local populations (Brower, 1999; Sites and Marshall, 2003). We find that the *C. ponderosae* species taxon is not a monophyletic group and haplotypes that represent this taxon are grouped with several other *Conophthorus* species taxa. Consequently, we hypothesize alternative taxa that correspond to evolutionary entities. Haplotypes are grouped based on monophyly and are recognized (ranked) based on the average percent sequence divergence observed for *Conophthorus* species taxa that are monophyletic and morphologically diagnosable. This is an objective ranking criterion based on a sequence divergence that is associated with diagnostic characters of species taxa (Mishler and Brandon, 1987; Sperling, 2003). These taxa (referred to in this study as evolutionary significant clades, ESCs) represent hypotheses of evolutionary entities that are testable with additional data (Hey et al., 2003).

In addition, we explore factors that may have influenced *Conophthorus* lineage diversification. Many *Conophthorus* species were described because of their association with different pine species (Hopkins, 1915), which suggests that host use may influence speciation as observed with other scolytids (Kelley et al., 1999; Kerdelhué et al., 2002). However, speciation and/or lineage diversification due to geographic isolation is well documented for many taxa (e.g., Mayr, 1970; Peterson and Denno, 1998). Both factors can contribute to lineage diversification but we expect that the factor with greater influence, if any, would have the strongest phylogenetic association with *Conophthorus* ESCs and deeper clades.

For this study, the 3′ half of the mtDNA cytochrome oxidase I gene (COI) is used to reconstruct a phylogeny of all recognized *Conophthorus* species, several selected *Conophthorus* populations that may represent additional species, and five outgroups. We then use this phylogeny to evaluate *Conophthorus* taxonomy and to assess the association of beetle ESCS and deeper clades with host and geography.

2. Materials and methods

2.1. Specimens and DNA sequence preparation

A total of 13 species from 43 populations and closely related outgroups (Table 2) were included in this study. The outgroup species are represented by specimens from three genera and include one species (*Pityophthorus schwerdtfergeri*) that feeds on both *Pinus* twigs and cones. Live beetles were excised from infested host cones with a knife and forceps and stored in 100% ethanol. A. Cognato and R. Bolaños identified all beetles using the diagnostic morphological characters of Wood (1982) and Bolaños (2002). Total genomic DNA was extracted from beetle thoraces with a silica-based spin column procedure (i.e., Qiamp, Qiagen, Santa Clara, CA), following the manufacturer's tissue protocol. A final elution volume of 200 µl of buffer EB was used. The remaining body parts were glued to a mounting board or pointed and pinned. These specimens are vouchered in the Texas A&M University Department of Entomology insect collection.

A region of approximately 830 nucleotides of mtDNA COI was amplified via the polymerase chain reaction (PCR) with primer pairs C1-J-2183 and C1-N-2611 (Cognato and Sperling, 2000; Simon et al., 1994) and C1-J-2441 and TR2-N-3014 (Simon et al., 1994) following the methods of Cognato and Sperling (2000). Each PCR contained: 35 µl ddH_2O, 5 µl of 10× *Taq* DNA polymerase buffer (Promega, Madison, WI), 4 µl of 25 mM Promega $MgCl_2$, 1 µl of 40 mM deoxynucleotide

Table 2
Collection data for *Conophthorus* and outgroup species

Species	Location	Latitude, longitude	Host	Host subgenus	Host subsection	Location >900	Location 200–300 km	Haplotype	ESC	Clade
C. apachecae Hopkins	MEX: Durango	25.21N 105.44W	*P. engelmannii*	*Pinus*	*Ponderosae*	1	1	A	I	γ
C. conicolens Wood	MEX: Michoacan	19.24N 102.08W	*P. pseudostrobus*	*Pinus*	*Ponderosae*	1	3	B	M	δ
C. coniperda (Schwarz)	1. USA:MD: Anne Arundel Co.	38:58:18N 76:30:11W	*P. strobus*	*Strobus*	*Strobi*	3	10	C	E	β
	2. USA:NY: Suffolk Co.	40:46:49N 72:54:47W	*P. strobus*	*Strobus*	*Strobi*	3	10	C	E	β
	3. USA:RI: Lincoln Co.	41:49:19N 71:25:11W	*P. strobus*	*Strobus*	*Strobi*	3	10	C	E	β
	4. USA:WV: Pendleton Co.	38:40:15N 79:29:29W	*P. rigida*	*Pinus*	*Australes*	3	10	C	E	β
	5. CAN:Ontario	45.00N 77.80W	*P. strobus*	*Strobus*	*Strobi*	3	11	C	E	β
	6. CAN:Ontario	45.00N 77.80W	*P. strobus*	*Strobus*	*Strobi*	3	11	C	E	β
C. echinatae Wood	USA:MI: Carter Co.	36:57:08N 91:09:51W	*P. echinata*	*Pinus*	*Australes*	3	12	D	F	β
C. edulis Hopkins	1. USA:UT: Kane Co.	37:04:42N 113:34:43W	*P. edulis*	*Strobus*	*Cembroides*	2	5	E	K	γ
	2. USA:AZ: Cochise Co.	32.00N 110.00W	*P. cembroides*	*Strobus*	*Cembroides*	2	4	F	K	γ
	3. MEX: Hidalgo	20.36N 99.08W	*P. cembroides*	*Strobus*	*Cembroides*	1	3	G	K	γ
C. mexicanus Wood	MEX: Hidalgo	20.05N 98.22W	*P. patula*	*Pinus*	*Oocarpae*	1	3	H	E	β
C. michoacanae Wood	MEX: Michoacan	19.25N 102.04W	*P. michoacana*	*Pinus*	*Ponderosae*	1	3	I	J	γ
C. monophyllae Hopkins	USA:CA: Riverside Co.	33:56:26N 117:23:51W	*P. monophylla*	*Strobus*	*Cembroides*	2	6	J	K	γ
C. ponderosae Hopkins	1. USA:CA: Calaveras Co.	38:13:56N 120:22:10W	*P. ponderosa*	*Pinus*	*Ponderosae*	2	7	L	A	α
	2. USA:CA: Siskiyou Co.	41:38:39N 121:58:11W	*P. ponderosa*	*Pinus*	*Ponderosae*	2	7	Q	A	α
	3. USA:ID: Bonner Co.	33:42:15N 116:43:30W	*P. ponderosa*	*Pinus*	*Ponderosae*	2	9	R	C	α
	4. USA:ID: Kootenai Co.	48:16:48N 116:33:29W	*P. monticola*	*Strobus*	*Strobi*	2	9	R	C	α
	5 CAN: British Columbia	48:37N 123:24W	*P. monticola*	*Strobus*	*Strobi*	2	9	X	C	α
	6. CAN: British Columbia	49:15N 123:04W	*P. contorta*	*Pinus*	*Contortae*	2	9	W	C	α
	7. CAN:British Columbia	49:15N 123:04W	*P. monticola*	*Strobus*	*Strobi*	2	9	W	C	α
	8. USA:CA: El Dorado Co. 1	38:45:08N 120:34:14W	*P. lamberitana*	*Strobus*	*Strobi*	2	7	M	C	α
	9. USA:CA: El Dorado Co. 2	38:45:08N 120:34:14W	*P. lamberitana*	*Strobus*	*Strobi*	2	7	M	C	α
	10. USA:CA: Mendicino Co.	39:00:33N 123:21:54W	*P. lamberitana*	*Strobus*	*Strobi*	2	7	O	C	α
	11. USA:CA: Riverside Co.	33:42:15N 116:43:30W	*P. ponderosa*	*Pinus*	*Ponderosae*	2	6	P	C	α
	12. USA:CA: San Bernardino Co.	34:08:23N 117:17:32W	*P. ponderosa*	*Pinus*	*Ponderosae*	2	6	P	C	α
	13. USA:CA: Fresno Co.	37:06:15N 119:19:00W	*P. lamberitana*	*Strobus*	*Strobi*	2	7	N	C	α
	14. USA:CA: Riverside Co.	48:16:48N 116:33:29W	*P. lamberitana*	*Strobus*	*Strobi*	2	6	T	D	α
	15. MEX: Baja California	30.45N 115.13W	*P. lamberitana*	*Strobus*	*Strobi*	2	6	Z	D	α
	16. USA:AZ: Coconino Co.	36:42:48N 112:12:56W	*P. ponderosa*	*Pinus*	*Ponderosae*	2	5	K	E	β
	17. USA:NV: White Pine Co.	39:00:48N 114:07:19W	*P. flexilis*	*Strobus*	*Strobi*	2	5	V	E	β
	18. USA:CO: Park Co.	39:22N 105:48W	*P. aristata*	*Strobus*	*Balfouriana*	2	8	S	E	β
	19. USA:NV: Clark Co.	36:12:21N 115:13:22W	*P. flexilis*	*Strobus*	*Strobi*	2	5	U	I	γ
	20. MEX: Durango	25.21N 105.44W	*P. arizonica*	*Pinus*	*Ponderosae*	1	1	AA	I	γ
	21. MEX:Mexico	19.02N 98.38W	*P. hartwegii*	*Pinus*	*Ponderosae*	1	3	Y	I	γ
C. radiatae Hopkins	USA:CA: Alameda Co.	37.57N 122.21W	*P. radiata*	*Pinus*	*Oocarpae*	2	7	AB	B	α
C. resinosae Hopkins	1. CAN: Ontario	45.00N 77.80W	*P. banksiana*	*Pinus*	*Contortae*	3	11	C	E	β
	2. CAN: Ontario	45.00N 77.80W	*P. resinosa*	*Pinus*	*Sylvestres*	3	11	C	E	β

(*continued on next page*)

Table 2 (*continued*)

Species	Location	Latitude, longitude	Host	Host subgenus	Host subsection	Location >900	Location 200–300 km	Haplotype	ESC	Clade
C. teocotum Wood	MEX: Michoacan	19.25N 102.04W	*P. teocote*	*Pinus*	*Ponderosae*	1	3	AC	L	δ
C. terminalis Flores & Bright	MEX: Neuvo Leon	24.50N 100.04W	not recorded			1	2	AD	H	β
Conophthorus sp. 1	MEX:Mexico	19.13N 98.48W	*P. leiophylla*	*Pinus*	*Leiophyllae*	1	3	AE	G	β
Conophthorus sp. 2	MEX:Mexico	19.30N 99.00W	*P. montezumae*	*Pinus*	*Ponderosae*	1	3	AF	E	β
Dendroterus striatus (LeConte)	USA:CA: San Diego Co.		*Bursera microphylla*							
Pityophthorus setosus Blackman	USA:CA: Monterey Co.		*P. radiata*							
Pityophthorus schwerdtfergeri (Schedl)	MEX:Chiapas		Not recorded							
Pseudopityophthorus pubipennis (LeConte)	USA:CA: Alameda Co.		*Quercus* sp.							
Pseudopityophthorus minutissimus (Zimmermann)	USA:NY: Suffolk Co.		*Quercus* sp.							

triphosphates (dNTPs), 2 μl of each 5 mM oligonucleotide primer, 0.2 μl Promega *Taq* DNA polymerase, and 1 μl DNA template (containing between 1 and 5 ng). The PCR was performed on a thermal cycler (MJ Research, MA) under the following conditions: 1 cycle for 3 min at 95 °C, 0.75 min at 45 °C, 1 min at 72 °C followed by 34 cycles of 0.5 min at 94 °C, 0.75 min at 45 °C, 1 min at 72 °C, and a final elongation cycle of 5 min at 72 °C.

Unincorporated dNTPs and oligonucleotides were removed from PCRs with a Qiaquick PCR Purification Kit (Qiagen, Santa Clara, CA) and were directly sequenced on an ABI 377 automated sequencer following a BigDye (Applied Biosystems, Foster City, CA) fluorescent chemistry reaction. Both sense and anti-sense strands were sequenced for all individuals and edited consensus sequences were submitted to GenBank (AY974092–AY97139).

2.2. Sequence analysis

Electropherograms were edited with Sequence Navigator (Applied Biosystems, Foster City, CA). PCR products were re-amplified and/or re-sequenced for electropherograms that had many base-call ambiguities, although this was an uncommon occurrence. Primer sequences and ambiguous nucleotides were removed from the 5′- and 3′-ends of each consensus sequence, which resulted in 785 nucleotides for cladistic analysis. The alignment of individual sequences was trivial because of amino acid conservation. No nucleotide insertions or deletions were observed. Nucleotide divergence, cladistic relationships, and branch support were analyzed with the program PAUP* (Swofford, 2002). Phylograms were generated under a parsimony optimality criterion. A heuristic search for the most-parsimonious phylograms was performed with 100 replicates of random step-wise addition of haplotypes and branch swapping via tree-bisection-reconnection. All other settings were default. Bootstrap proportions were determined with 500 replicates and default PAUP* settings. Branch support (Bremer, 1994) for individual nodes was assessed using constraint trees generated with TreeRot (Sorensen, 1996). Nucleotide changes were parsimoniously optimized on the resulting tree and graphed using MacClade 4 (Maddison and Maddison, 2000) to compare the frequency of changes to a Poisson distribution (Olmstead et al., 1998). Similarity to this distribution would suggest that nucleotide change is stochastic and equiprobable, and therefore consistent with a Jukes–Cantor model of nucleotide substitution (Jukes and Cantor, 1969; Olmstead et al., 1998). To test for potential saturation of character change, branch lengths were estimated with a Jukes–Cantor model and plotted against branch lengths estimated with a Tamura–Nei model of nucleotide substitution, which accounts for unequal

nucleotide frequency, variation in substitution rates, and transition bias (Tamura and Nei, 1993). An asymptotic relationship between models is interpreted as saturation of character change.

2.3. Host, geographic, and operational taxonomic unit phylogenetic patterns

Pine host species based on the taxonomy of Critchfield and Little (1966) were recorded for each operational taxonomic unit (OTU) (Table 2). We also recorded the respective sub-genera and sub-sections for each OTU, because phylogenetic patterns may be more apparent at higher taxonomic levels. Monophyly of most of these taxa is well supported except for the cembroides sub-section, which is not a problem for this study because the pinyon hosts of our sampled beetles are monophyletic (Liston et al., 1999). Pattern of host use and estimation of ancestral states were inspected with MacClade 4 under ACCTRAN character state optimality (Maddison and Maddison, 2000).

Geographic distance was used to group *Conophthorus* specimen localities (Table 2), which allowed localities to be nested by proximity. Latitude and longitude coordinates were used to estimate distances (kilometers) between collection sites with a web-based distance calculator (http://www.indo.com/cgi-bin/dist). This program corrected for global circumference. These distances were arranged into a pair-wise distance matrix (not shown) that was further modified into a NEXUS format (Maddison et al., 1997) for use with PAUP*. Unweighted pair group method with arithmetic mean (UPGMA) analysis was used to group the populations based on geographic proximity (not shown). Two levels of proximity were chosen to investigate the effect of area size on lineage diversity. These areas are greater than the maximum distance (~50 km) seasonally traveled by bark beetles (Jactel and Gaillard, 1991). Areas of >900 km proximity and within 200–300 km proximity were grouped. Three areas (1, 2, and 3) which approximated distributions in Mexico, Western North America (NA), and Eastern NA were designated as areas of >900 km proximity. Fifteen areas were designated as areas within 200–300 km proximity but generalization to geopolitical areas was not made. These locations were recorded for the OTUs as character states (Table 2) and patterns of geographic location were inspected with MacClade 4 under ACCTRAN character state optimality (Maddison and Maddison, 2000).

A null distribution of random host and geographic patterns was generated for the resulting phylogeny (Havery and Pagel, 1991). Using MacClade 4, character states for each OTU were randomized for 1000 replications. These random character states were mapped on the phylogeny, the number of character state changes was calculated for each replicate, and the mean and standard deviation was calculated. Host and geographic patterns were deemed random, if the value of the original character state change fell within the standard deviation.

OTUs were grouped as haplotypes, members of evolutionary significant clades, and members of larger groups of clades (Table 2). Haplotypes were initially considered as OTUs because of the uncertain species limits (Vrana and Wheeler, 1992) and were determined as unique nucleotide sequences. As discussed in the introduction, monophyletic groups of haplotypes and/or species were considered because species limits are questionable for some species taxa. Current taxonomy may not reflect evolutionary patterns between *Conophthorus* species taxa, their hosts and geographic proximity. The average nucleotide divergence under a Jukes–Cantor model for all species excluding those that have poor diagnostic morphological characters (*C. coniperda*, *C. ponderosae*, and *C. resinosae*) is 1.0% (Fig. 1). Thus evolutionarily significant clades were determined as haplotypes or clades of haplotypes that exhibited >1.0% mean nucleotide difference as compared to a sister haplotype or clade (Table 2). These evolutionarily significant clades were further grouped into larger clades that exhibited >2.5% mean nucleotide difference as compared to a sister clade (Table 2). Random associations (χ^2 values) between location, host, and clades (Table 2) were tested using 1000 permutations in Monte Carlo simulation (Posada, 2001). Random associations between haplotypes, locations or host were not tested because most haplotypes are represented by a single individual.

Using the Mantel test (Casgrain and Legendre, 2001), isolation by distance was tested against pair-wise nucleotide differences under a JC model and geographic distance for all individuals. Geographic distances (kilometers) were estimated with the same web-based distance calculator as above.

3. Results

3.1. Cladistic analysis

Thirty equally parsimonious trees were found for the *Conophthorus* individuals and outgroup species (Fig. 1). The strict consensus of these trees was mostly resolved except for clades with zero branch support (Table 3, Fig. 1). Many clades exhibited relatively high branch support values including many internal branches (Table 3). *Conophthorus* is a well-supported monophyletic group that shares a sister-taxon relationship with *Pityophthorus* (Table 3, Fig. 1). Mexican species, *C. teocotum* and *C. conicolens* (clade δ), are basal and the remaining species are divided into three clades (α, β, and γ; Fig. 1). Several species are not monophyletic, and *C. ponderosae* represents an extreme case with individuals found in all three clades. Clade α contains *C. radiatae* and *C. ponderosae*

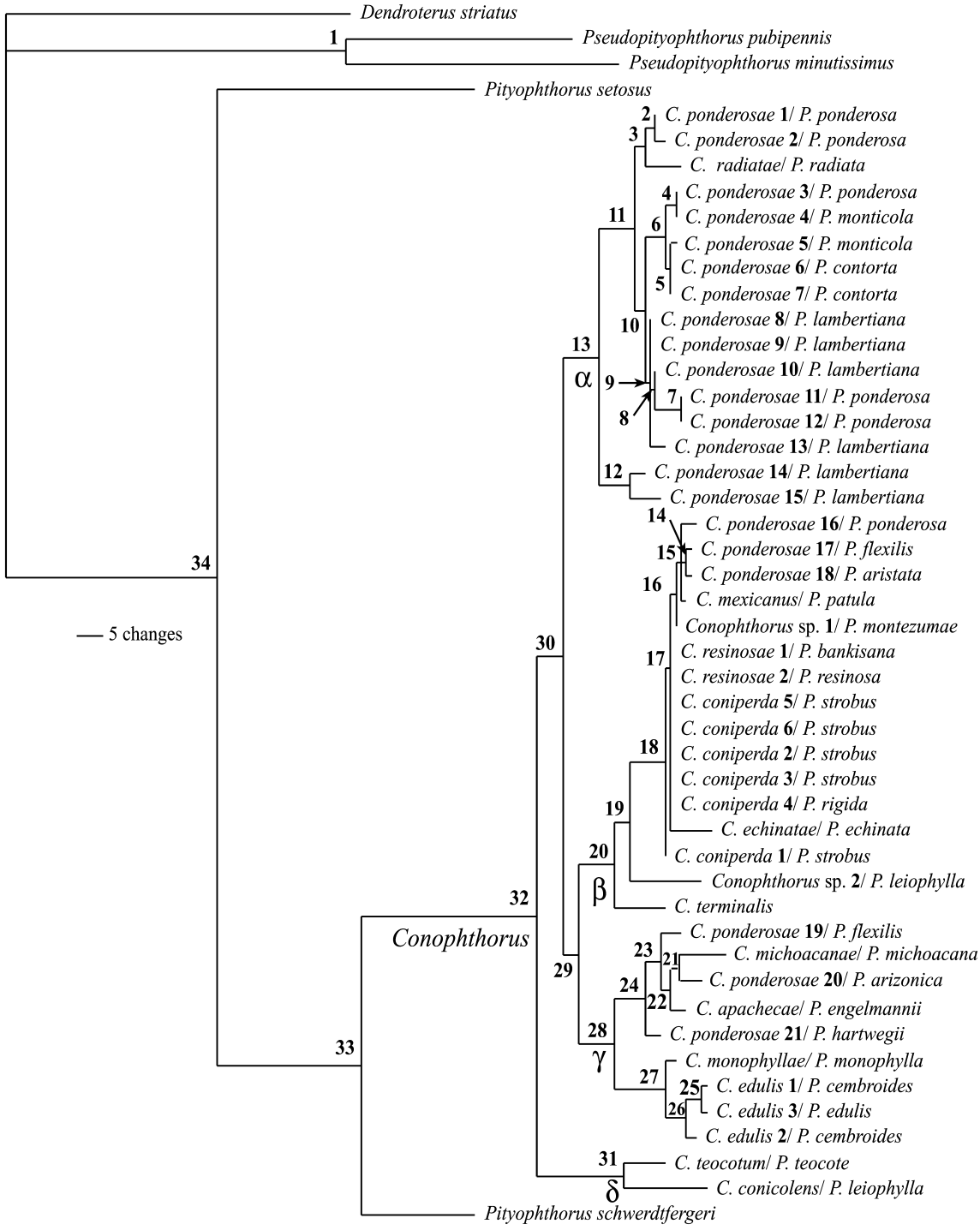

Fig. 1. Phylogram of *Conophthorus* individuals/hosts inferred from 785 nucleotides of mtDNA COI gene. The tree is 1 of 30 most-parsimonious trees found in a heuristic search using PAUP* (see text for details of phylogeny reconstruction). Numbers above branches refer to specific clades and branch support values given in Table 3. Clades 15, 16, 17, and 20 are unresolved in a strict consensus of the most-parsimonious trees. Clades designated by symbols below branches are discussed in the text. CI = 0.83, RI = 0.90, RC = 0.75.

individuals collected from localities from the Pacific Northwest of North America and from four hosts (Table 2, Fig. 1). Clade β contains the Eastern NA *Conophthorus* species, *C. ponderosae* from the Rocky Mountains, and several Mexican species. Little to no nucleotide variation was observed for the Eastern NA *Conophthorus*, and their relationships were unresolved (Table 3, Fig. 1). Clade γ contains two clades, one of which contains *C. ponderosae* from Mexico and *C. michoacanae*, while the other clade contains *Conophthorus* species that feed on pinyon pine.

Table 3
Branch support and bootstrap values for the *Conophthorus* phylogram (Fig. 1)

Clade	Bootstrap value	Partition branch support			
		First	Second	Third	Total
1	100	5	−1	16	20
2	89	0	0	2	2
3	65	0	0	2	2
4	85	0	0	2	2
5	65	0	0	1	1
6	95	0.3	0	3.7	4
7	98	0.5	0	4.5	5
8	<50	0.3	0	0.7	1
9	<50	1	0	0	1
10	64	0.8	0	1.2	2
11	92	1	1	3	5
12	99	2	0	4	6
13	86	−1	0	5	4
14	<50	0	0	1	1
15	<50	0	−0.5	0.5	0
16	<50	0	−0.2	0.2	0
17	<50	0	0	0	0
18	99	0	0	5	5
19	<50	1	0	0	1
20	<50	1	1	2	4
21	<50	0	0	0	0
22	55	0	0	1	1
23	<50	0	0	1	1
24	76	0	0	1	1
25	86	1	0.4	1.6	3
26	80	1	1	1	3
27	99	0	0.1	3.9	4
28	90	1	0	4	5
29	68	0	0	2	2
30	65	2.4	0	0.6	3
31	100	2	5	6	13
32	100	7	2	13	22
33	79	−2	0	8	6
34	85	1.5	2	4.5	8
Total		25.8	10.8	101.4	

Branch support is partitioned by codon position.

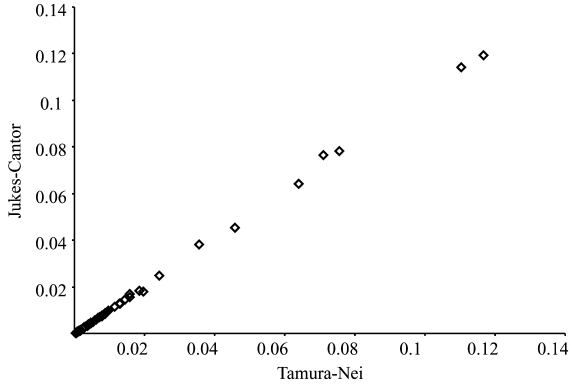

Fig. 2. Non-asymptotic relationship between Jukes–Cantor and Tamura–Nei estimation of COI nucleotide change inferred from the *Conophthorus* phylogram (Fig. 1).

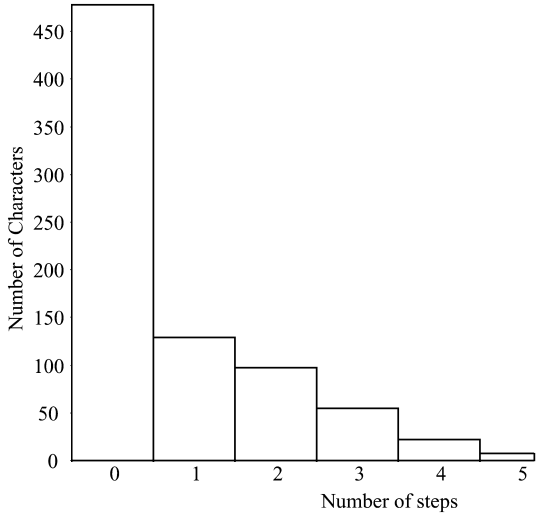

Fig. 3. Distribution of nucleotide sites exhibiting parsimony inferred change for mtDNA COI. The distribution fits a Poisson distribution ($\chi^2 = 0.00166$, $p = 0.9919$) and nucleotide change is consistent with a Jukes–Cantor model of stochastic and equiprobable nucleotide evolution.

3.2. Nucleotide and amino acid residue patterns

Based in part on the strict consensus tree (Fig. 1), several patterns of nucleotide change were inferred. Potential saturation of nucleotide change was not shown by an asymptotic relationship between Jukes–Cantor and Tamura–Nei models of nucleotide substitution (Fig. 2). These data also demonstrated a fit to a Poisson distribution of character change ($\chi^2 = 0.00166$, df = 6, $p = 0.9919$) (Fig. 3). These results indicate that nucleotide change per site is stochastic and equiprobable. Thus the parsimony optimality criterion for character change is appropriate given that the majority of variable sites exhibit a low rate of change (Olmstead et al., 1998), and the data likely reflect an overall phylogenetic pattern among *Conophthorus* species.

First and second positions exhibit low rates of change predicted by a model for amino acid conservation among insects (Lunt et al., 1996). As expected, the highest rate of change was exhibited in the third positions. Transition/transversion ratios were skewed from neutral expectations of a Jukes–Cantor model of equiprobable change. A prevalence of transitions occurred overall and for each codon position (Table 4). A skew in transitions has been observed for other taxa that are relatively closely related as compared to other more distantly related taxa (e.g., Caterino and Sperling, 1999; Cognato and Sperling, 2000; Rand et al., 2000). The taxa included in this study can be considered closely related as evidenced by the low mean branch lengths (Table 4). Character support for the *Conophthorus* phylogram (Fig. 3) mostly derives from third positions, which demonstrate 4–10 times more branch support than first and second positions, respectively (Table 3). This is not surprising given that 70% of informative

Table 4
Nucleotide divergence patterns observed for codon positions for COI for *Conophthorus* and outgroup species

	Total sites	Variable sites	Informative sites	Number of steps	CI, RI	Mean branch length	ti/tv
Total	794	308	209	639	0.67, 0.78	0.009	5.39
First	265	74	46	135	0.67, 0.79	0.006	2.32
Second	264	21	16	36	0.75, 086	0.0014	2.95
Third	265	213	147	468	0.64, 0.77	0.023	5.74

Mean ti/tv ratios were calculated from average pair-wise OTU comparisons.

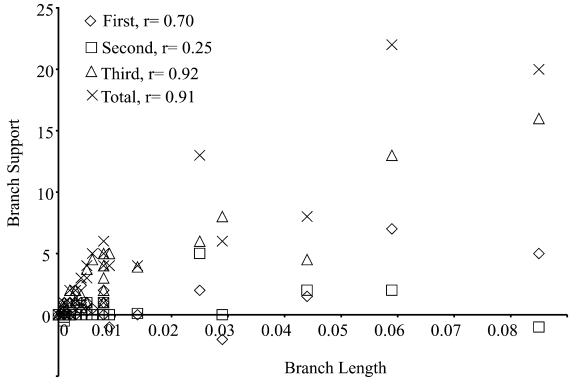

Fig. 4. Relationships between partition branch support values and branch length. First, third, and total *r* values are significant at $\alpha = 0.05$.

sites occur at third positions (Table 4). Branch support was significantly correlated with first and third position branch length and no correlation was observed for the second position (Fig. 4). Overall, these patterns of nucleotide change have been observed for other bark beetle and insect species (e.g., Caterino et al., 2000; Cognato and Vogler, 2001).

Amino acids translated from the nucleotide sequence exhibit 62 variable and 37 parsimony informative sites. A mean change of 2.45 amino acids occurred for each branch of the phylogram however, this change was not evenly distributed over the tree. For example, the *Conophthorus* and δ clades were associated with 9 and 11 amino acid changes, respectively. The other major clades, α, β, and γ, were associated with only four, one, and zero changes, respectively. Interestingly, amino acid change was also observed among a few terminal lineages. For example, *C. mexicanus*, *C. michoacanae*, and *C. radiatae* exhibited one change; *C. echinatae* exhibited three changes, and *C.* species 2 exhibited, five changes. These observations were not the spurious results of base-pair ambiguities of the raw sequence data (see, Materials and methods).

3.3. Geographic and host use patterns

As mapped on the phylogram, geographic and host use character states were not distributed randomly among individuals (significant at $\alpha = 0.02$). Individuals were isolated by distance (Mantel $r = 0.312$, $p = 0.001$) and mostly grouped by geographic proximity (Figs. 5A and B). Ancestral states for the geographic proximity >900 km were well resolved for the phylogram and Mexico was inferred as the origin of *Conophthorus* (Fig. 5A). A more specific location could not be determined (Fig. 5B). There was a less obvious pattern with host use and gains and losses of specific hosts occurred throughout the phylogeny (Figs. 6A and B). An ancestral host taxon either for *Pinus* subgenera or subsections could not be inferred for *Conophthorus* (Figs. 6A and B). Groups of related OTUs, whether smaller ESCs or larger clades, showed significant associations with either small or larger areas of geographic proximity (Table 5). No associations were observed between taxa and hosts in the context of the entire phylogeny. This lack of association with hosts was generally observed within areas >900 km with one exception, ESCs associated with host subsections within Western NA (Table 5). Associations between ESCs, hosts, and geographic proximities for specific clades (α, β, and γ) were not significant, save the association between ESCs and the 200–300 km geographic proximity observed in clade α.

4. Discussion

DNA sequence variation and the reconstructed phylogeny presented in this study show strong support for the monophyly of *Conophthorus* (Table 3, Fig. 1). The genus is the sister taxon of *Pityophthorus*, and based on an arthropod molecular clock (Brower, 1994) this divergence occurred approximately 4 million years ago, soon after the beginning of the Pliocene. The phylogeny also suggests that *P. schwerdtfergeri* is the sister species to *Conophthorus.* This hypothesis deserves further investigation with the addition of more specimens of the very diverse genus *Pityophthorus* (>200 species) (Wood and Bright, 1992). The sister relationship of *P. schwerdtfergeri* to *Conophthorus* would suggest that pine cone feeding occurred before the development of the taxonomic characters that define *Conophthorus* (Hopkins, 1915).

Conophthorus species were subdivided by Hopkins (1915), however there is no evidence that these categories were treated as formal taxa. Furthermore, Hopkins' groups should not be used for taxonomy because they are not monophyletic (Fig. 1). Generic subdivision is sometimes needed to aid communication of important pest

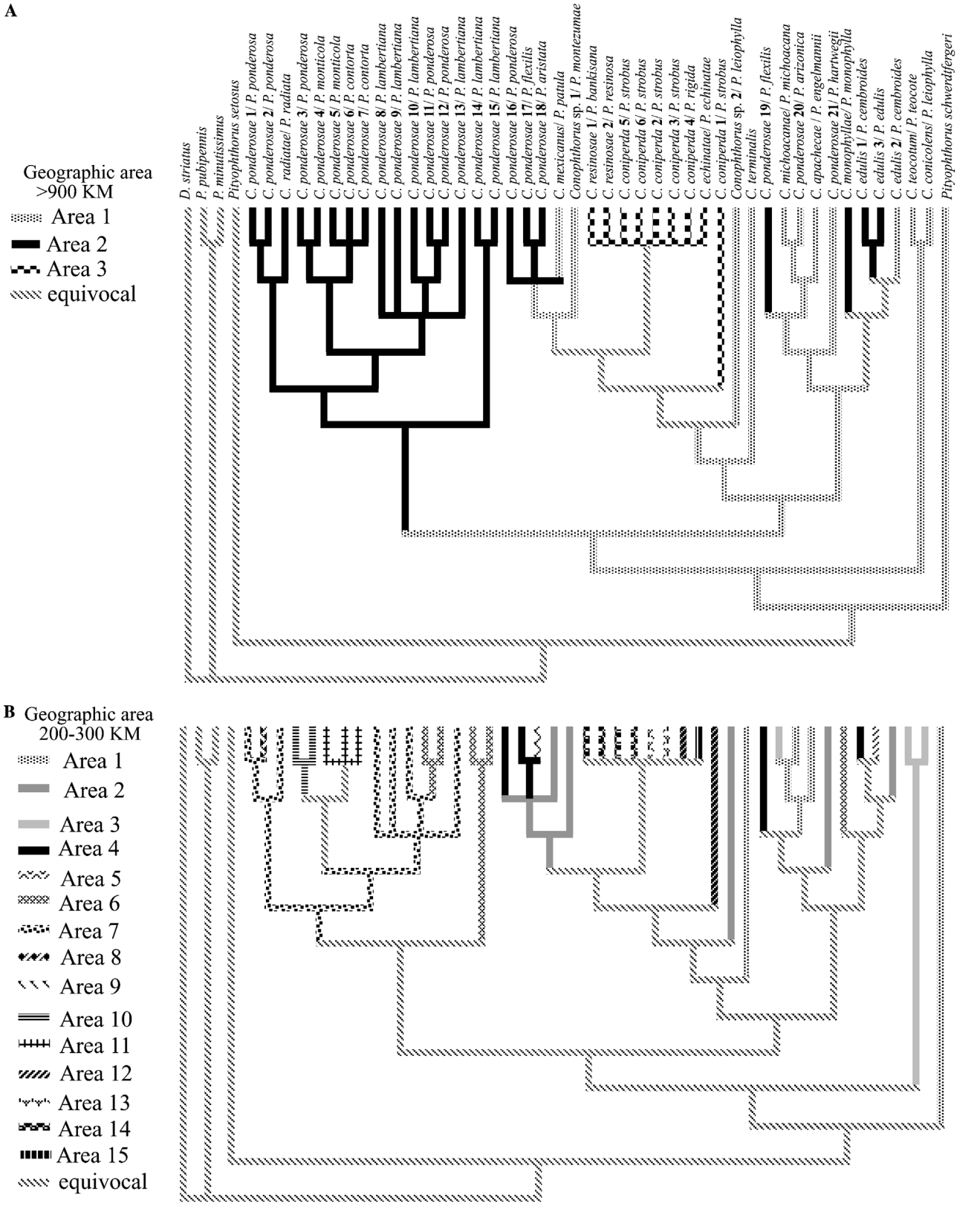

Fig. 5. Patterns of geographic proximity inferred from the *Conophthorus* phylogram. Accelerated transformation was assumed for all character states. (A) Geographic proximities greater than 900 km. (B) Geographic proximities between 200 and 300 km.

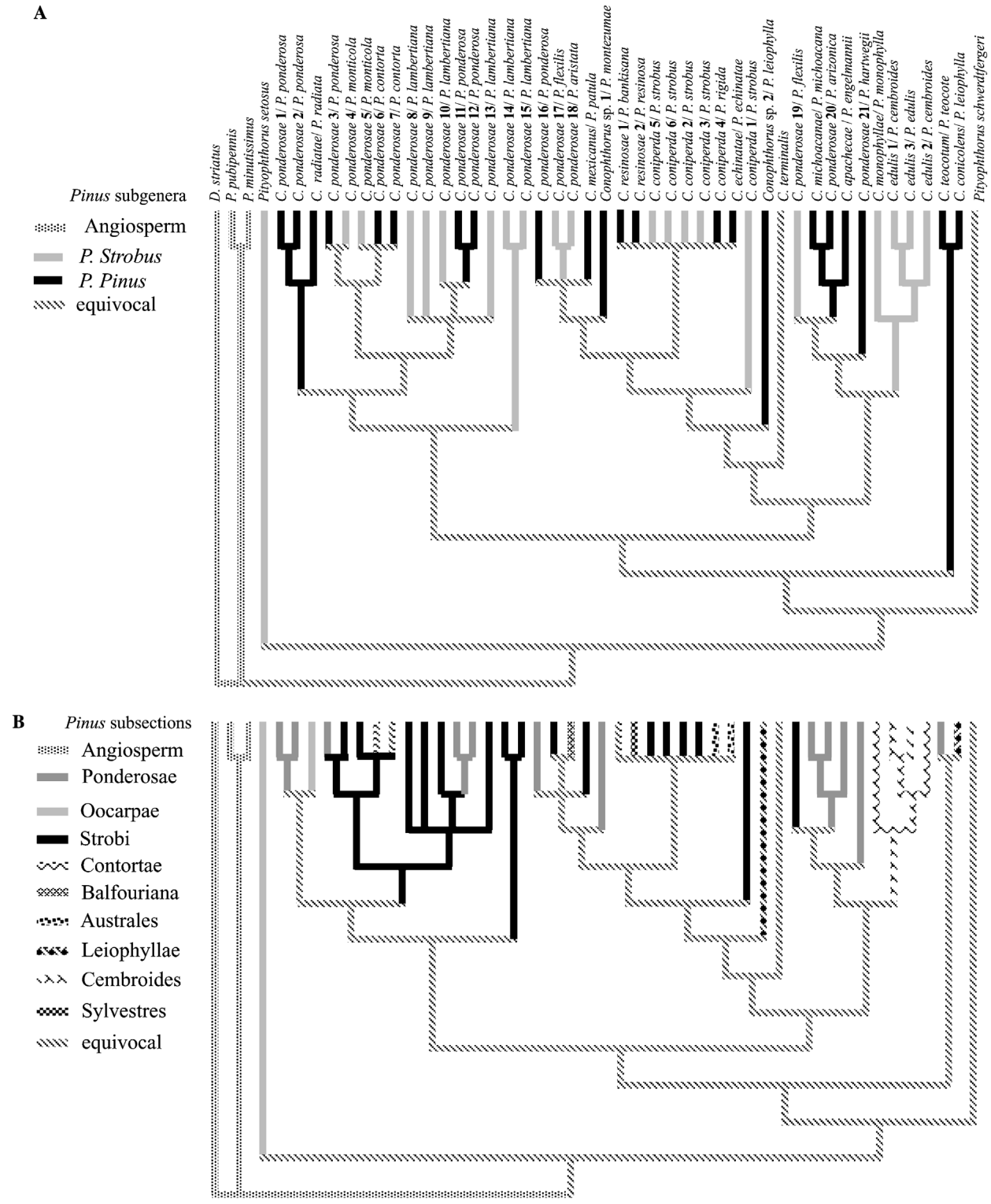

Fig. 6. Patterns of host use inferred from the *Conophthorus* phylogram. Accelerated transformation was assumed for all character states. (A) *Pinus* subgenera and Angiosperm outgroup. (B) *Pinus* subsections and Angiosperm outgroup.

species. Clades α, β, γ, and δ are candidates for recognition as species groups or subgenera (Fig. 1) but only if these clades are resolved with the addition of other kinds of data (whether morphological or nuclear gene sequences) and further phylogenetic analysis (Cognato and Vogler, 2001).

The *Conophthorus* phylogram supports several synonymies made by past taxonomic studies (Table 1) (Wood and Bright, 1992). Many species were described based on host association with little or no morphological evidence (Hopkins, 1915). This study suggests that, in general,

Table 5
Associations between *Conophtorus* taxa, hosts, and geographic regions

		Host subgenera	Host subsections	Area 200–300 km	Area >900 km
	ESC	0.098, 0.008	0.015, 0.004	<0.001, <0.001*	<0.001, <0.001*
	Clades	0.58, 0.005	0.162, 0.004	0.0017, 0.0004*	<0.001, <0.001*
Within area 1, >900 km	ESC	0.50, 0.016	0.25, 0.014	NA	NA
Within area 2, >900 km	ESC	0.14, 0.011	0.002, 0.0014*	NA	NA
Within area 3, >900 km	ESC	1.0, 0.0	0.48, 0.016	NA	NA
Within clade α	ESC	0.10, 0.009	0.046, 0.007	0.001, 0.0009*	NA
Within clade β	ESC	0.48, 0.015	0.32, 0.014	0.17, 0.012	0.68, 0.015
Within clade γ	ESC	0.064, 0.007	0.07, 0.008	0.89, 0.01	0.31, 0.015

First number in each column is Monte Carlo significance, followed by standard error. *Significantly different from random at $p < 0.01$.
First two comparisons consider the entire phylogeny and all geographic areas. The remaining comparisons only consider associations within specific areas or clades. NA = not applicable. Clades and ESCs are defined in text and Table 2.

host association is not important for taxonomy or speciation, as indicated by the lack of phylogenetic concordance with hosts (Figs. 6A and B). This finding contrasts with other studies that suggest that speciation and/or lineage diversification in scolytids and other insects are associated with shifts to novel hosts, with or without different secondary chemicals (Becerra, 1997; Kelley et al., 2000; Jordal et al., 2004; Kerdelhué et al., 2002). Although *Pinus* species have variable types and amounts of secondary chemicals (Byers, 1995; Gijzen et al., 1993), perhaps *Conophthorus* species can metabolize novel chemicals without deleterious effects, as observed in other scolytids. Host infidelity and a lack of phylogenetic concordance with hosts have been observed for the pinyon pine *Ips* bark beetle (Cognato et al., 2003). However, hosts may have microevolutionary effects at a local scale as observed among other insects (Mopper, 1996) and as suggested by the association between ESCs and *Pinus* subsections in Western NA (Table 5).

Geographic isolation likely is an important factor for *Conophthorus* lineage diversification because phylogenetically related ESCs are most often associated in the same geographic proximity (Table 3) and there is general concordance between phylogeny and geographic area (Fig. 5A). Distances between 200 and 300 km appear to be enough to promote lineage diversification (Table 5). Geographic distances between populations are key to the process of allopatric speciation (Mayr, 1970). Glaciated North American and Europe landscapes during the Pleistocene have been implicated as important isolating factors that generate lineage diversity among bark beetle species (Cognato et al., 1999, 2003; Kelley et al., 1999; Stauffer et al., 1999). The Pleistocene topography likely also helped to generate *Conophthorus* lineage diversity, especially in Western NA where mountainous terrain allowed latitudinal and altitudinal separation of host ranges (Webb and Bartlein, 1992). Clade α contains resolved groups of *C. ponderosae* from Western NA that are separated by relatively large branch lengths (Fig. 1). The diversification of this lineage would have begun approximately 1.1 million years ago, which was well into the Pleistocene. In comparison, clade 18 is poorly resolved and exhibits little lineage diversification (Fig. 1). These Eastern NA species would likely have experienced less isolation during the Pleistocene due to latitudinal habitat shifts over the relativity flat terrain (Delcourt and Delcourt, 1981). Isolation of hosts *P. radiata* and *P. echinata* (west of the Mississippi River) and associated pine cone beetles likely contributed to the speciation of *C. radiatae* and *C. echinatae*, respectively. Based on the terminal branch lengths of *C. radiatae* and *C. echinatae*, these isolation events occurred approximately 40,000 years ago in the middle of two glacial maxima (Delcourt and Delcourt, 1981). Isolation may be compounded by pine cone beetle mating behavior because females may be inseminated before emerging from brood cones (Schaefer, 1962; Williamson et al., 1966) and emerging beetles tend to infest cones of the same or nearby trees (Henson, 1962). Thus, these behaviors increase the probability of mating with kin, which would result in greater genetic isolation among populations.

Mexico appears to have been the center for the origin of *Conophthorus* species, based on the reconstruction of ancestral states for geographic areas (Fig. 5A). Also, pine cone beetle lineages (clades β and γ) derived from Mexican lineages appear to have diversified in Eastern and Western NA (Fig. 1). This hypothesis is concordant with observations that Mexico is a biodiversity "hotspot" for other taxa including *Pinus* (Dinerstein et al., 1995). Our results also suggest that a higher diversity of *Conophthorus* occurs in Mexico. For example, *Conophthorus* sp. 2, which was collected from *P. leiophylla*, exhibits a branch length of 1.9% sequence difference and of these, five amino acids substitutions are observed. These differences are comparable or exceed the differences observed for recognized species such as *C. echinatae*, *C. michoacana*, and *C. radiatae* (Fig. 1). Genetic distance compared between sister taxa may be used to identify cryptic species (Sperling, 2003) and other scolytid studies have used branch length as a measure for taxonomic decisions (Jordal et al., 2004; Kerdelhué et al., 2002). Conversely,

cryptic species, i.e., those not suggested by morphological or DNA data, may also be underestimated. For example, *C. coniperda* and *C. resinosae* exhibit little to no sequence difference (Fig. 1) despite cytogenetic, allozyme, and behavioral differences (de Groot and Ennis, 1990; de Groot, 1991; de Groot and Borden, 1991; de Groot et al., 1992). The exact mechanisms that cause these differences are unknown but the phylogram (Fig. 1) suggests that they evolved at a rate greater than the mitochondrial mutation rate. Alternatively, their similarity with respect to mtDNA may derive from recent introgression in spite of differences in the rest of the genome. These findings suggest that the absence of sequence difference does not preclude the existence of different species.

Our results will allow pine seed producers and chemical ecologists to better monitor and control pest *Conophthorus* populations. Managers and researchers should view *Conophthorus* populations as distinct evolutionary entities with potentially unique responses to semiochemical control. Evidence that *C. ponderosae* is polyphyletic, as well as disproportionate phylogenetic branch lengths among individuals, indicates a need for a major revision of *Conophthorus* species. Once species limits are revised and formally named, control and research should become easier, because a phylogenetic framework can be used to predict the biological attributes of species. However, this mitochondrial data set is only one character type, and phylogenetic revisions are much improved when they are based on multiple data sets (e.g., Cognato and Vogler, 2001; Damgaard and Cognato, 2005). Hence, future studies should include a larger range of character types.

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