Molecular Phylogenetics and Evolution 52 (2009) 498–511

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Reticulate evolution and incomplete lineage sorting among the ponderosa pines

Ann Willyard [a,b,c], Richard Cronn [d], Aaron Liston [a,*]

[a] Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331, USA
[b] Department of Biology, University of South Dakota, 414 E. Clark St, Vermillion, SD 57069, USA
[c] Biology Department, Hendrix College, 1600 Washington Ave., Conway, AR 72032, USA
[d] Pacific Northwest Research Station, USDA Forest Service, 3200 SW Jefferson Way, Corvallis, OR 97331, USA

ARTICLE INFO

Article history:
Received 6 January 2009
Revised 10 February 2009
Accepted 13 February 2009
Available online 26 February 2009

Keywords:
Pinus
Pine
Hybridization
Low copy nuclear loci
Reticulate evolution
LEA
WD-40
trnG

ABSTRACT

Interspecific gene flow via hybridization may play a major role in evolution by creating reticulate rather than hierarchical lineages in plant species. Occasional diploid pine hybrids indicate the potential for introgression, but reticulation is hard to detect because ancestral polymorphism is still shared across many groups of pine species. Nucleotide sequences for 53 accessions from 17 species in subsection *Ponderosae* (*Pinus*) provide evidence for reticulate evolution. Two discordant patterns among independent low-copy nuclear gene trees and a chloroplast haplotype are better explained by introgression than incomplete lineage sorting or other causes of incongruence. Conflicting resolution of three monophyletic *Pinus coulteri* accessions is best explained by ancient introgression followed by a genetic bottleneck. More recent hybridization transferred a chloroplast from *P. jeffreyi* to a sympatric *P. washoensis* individual. We conclude that incomplete lineage sorting could account for other examples of non-monophyly, and caution against any analysis based on single-accession or single-locus sampling in *Pinus*.

1. Introduction

Many species in the genus *Pinus* are characterized by large effective population sizes, limited interspecific divergence, and century-long fertile life spans (Syring et al., 2007). These factors appear to have created a remarkable evolutionary web that is profoundly affected by incomplete lineage sorting. Hybridization between distinct *Pinus* species may also have created reticulate rather than strictly hierarchical patterns of descent (e.g. Matos and Schaal, 2000). Despite persistent attempts to classify pine species based on morphological, biochemical, cytological, and molecular characters, the taxonomic relationships among many species remain unsolved. There is a growing understanding of the causes.

First, *Pinus* is an ancient genus, diverging from other extant genera at least 100 million years ago in the Cretaceous (Alvin, 1960). Nevertheless, integration of genetic and fossil evidence indicates that many of the roughly 100 species of pine arose rather recently, especially when measured in generations rather than years. For example, the stem lineage of the 17 species in subsection *Ponderosae* (Pinaceae, *Pinus*, subgenus *Pinus*, section *Trifoliae*) diverged within the last 15 million years, and the crown divergence began around 5 million years ago (Willyard et al., 2007). Using an average generation time of 50 years (Syring et al., 2007), this 17-species clade began diverging some 300,000 generations ago and the crown divergence occurred on the order of 100,000 generations ago. Thus, the first factor confounding their evolutionary relationships is that pine *species* may be relatively young, even though the genus is ancient and individuals are generally long-lived.

Second, *Pinus* classification is bedeviled by plastic, homoplasious, and highly variable morphological character states. For example, serotinous cones are variable within and among populations (Borchert, 1985) and have evolved independently in several lineages (Gernandt et al., 2005). Abundant within-population genetic variation appears to be the norm. Using evidence from quantitative traits, allozymes, or molecular markers, more than 90% of variation is generally contained within vs. among populations, although a few pine species exhibit higher among-population differentiation (Ledig, 1998; Sorensen et al., 2001). The interplay between homoplasy and intraspecific variability makes delineations between some groups of pine species challenging, encouraging some to propose hybrid origins to explain the enigma. An important example may be *P. densata* (subgenus *Pinus*, section *Pinus*), often cited as a diploid hybrid species (Ma et al., 2006 and references therein).

A third factor affecting pine evolutionary relationships is that lineage sorting between pine species is often incomplete. When molecular sequences for multiple individuals are sampled per species, many conspecific samples lack allelic monophyly. This has been attributed to incomplete lineage sorting (Syring et al.,

* Corresponding author. Fax: +1 541 737 3573.
E-mail addresses: Ann.Willyard@USD.edu (A. Willyard), rcronn@fs.fed.us (R. Cronn), listona@science.oregonstate.edu (A. Liston).

1055-7903/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2009.02.011

2007). Because loci coalesce at different rates and are affected by stochastic processes (Carstens and Knowles, 2007), incomplete lineage sorting creates conflicting topologies for pine species from unlinked loci (Syring et al., 2005). Many of the factors that delay allelic coalescence are present in woody trees (and specifically in *Pinus*): predominantly outcrossed mating, high within-species (and within-population and within-individual) mean heterozygosity, long generation time, and large effective population sizes where alleles are rarely purged (Rosenberg, 2003). Incomplete lineage sorting may be especially troublesome in *Pinus* because speciation has been rapid relative to effective population sizes (Willyard et al., 2007).

Fourth, hybridization between diverged lineages is thought to be an important factor in the evolution of many plants (Arnold, 1997; Grant, 1981; Stebbins, 1950), an idea supported by empirical data (Arnold, 1993; Cronn and Wendel, 2004; Doyle et al., 2004). Interspecific gene flow has been proposed to facilitate adaptive radiations in plants (Seehausen, 2004), the invasion of novel habitats by nascent species (Petit et al., 2003; Rieseberg et al., 2003; Stebbins, 1959) and the invasion of new habitats by exotic plants (Ellstrand and Schierenbeck, 2000). Many researchers have speculated that reticulate evolution, facilitated by introgression, may have influenced the observed complexities among pine species.

Putative natural hybrids have been studied between many sympatric pine species, including *P. banksiana* and *P. contorta* (mitochondrial restriction sites; Dong and Wagner, 1993); *P. brutia* and *P. halepensis* (chloroplast microsatellite loci; Bucci et al., 1998); *P. coulteri* and *P. jeffreyi* (morphology; Libby, 1958; Zobel, 1951); *P. jeffreyi* and *P. ponderosa* (morphology; Haller, 1962); *P. edulis* and *P. monophylla* (morphology; Lanner and Phillips, 1992); *P. hartwegii* and *P. montezumae* (morphology and chloroplast; Matos, 1995; Matos and Schaal, 2000); *P. montezumae* and *P. pseudostrobus* (chloroplast microsatellite loci; Delgado et al., 2007); *P. mugo* and *P. sylvestris* (morphology; Christensen and Dar, 1997); and *P. palustris* and *P. taeda* (morphology; Namkoong, 1966). Artificial hybridizations between many pine species within taxonomic subsections yield fertile seeds (Critchfield, 1986), demonstrating that incomplete mating barriers between allopatric *Pinus* species are common and suggesting that geographic distance is a major barrier to natural hybridization among pine species. However, not all hypotheses of natural pine hybridization have withstood genetic analysis. For example, trees long suspected of representing a hybrid swarm between *P. arizonica* and *P. ponderosa* (Epperson et al., 2003) instead represent a unique third taxon (Epperson et al., 2009).

There is also substantial evidence that pine species are capable of rather rapid migrations (Petit et al., 2004). The combination of incomplete mating barriers and the opportunity for secondary contact via migration may have allowed infrequent, but evolutionarily significant, introgression within *Pinus*. In fact, the long-term retention of ancestral polymorphism observed in *Pinus* could be partially driven by migrant alleles from occasional interspecific hybridization. These alleles would increase the diversity within populations and slow the process of allelic coalescence. This may mean that reticulate evolution is an important driving force behind pine genetic patterns. Detection of reticulation is not at all straightforward, but is vital because assuming a hierarchical relationship for groups with a net-like rather than tree-like history may lead to erroneous conclusions (Doolittle and Bapteste, 2007; Posada and Crandall, 2001; McDade, 1992, 1990). Conflicting relationships for the *Ponderosae* have been reported based on samples of different characters and exemplars (Eckert and Hall, 2006; Gernandt et al., 2005; Krupkin et al., 1996; Liston et al., 1999). However, phylogenetic incongruence can be due to incomplete lineage sorting, recombination, natural selection, random lineage sorting, homoplasy, errors in phylogenetic inference, and reticulate evolution. Estimates of intraspecific genetic diversity are useful for interpreting causes of incongruence because a large effective population size (N_e) suggests that incomplete lineage sorting may provide the entire explanation for lack of monophyly and even for incongruent results, as genomes in diverse species may be "mosaics of conflicting genealogies" (Pollard et al., 2006). On the other hand, incongruence among species with limited diversity may suggest the involvement of other mechanisms, such as reticulate evolution or drift. Despite their large *current* effective population sizes, it is possible that some pine species have undergone genetic bottlenecks in the past that were severe enough for drift to affect the lineage (Ledig, 2000).

1.1. Ponderosae taxonomy

Nineteen species of *Ponderosae* are sometimes recognized, but we synonymize *P. nubicola* J.P. Perry with *P. pseudostrobus,* and *P. donnell-smithii* Masters with *P. hartwegii* (Farjon and Styles, 1997). Two taxa have sometimes been treated within *P. ponderosa* (*P. arizonica* and *P. washoensis*), but are currently recognized as distinct species. Rehfeldt (1999a) provided evidence for elevating *P. arizonica* from its varietal status under *P. ponderosa*. The narrowly endemic *P. washoensis* is included in the Flora of North America (Kral, 1993). *Pinus ponderosa* var. *scopulorum* and *P. arizonica* var. *stormiae* are sometimes treated at the species level. Our sampling scheme recognizes these two taxa as varieties (Table 1).

The 17 current species are divided into two major groups: the California big-coned pines (*Sabinianae*; Loudon, 1838) and *Ponderosae sensu stricto* (*s.s.*; Table 1). Three species (*P. sabiniana*, *P. coulteri*, and *P. torreyana*) are traditionally grouped in the *Sabinianae* based on shared morphology (Little and Critchfield, 1969; Price et al., 1998). Chloroplast results (Gernandt et al., 2009) support proposals based on heptane biochemistry (Mirov, 1961) and seed fatty acids (Wolff et al., 2000) that *P. jeffreyi*, despite its superficial resemblance to *P. ponderosa*, belongs with the *Sabinianae*. We use *Sabinianae* to refer to all four species of California big-coned pines (Table 1).

Floristic treatments (Farjon and Styles, 1997; Martínez, 1948; Perry, 1991; Price et al., 1998) have suggested subdividing *Ponderosae s.s.* species in very different ways (Table 1), but these groups have not been supported by phylogenetic analyses (Gernandt et al., 2005).

1.2. Experiment design

Given the complexity of previous taxonomic delineations and the potential for a mosaic genome in some pines due to the potential for hybridization, it is vital to sample multiple individuals within each species and to also sample loci that segregate independently. We included a comprehensive sample of *Ponderosae* species with two to six individuals per species that represent the geographic range of each taxon wherever possible. Three independent gene regions were used: two unlinked low-copy nuclear loci and a locus from the separately-segregating chloroplast organelle. These regions can be used to infer independent gene trees or networks to increase the opportunity to detect incongruence patterns that may be attributed to interspecific hybridization. Paternally inherited *Pinus* chloroplasts can provide powerful markers for detecting introgression when used in conjunction with nuclear markers. However, organelle genealogies are predominantly uniparental and therefore susceptible to introgression (Liston et al., 2007). If substitution rates are comparable, the fourfold smaller effective population size (due to haploidy and uniparental inheritance of chloroplasts) leads to faster coalescence for chloroplast than nuclear loci (Birky et al., 1983). However, mean *Pinus* substitution rates are about threefold faster in nuclear than chloroplast loci (roughly 0.12 vs. 0.04 substitutions per site per year, respectively (Willyard

Table 1
Classification of *Ponderosae* taxa.

Taxon and author	This study	Martínez (1948)	Perry (1991)	Farjon and Styles (1997)	Price et al. (1998)
P. arizonica var. *arizonica* Engelmann	*Ponderosae s.s.*	Ponderosae	Ponderosae	Ponderosae	Ponderosa
P. arizonica var. stormiae Martínez	*Ponderosae s.s.*	Ponderosae	Ponderosae	Ponderosae	Ponderosae
P. cooperi Blanco	*Ponderosae s.s.*	Montezumae (*P. lutea*)	Montezumae	Ponderosae (*P. arizonica* var. *cooperi*)	Ponderosae
P. coulteri D. Don	*Sabinianae*	Coulteri	n/a	n/a	Sabinianae
P. devoniana Lindley	*Ponderosae s.s.*	Montezumae (*P. michoacana*)	Michoacana (*P. michoacana*)	Pseudostrobi	Montezumae
P. douglasiana Martínez	*Ponderosae s.s.*	Pseudostrobus	Montezumae	Pseudostrobi	Pseudostrobus
P. durangensis Martínez	*Ponderosae s.s.*	Montezumae	Ponderosae	Oocarpae	Ponderosae
P. engelmannii Carrière	*Ponderosae s.s.*	Ponderosae	Ponderosae	Ponderosae	Ponderosae
P. hartwegii Lindley	*Ponderosae s.s.*	Montezumae	Rudis	Ponderosae	Montezumae
P. jeffreyi Balfour	*Sabinianae*	Ponderosae	Ponderosa	Ponderosae	Ponderosae
P. maximinoi M.E. Moore	*Ponderosae s.s.*	Pseudostrobus (*P. tenuifolia*)	Pseudostrobus	Pseudostrobi	Pseudostrobus
P. montezumae Lambert	*Ponderosae s.s.*	Montezumae	Montezumae	Pseudostrobi	Montezumae
P. ponderosa var. *ponderosa* Douglas ex P. & C. Lawson	*Ponderosae s.s.*	Ponderosae	n/a	Ponderosae	Ponderosae
P. ponderosa var. *scopulorum* Engelmann	*Ponderosae s.s.*	n/a	n/a	n/a	Ponderosae
P. pseudostrobus Lindley	*Ponderosae s.s.*	Pseudostrobus	Pseudostrobus	Pseudostrobi	Pseudostrobus
P. sabiniana Douglas ex D.Don	*Sabinianae*	n/a	n/a	n/a	Sabinianae
P. torreyana Parry ex Carrière	*Sabinianae*	n/a	n/a	n/a	Sabinianae
P. washoensis Mason & Stockwell	*Ponderosae s.s.*	n/a	n/a	n/a	Ponderosae
P. yecorensis Debreczy & Rácz	*Ponderosae s.s.*	n/a	n/a	n/a	n/a

et al., 2007), suggesting that without considering recombination, phylogenetic informativeness for some nuclear loci may be comparable to chloroplast loci in *Pinus*. We explore this possibility by selecting nuclear loci with the highest level of divergence that successfully amplify and align across this taxonomic subsection. Our loci contain large regions of potentially neutral introns using primers anchored in or near exons (Liston et al., 2007; Syring et al., 2005; Willyard et al., 2007).

2. Materials and methods

2.1. Plant materials

We sampled 53 accessions representing 17 *Ponderosae* species (Table 2, Fig. 1). Based on a *Pinus* phylogeny (Syring et al., 2005), we selected *P. contorta* (sect. *Trifoliae*, subsect. *Contortae*) as the outgroup. Specimen vouchers were deposited in herbaria; abbreviations follow Holmgren and Holmgren (1998; Table 2). Haploid genomic DNA was isolated from megagametophytes of single seeds (allowing amplification of low-copy nuclear loci without cloning) using the FastDNA Kit® (Qbiogene, Irvine, CA, USA).

2.2. Nuclear loci

We selected two low-copy nuclear regions originally identified from expressed sequences (ESTs) of *P. taeda*. Previously, we surveyed many published *Pinus* ESTs to identify longer genomic amplicons that span introns (Willyard et al., 2007). We chose two of these loci that amplify well and yield alignable nucleotide sequences across *Ponderosae*.

The *LEA-like* locus is based on EST *IFG8612* (GenBank Accession No. AA739606), linkage-mapped to *P. taeda* linkage group 3 (Krutovsky et al., 2004). This amplicon has highest similarity (BLASTN; nonredundant nucleotides; http://130.14.29.110/BLAST/) to a late embryogenesis abundant-like (*LEA-like*) locus in *Pseudotsuga menziesii* (GenBank Accession No. AJ012483). We used the published translation from *Pseudotsuga* to infer that our amplicon has 53 bps of exon with the remainder intronic. Primers were designed for subgenus *Pinus*: 8612F1: TGT TAG CAT GCA ATC AAT CAC; 8612R5: TTG TTC CAG ACG CTA TTT CT.

WD-40 is based on EST *IFG8898* (GenBank Accession No. AA739796), mapped to *P. taeda* linkage group 4 (Temesgen et al., 2001). Based on our translation of the *P. taeda* cDNA for *WD-40*, we infer that our amplicon contains two exons (137 bps) with the remainder intronic. Our translation has highest similarity (BLASTP; nonredundant proteins) to *Arabidopsis thaliana* plasma membrane intrinsic protein (*WD-40*; GenBank Accession No. NP_175413). We used published primers (Temesgen et al., 2001) 8898F (ATG GGG GTG CAG CAT AAA C) and 8898R (GGG ATG GCA ACA ACA AAA A).

For both nuclear loci, 40 μl PCR reactions contained ca. 50 ng of DNA template, 0.4 mM each primer, 0.2 mM dNTPs, 0.1 μg/μl BSA, and 2 units of *Taq* polymerase in supplied buffer (Fisher Scientific, Pittsburgh, PA, USA). Reactions contained 1.5 mM $MgCl_2$ for *LEA-like* and 2.0 mM $MgCl_2$ for *WD-40*. The thermocycler program preheated at 80 °C for 2 min, and then ran 35 cycles of denaturing for 1 min at 94 °C, annealing for 1 min, and extending for 1.5 min at 72 °C, with a 10 min final extension at 72 °C. Optimized PCR annealing temperature was 55 °C for *LEA-like* and 60 °C for *WD-40*.

2.3. Chloroplast locus

The *trnG*^{UCC} intron was PCR-amplified using published primers 3′trnG (GTA GCG GGA ATC GAA CCC GCA TC) and 5′trnG2G (GCG GGT ATA GTT TAG TGG TAA AA) (Shaw et al., 2005). The *Pinus* amplicon aligns with positions 8857 through 9610 of the *P. thunbergii* chloroplast sequence (GenBank Accession No. D17510; Liston et al., 2007). PCR used 20 μl reactions with ca. 30 ng of DNA template, 0.2 mM each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.1 μg/μl BSA, and 1 unit of *Taq* polymerase in supplied buffer (Fisher Scientific, Pittsburgh, PA, USA). The thermocycler program preheated at 80 °C for 2 min, and then ran 35 cycles of denaturing for 1 min at 95 °C, annealing with a ramp from 50 to 60 °C for 1 min, and extending for 1 min at 65 °C, with a 10 min extension at 65 °C.

2.4. Sequencing

Products were cleaned with an ethanol precipitation, sequenced using BigDye® v. 3.1 (Applied Biosystems, Foster City, CA, USA), and visualized on an Applied Biosystems 3730 Genetic Analyzer. DNA from single seeds was insufficient for direct PCR in two accessions. These samples were pre-amplified using whole-genome multiple displacement following the protocol of Hosono et al. (2003) with

Table 2
Plants sampled: geographic source, generalized map location (see Fig. 1), specimen voucher, and GenBank accessions. Herbarium abbreviations follow Holmgren and Holmgren (1998).

Taxon	Collecting locality	Map No.	Latitude	Longitude	Accession/specimen voucher	GenBank Accession Nos.		
						LEA-like	*WD-40*	*trnG*
P. arizonica var. *arizonica*	USA: NM: Silver City	17	33.13°N	−108.00°W	ARIZ01/Rehfeldt s.n. (OSC)	FJ395915	FJ395961	FJ395865
	USA: AZ: Saguaro National Park	18	32.20°N	−110.53°W	ARIZ02/Rehfeldt s.n. (OSC)	FJ395916	FJ395962	FJ395866
P. arizonica var. stormiae	México: COAH: Paila	21	25.50°N	−102.50°W	ARIZ03/Henrickson 24294 (OSC)	FJ395917	FJ395963	FJ395867
P. cooperi	México: DGO: El Salto	22	23.80°N	−105.40°W	COOP01S1/Inst. Forest Genetics s.n. (OSC)	n/a	FJ395964	n/a
					COOP01S2/Inst. Forest Genetics s.n. (OSC)	FJ395918	FJ395965	FJ395868
					COOP01S3/Inst. Forest Genetics s.n. (OSC)	FJ395919	FJ395966	FJ395869
P. coulteri	USA: CA: Santa Barbara	12	34.92°N	−116.92°W	COUL01/Wisura s.n. (RSA)	FJ395920	FJ395967	FJ395870
	USA: CA: San Gabriel Mts.	11	34.35°N	−117.98°W	COUL02/Wisura s.n. (RSA)	FJ395921	FJ395968	FJ395871
	USA: CA: Anza-Borrego	16	33.50°N	−116.50°W	COUL03/Simpson s.n. (OSC)	FJ395922	FJ395969	FJ395872
P. devoniana	México: HGO: Huasca	23	20.20°N	−98.60°W	DEVO01/Gernandt s.n. (OSC)	FJ395923	FJ395970	FJ395873
	México: MIC: Jujucato	25	19.42°N	−101.82°W	DEVO02/Hernandez s.n. (OSC)	FJ395924	FJ395971	FJ395874
	México: GRO: Filo de Caballos	28	17.65°N	−99.84°W	DEVO03/Syring 1009 (OSC)	FJ395925	FJ395972	FJ395875
P. douglasiana	México: JAL: Atenquique	24	19.53°N	−103.52°W	DOUG01/Inst. Forest Genetics s.n. (OSC)	FJ395926	n/a	FJ395876
	México: GRO: Yerba Santa	29	16.97°N	−98.58°W	DOUG03S1/Syring 1015 (OSC)	n/a	FJ395973	FJ395877
					DOUG03S2/Syring 1015 (OSC)	n/a	FJ395974	n/a
	México: SIN: Concordia	22	23.48°N	−105.85°W	DOUG04/Ferguson 1872 (ARIZ)	n/a	FJ395975	FJ395878
P. durangensis	México: CHIH: San Juanita	20	28.00°N	−107.58°W	DURA01/Hjerting & Odum 10 (E)	FJ395927	n/a	FJ395879
P. engelmannii	USA: AZ: Cave Creek	18	31.72°N	−110.78°W	ENGE01/Rehfeldt s.n. (OSC)	FJ395928	FJ395976	FJ395880
	USA: AZ: Florida Canyon	18	31.73°N	−110.83°W	ENGE02/Rehfeldt s.n. (OSC)	FJ395929	FJ395977	FJ395881
P. hartwegii	Guatemala: Quetzaltenango	32	14.08°N	−91.52°W	DONN01/Sander s.n. (OSC)	FJ395932	FJ395978	n/a
					DONN02/Sander s.n. (OSC)	FJ395930	FJ395979	FJ395882
	México: MEX: Temascaltepec	26	19.12°N	−99.76°W	HART01/Gernandt & Sherwood 445 (OSC)	FJ395931	FJ395980	FJ395883
	México: GRO: Yerba Santa	28	17.52°N	−99.96°W	HART07/Syring 1016 (OSC)	FJ395933	FJ395981	FJ395884
P. jeffreyi	USA: CA: San Gabriel Mts.	14	34.03°N	−117.92°W	JEFF01/Wisura et al. s.n. (OSC)	FJ395934	FJ395982	FJ395885
	USA: CA: Bishop	10	37.37°N	−118.39°W	JEFF04/Kazmierski s.n. (OSC)	FJ395935	FJ395983	FJ395886
	USA: CA: Susanville	6	40.30°N	−120.87°W	JEFF06/Laws s.n. (OSC)	FJ395936	FJ395984	FJ395887
	USA: CA: Warner Mts.	4	41.17°N	−120.28°W	JEFF12/Willyard 1018 (OSC)	n/a	FJ395985	FJ395888
	USA: NV: Mt. Rose	8	39.33°N	−119.88°W	JEFF13/Willyard 1019 (OSC)	FJ395937	FJ395986	FJ395889
	USA: NV: Reno	8	39.24°N	−119.84°W	JEFF14/Willyard 1020 (OSC)	FJ395938	FJ395987	FJ395890
P. maximinoi	México: OAX: San Jeronomo	27	17.82°N	−97.83°W	MAXI01/Hernandez s.n. (OSC)	FJ395939	FJ395988	FJ395891
	Honduras: COM: Minas de Oro	33	13.53°N	−86.56°W	MAXI03/Simpson s.n. (OSC)	FJ395940	FJ395989	FJ395892
	Guatemala: Alta Verapaz	30	15.47°N	−90.37°W	MAXI05/Escobras s.n. (OSC)	n/a	FJ395990	FJ395893
P. montezumae	México: HGO: Epazoyucan	23	20.11°N	−98.61°W	MONZ01/Gernandt 416 (MEXU)	FJ395941	FJ395991	FJ395894
	Guatemala: HUE: Malacatancito	31	15.22°N	−91.52°W	MONZ02/Escobras s.n. (OSC)	FJ395942	FJ395992	FJ395895
P. ponderosa var. *ponderosa*	USA: WA: Curlew	1	48.88°N	−118.77°W	POND02/Berdeen s.n. (OSC)	FJ395943	FJ395993	FJ395896
	USA: CA: Big Bear Lake	13	34.15°N	−116.85°W	POND04/USFS Camino S. O. s.n. (OSC)	FJ395944	FJ395994	FJ395897
	USA: CA: Warner Mts.	4	41.03°N	−120.32°W	POND33/Willyard 1021 (OSC)	n/a	FJ395996	FJ395899
	USA: OR: Abert Rim	3	42.38°N	−120.23°W	WASH12/Willyard 1007 OSC	FJ395946	FJ395997	FJ395901
	USA: OR: Blue Mts.	2	44.07°N	−118.78°W	WASH15/Willyard 1025 (OSC)	FJ395947	FJ395998	FJ395900
P. ponderosa var. *scopulorum*	USA: UT: Price Canyon	7	39.77°N	−110.92°W	POND10/McArthur s.n. (OSC)	FJ395945	FJ395995	FJ395898
P. pseudostrobus	Guatemala: HUE: Patio de Bolas	31	15.38°N	−91.43°W	PSEU03/Escobras s.n. (OSC)	FJ395948	FJ395999	FJ395902
	México: GRO: Filo de Caballos	28	17.65°N	−99.84°W	PSEU04/Syring 1010 (OSC)	n/a	FJ396000	FJ395903
P. sabiniana	USA: CA: Weaverville	5	40.73°N	−122.94°W	SABI01/Syring s.n. (OSC)	FJ395949	FJ396001	FJ395904
	USA: CA: Clearlake	9	39.18°N	−122.70°W	SABI02/O'Brien s.n. (OSC)	FJ395950	FJ396002	FJ395905
	USA: CA: Redding	5	40.55°N	−122.46°W	SABI04/Willyard 984 (OSC)	FJ395951	FJ396003	FJ395906
P. torreyana	USA: California: Santa Rosa Is.	15	33.95°N	−120.10°W	TORR01/Liston 1236 (OSC)	FJ395952	FJ396004	FJ395907
P. washoensis	USA: CA: Warner Mts. 1	4	41.16°N	−120.12°W	WASH01/USFS Camino S. O. s.n. (OSC)	FJ395953	FJ396005	FJ395908
	USA: CA: Babbitt Peak	8	39.43°N	−120.08°W	WASH02/USFS Camino S. O. s.n. (OSC)	FJ395954	FJ396006	FJ395909
	USA: NV: Mt. Rose	8	39.33°N	−119.52°W	WASH03/Rehfeldt s.n. (OSC)	FJ395955	FJ396007	FJ395910
	USA: CA: Warner Mts. 2	4	41.18°N	−120.12°W	WASH04/Rehfeldt s.n. (OSC)	FJ395956	FJ396008	FJ395911
	USA: CA: Warner Mts. 3	4	41.17°N	−120.25°W	WASH13/Willyard 1023 (OSC)	FJ395957	FJ396009	FJ395912
	USA: CA: Warner Mts. 4	4	41.17°N	−120.25°W	WASH14/Willyard 1024 (OSC)	FJ395958	FJ396010	FJ395913
P. yecorensis	México: SON: Yecora	19	28.38°N	−108.87°W	YECO02/Ferguson 2422 (ARIZ)	FJ395959	FJ396011	n/a
P. contorta	outgroup	n/a	n/a	n/a	CONT06, CONT40	FJ395914	FJ395960	FJ395864

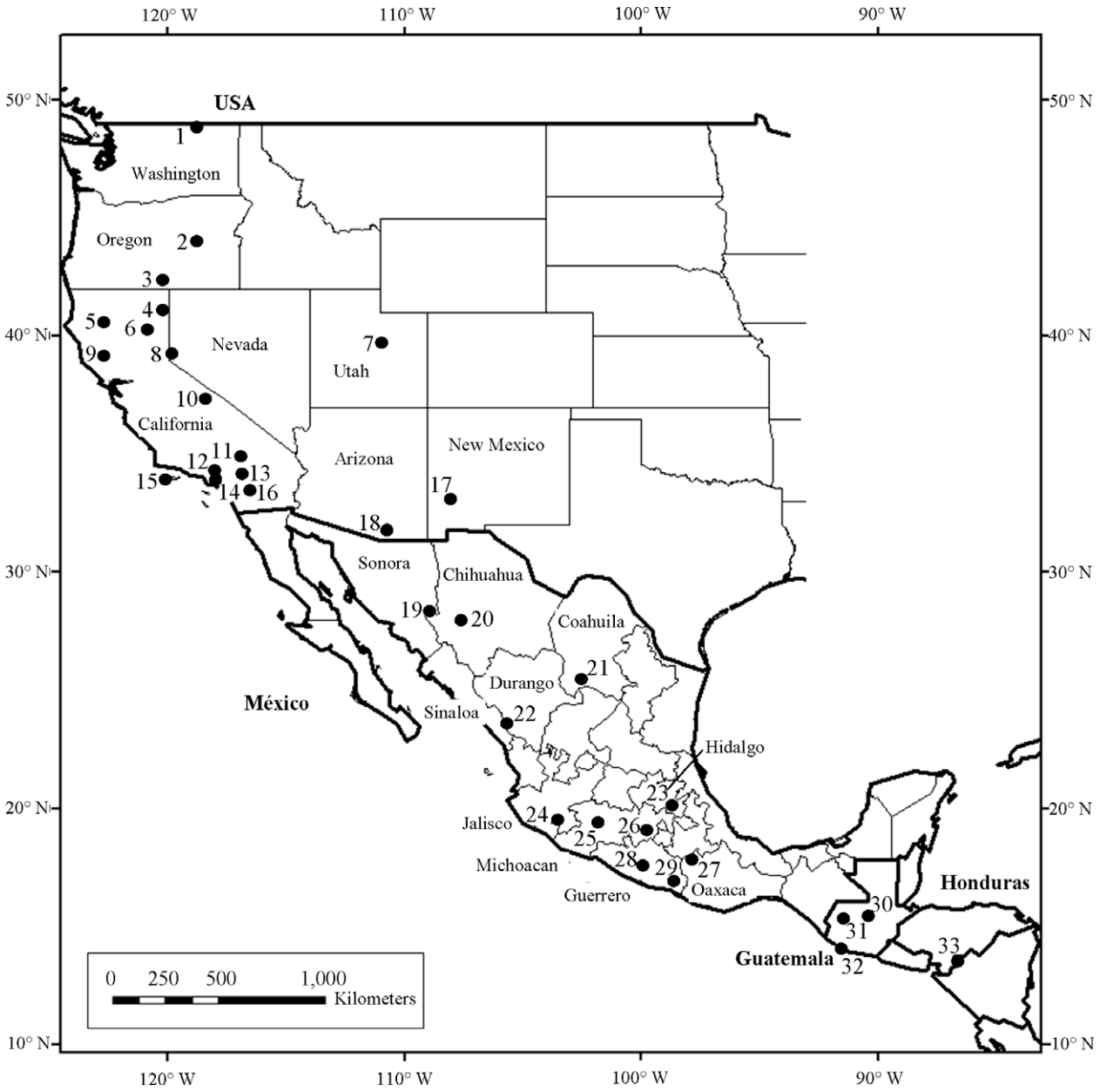

Fig. 1. Generalized collection locales for plant materials in United States of America, México, Guatemala, and Honduras (WGS 84). Map numbers correspond to Table 2.

phi29 DNA polymerase, pyrophosphatase (New England Biolabs, Ipswitch, MA, USA), and random hexamer primers (Operon, Huntsville, AL, USA). The whole-genome product was used as template for PCR. For five samples, DNA was isolated from an excised gel band with Ultra Clean™ DNA purification kit (MoBio Laboratories, Carlsbad, CA) prior to sequencing. Nucleotide sequences are available in GenBank (Table 2).

2.5. Analysis of nuclear loci

Forward and reverse nucleotide reads were assembled using CodonCode (vers. 1.4.6; CodonCode Corporation, Dedham, MA) and edited by hand to create a consensus sequence. Alignments were made by eye to minimize the number of inferred indels. Each locus was analyzed independently. For the Bayesian analysis, a nucleotide substitution model was selected with the Akaike Information Criterion (AIC) using MrModeltest (vers. 2.0; Nylander et al., 2004). Gaps were treated as missing data in the nucleotide partition and coded as present/absent with the simple indel coding method (Simmons and Ochoterena, 2000) using SeqState (vers. 1.4; Müller, 2005). Indel characters were analyzed using an equal-rate binary model. Three partitions were defined: nonsynonymous (approximated with 1st and 2nd codon positions of inferred exons); synonymous (3rd codon positions plus noncoding sequences); and indel characters. We assessed the usefulness of partitions with AIC scores (Akaike, 1974) and a comparison of the number of supported nodes.

We performed two runs using MrBayes (vers. 3.1; Ronquist and Huelsenbeck, 2003) for each locus. Each run used four simultaneous chains and 10 million generations of Metropolis-coupled Monte Carlo simulations, sampling every 1000 generations to save 10,000 trees per run, with default settings for chain heating and rates allowed to vary by partition. We assessed convergence and chose the number of samples to discard as burn-in based on stationarity of a plot of the generation versus log-likelihood for each run. We also compared plots of tree distances and split frequencies

between two runs using the *Comparetree* function in MrBayes. One majority-rule consensus tree was built for each locus by combining trees generated by two runs, discarding the first 1000 trees from each run. Branch lengths were estimated by averaging across all retained trees. Nodes with less than 0.95 posterior probabilities were collapsed. Alignments and trees are available at TreeBase (study accession number S2297; matrix accession number M4362).

Each nuclear locus was also analyzed with the parsimony criterion using PAUP* (vers. 4.0b10; Swofford, 2002). The heuristic search used stepwise addition of starting trees, 1000 random additions, with 50,000 trees retained, and tree-bisection-reconnection branch-swapping. Nonparametric bootstrap was performed with 100 replicates, holding 50 trees each for a random addition of 1000 replicates. Strict consensus trees were compared with Bayesian consensus trees and Bayesian-supported nodes with parsimony bootstraps less than 50 were collapsed.

Because phylogenetic models assume a hierarchical, bifurcating tree that may not apply to these lineages, we explored an alternative network method that allows reticulate evolution. Nuclear networks were created with neighbor-net (Huson and Bryant, 2006) using SplitsTree (vers. 4.8). For distance calculations, we chose the nucleotide substitution model favored by AIC (see Bayesian phylogeny results). Because the GTR model preferred for *LEA-like* is not available in SplitsTree, we chose the most parameterized model. Thus, for both loci, distances were computed under maximum likelihood with an HKY85 model, transitions: transversions weighted 2:1, and gamma, proportion of invariable sites, and base frequencies estimated empirically.

If detected, genetic recombination or a departure from neutrality can offer alternative explanations for incongruence in a phylogenetic analysis. Evidence of recombination was evaluated using alignments with all gaps removed (Posada, 2002), excluding the divergent *P. maximinoi* (Oaxaca) sequence from *LEA-like*, using the Phi test (Bruen et al., 2006) in SplitsTree and RDP, GENECONV, Chimaera, MaxChi, BootScan, SisScan, 3Seq, and LARD methods in RDP3 (vers. 3.22; Martin et al., 2005). For species with three or more accessions per locus, we tested departure from neutrality with: *Fu and Li D* and *F* (outgroup option; Fu and Li, 1993), *Fay and Wu H* (outgroup option; Fay and Wu, 2000), and *Tajima D* (Tajima, 1989) using DnaSP (vers. 4.10.9; Rozas et al., 2003). Significance at the 0.95 level was adjusted for multiple tests (Rice, 1989). We also tested *interspecific* comparisons using all accessions for each locus. For this test, significance was estimated from coalescent simulations (no recombination, moderate recombination, or free recombination) with 15, 25, 50, and 100 bp sliding windows. Interspecific tests were repeated with alignments that excluded all gaps and all missing data.

When lineage sorting is incomplete, multiple accessions of a species fail to resolve as monophyletic, and this pattern is difficult to distinguish from reticulate ancestry. We used population genetic theory to address the extent to which polyphyly could be explained in the *Ponderosae* data set by this phenomenon rather than reticulate evolution. A rough approximation of the coancestry coefficient θ_w (Watterson, 1975) was calculated in DnaSP for three species with five or more samples per locus (*P. jeffreyi, P. ponderosa,* and *P. washoensis*). The mean θ_w for two loci was used to estimate effective population size (N_e) for each species using the formula $N_e = \theta_w/(4\,\mu\,G)$, assuming generation time $G = 50$ years. Because nuclear mutation rates vary widely, we calculated N_e using the mean rate for *Pinus* across nine nuclear loci ($\mu = 0.70 \times 10^{-9}$ substitutions per site per year) and for the mean plus and minus one standard deviation (SD = 0.27×10^{-9} substitutions per site per year; Willyard et al., 2007). We estimated the number of years for each species until allelic monophyly is more likely than paraphyly using the formula: $1.665 \times 2\,N_e\,G$ (Rosenberg, 2003).

As another coarse estimate of the expectation that gene tree topologies could arise through random lineage sorting, we simulated 1000 trees for each of three levels of effective population size (30, 100, and 200×10^3) using the option in Mesquite (vers.2, beta 2; Maddison and Maddison, 2006) to generate gene trees within a species tree using a simple coalescence model (i.e. for a neutral gene and a constant population size). For this test, we used a species tree that unites two polytomies (i.e. four *Sabinianae* and 13 *Ponderosae s.s.*). The symmetric distance (Penny and Hendy, 1985) was calculated in PAUP* for three 'clouds' of 1000 simulated trees, and for each consensus gene tree (with poorly-supported nodes collapsed as described above) against each cloud of simulated trees. We compared the distribution of symmetric distances for each gene tree to each cloud versus the distribution *within* each cloud.

2.6. Analysis of chloroplast locus

Nucleotide sequences of *trnG* were aligned by hand and haplotype networks were created using median joining (Bandelt et al., 1999) in Network vers. 4.5.0.0, www.fluxus-engineering.com.

3. Results

3.1. Nuclear alignments

Sequences from 45 individuals representing 17 species for *LEA-like* were aligned with a length of 1630 bps and 3.05% missing data. The inferred intron varied from 837 to 1515 bps. The simple indel coding method inferred 71 indel characters, 33 shared and 38 singletons. Sequences from 51 individuals representing 16 species for *WD-40* aligned across 1182 bps with 4.43% missing data. The length of the inferred intronic regions varied from 1000 to 1116 bps. We coded 18 indel characters for *WD-40*, 14 shared and four singletons. Alignment of sequences from 49 individuals representing 16 species for the *trnG* intron required one indel in a mononucleotide repeat, for an aligned length of 722 bps.

The *LEA-like* sequence for *P. maximinoi* (Oaxaca) is highly divergent, resolving as sister to the remaining *Ponderosae* on the gene tree and yielding a θ_w in relation to the Honduras accession nearly three SD from the mean (data not shown). Both accessions of *P. maximinoi* were verified by repeated amplification and resequencing. High intraspecific divergence in both nuclear loci for *P. montezumae* (data not shown) was also verified by repeated amplification and resequencing. The *LEA-like* sequence for *P. sabiniana* (Clearlake) contains several small indels in the inferred exon, suggesting that our PCR amplicon may be a pseudogene. We chose to retain this sample in our analyses.

3.2. Bayesian phylogeny

The AIC favored GTR + G for *LEA-like* and HKY + G for *WD-40*. For both loci, two independent Bayesian runs yielded majority-rule consensus trees with identical topologies (Figs. 2 and 3). For two runs, the average standard deviations of split frequencies were 0.003840 and 0.003971 for *LEA-like* and *WD-40* loci, respectively.

Analyses with and without indel coding resulted in identical topologies, differing only in posterior probabilities; some nodes were moved above or below our threshold of 0.95 for well-supported nodes. Inclusion of indel coding resolved more deep nodes for *LEA-like* and more highly-supported nodes for *WD-40*. Topologies were identical and branch lengths were nearly identical for both partitioning schemes for each locus. For *LEA-like* (53 exonic bps), the two-partition model (nucleotides and indel-codes) was preferred, but for *WD-40* (using 103 bps from the first exon), three partitions (synonymous, nonsynonymous, and indel coding) per-

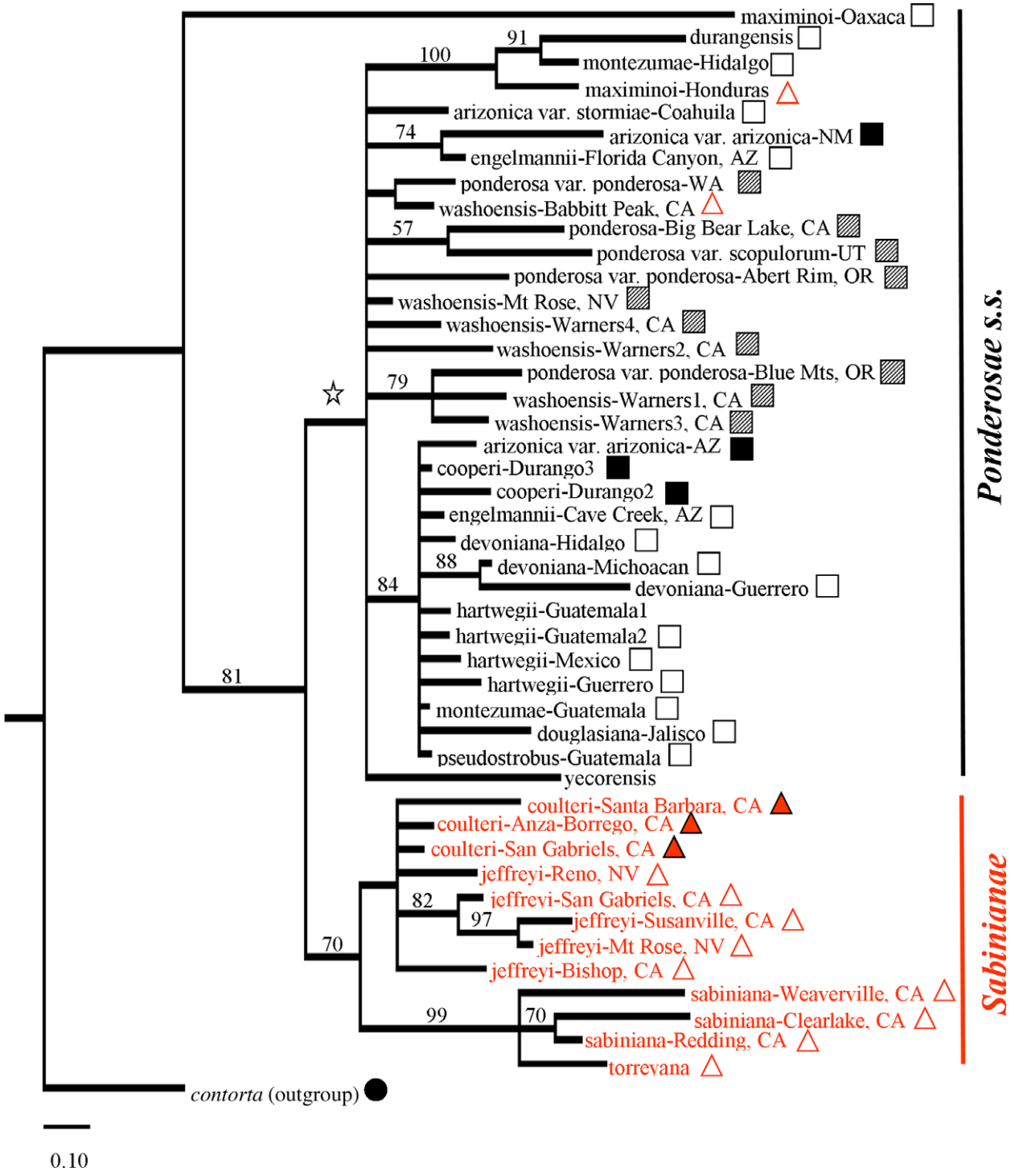

Fig. 2. Bayesian majority-rule consensus tree for *LEA-like* showing only nodes with 0.95 posterior probabilities or higher. Numbers are bootstrap proportions from parsimony analysis. Branches are proportional to length; scale bar is substitutions per site. See Fig. 6a for *trnG* haplotype symbols. Node marked with a star had a parsimony bootstrap less than 50, but the Bayesian posterior probability was 0.98.

formed better. We present only trees inferred with the inclusion of indel coding and the preferred partition.

3.3. Parsimony phylogeny

The parsimony strict consensus gene trees inferred similar topologies to their Bayesian counterparts except that in *WD-40*, eight nodes with 0.95 or greater posterior probability received bootstrap support below 50. We collapsed these nodes as well. Based on this criterion, two nodes would collapse in *LEA-like*. However, one node with a low bootstrap proportion (highlighted with a star in Fig. 2) received a Bayesian posterior probability of 0.98. Because the low support from parsimony may be an artifact, we elected to show this node in Fig. 2.

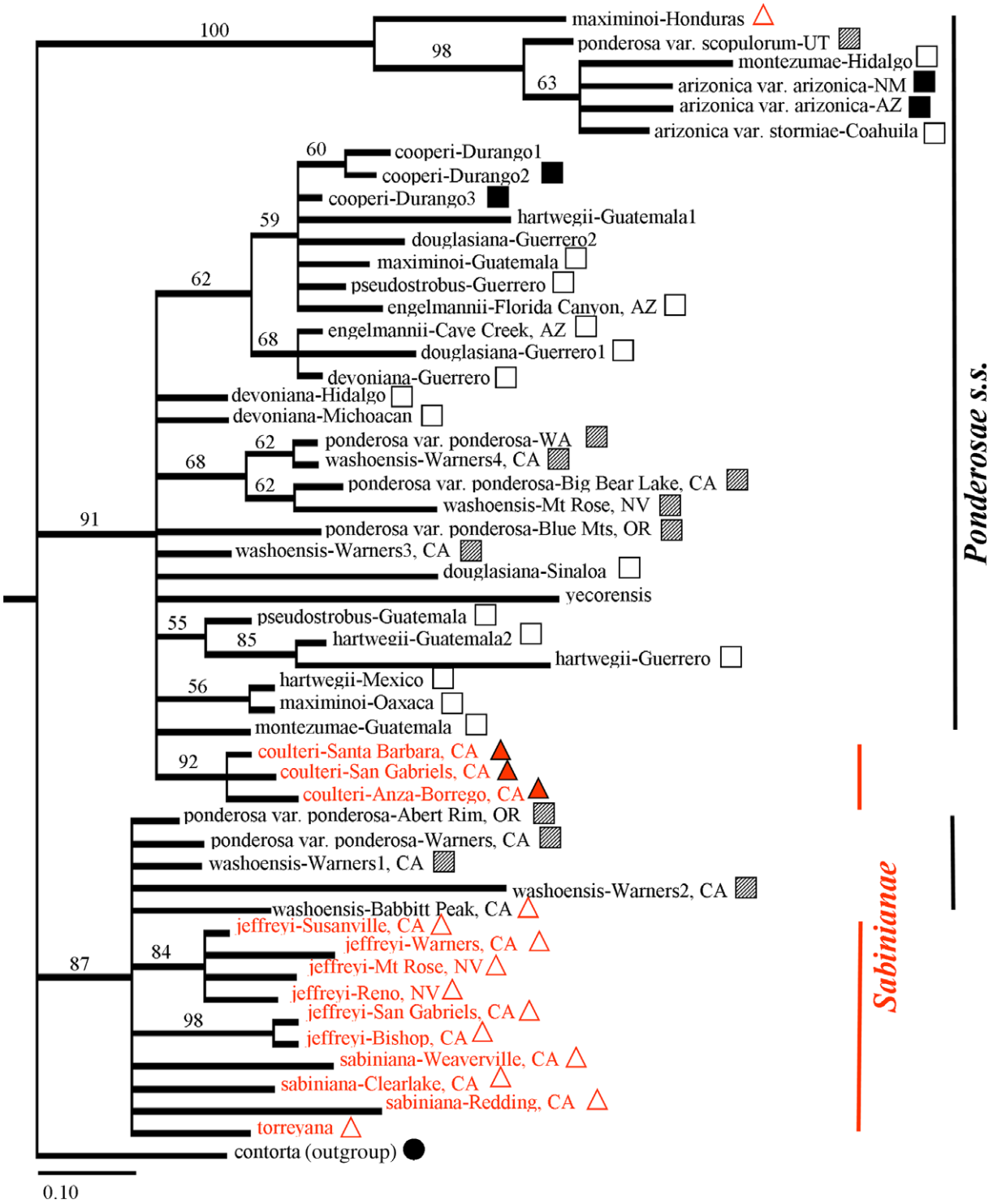

Fig. 3. Bayesian majority-rule consensus tree for *WD-40* showing only nodes with 0.95 posterior probabilities or higher and supported by parsimony bootstrap greater than 50. Numbers are bootstrap proportions from parsimony analysis. Branches are proportional to length; scale bar is substitutions per site. See Fig. 6a for *trnG* haplotype symbols.

3.4. Nuclear gene trees and networks

The nuclear gene trees (Figs. 2 and 3) contain numerous polytomies. If the basal reticulations on the *LEA-like* and *WD-40* networks (Figs. 4 and 5) are viewed as ambiguities rather than lateral transfers, then these networks appear similar to their respective gene trees. A *LEA-like* network that excludes *P. maximinoi* (Oaxaca) displayed a similar level of resolution (results not shown). *LEA-like* resolves *P. maximinoi* (Oaxaca) as an outlier to two clades: one contains *Ponderosae s.s.*, the other with the four *Sabinianae* (including *P. jeffreyi* as expected; Figs. 2 and 4). In contrast, *WD-40* resolves three clades (Figs. 3 and 5). One unites all *P. arizonica* samples with *P. ponderosa* var. *scopulorum*, *P. montezumae* (Hidalgo), and *P. maximinoi* (Honduras). The second clade contains the remaining *Ponderosae s.s.* plus a derived, monophyletic grouping of all three samples of *P. coulteri*. The third clade contains all *Sabin-*

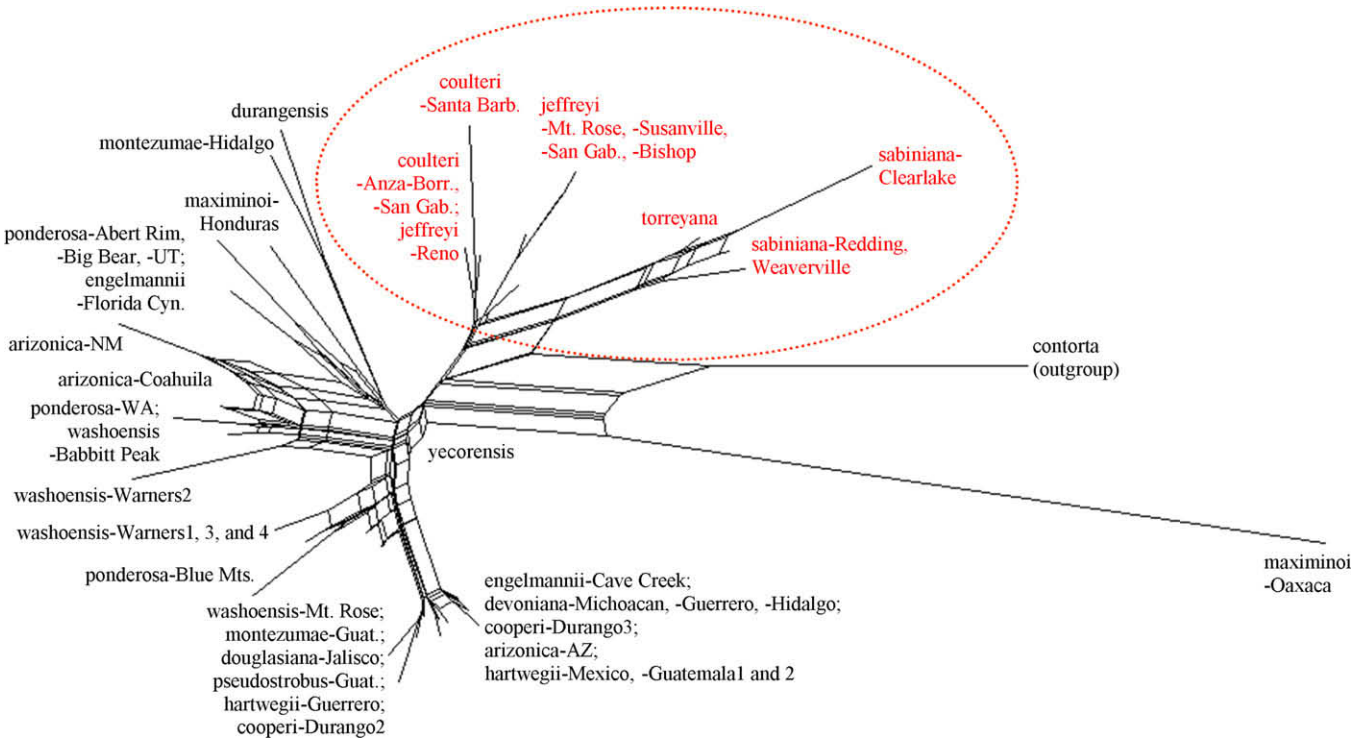

Fig. 4. *LEA-like* neighbor-net network created using maximum likelihood distances. Oval highlights clade that contains four *Sabinianae* species.

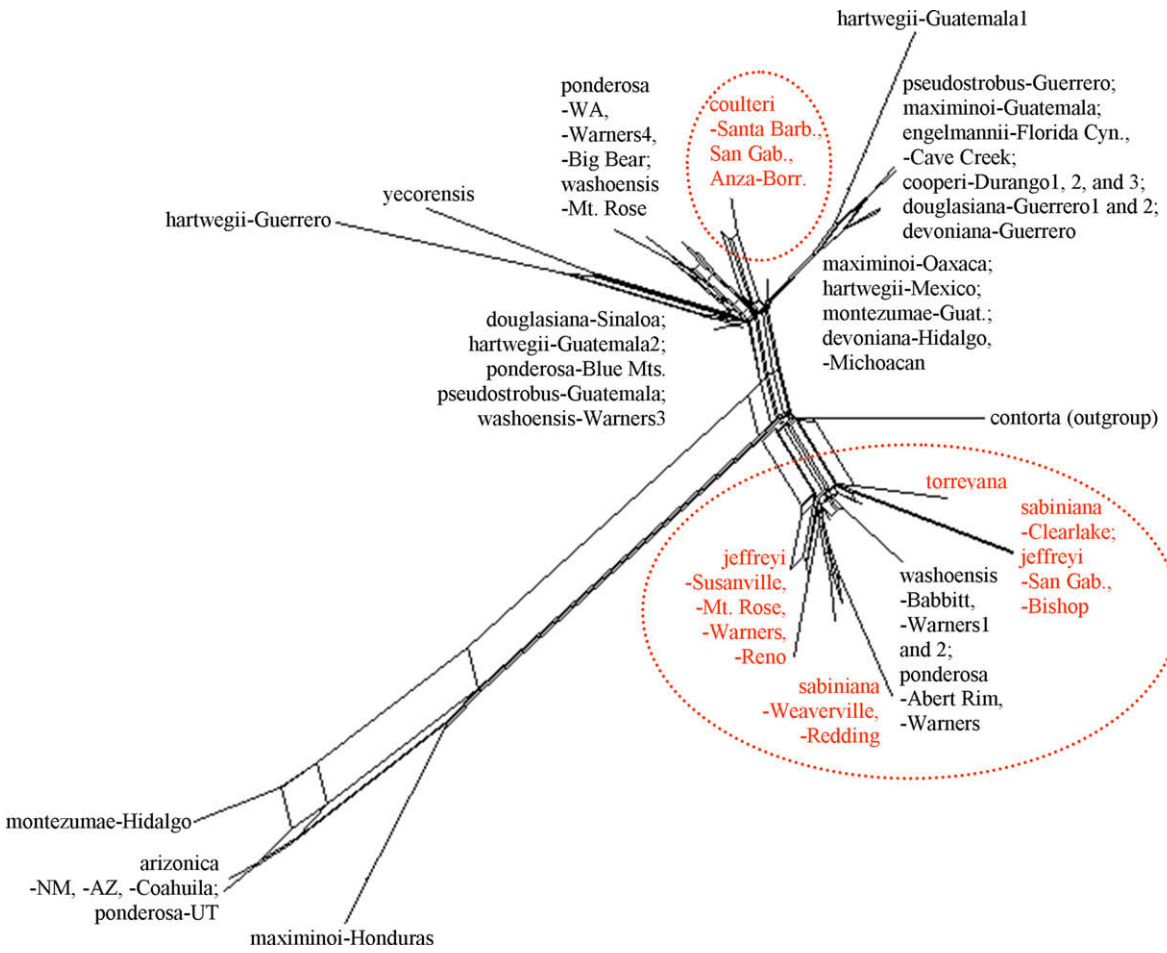

Fig. 5. *WD-40* neighbor-net network created using maximum likelihood distances. Ovals highlight two clades that contain the four *Sabinianae* species.

ianae except *P. coulteri*, and also contains five accessions classified in *Ponderosae s.s.*: two *P. ponderosa*, and three *P. washoensis*.

3.5. Chloroplast haplotype network

We excluded position 492 in the *trnG* alignment due to ambiguities in several sequences. Six phylogenetically informative substitutions (positions 91, 180, 548, 563, 568, and 604 in the alignment) yield six haplotypes that differ by one or two substitutions: CTCGAC (OUTGROUP); CTCGTC (MAIN SABINIANAE); ATCTTC (COULTER); ATTTTC (NORTHERN SIERRA MADRE); CTTGTC (MEXICAN); and CCTGTA (PONDEROSA) (Fig. 6). The OUTGROUP haplotype differs from MAIN SABINIANAE by one substitution (position 568), and the PONDEROSA haplotype differs from MEXICAN by two unique substitutions (positions 180 and 604), but the main cycle of the network cannot be automatically resolved into a tree because two alternate three-step paths (both involving positions 91, 548, and 563) connect MAIN SABINIANAE and NORTHERN SIERRA MADRE haplotypes with either COULTER or MEXICAN as the intermediate. Plotting *trnG* haplotypes on nuclear gene trees (Figs. 2 and 3) suggests a substantially different chloroplast lineage. However, the *trnG* network cycle can be arbitrarily broken (Fig. 6b) to yield a topology with a *Sabinianae-Ponderosa s.s.* divergence similar to that recovered from cpDNA phylogeny (Gernandt et al., 2009).

For 13 species, only one chloroplast haplotype was observed. However, two accessions of *P. arizonica* var. *arizonica* carry the NORTHERN SIERRA MADRE, while *P. arizonica* var. *stormiae* (Coahuila) shares the MEXICAN haplotype. *Pinus maximinoi* (Honduras) and *Pinus washoensis* (Babbitt Peak) share the MAIN SABINIANAE haplotype.

3.6. Recombination

Excluding the divergent *P. maximinoi* (Oaxaca) accession and using alignments with all gaps removed, the Phi test finds significant evidence for recombination for *LEA-like* ($P = 0.037$), but not for *WD-40* ($P = 0.915$). With all missing data removed from *LEA-like*, the Phi test is not significant ($P = 0.376$).

3.7. Neutrality

After correcting for multiple tests, no *intraspecific* test revealed a significant departure from neutrality at either locus. Negative departure is indicated for *LEA-like* at all *interspecific* tests

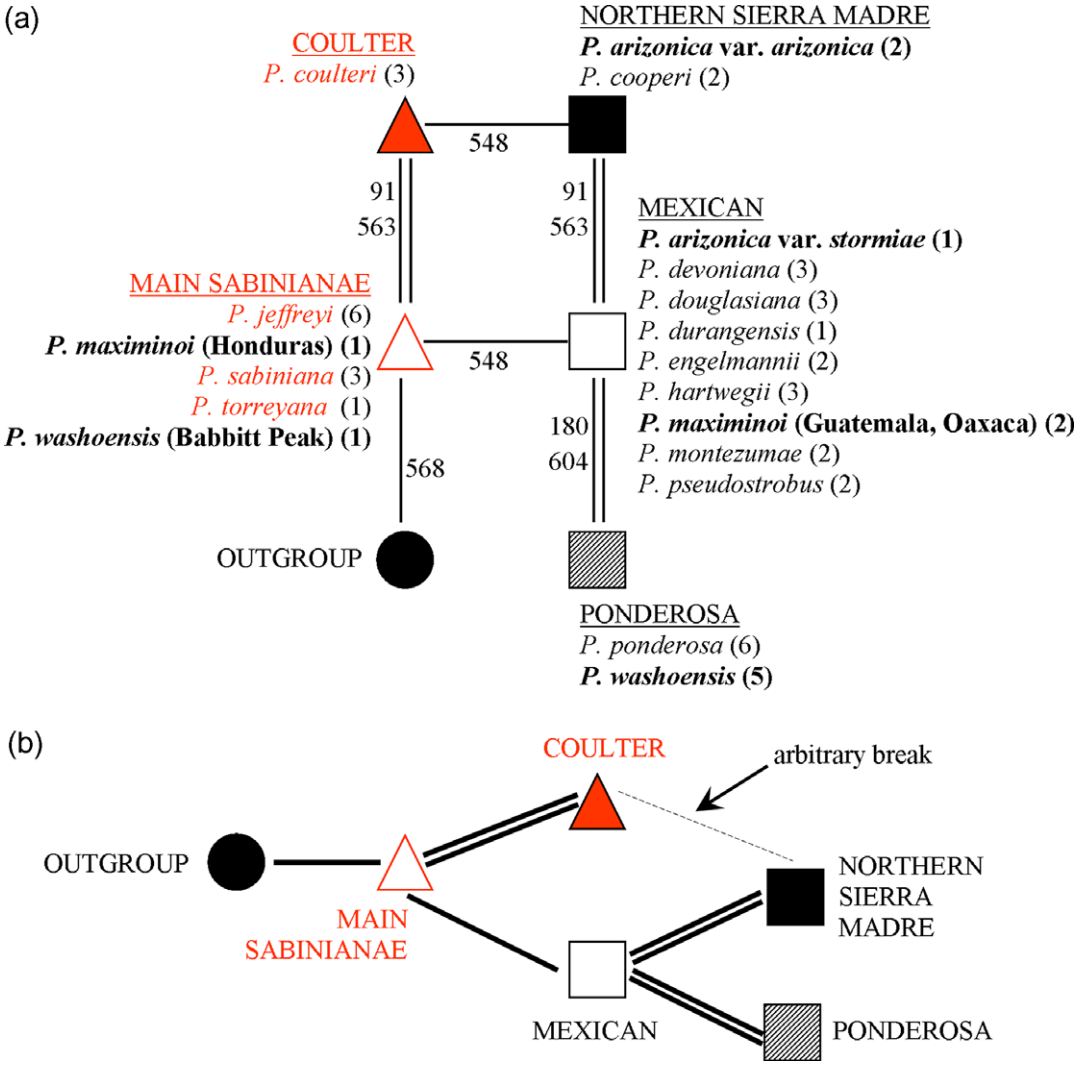

Fig. 6. (a) Chloroplast *trnG* haplotype network. Single and double lines represent one and two nucleotide substitutions, respectively, at six aligned positions: 91, 180, 548, 563, 568, and 604. Number of accessions in parenthesis. Species with more than one haplotype are in bold. (b) Arbitrarily resolved network (see Section 3.5).

Table 3
Projected coalescence times for three species. Effective population sizes (N_e) are inferred from the mean intraspecific coancestry coefficient (θ_w) for two loci assuming a generation time $G = 50$. Years for monophyly to be more likely than paraphyly (million years to coalesce) are estimated with the formula $1.665 \times 2\,N_e\,G$ by bracketing the mean mutation rate ($\mu = 0.70 \times 10^{-9}$ substitutions per site per year) with the rate plus and minus one SD (0.27×10^{-9}) as described in Section 2.5.

	LEA-like		*WD-40*		Mean	N_e ($\times 10^3$)	Million years to coalesce
	n	θ_w	n	θ_w	θ_w	μ: +1 SD/mean/−1 SD	
P. jeffreyi	5	0.00433	6	0.00447	0.00440	51/31/23	9/5/4
P. washoensis	6	0.01020	6	0.00650	0.00835	97/60/43	16/10/7
P. ponderosa	5	0.01642	6	0.01211	0.01427	166/102/74	28/17/12

($P < 0.02$), consistent with positive selection. However, these tests are not significant when all missing data are removed.

3.8. Time to monophyly

For three species with five or more samples, we used intraspecific diversity to infer years until allelic monophyly is more likely than paraphyly. These calculations suggest that N_e ranges from ca. 23×10^3 to 166×10^3 and years to coalesce from ca. 4 to 28 million years (Table 3).

3.9. Coalescent simulations

Distributions of symmetric distances *within* a 1000-tree cloud of simulated trees are nearly identical at three levels of N_e (Fig. 7a). The *WD-40* consensus gene tree differs more from a cloud of simulated trees (symmetric distances range from 68 to 70) than does the *LEA-like* consensus gene tree (symmetric distances range from 64 to 66; Fig. 7b). However, ranges of mean distances from either consensus gene tree *to* the cloud (Fig. 7b) are far below the range of distances *within* each cloud of trees (94–102; Fig. 7a).

4. Discussion

Our tests did not reveal evidence for genetic recombination or for a departure from neutrality. Thus, neither of these mechanisms can explain the lack of monophyletic species observed across the nuclear gene trees. Projections based on intraspecific genetic diversity and on coalescent simulations hint that the retention of ancestral polymorphism may explain most incongruence in this *Ponderosae* data set. Mean coalescence times for two species (*P. ponderosa* and *P. washoensis*; Table 3) approach the inferred stem age (about 15 million years) for the entire 17-species subsection and all three species meet or exceed the inferred crown age (about 5 million years) (Willyard et al., 2007), suggesting that allelic monophyly may be unlikely for many loci across their nuclear genomes. Similarly, coalescent simulations for our 17-species phylogenies reveal that tree-to-tree distances within a cloud of simulated trees are large enough to contain the distances of each gene tree to the cloud. Thus, our phylogenetic trees combine two features which increase the probability of incongruence: numerous tips and species with large effective population sizes. For these phylogenies, reticulate evolution need not be invoked to explain most instances of incongruence. In some data sets, the removal of a putative hybrid allele can improve the resolution. For our *Ponderosae* gene trees, excluding the divergent *P. maximinoi* (Oaxaca) accession yields a nearly identical topology (results not shown).

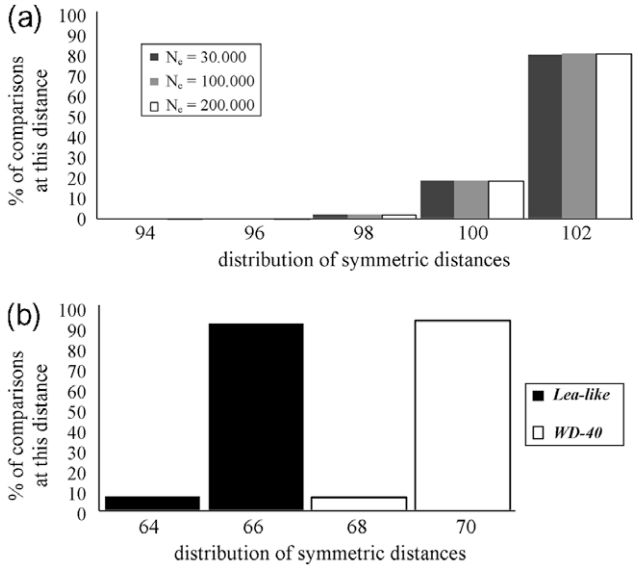

Fig. 7. Distribution of symmetric distances for gene trees simulated using a simple coalescent model within a species tree; (a) within 'clouds' of gene trees simulated for three levels of effective population size (N_e); (b) from the *LEA-like* consensus gene tree and from the *WD-40* consensus gene tree to the cloud of trees simulated using N_e = 100,000.

A lack of allelic monophyly was also observed in a *Ponderosae* phylogeny based on different taxonomic sampling that used sequences of chloroplast noncoding regions (Gernandt et al., 2009). Despite their morphological and ecological distinctiveness, a pattern of molecular polyphyly may be expected for these species because effective population sizes are large relative to the number of generations since divergence. In the *Ponderosae*, this pattern extends rather deeply into the gene trees, and none of the previously proposed subdivisions within *Ponderosae s.s.* (Table 1) are resolved in either gene tree. This suggests that these groupings are sufficiently young that incomplete lineage sorting could explain conflicting placements within *Ponderosae s.s.*

However, we found support for the traditional *Sabinianae-Ponderosae s.s.* clades (Figs. 2–5 and 6b). Across this deep node, conflicts between three independent genomic regions are less likely to be attributable to incomplete lineage sorting, and can thus be used to identify potential examples of reticulate evolution. The most dramatic example of incongruence in our data set is the resolution of a monophyletic *P. coulteri* clade within *Ponderosae s.s.* in *WD-40* (Figs. 3 and 5). In addition, five *Ponderosae s.s.* resolve with *Sabinianae* in *WD-40* (Figs. 3 and 5) and two *Ponderosae s.s.* share the MAIN SABINIANAE chloroplast haplotype (Fig. 6).

4.1. Pinus coulteri

The very distinctive Coulter pine is allied with two other California big-cone pines (*P. sabiniana* and *P. torreyana*; Price et al., 1998). Unique allozyme alleles are observed in some *P. coulteri* populations, and hybridization with *P. jeffreyi* was proposed as a potential source for these alleles (Ledig, 2000). In the present study, three accessions of *P. coulteri* resolve with *Ponderosae s.s.* in *WD-40* as a monophyletic clade with a moderate branch length. At this level of sampling (three *P. coulteri*; 48 others), monophyly due to random branching is highly unlikely ($P < 0.01$; Rosenberg, 2007). *Pinus coulteri*'s intraspecific diversity can be roughly

approximated using the mean of two loci for three accessions in this study as $\theta_w = 0.00729$. This diversity is relatively low, suggesting that *P. coulteri* is unlikely to have maintained ancient *WD-40* alleles across its genome. However, differential retention of ancestral alleles can occur due to locus-specific effects during a genetic bottleneck.

The *WD-40* gene tree topology could be explained by hybridization. Artificial and a few putative natural *P. coulteri* × *P. jeffreyi* hybrids have been viewed as crossing the *Sabinianae-Ponderosae s.s.* division (Critchfield, 1966), but our results confirm that *P. coulteri* and *P. jeffreyi* are rather closely related members of the *Sabinianae*. *Pinus coulteri* has not been successfully crossed with any extant *Ponderosae s.s.* species (Critchfield, 1966). Further, the topology argues for 'ancient' rather than 'recent' introgression because the node representing the most recent common ancestor of the "*ponderosae s.s.*-style" *P. coulteri* alleles is relatively deep in the *WD-40* gene tree.

We propose that *P. coulteri* might have retained *WD-40* alleles from an unknown *Ponderosae s.s.* parent and that this could account for the unique allozyme alleles as well. Two alternatives can be envisioned: (i) introgression after *P. coulteri* had speciated; or (ii) *P. coulteri* arising as a diploid hybrid species. In either case, hybridization was likely followed by one or more bottlenecks that purged part of the genome. *Pinus coulteri*'s massive and well-armed cones may have contributed to reproductive isolation. Adaptation to different seed predators (Borchert, 1985) might have allowed *P. coulteri* to move into hotter and drier foothill habitats than either of its parents (or its introgressing partner), providing reproductive isolation by allopatry. Scenarios of either introgression or hybrid speciation fit a theoretical framework for hybridization as an evolutionary stimulus (Anderson and Stebbins, 1954) and we are conducting further sampling to address the origin of *P. coulteri*.

4.2. Pinus ponderosa and P. washoensis

Throughout much of its geographic range, *P. jeffreyi* meets lower-altitude *P. ponderosa* or higher-altitude *P. washoensis* at the margins of each species' zone. Morphological traits support a few natural hybrids between *P. jeffreyi* and *P. ponderosa* (Haller, 1962), and the nature of this putative introgression is more interesting in light of our current understanding that *P. jeffreyi* is part of the *Sabinianae* lineage. In *LEA-like*, all accessions of *P. ponderosa* and *P. washoensis* resolve as expected within *Ponderosae s.s.* Our *trnG* network supports a transfer of the MAIN SABINIANAE chloroplast haplotype into one individual of *P. washoensis* (Babbitt Peak), which harbors a haplotype that is three substitutions removed from its conspecifics (Fig. 6). *WD-40* resolves the Babbitt Peak accession plus two other *P. washoensis* and two *P. ponderosa* individuals with the *Sabinianae*. The remaining 'misplaced' individuals carry the expected PONDEROSA chloroplast haplotype (Fig. 6a). This supports a low level of ongoing introgression between *P. jeffreyi* and *P. ponderosa* and between *P. jeffreyi* and *P. washoensis*.

Studies that place a *P. washoensis* allele sister to *P. jeffreyi* or sister to *P. sabiniana* (Patten and Brunsfeld, 2002; Prager et al., 1976), or that place *P. jeffreyi* within *Ponderosae s.s.* (Eckert and Hall, 2006) may be footprints of introgression. We hypothesize that reticulate ancestry might contribute to a preference for high-altitude sites in *P. washoensis* (Haller, 1965; Mason and Stockwell, 1945). All species of *Sabinianae* grow in colder climates or on ultramafic soil (*P. jeffreyi*; Haller, 1962) or in more arid habitats (*P. coulteri*, *P. sabiniana*, and *P. torreyana*) than *P. ponderosa*. Perhaps high-altitude Washoe pines represent a lineage that has retained more of the introgressed *Sabinianae* genome because of traits that are more useful in harsh climates. Additional information from the maternally inherited mitochondrial genome (Godbout et al., 2005) may be useful to detect recent hybridization, and we are currently assessing introgression in *P. washoensis* using nuclear microsatellite loci for population-level samples.

The lack of reciprocal monophyly for *P. ponderosa* and *P. washoensis* and their shared chloroplast haplotype might be interpreted as support for the conclusion that the narrowly endemic *P. washoensis* is synonymous with the wide-ranging *P. ponderosa* (Brayshaw, 1997; Lauria, 1997; Niebling and Conkle, 1990; Rehfeldt, 1999b). However, we note that *none* of the *Ponderosae* species achieve monophyly in both of our gene trees.

4.3. Incomplete lineage sorting or reticulation?

Anomalous results for *P. maximinoi* and *P. montezumae* could be explained by incomplete lineage sorting, but there are indications that future studies designed to detect reticulate ancestry may be fruitful. *Pinus maximinoi* encompasses an unusual range of genetic diversity across its wide distribution. In our study, *P. maximinoi* (Oaxaca) is highly divergent from other *LEA-like* sequences, but this accession is unremarkable in *WD-40*, and it is the *P. maximinoi* (Honduras) allele that resolves unexpectedly in *WD-40*. Artificial hybrids can be created between *P. maximinoi* and *P. taeda* (subsect. *Australes*; Dvorak et al., 2000). These clues suggest that our results could be due to misidentifications, cryptic species, or introgression (perhaps even outside its taxonomic subsection).

Although our two *P. montezumae* accessions do not sort across the *Sabinianae-Ponderosae s.s.* divergence, the sequences resolve in very different locations on both nuclear gene trees despite sharing a *trnG* haplotype (Fig. 6a). Because *P. montezumae* can be difficult to distinguish from *P. devoniana*, *P. hartwegii*, and *P. pseudostrobus* (Perry, 1991), misidentification cannot be ruled out. Alternatively, the extreme variability may be attributed to the complex patterns of interspecific hybridization reported for *P. montezumae* with *P. pseudostrobus* and with *P. hartwegii* (Delgado et al., 2007; Matos and Schaal, 2000). Recent introgression would not explain our placement of the Hidalgo accession because the geographic ranges of the species that *P. montezumae* joins (*P. durangensis* and *P. arizonica*) do not extend into Hidalgo. Incomplete lineage sorting could create the patterns we observe, but in light of strong evidence for ongoing localized hybridization, our results do not exclude *ancient* introgression between *P. montezumae* and *previously* sympatric species.

4.4. Species tree

Because the gene trees inferred by *LEA-like* and *WD-40* are different, we do not present a combined species phylogeny. Our two nuclear gene trees are too incongruent to calculate "Concordance Factors" for internal nodes (Baum, 2007) using BUCKy (Ané et al., 2007). We note that any quantitative assessment of discordance is obscured by the astronomical number of potential topologies possible for a 53-tip tree (more than 7×10^{81}; Felsenstein, 2004). Although networks help visualize the extent to which gene trees are incongruent (Figs. 4 and 5; McBreen and Lockhart, 2006), they do not help distinguish between incomplete lineage sorting and reticulation. As a further example, the numerous reticulations suggested by T-Rex (data not shown; Makarenkov and Lapointe, 2004) are difficult to interpret because they are inferred on neighbor-joining trees that resolve different nodes than our Bayesian and Parsimony gene trees.

We suggest that the low resolution observed here and in other species-level *Ponderosae* phylogenies (Gernandt et al., 2005, 2009) is reflective of the recency of species divergence. Despite the wide range of mutation rates among *Pinus* loci (Willyard et al., 2007), this low resolution is likely to be mirrored across nuclear and chloroplast genomes.

5. Conclusions

The inference of hybrid ancestry in natural species is a challenging but worthwhile endeavor. Comparing phylogenetic hypotheses based on independent genomic regions is an important method to detect potential reticulations, but requires the exclusion of other causes of incongruence. In this data set, we did not detect genetic recombination or a departure from neutrality. However, our rough calculations suggest that incomplete lineage sorting is a major source of the incongruence observed at all but the deepest nodes of the *Ponderosae* tree. Phylogenetic inferences like the one presented here for reticulate evolution in *P. coulteri* and among *P. jeffreyi*, *P. ponderosa*, and *P. washoensis*, do not meet the standard of resynthesizing a hybrid species (Rieseberg et al., 2003). Further, our imposition of a tree-like hierarchy on obviously not-quite-tree-like relationships is perilous. Nevertheless, if these methods are used and interpreted cautiously, valuable clues can be gleaned about potential examples of reticulate evolution that are worthy of future study. This can be accomplished without the large number of loci that will apparently be required (Maddison and Knowles, 2006) to infer species relationships from multiple gene trees. In particular, a phylogenetic overview like the one created here from independent genomic regions is crucial to the identification of the potential gene-flow 'players', which may be taxonomically distant as well as geographically remote in their current distributions. It is clear from our results that species-level diagnosis based on either single-accession sampling or single-locus sampling in *Pinus* is inadequate.

Acknowledgments

We thank Mariah Parker-DeFeniks, Anthony Shireman, Carla Streng, and Kathleen Farrell for laboratory work. The many collectors who generously contributed plant material are gratefully acknowledged in our GenBank records. We are indebted to David Gernandt for sharing unpublished information as well as helpful comments. We thank Tim Cowman, John R. Haller, Mathew Horning, Susan Huber, Ronald Lanner, Molly Nepokroeff, Bernard Pfeil, Joe Reynoldson, and Conrad Schoch, and two anonymous reviewers for valuable guidance. Funding was provided by National Science Foundation grant DEB 0317108 to Aaron Liston and Richard Cronn and by USDA Forest Service Pacific Northwest Research Station.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.02.011.

References

Akaike, H., 1974. A new look at the statistical model identification. IEEE Trans. Automatic Control 19, 716–723.
Alvin, K.L., 1960. Further conifers of the Pinaceae from the Wealden Formation of Belgium. Institut Royal des Sciences Naturelles de Belgique. Mémoires 146, 1–39.
Anderson, E., Stebbins Jr., G.L., 1954. Hybridization as an evolutionary stimulus. Evolution 8, 378–388.
Ané, C., Larget, B., Baum, D.A., Smith, S.D., Rokas, A., 2007. Bayesian estimation of concordance among gene trees. Mol. Biol. Evol. 24, 412–426.
Arnold, M.L., 1993. *Iris nelsonii* (Iridaceae): origin and genetic composition of a homoploid hybrid species. Am. J. Bot. 80, 577–583.
Arnold, M.L., 1997. Natural Hybridization and Species Concepts. Oxford Univ. Press, New York, NY.
Bandelt, H.-J., Forster, P., Röhl, A., 1999. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. 16, 37–48.
Baum, D.A., 2007. Concordance trees, concordance factors, and the exploration of reticulate genealogy. Taxon 56, 417–426.
Birky Jr., C.W., Maruyama, T., Fuerst, P., 1983. An approach to population and evolutionary genetic theory for genes in mitochondria and chloroplasts, and some results. Genetics 103, 513–527.
Borchert, M., 1985. Serotiny and cone-habit variation in populations of *Pinus coulteri* (Pinaceae) in the southern Coast Ranges of California. Madroño 32, 29–48.
Brayshaw, T.C., 1997. Washoe and ponderosa pines on Promontory Hill near Merritt, B.C., Canada. Ann. Naturhist. Mus. Wien 99B, 673–680.
Bruen, T.C., Philippe, H., Bryant, D., 2006. A simple and robust statistical test for detecting the presence of recombination. Genetics 172, 2665–2681.
Bucci, G., Anzidei, M., Madaghiele, A., Vendramin, G.G., 1998. Detection of haplotypic variation and natural hybridization in *halepensis*-complex pine species using chloroplast simple sequence repeat (SSR) markers. Mol. Ecol. 7, 1633–1643.
Carstens, B.C., Knowles, L., 2007. Estimating species phylogeny from gene-tree probabilities despite incomplete lineage sorting: an example from *Melanoplus* grasshoppers. Syst. Biol. 56, 400–411.
Christensen, K.I., Dar, G.H., 1997. A morphometric analysis of spontaneous and artificial hybrids of *Pinus mugo* ×*sylvestris* (Pinaceae). Nordic J. Bot. 17, 77–86.
Critchfield, W.B., 1966. Crossability and relationships of the California big-cone pines. USDA Forest Service Research Paper NC-6, pp. 36–44.
Critchfield, W.B., 1986. Hybridization and classification of the white pines (*Pinus* section *Strobus*). Taxon 35, 647–656.
Cronn, R., Wendel, J.F., 2004. Cryptic trysts, genomic mergers, and plant speciation. New Phytol. 161, 133–142.
Delgado, P., Salas-Lizana, R., Vásquez-Lobo, A., Wegier, A., Anzidei, M., Alvarez-Buylla, E.R., Vendramin, G.G., Piñero, D., 2007. Introgressive hybridization in *Pinus montezumae* Lamb and *Pinus pseudostrobus* Lindl. (Pineaceae): morphological and molecular (cpSSR) evidence. Int. J. Plant Sci. 168, 861–875.
Dong, J., Wagner, D.B., 1993. Taxonomic and population differentiation of mitochondrial diversity in *Pinus banksiana* and *Pinus contorta*. Theor. Appl. Genet. 86, 573–578.
Doolittle, W.F., Bapteste, E., 2007. Pattern pluralism and the Tree of Life hypothesis. Proc. Natl. Acad. Sci. USA 104, 2043–2049.
Doyle, J.J., Doyle, J.L., Rauscher, J.T., Brown, A.H.D., 2004. Diploid and polyploid reticulate evolution throughout the history of the perennial soybeans (*Glycine* subgenus *Glycine*). New Phytol. 161, 121–132.
Dvorak, W.S., Hamrick, J.L., Furman, B.J., Hodge, G.R., Jordan, A.P., 2000. Conservation efforts for *Pinus maximinoi* in Mesoamerica and its potential as a hybrid with *Pinus taeda* in South America. In: CAMCORE (Ed.), Conservation and testing of tropical and subtropical forest tree species. North Carolina State Univ., Raleigh, NC, p. 16.
Eckert, A.J., Hall, B.D., 2006. Phylogeny, historical biogeography, and patterns of diversification for *Pinus* (Pinaceae): phylogenetic tests of fossil-based hypotheses. Mol. Phylogenet. Evol. 40, 166–182.
Ellstrand, N.C., Schierenbeck, K.A., 2000. Hybridization as a stimulus for the evolution of invasiveness in plants? Proc. Natl. Acad. Sci. USA 97, 7043–7050.
Epperson, B., Chung, M., Telewski, F., 2003. Spatial pattern of allozyme variation in a contact zone of *Pinus ponderosa* and *P. arizonica* (Pinaceae). Am. J. Bot. 90, 25–31.
Epperson, B., Telewski, F., Willyard, A., 2009. Chloroplast diversity in a putative hybrid swarm of *Ponderosae*. Am. J. Bot. 96.
Farjon, A., Styles, B., 1997. Flora Neotropica, Monograph 75, *Pinus* (Pinaceae). The New York Botanical Garden, New York, NY, USA.
Fay, J.C., Wu, C.-I., 2000. Hitchhiking under positive Darwinian selection. Genetics 155, 1405–1413.
Felsenstein, J., 2004. Inferring Phylogenies. Sinauer Assoc. Inc., Sunderland, MA.
Fu, Y.-X., Li, W.-H., 1993. Statistical tests of neutrality of mutations. Genetics 133, 693–709.
Gernandt, D.S., Hernández-León, S., Salgado-Hernández, E., Pérez de la Rosa, J.A., 2009. Phylogenetic relationships of *Pinus* subsection *Ponderosae* inferred from rapidly evolving cpDNA regions. Sys. Bot. 34.
Gernandt, D.S., Gaeda López, G., García, S.O., Liston, A., 2005. Phylogeny and classification of *Pinus*. Taxon 54, 29–42.
Godbout, J., Jaramillo-Correa, J.P., Beaulieu, J., Bousquet, J., 2005. A mitochondrial DNA mnisatellite reveals the postglacial history of jack pine (*Pinus banksiana*), a broad-range North American conifer. Mol. Ecol. 14, 3497–3512.
Grant, V., 1981. Plant Speciation, second ed. Columbia Univ. Press, New York, NY.
Haller, J.R., 1962. Variation and hybridization in ponderosa and Jeffrey pines. Univ. Calif. Publ. Bot. 34, 123–165.
Haller, J.R., 1965. *Pinus washoensis* in Oregon: taxonomic and evolutionary implications. Am. J. Bot. 52, 646.
Holmgren, P.K., Holmgren, N.H., 1998 [continuously updated]. Index Herbariorum: a global directory of public herbaria and associated staff. New York Botanical Garden's Virtual Herbarium. Available from: <http://sweetgum.nybg.org/ih/>.
Hosono, S., Faruqi, A.F., Dean, F.B., Du, Y., Sun, Z., Wu, X., Du, J., Kingsmore, S.F., Egholm, M., Lasken, R.S., 2003. Unbiased whole-genome amplification directly from clinical samples. Genome Res. 13, 954–964.
Huson, D.H., Bryant, D., 2006. Application of phylogenetic networks in evolutionary studies. Mol. Biol. Evol. 23, 254–267.
Kral, R., 1993. *Pinus*. In: Flora of North America Editorial Committee (Ed.), Flora of North America, vol. 2. Oxford Univ. Press, New York, NY, pp. 373–398.
Krupkin, A.B., Liston, A., Strauss, S.H., 1996. Phylogenetic analysis of the hard pines (*Pinus* subgenus *Pinus*, Pinaceae) from chloroplast DNA restriction site analysis. Am. J. Bot. 83, 489–498.
Krutovsky, K.V., Troggio, M., Brown, G.R., Jermstad, K.D., Neale, D.B., 2004. Comparative mapping in the Pinaceae. Genetics 168, 447–461.
Lanner, R.M., Phillips, A.M., 1992. Natural hybridization and introgression of pinyon pines in northwestern Arizona. Int. J. Plant Sci. 153, 250–257.

Lauria, F., 1997. The taxonomic status of *Pinus washoensis* H. Mason & Stockw. (Pinaceae). Ann. Naturhist. Mus. Wien 99B, 655–671.
Ledig, F.T., 1998. Genetic variation in *Pinus*. In: Richardson, D.M. (Ed.), Ecology and Biogeography of *Pinus*. Cambridge Univ. Press, Cambridge, UK, pp. 251–280.
Ledig, F.T., 2000. Founder effects and the genetic structure of Coulter pine. J. Hered. 91, 307–315.
Libby, W.J., 1958. The backcross hybrid Jeffrey × (Jeffrey × Coulter) pine. J. For. 56, 840–842.
Liston, A., Parker-Defeniks, M., Syring, J.V., Willyard, A., Cronn, R., 2007. Interspecific phylogenetic analysis enhances intraspecific phylogeographic inference. A case study in *Pinus lambertiana*. Mol. Ecol. 16, 3926–3937.
Liston, A., Robinson, W.A., Piñero, D., Alvarez-Buylla, E.R., 1999. Phylogenetics of *Pinus* (Pinaceae) based on nuclear ribosomal DNA internal transcribed spacer region sequences. Mol. Phylogenet. Evol. 11, 95–109.
Little, E.L., Critchfield, W.B., 1969. Subdivisions of the genus *Pinus* (Pines). USDA Forest Service Misc. Pub. 1144.
Loudon, J., 1838. Arboretum et fruticetum britannicum. James Ridgeway and Sons, London.
Ma, X.-F., Szmidt, A.E., Wang, X.-R., 2006. Genetic structure and evolutionary history of a diploid hybrid pine *Pinus densata* inferred from the nucleotide variation at seven gene loci. Mol. Biol. Evol. 23, 807–816.
Maddison, W.P., Maddison, D., 2006. Mesquite: a modular system for evolutionary analysis, vers. 1.12. Available from: <http://mesquiteproject.org>.
Maddison, W.P., Knowles, L.L., 2006. Inferring phylogeny despite incomplete lineage sorting. Syst. Biol. 55, 21–30.
Makarenkov, V., Lapointe, F.-J., 2004. A weighted least-squares approach for inferring phylogenies from incomplete distance matrices. Bioinformatics 20, 2113–2121.
Martin, D.P., Williamson, C., Posada, D., 2005. RDP2: recombination detection and analysis from sequence alignments. Bioinformatics 21, 260–262.
Martínez, M., 1948. Los Pinos Mexicanos, Segunda Edicion. Ediciones Botas, Mexico City, Mexico.
Mason, H.L., Stockwell, W.P., 1945. A new pine from Mount Rose, Nevada. Madroño 8, 61–63.
Matos, J.A., 1995. *Pinus hartwegii* and *P. rudis*: a critical assessment. Syst. Bot. 20, 6–21.
Matos, J.A., Schaal, B.A., 2000. Chloroplast evolution in the *Pinus montezumae* complex: a coalescent approach to hybridization. Evolution 54, 1218–1233.
McBreen, K., Lockhart, P.J., 2006. Reconstructing reticulate evolutionary histories of plants. Trends Ecol. Evol. 11, 398–404.
McDade, L., 1990. Hybrids and phylogenetic systematics I. Patterns of character expression in hybrids and their implications for cladistic analysis. Evolution 44, 1685–1700.
McDade, L., 1992. Hybrids and phylogenetic systematics II. The impact of hybrids on cladistic analysis. Evolution 46, 1329–1346.
Mirov, N.T., 1961. Composition of gum turpentines of pines. USA For. Service Tech. Bull. 1239.
Müller, K., 2005. SeqState—primer design and sequence statistics for phylogenetic DNA data sets. Appl. Bioinform. 4, 65–69.
Namkoong, G., 1966. Statistical analysis of introgression. Biometrics 22, 488–502.
Niebling, C.R., Conkle, M.T., 1990. Diversity of Washoe pine and comparisons with allozymes of ponderosa pine races. Can. J. For. Res. 20, 298–308.
Nylander, J.A.A., Ronquist, F., Huelsenbeck, J.P., Nieves-Aldrey, J.L., 2004. Bayesian phylogenetic analysis of combined data. Syst. Biol. 53, 47–67.
Patten, A., Brunsfeld, S., 2002. Evidence of a novel lineage within the *Ponderosae*. Madroño 49, 189–192.
Penny, D., Hendy, M.D., 1985. The use of tree comparison metrics. Syst. Zool. 34, 75–82.
Perry Jr., J.P., 1991. The Pines of Mexico and Central America. Timber Press, Portland, OR, USA.
Petit, R.J., Bialozyt, R., Garnier-Gére, P., Hampe, A., 2004. Ecology and genetics of tree invasions: from recent introductions to Quaternary migrations. For. Ecol. Manag. 197, 117–137.
Petit, R.J., Bodénès, C., Ducousso, A., Roussel, G., Kremer, A., 2003. Hybridization as a mechanism of invasion in oaks. New Phytol. 161, 151–164.
Pollard, D.A., Iyer, V.N., Moses, A.M., Eisen, M.B., 2006. Widespread discordance of gene trees with species tree in *Drosophila*: evidence for incomplete lineage sorting. PLoS Gen. 2, 1634–1646.
Posada, D., 2002. Evaluation of methods for detecting recombination from DNA sequences: empirical data. Mol. Biol. Evol. 19, 708–717.
Posada, D., Crandall, K.A., 2001. Intraspecific gene genealogies: trees grafting into networks. Trends Ecol. Evol. 16, 37–45.
Prager, E.M., Fowler, D.P., Wilson, A.C., 1976. Rates of evolution in conifers (Pinaceae). Evolution 30, 637–649.
Price, R.A., Liston, A., Strauss, S.H., 1998. Phylogeny and Systematics of *Pinus*. In: Richardson, D.M. (Ed.), Ecology and Biogeography of *Pinus*. Cambridge Univ. Press, Cambridge, UK, pp. 49–68.
Rehfeldt, G.E., 1999a. Systematics and genetic structure of *Ponderosae* taxa (Pinaceae) inhabiting the mountain islands of the Southwest. Am. J. Bot. 86, 741–752.
Rehfeldt, G.E., 1999b. Systematics and genetic structure of Washoe pine: applications in conservation genetics. Silvae Genet. 48, 167–173.
Rice, W.R., 1989. Analyzing tables of statistical tests. Evolution 43, 223–225.
Rieseberg, L.H., Raymond, O., Rosenthal, D.M., Lai, Z., Livingstone, K., Nakazato, T., Durphy, J.L., Schwarzbach, A.E., Donovan, L.A., Lexer, C., 2003. Major ecological transitions in wild sunflowers facilitated by hybridization. Science 301, 1211–1216.
Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.
Rosenberg, N.A., 2003. The shapes of neutral gene genealogies in two species: probabilities of monophyly, paraphyly, and polyphyly in a coalescent model. Evolution 57, 1465–1477.
Rosenberg, N.A., 2007. Statistical tests for taxonomic distinctiveness from observations of monophyly. Evolution 61, 317–323.
Rozas, J., Sánchez-DelBarrio, J.C., Messeguer, X., Rozas, R., 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19, 2496–2497.
Seehausen, O., 2004. Hybridization and adaptive radiation. Trends Ecol. Evol. 19, 198–207.
Shaw, J., Lickey, E.B., Beck, J.T., Farmer, S.B., Liu, W., Miller, J., Siripun, K.C., Winder, C.T., Schilling, E.E., Small, R.L., 2005. The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. Am. J. Bot. 92, 142–166.
Simmons, M.P., Ochoterena, H., 2000. Gaps as characters in sequence-based phylogenetic analyses. Syst. Biol. 49, 369–381.
Sorensen, F.C., Mandel, N.L., Aagaard, J.E., 2001. Role of selection versus historical isolation in racial differentiation of ponderosa pine in southern Oregon: an investigation of alternative hypotheses. Can. J. For. Res. 31, 1127–1139.
Stebbins, G.L., 1950. Variation and Evolution in Plants. Columbia Univ. Press, New York, NY.
Stebbins, G.L., 1959. The role of hybridization in evolution. Proc. Am. Phil. Soc. 103, 231–251.
Swofford, D., 2002. PAUP*: phylogenetic analysis using parsimony (* and other methods), vers. 4.0 b10. Sinauer Assoc. Inc., Sunderland, MA, USA.
Syring, J., Farrell, K., Businský, R., Cronn, R., Liston, A., 2007. Widespread genealogical nonmonophyly in species of *Pinus* subgenus *Strobus*. Syst. Biol. 56, 163–181.
Syring, J., Willyard, A., Cronn, R., Liston, A., 2005. Evolutionary relationships among pine (Pinaceae) subsections inferred from multiple low-copy nuclear loci. Am. J. Bot. 92, 2086–2100.
Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123, 585–595.
Temesgen, B., Brown, G.R., Harry, D.E., Kinlaw, C.S., Sewell, M.M., Neale, D.B., 2001. Genetic mapping of expressed sequence tag polymorphism (ESTP) markers in loblolly pine (*Pinus taeda*). Theor. Appl. Genet. 102, 664–675.
Watterson, G.A., 1975. On the number of segregating sites in genetical models without recombination. Theor. Pop. Biol. 7, 256–276.
Willyard, A., Syring, J., Gernandt, D.S., Liston, A., Cronn, R., 2007. Fossil calibration of molecular divergence infers a moderate mutation rate and recent radiations for *Pinus*. Mol. Biol. Evol. 24, 90–101.
Wolff, R.L., Pédrono, F., Pasquier, E., Marpeau, A.M., 2000. General characteristics of *Pinus* spp. seed fatty acid compositions, and importance of Δ5-olefinic acids in the taxonomy and phylogeny of the genus. Lipids 35, 1–22.
Zobel, B., 1951. The natural hybrid between Coulter and Jeffrey pines. Evolution 5, 405–413.