

Phylogeny of *Fomitopsis pinicola*: a species complex

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Abstract: Fungal species with a broad distribution may exhibit considerable genetic variation over their geographic ranges. Variation may develop among populations based on geographic isolation, lack of migration, and genetic drift, though this genetic variation may not always be evident when examining phenotypic characters. *Fomitopsis pinicola* is an abundant saprotrophic fungus found on decaying logs throughout temperate regions of the Northern Hemisphere. Phylogenetic studies have addressed the relationship of *F. pinicola* to other wood-rotting fungi, but pan-continental variation within *F. pinicola* has not been addressed using molecular data. While forms found growing on hardwood and softwood hosts exhibit variation in habit and appearance, it is unknown if these forms are genetically distinct. In this study, we generated DNA sequences of the nuc rDNA internal transcribed spacers (ITS), the *TEF1* gene encoding translation elongation factor 1- α , and the *RPB2* gene encoding the second largest subunit of RNA polymerase II for collections across all major geographic regions where this fungus occurs, with a primary focus on North America. We used Bayesian and maximum likelihood analyses and evaluated the gene trees within the species tree using coalescent methods to elucidate evolutionarily independent lineages. We find that *F. pinicola* sensu lato encompasses four well-supported, congruent clades: a European clade, southwestern US clade, and two sympatric northern North American clades. Each clade represents distinct species according to phylogenetic and population-genetic species

concepts. Morphological data currently available for *F. pinicola* do not delimit these species, and three of the species are not specific to either hardwood or softwood trees. Originally described from Europe, *F. pinicola* appears to be restricted to Eurasia. Based on DNA data obtained from an isotype, one well-defined and widespread clade found only in North America represents the recently described *Fomitopsis ochracea*. The remaining two North American clades represent previously undescribed species.

Key words: Bayesian, coalescent, maximum likelihood, phylogenetic species, polyporoid clade, species tree

INTRODUCTION

The variable phenotypic appearance of *Fomitopsis pinicola* (Sw. n: Fr.) P. Karst as well as its collection on a variety of host species (Mounce 1929) covering a broad geographic range (Spaulding 1961, Anonymous 1979, Quiniones 1980, Schmid-Heckel 1988, Teng 1996, Filsińska 1997, Hermansson 1997, Pande and Rao 1998, Chen 2002, Cho and Shin 2004, Legon et al. 2005, Kobayashi 2007) has led to speculation that cryptic species may be present. Fries (1821) described two species, *Polyporus marginata* being lighter in color and unguulate, and *Polyporus pinicola* with a cap color tending to black and cinnamon. The host range of *P. marginata* included *Fagus*, *Betula*, *Pinus*, and *Pyrus*, while that of *P. pinicola* included *Abies*, and *Betula*. Saccardo (1888) described a third species, *Fomes unguulates*, having a cap with thick, concentric reddish-ochre colored furrows, which was collected on conifers in the Italian Alps. Mounce (1929) found various forms and colors of sporocarps on *Tsuga* in the United States. Her extensive collections and work with crosses of single spore isolates lead her to agree with Hedgecock (1914), Lloyd (1915), Murrill (1908), and Overholts (1915) that the three species described by Saccardo were forms of the same species. Mounce discovered that, both within and between North America and Europe, monosporous mycelia of *F. pinicola* isolated from sporophores collected on deciduous hosts were mutually fertile with monosporous mycelia isolated from sporophores from coniferous hosts. She concluded that *F. marginalis* and *F. pinicola* were the same species and that European and American forms of the fungus were identical to each other. Further work with monosporous pairings lead to the discovery of two intersterile populations in North America (Mounce and Macrae 1938). Describing them as separate species

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was rejected when both populations were found to form fertile spores when crossed with isolates from Europe. Though inhabiting a large geographic range, European populations of *F. pinicola* were found to be members of one intersterility group (Högberg et al. 1999).

A recently described species, *F. ochracea*, originally collected in Alberta, Canada on *Populus tremuloides* (Ryvarden and Stokland 2008), was delimited from *F. pinicola* based largely on pore color and spore morphology. Ryvarden and Stokland (2008) reported that the pore surface of *F. ochracea* did not exhibit a change from cream to citrus yellow when bruised, as seen in *F. pinicola*, and that the basidiospores were globose to broadly ellipsoid, rather than cylindrical. They noted that the smooth ochraceous pileus of *F. ochracea* is easily separated from that of *F. pinicola*. They also obtained a sequence from the holotype and compared it to sequences of several *F. pinicola* from North America but did not make formal comparisons to other collections.

In this study we evaluated the use of sequence data from three nuclear and two mitochondrial loci to assess whether *F. pinicola* comprises a species complex. Species boundaries in a species complex can be determined by various criteria depending on the species concept in use. Molecular techniques have given rise to the phylogenetic species concept, and promising statistical-molecular species delimitation methods include genealogical concordance and coalescent species delimitation. Coalescent theory is a branch of population genetics that models genetic drift backward through time, tracing polymorphism in a gene back to a most recent common ancestral allele. This process models divergence between alleles from the present, when genes are sampled, back to the time when allelic genes diverged (Degnan and Salter 2005). At this point all lineages of the gene have coalesced and the resulting product is a gene genealogy. This process accommodates incomplete lineage sorting that can be problematic in conventional phylogenetic analyses and has been successful in delimiting cryptic species in lichens (Leavitt et al. 2011, 2012a, 2012b) but has not been applied widely in the fungi. Cryptic species are proving to be extremely common in higher fungi, particularly those with wide geographic distributions or host ranges. Among existing approaches, coalescent methods offer the greatest potential resolution of evolutionarily independent lineages where reciprocal monophyly of the majority of loci has not yet been achieved. Understanding the patterns and processes that create fungal biodiversity cannot be achieved without highly resolved species delimitation and estimation of speciation histories. Hence, methods such as coalescent species delimitation have the potential to

provide much greater insight into fungal speciation. The present study of *F. pinicola* illustrates this potential.

Although principles of the biological species concept and the phylogenetic species concept were also considered, our study centered on coalescent theory, using a Bayesian hierarchical model (Liu and Pearl 2007) to estimate the phylogeny of the species complex in *F. pinicola*. We tested the hypothesis that *F. pinicola* constitutes a species complex using complementary methods: phylogenetic and recently developed coalescent population genetic methods. In doing so, we also sought to test whether distinct species within the *F. pinicola* complex correspond to geographic regions, host plants, or sporocarp color forms. This paper focuses primarily on evolutionary questions, and a parallel paper will address taxonomic descriptions.

MATERIALS AND METHODS

Sample collections.—To encompass the largest possible genetic diversity, fresh fruiting bodies collected by us were augmented with data collected from herbarium specimens and cultures, which allowed us to increase the geographical range of the dataset. Sporophores were collected from decaying softwood and hardwood substrates in various habitat types including circumpolar subarctic boreal forests, coastal temperate rainforests, northern mixed hardwood forests, and southern coniferous forests at high elevations (TABLE I).

Herbaria and culture collections.—Dried specimens of *F. pinicola* were sampled from the Gary A. Laursen Herbarium, University of Washington, formerly located at the University of Alaska, Fairbanks; the Center for Forest Mycology Research Herbarium, US Forest Service, Madison, Wisconsin; the US National Fungus Collections (BPI), Beltsville, Maryland; the National Herbarium of the Netherlands, University of Leiden Branch, Leiden, the Netherlands; and the Royal Ontario Museum Fungarium, Ontario, Canada. Fungal cultures were obtained from private and public culture collections including the Center for Forest Mycology Research Culture Collection, US Forest Service, Madison, Wisconsin. An attempt to include the type specimen of *F. pinicola* in this study was unsuccessful as the type exists as an illustration only and no biological specimen is known (Ryvarden 1991).

DNA isolation.—Tissue samples for DNA extraction were removed from the interior of fruiting body pilei. Tissue specimens were then either ground in a detergent solution of cetyl trimethyl ammonium bromide (CTAB) in glass tissue grinders (Kimble Chas Kontes, size 24), between frosted glass slides (VWR), or under liquid nitrogen in a porcelain mortar. Tissue samples for DNA extraction taken from cultures were removed by gently scraping hyphae from the surface of the agar plate and then grinding the sample under CTAB between two glass slides. Difficult cultures were grown in 1% malt liquid media, freeze dried, and ground under liquid nitrogen. After grinding the tissue by one of the preceding

TABLE I. Collection of *Fomitopsis* species complex used in phylogenetic analysis, origin, repository and GenBank numbers

Taxon name	Collection No.	Country	State/province/district	Collector	Host	Herbarium code	GenBank accession Nos.		
							ITS	RPB2	TEFI
<i>F. ochracea</i>	DLL-3	USA	Minnesota	D.L. Lindner	<i>Populus</i>	CFMR	KF169588	KF169657	KF178313
<i>F. ochracea</i>	DLL-4	USA	Minnesota	D.L. Lindner	<i>Populus</i>	CFMR	KF169589	KF169658	KF178314
<i>F. ochracea</i>	FP-125083-T	USA	New Hampshire	A.L. Shigo	<i>Tsuga</i>	CFMR	KF169590	KF169659	KF178315
<i>F. ochracea</i>	HHB-17611	USA	Alaska/Kenai	H.H. Burdsall	<i>Picea</i>	CFMR	KF169591	KF169660	KF178316
<i>F. ochracea</i>	HHB-19667	USA	Tennessee	H.H. Burdsall	<i>Picea</i>	CFMR	KF169592	KF169661	KF178317
<i>F. ochracea</i>	HHB-19670	USA	Tennessee	H.H. Burdsall	<i>Picea</i>	CFMR	KF169593	KF169662	KF178318
<i>F. ochracea</i>	HHB-19692	USA	Tennessee	H.H. Burdsall	<i>Picea</i>	CFMR	KF169594	KF169663	KF178319
<i>F. ochracea</i>	HHB-3331-Sp	USA	Michigan	H.H. Burdsall	<i>Acer</i>	CFMR	KF169595	KF169664	KF178320
<i>F. ochracea</i>	JEH-12C	USA	Alaska	J.E. Haight	<i>Tsuga</i>	CFMR	KF169596	KF169665	KF178321
<i>F. ochracea</i>	JEH-12E	USA	Alaska	J.E. Haight	<i>Tsuga</i>	CFMR	KF169597	KF169666	KF178322
<i>F. ochracea</i>	JEH-12F	USA	Alaska	J.E. Haight	<i>Tsuga</i>	CFMR	KF169598	KF169667	KF178323
<i>F. ochracea</i>	JEH-13A	USA	Alaska	J.E. Haight	<i>Tsuga</i>	CFMR	KF169599	KF169668	KF178324
<i>F. ochracea</i>	JEH-13B	USA	Alaska	J.E. Haight	<i>Tsuga</i>	CFMR	KF169600	KF169669	KF178325
<i>F. ochracea</i>	JEH-13D	USA	Alaska	J.E. Haight	<i>Tsuga</i>	CFMR	KF169601	KF169670	KF178326
<i>F. ochracea</i>	JEH-37	USA	Alaska	J.E. Haight	<i>Tsuga</i>	CFMR	KF169602	KF169671	KF178327
<i>F. ochracea</i>	JEH-38	USA	Alaska	J.E. Haight	<i>Tsuga</i>	CFMR	KF169603	KF169672	KF178328
<i>F. ochracea</i>	JEH-79	Canada	Alberta	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169604	KF169673	KF178329
<i>F. ochracea</i>	JEH-80-ss1	Canada	Alberta	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169605	KF169674	KF178330
<i>F. ochracea</i>	JEH-81	Canada	Alberta	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169606	KF169675	KF178331
<i>F. ochracea</i>	JEH-83-ss2	Canada	Alberta	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169607	KF169676	KF178332
<i>F. ochracea</i>	JEH-85	Canada	Alberta	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169608	KF169677	KF178333
<i>F. ochracea</i>	JEH-87-ss5	Canada	British Columbia	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169609	KF169678	KF178334
<i>F. ochracea</i>	JEH-87-ss7	Canada	British Columbia	J.E. Haight	<i>Populus</i>	CFMR	KF169610	KF169679	KF178335
<i>F. ochracea</i>	JEH-88	Canada	Alberta	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169611	KF169680	KF178336
<i>F. ochracea</i>	JEH-91	Canada	Alberta	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169612	KF169681	KF178337
<i>F. ochracea</i>	KTS-28	USA	Vermont	K.T. Smith	<i>Picea</i>	CFMR	KF169613	KF169682	KF178338
<i>F. ochracea</i>	LT-12	USA	Alaska	L. Trummer	Not recorded	CFMR	KF169614	KF169683	KF178339
<i>F. ochracea</i>	LT-16	USA	Alaska	L. Trummer	Not recorded	CFMR	KF169615	KF169684	KF178340
<i>F. ochracea</i>	LT-18	USA	Alaska	L. Trummer	Not recorded	CFMR	KF169616	KF169685	KF178341
<i>F. ochracea</i>	LT-19	USA	Alaska	L. Trummer	Not recorded	CFMR	KF169617	KF169686	KF178342

TABLE I. Continued

Taxon name	Collection No.	Country	State/province/district	Collector	Host	Herbarium code	GenBank accession Nos.		
							ITS	RPB2	TEFI
<i>F. ochracea</i>	LT-17	USA	Alaska	L. Trummer	Not recorded	CFMR	KF169618	KF169687	KF178343
<i>F. ochracea</i>	48800	Canada	Newfoundland	L. Ryvarden	<i>Picea mariana</i>	TRTC	KF169619	KF169688	KF178344
<i>F. ochracea</i>	PEL-Lk-6-1	USA	Minnesota	D.L. Lindner	<i>Populus</i>	CFMR	KF169620	KF169689	KF178345
<i>F. pinicola</i>	LT-323	Estonia	Tartumaa	D.L. Taylor	<i>Picea</i>	CFMR	KF169651	KF169720	KF178376
<i>F. pinicola</i>	LT-319	Estonia	Tartumaa	D.L. Taylor	<i>Picea</i>	CFMR	KF169652	KF169721	KF178377
<i>F. pinicola</i>	FCUG 2034	Sweden	Not recorded	K-H. Larsson	Not recorded	GB	KF169653	KF169722	KF178378
<i>F. pinicola</i>	FCUG 2056	Sweden	Not recorded	K-H. Larsson	Not recorded	GB	KF169654	KF169723	KF178379
<i>F. pinicola</i>	HK-19330	Russia	South Ural	H. Koiranta	<i>Picea</i>	H	KF169655	KF169724	KF178380
<i>F. pinicola</i>	TS-Fp-24	Russia	Moscow	T. Semenova	<i>Picea</i>	MW	KF169656	KF169725	KF178381
<i>F. sp.</i>	32TT	USA	Washington	C.G. Shaw	<i>Dendroctonus</i>	CFMR	KF169621	KF169690	KF178346
<i>F. sp.</i>	CS-1	USA	Oregon	C.L. Schmitt	<i>Abies</i>	CFMR	KF169622	KF169691	KF178347
<i>F. sp.</i>	DR-301	USA	Michigan	D.L. Richter	<i>Picea</i>	CFMR	KF169623	KF169692	KF178348
<i>F. sp.</i>	DR-366	USA	Michigan	D.L. Richter	<i>Acer</i>	CFMR	KF169624	KF169693	KF178349
<i>F. sp.</i>	DR-472	USA	Michigan	D.L. Richter	<i>Populus</i>	CFMR	KF169625	KF169694	KF178350
<i>F. sp.</i>	JAG-08-19	USA	Idaho	J.A. Glaeser	Not recorded	CFMR	KF169626	KF169695	KF178351
<i>F. sp.</i>	JAG-08-20	USA	Idaho	J.A. Glaeser	Not recorded	CFMR	KF169627	KF169696	KF178352
<i>F. sp.</i>	JAG-08-25	USA	Idaho	J.A. Glaeser	Not recorded	CFMR	KF169628	KF169697	KF178353
<i>F. sp.</i>	JEH-78	Canada	Alberta	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169629	KF169698	KF178354
<i>F. sp.</i>	JEH-82	Canada	Alberta	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169630	KF169699	KF178355
<i>F. sp.</i>	JEH-86	Canada	Alberta	J.E. Haight	<i>Populus tremuloides</i>	CFMR	KF169631	KF169700	KF178356
<i>F. sp.</i>	JEH-146	USA	Wisconsin	A.D. Parker	<i>Larix</i>	CFMR	KF169632	KF169701	KF178357
<i>F. sp.</i>	JEH-147	USA	Wisconsin	A.D. Parker	<i>Larix</i>	CFMR	KF169633	KF169702	KF178358
<i>F. sp.</i>	KM-1	USA	Oregon	K. Mallams	<i>Abies</i>	CFMR	KF169634	KF169703	KF178359
<i>F. sp.</i>	LT-5	USA	Alaska	L. Trummer	Not recorded	CFMR	KF169635	KF169704	KF178360
<i>F. sp.</i>	MJL-112-Sp	USA	New York	M.J. Larsen	<i>Abies</i>	CFMR	KF169636	KF169705	KF178361
<i>F. sp.</i>	FP-105760-T	USA	Idaho	R.W. Davidson	<i>Pinus</i>	CFMR	KF169637	KF169706	KF178362
<i>F. sp.</i>	FP-133890-T	USA	Montana	M.J. Larsen	Conifer	CFMR	KF169638	KF169707	KF178363
<i>F. sp.</i>	FP-125086-T	USA	New Hampshire	A.L. Shigo	<i>Tsuga</i>	CFMR	KF169639	KF169708	KF178364
<i>F. sp.</i>	JS-22	USA	Maine	J. Schilling	<i>Picea</i>	CFMR	KF169640	KF169709	KF178365
<i>F. sp.</i>	FP-105881-R	USA	Colorado	T.E. Hinds	<i>Pinus</i>	CFMR	KF169641	KF169710	KF178366

TABLE I. Continued

Taxon name	Collection No.	Country	State/province/district	Collector	Host	Herbarium code	GenBank accession Nos.		
							ITS	<i>RPB2</i>	<i>TEF1</i>
<i>F. sp.</i>	JEH-142-ss12	USA	New Mexico	D.L. Taylor	Not recorded	CFMR	KF169642	KF169711	KF178367
<i>F. sp.</i>	JEH-142-ss14	USA	New Mexico	D.L. Taylor	Not recorded	CFMR	KF169643	KF169712	KF178368
<i>F. sp.</i>	JEH-142-ss5	USA	New Mexico	D.L. Taylor	Not recorded	CFMR	KF169644	KF169713	KF178369
<i>F. sp.</i>	JEH-142-ss6	USA	New Mexico	D.L. Taylor	Not recorded	CFMR	KF169645	KF169714	KF178370
<i>F. sp.</i>	JW24-525-0-sap	USA	South Dakota	J.J. Worrall	<i>Pinus</i>	CFMR	KF169646	KF169715	KF178371
<i>F. sp.</i>	JW24-549B-1-sap	USA	South Dakota	J.J. Worrall	<i>Pinus</i>	CFMR	KF169647	KF169716	KF178372
<i>F. sp.</i>	JW18-240-1-sap	USA	Colorado	J.J. Worrall	<i>Pseudotsuga</i>	CFMR	KF169648	KF169717	KF178373
<i>F. sp.</i>	JW-F.pinicola-#2	USA	Colorado	J.J. Worrall	<i>Picea</i>	CFMR	KF169649	KF169718	KF178374
<i>F. sp.</i>	RLG-10752-Sp	USA	Arizona	R.L. Gilbertson	<i>Pseudotsuga</i>	CFMR	KF169650	KF169719	KF178375

methods, the DNA samples were stored overnight at -20°C before 2 h incubation in a water bath at 65°C . Following centrifugation at 14 000 RCF, the supernatant was drawn off and mixed with an equal amount of 100% isopropanol and incubated overnight at 0°C . The solution was centrifuged at 0°C for 30 min. The liquid was then drawn off and the precipitate was washed in 70% ethanol and then allowed to dry for 15 min before being dissolved in 50 μl of molecular grade water. The resulting DNA was cleaned using a GeneClean Kit (Bio 101, Carlsbad, California) with glass milk as described in Taylor and Bruns (1997). A subset of challenging samples was extracted using Qiagen Plant DNEasy kits (QIAGEN, Germantown, Maryland).

Target loci.—We targeted the following loci for amplification and sequencing: nuc rDNA ITS1-5.8S-ITS2 region (ITS) and genes for translation elongation factor 1- α (*TEF1*), RNA polymerase II subunit 2 (*RPB2*), ATP synthase subunit 6 (*ATP6*), and the mitochondrial large subunit rRNA gene (mt-LSU). *RPB2* and *TEF1* belong to different scaffolds on the JGI *F. pinicola* genome, suggesting that they are unlinked independent loci. In addition, given the topological conflict observed between ITS and either *RPB2* or *TEF1*, we conclude that these three regions are effectively independent. The ITS primers ITS1F and ITS4 produced strong PCR amplification and clean sequences. In contrast, AFTOL versions of the primers for *RPB2* and *TEF1* resulted in poor amplification from *F. pinicola*, and therefore new primers were designed for *TEF1*, *RPB2*, *ATP6*, and mt-LSU as follows. First, target regions from closely related fungi, such as other species of *Fomitopsis*, *Piptoporus*, *Trametes*, and *Ganoderma*, were downloaded from GenBank. These sequences were then used in blast searches against the draft genome of *F. pinicola* using tools on the DOE Fungal Genome Portal (<http://genome.jgi.doe.gov/programs/fungi/index.jsf>). The corresponding regions of the *F. pinicola* genome were extracted and aligned to those of related fungi using MUSCLE (Edgar 2004). The positions of standard AFTOL primers were then located on the resulting alignments. Considerable mismatches with *F. pinicola* were noted. In most cases, we were able to simply exchange mismatching bases with a matching base in *F. pinicola*, using the same primer positions, to design *Fomitopsis*-specific primers. In a few cases, new positions and novel primers were chosen. We used degenerate bases in situations where there were variable positions within the genus *Fomitopsis*. Annealing temperatures and potential for self and cross-dimers were evaluated using NetPrimer (Premier Biosoft). Possible primers with dimers stronger than -8kcal/mol or NetPrimer scores below 80 were discarded. Primers used in this study are listed (SUPPLEMENTARY TABLE I).

Polymerase chain and sequencing reactions.—Polymerase Chain Reactions (PCR) were run using GoTaq DNA polymerase kits (Promega, Madison, Wisconsin). Reactions with a total volume of 15 μl were set up containing 3 μl of $5\times$ GoTaq buffer and with concentrations of 200 μM each dNTPs, 0.2 μM forward and reverse primers, 0.375 U *Taq* DNA polymerase and 3 μl of sample DNA. PCR protocols were run on a MJ Research PTC-200 Thermo Cycler at annealing temperatures specific for each primer (SUPPLEMENTARY TABLE II). PCR

products were run on a 1.8% agarose gel and stained with ethidium bromide to visualize the bands and were purified using an ExoSAP-IT Kit (GE Healthcare). Sequencing reactions were carried out in-house using the Applied Biosystems Big Dye 3.1 Terminator Cycle Sequencing Kits (Invitrogen) and were purified using a CleanSEQ Sequence Cleanup Kit (Agencourt). The in-house protocol for sequencing reactions was 96 C for 30 s, 50 C for 30 s, 60 C for 3 min, and the cycle repeated 30 × before ending at 8 C until removed.

Cycle sequence products were run on an ABI 3730xl DNA Analyzer (Life Technologies, Grand Island, New York) at the University of Wisconsin Biotechnology Center (Madison, Wisconsin). A subset of raw PCR products was sent to Functional Biosciences (Madison, Wisconsin) for sequencing. We obtained 161 passing ITS sequences, which along with additions downloaded from GenBank, resulted in 203 ITS sequences. Our sequences plus additions from GenBank yielded 132 *TEF1* sequences and 112 *RPB2* sequences. In total, 60 collections were sequenced in common over the three gene regions (TABLE I).

Analysis.—Raw sequences were processed using CodonCode Aligner 3.7.1.1 (CodonCode, Centerville, Massachusetts). Sequences were imported, bases were called, and Phred scores were obtained based on the default settings. Forward and reverse sequences from each primer pair were then assembled automatically. End clipping was accomplished by setting a minimum threshold of a 1% error rate, corresponding to a consensus quality score of 20, and terminal bases below the minimum threshold were then trimmed using the clip ends feature. The same minimum threshold was used to evaluate the rest of the sequence, though some user discretion was used in cases of a short region of low quality bases that could still easily be aligned. Processed sequences were initially aligned using ClustalW (Thompson et al. 1994) and then adjusted manually. Sequence alignments and all other phylogenetic information generated in this study were deposited in TreeBASE (<http://purl.org/phylo/treebase/phylovs/study/TB2:S18816>).

Phylogenetic inference.—To determine the best model of nucleotide substitution, we used the program MrModeltest 2.3 (Nylander 2004). Phylogenetic tree construction was carried out using maximum likelihood in RAxML 7.3.0 (Stamatakis 2006) and Bayesian analyses in MrBayes 3.2 (Hulsenbeck and Ronquist 2001). Initially a pilot dataset of 24 sequences for each gene was analyzed using MrBayes to evaluate numbers of parsimony informative characters within *F. pinicola*. During this evaluation it was found that amplification with primers ATP6-Fp1 and ATP6-2*, ML_5Fp1 and cML5_Fp, ML5_Fp and ML6_Fp, and ML_7 and ML_8 yielded aligned datasets with no variability. Therefore, the *ATP6* and mtLSU genes (SUPPLEMENTARY TABLE III) were dropped from further consideration in this study. Once the variability of a gene was determined to be informative (i.e. sufficiently variable to support phylogenetic inference), additional samples were sequenced. These larger datasets were analyzed independently for each gene using maximum likelihood in RAxML with 1000 rapid bootstrap replicates and Bayesian inference in MrBayes (nst = 2, rates = gamma and 3 000 000

generations). Sequences for *Fomitopsis palustris*, *Fomitopsis meliae*, and *Piptoporus betulina* were downloaded from GenBank and added to the ITS and *RPB2* alignments for use as outgroups. However, these sequences proved difficult to align unambiguously and produced long branches in the gene trees (data not shown). A suitable outgroup also could not be found for use with *TEF1*. Therefore, we were forced to use midpoint rooting for all three genes. After the gene analyses a 1739 base pair concatenated multigene alignment was constructed using MEGA (Tamura et al. 2011) consisting of 60 collections for which we had sequences from all three final loci, namely *RPB2*, *TEF1*, and ITS. This concatenated alignment was partitioned by gene, by codon position (1st, 2nd, 3rd) vs. noncoding region (spacers and introns), and analyzed using MrBayes. The model for the run was nst = 6, rates = invgamma, and 3 000 000 generations.

Coalescent species trees.—Phylogenetic approaches to tree-building are well suited to identifying well-supported clades and relationships among them above the species level. However, these approaches assume a non-reticulated, dichotomously branching history, and may fail to accurately depict genetic groupings when all alleles have not sorted to reciprocal monophyly. We therefore also applied population genetic approaches that attempt to estimate species boundaries based upon independent and potentially conflicting genealogical histories. First, the multispecies coalescent tree was estimated using MrBayes 3.2. Note that coalescent analysis is a new feature of MrBayes, not based on the standard method of comparing probabilities of randomly permuted phylogenetic trees. The 1739 base pair concatenated alignment was run using species partitions based on well-supported groups identified in the concatenated Bayesian phylogenetic analysis. The analysis model was run with settings as described in the program manual (Ronquist et al. 2011): speciespartitions = species, unlink topology = (all), prset topologypr = speciestree, prset brlenspr = clock:speciestree, prset popvarpr = variable, prset popsizpr = lognormal (4.6, 2.3), and using nst = 6, rates = gamma, and run for 5 000 000 generations.

Second, to test the species boundaries suggested by the coalescent analysis in MrBayes, the 60 collection, 3-gene dataset was further analyzed using the program BPP (Rannala and Yang 2003, Yang and Rannala 2010) in the species delimitation mode using rjMCMC algorithms. This method accommodates the species phylogeny as well as lineage sorting due to ancestral polymorphism. Because little was known about population size, a diffuse prior was used (Yang and Rannala 2010). Therefore, a gamma prior $G(2, 1000)$, with mean $2/1000 = 0.002$, was used for the population size parameters (θ s). The age of the root in the species tree (τ_0) was assigned the gamma prior $G(2, 1000)$, while the other divergence time parameters were assigned the Dirichlet prior (Yang and Rannala 2010: equation 2). Choosing one of the interior nodes for collapsing generated a starting tree. Each analysis was run using both algorithm 0 and algorithm 1 (Yang and Rannala 2010). The algorithms approach the new species divergence time by scanning for the most recent coalescent event and use alternate fine-tuning parameters when determining the acceptance ratio for splitting or joining a pair of

taxa. In addition, at least three different starting trees were used with each algorithm to confirm consistency between runs. Posterior probabilities were further confirmed by artificially splitting a single phylogenetic clade into sister populations, creating a new guide tree containing these sister species, and observing whether the artificial nodes thus created collapsed during the species delimitation runs. Upon completion of each run, a posterior probability is assigned to each node of the user supplied guide tree, which is the probability of a speciation event. A probability of ≥ 0.95 may be interpreted as a species split, because a probability of 1 means that the rjMCMC algorithm supported that node each time it was evaluated (Leaché and Fujita 2010).

Population genetics parameter estimation.—Haplotype reconstruction. Cases of ambiguous base calls were commonly found in *RPB2* and *TEF1* gene sequences, indicating individuals heterozygous for that SNP. To determine if haplotypes were shared among the clades, the haplotypes underlying the heterozygous sequences were estimated using the program PHASE 2.1 (Stephens et al. 2001, Stephens and Donnelly 2003), after using SEQPHASE (Flot 2010) to construct the input files. Program default values were used for number of iterations, thinning interval and number of burn-in generations. Haplotype frequency was checked between runs for consistency. Goodness-of-fit results were checked between two runs and then the program was set to automatically run five independent runs using the $-x$ option to output the haplotype estimate corresponding to the run with the best average goodness-of-fit. Due to the large number of SNPs, each clade identified in the concatenated Bayesian analysis was run in PHASE separately. The PHASE output files were processed in SEQPHASE and the resulting FASTA files were aligned using MEGA and saved as a NEXUS file. A haplotype tree was produced using MrBayes (nst = 2, rates = equal, and 1 000 000 generations) and analyzed for the presence of shared haplotypes between clades.

Sequence polymorphism.—To measure DNA polymorphism and nucleotide divergence among the sequences in our dataset, the haplotype alignments for *RPB2*, *TEF1*, and ITS were concatenated and aligned using SeAl (Rambaut 2002) and analyzed in DnaSP (Librado and Rozas 2009). Nucleotide divergence was measured between each pair of clades (input to DnaSP as “populations”). Estimates of divergence included total number of polymorphic sites, number of fixed differences, the number of polymorphic sites present in the one clade but monomorphic in the second clade, total shared mutations between clades, average number of nucleotide differences between clades (K), nucleotide divergence (D_{xy}), and the number of net nucleotide substitutions per site between clades (D_a).

RESULTS

Sequence utility.—The *TEF1* and *RPB2* genes displayed considerable intraspecific variability in *F. pinicola*. Out of 557 total characters in the *TEF1* alignment, 32 were parsimony informative. The *RPB2* gene sequence yielded an alignment of 638 bases, of which 37

positions were parsimony informative. The ITS alignment was 544 characters with only 14 parsimony informative characters.

Gene trees.—Phylogenetic gene trees for *RPB2*, *TEF1*, and ITS produced using MrBayes each supported the presence of several distinct, well-supported clades within *F. pinicola* (FIG. 1). North American clades A and B (NAA and NAB) and the Southwest clade (SW) occur in North America, and a fourth clade (EUR) occurs in Europe. However, the delimitation of some clades and the relationships between them differed among loci. This is expected in cases of recent divergence where weak phylogenetic signal due to limited time for accumulation of mutations, incomplete lineage sorting, and/or occasional introgression prevent perfect concordance among gene trees. Nevertheless, in the combined analysis strong support for four clades was manifest.

The *RPB2* tree (FIG. 1A) supported NAB (pp 1.0) as separate from the other clades. The majority of the EUR (pp 1.0) is on a branch within the NAA clade (pp 0.95). The *TEF1* tree (FIG. 1B) showed a basal split of NAA, EUR, and SW from NAB (pp 1.0). While NAA had low support, the majority of SW formed a clade (pp 1.0), and support for the EUR clade was strong (pp 1.0). The ITS tree (FIG. 1C) supported clades NAB (0.96) and EUR (pp 1.0). The SW fell within the NAA lineage (pp 0.95). Phylogenetic gene trees for *RPB2*, *TEF1*, and ITS produced using RAxML (SUPPLEMENTARY FIGS. 1–3) had topologies and branch support values similar to the gene trees produced using MrBayes. The 203 sequence ITS tree (SUPPLEMENTARY FIG. 4, SUPPLEMENTARY TABLE IV) including GenBank sequences had a similar topology to that of FIG. 1C but with a larger dataset covering an extended geographical area.

Species tree.—The coalescent species analysis of the combined data run in MrBayes produced a tree of four clades (FIG. 1D) with posterior probabilities of 100% for each: NAA, NAB, SW, and EUR. The tree had a similar topology to the three gene trees, but the coalescent analysis provided a more distinct branching pattern and better definition for the boundary of each clade. The most basal node still separated NAB from the rest of the clades, but the EUR clade was distinctly separated from NAA and SW. In addition, the SW clade may include a subdivision (pp 1.0) not supported in the gene trees.

Testing species boundaries.—We ran BPP on a four species guide tree [(((EUR, NAA), SW), NAB)], which produced results supporting the clades diagnosed in the coalescent species tree. Each of the three nodes had a posterior probability of 100%. Artificially splitting North American clade B into sister species created

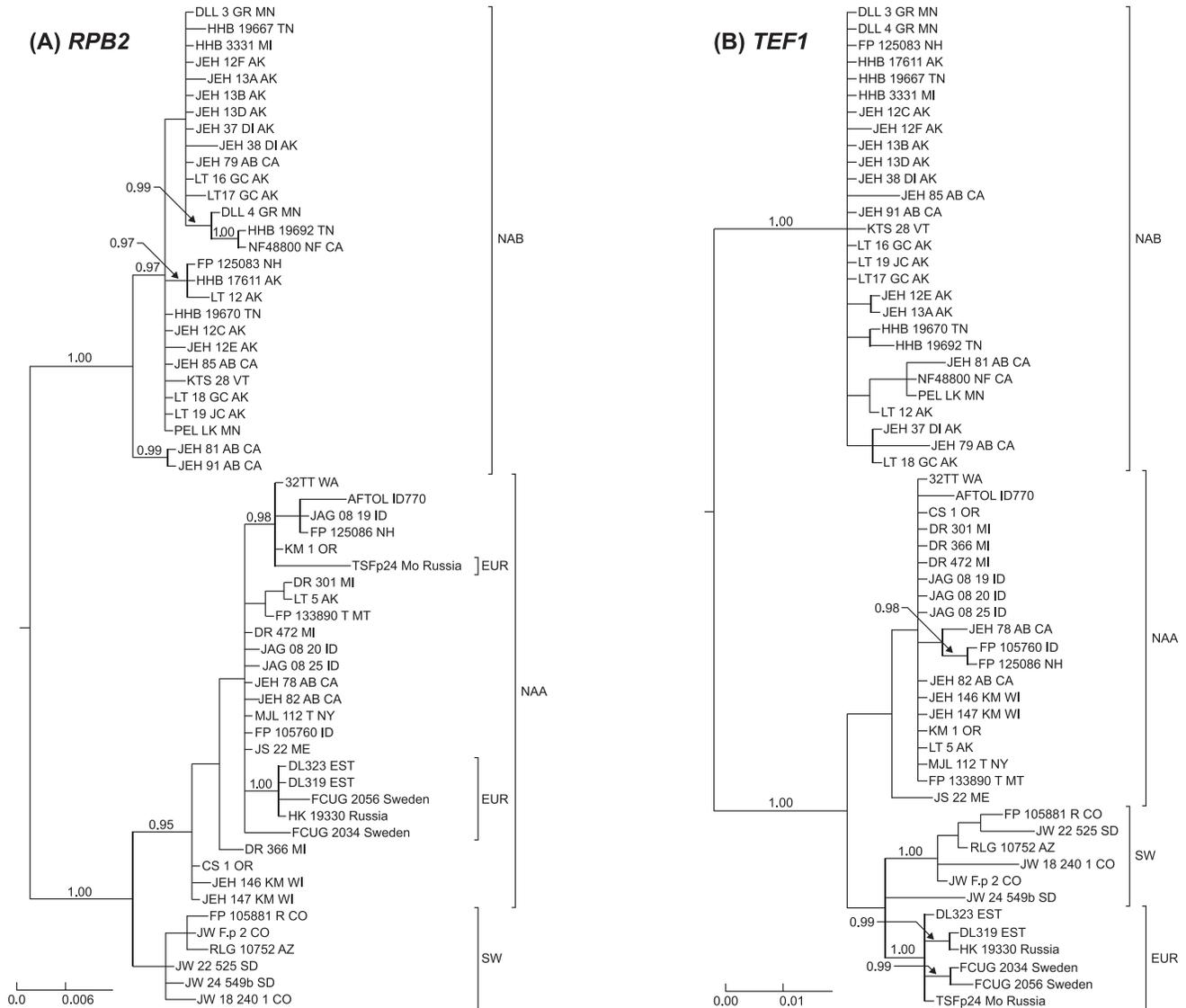


FIG. 1. Bayesian gene trees for *RPB2* (A), *TEF1* (B), ITS (C), and a Bayesian coalescent species tree (D) from a combined, concatenated, and partitioned dataset. The trees are midpoint rooted and branch support values (PP \geq 0.95) are shown on individual branches.

a guide tree with five species: (((EUR, NAA), SW), (NAB_a, NAB_b)). The three nodes, which were the same as those in the four-species guide tree again had 100% posterior probability, while the artificial node had posterior probabilities of 23%, 48%, and 14% over three runs with different starting trees. These analyses strongly support the genetic distinctiveness of the four clades recovered by other phylogenetic and coalescent methods above.

Intraclade diversity.—There were no shared haplotypes among clades and each clade had fixed differences, although the numbers of fixed differences varied considerably across pairs of clades. The numbers of fixed differences between NAB and NAA, NAB and EUR,

and NAB and SW were significantly higher than the number of fixed differences between NAA and EUR or NAA and SW (TABLE II). The number of shared mutations between NAB and the other clades for each gene was zero, with the exception of *RPB2* in the comparison of NAB and EUR. Pairwise nucleotide divergence values (K , D_{xy} , and D_a) were also higher between NAB and the other clades than for comparisons between the other clades. The highest number of shared mutations and the lowest values of pairwise nucleotide divergence occurred between clades NAA and EUR. Haplotype diversity for *F. pinicola* (TABLE III) was higher in EUR and SW (average EUR H_d 0.728, average SW H_d 0.732) than that for either NAB or NAA (0.498 and 0.397, respectively). The values for

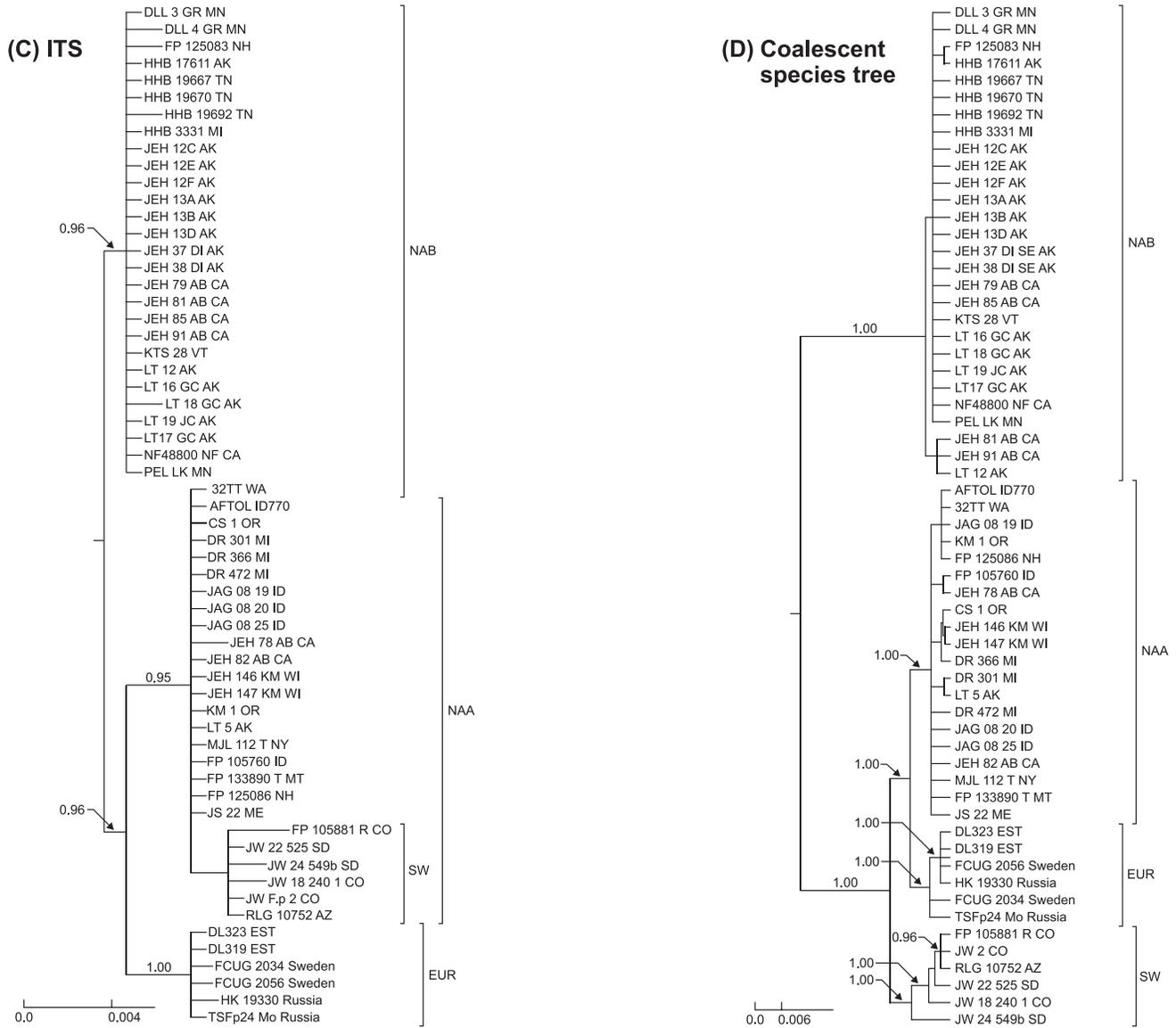


FIG. 1. Continued.

Watterson's θ_w (θ_w /site) and average pairwise nucleotide diversity (π), were low and similar in each clade. Analysis of the haplotype tree, which had a topology identical to that found in the gene trees, confirmed that haplotypes were not shared between any of the four clades.

Geography, color, and hosts.—Two clades, NAA and NAB, occurred in an area of North America ranging from the Alaska Interior to Maine and extending south along areas of high elevation as far as Oregon, Montana, and Tennessee; SW occurred in an area ranging from Arizona to western South Dakota, and EUR was specific to Europe and Asia. Individuals from NAA and NAB were found to inhabit the same range and occasionally the same site. Members of NAA and

NAB colonized both hardwoods and softwoods. Members of SW were recorded only from conifers, although we have also found *F. pinicola* sensu lato on hardwoods in this region, though we lack sequence data for these specimens. Members of the EUR clade represent specimens ranging from Sweden to Siberia and were collected on both hardwoods and softwoods. Collections were found in every clade that exhibited context colors commonly described in *F. pinicola*: cream, gray, red, brown, and black.

DISCUSSION

Results of three independent MrBayes analyses of the ITS, *TEF1*, and *RPB2* genes indicated the presence of four genetically distinct lineages within the *F. pinicola*

TABLE II. Nucleotide divergence among clades in the *Fomitopsis pinicola* species complex

Clade and locus	No. of polymorphic sites	No. of fixed differences	Polymorphic in clade 1 but monomorphic in clade 2	Polymorphic in clade 2 but monomorphic in clade 1	Shared mutations	<i>K</i>	<i>D_{xy}</i>	<i>Da</i>
North America B vs. North America A								
<i>RPB2</i>	60	14	28	20	0	12.189	0.03836	0.03211
<i>TEF1</i>	23	16	5	2	0	7.866	0.03430	0.03352
ITS	7	0	4	3	0	1.437	0.00547	0.00488
Mean						7.164	0.0260	0.0235
North America B vs. European								
<i>RPB2</i>	59	19	24	14	4	10.669	0.04248	0.03572
<i>TEF1</i>	29	15	5	9	0	6.834	0.03892	0.03516
ITS	10	1	4	5	0	1.255	0.00598	0.00413
Mean						6.253	0.02913	0.0250
North America B vs. Southwest								
<i>RPB2</i>	46	16	28	4	0	8.652	0.03514	0.03085
<i>TEF1</i>	28	16	5	8	0	6.442	0.03971	0.03552
ITS	12	4	4	4	0	1.740	0.00956	0.00794
Mean						5.611	0.02814	0.02477
North America A vs. European								
<i>RPB2</i>	32	0	13	12	7	5.024	0.00892	0.00109
<i>TEF1</i>	10	0	0	9	1	2.937	0.00964	0.00619
ITS	6	0	1	3	2	1.783	0.00574	0.00376
Mean						3.248	0.0081	0.00368
North America A vs. Southwest								
<i>RPB2</i>	25	0	19	5	1	6.229	0.01449	0.00907
<i>TEF1</i>	15	4	0	10	1	3.920	0.01407	0.00987
ITS	8	1	3	4	0	1.298	0.00410	0.00235
Mean						3.816	0.01089	0.00710
European vs. Southwest								
<i>RPB2</i>	32	7	19	5	1	8.607	0.01993	0.01361
<i>TEF1</i>	21	0	9	11	1	5.609	0.01264	0.00504
ITS	9	0	5	4	0	2.623	0.00666	0.00365
Mean						5.613	0.01308	0.00743

Abbreviations: *K* = average number of nucleotide differences among clades, *D_{xy}* = nucleotide divergence, *Da* = number of net nucleotide substitutions per site between clades.

species complex. Some of these lineages had high support in individual gene trees (FIG. 1), while a few lineages were not as well supported. This incongruity among gene trees is expected in recently divergent species because long periods of time need to elapse

to overcome incomplete lineage sorting. The ability to accommodate incomplete lineage sorting resulting from the independent evolutionary histories of each gene make coalescent analysis a powerful complement to genealogical concordance. The coalescent species

TABLE III. Nucleotide polymorphism of clades in the *Fomitopsis pinicola* species complex

Clade	Locus	<i>n</i>	<i>h</i>	<i>S</i>	<i>Hd</i>	θ_w /site	π
North American B							
	<i>RPB2</i>	64	34	26	0.928	0.00903	0.0054
	<i>TEF1</i>	64	5	5	0.333	0.00209	0.00105
	ITS	64	5	4	0.234	0.00157	0.00046
	Mean				0.498	0.004	0.002
North American A							
	<i>RPB2</i>	30	17	20	0.933	0.00809	0.00689
	<i>TEF1</i>	30	2	2	0.129	0.00092	0.00047
	ITS	30	2	3	0.129	0.00141	0.00072
	Mean				0.397	0.003	0.003
European							
	<i>RPB2</i>	16	9	20	0.892	0.00945	0.00878
	<i>TEF1</i>	16	6	10	0.867	0.00542	0.00659
	ITS	16	3	5	0.425	0.00282	0.00322
	Mean				0.728	0.006	0.006
Southwest							
	<i>RPB2</i>	14	4	6	0.659	0.00296	0.00386
	<i>TEF1</i>	14	4	12	0.747	0.00681	0.00857
	ITS	14	5	4	0.791	0.00234	0.00278
	Mean				0.732	0.004	0.005

Abbreviations: *n* = number of phased haploid sequences, *h* = number of haplotypes, *S* = number of segregating sites, *Hd* = haplotype diversity, θ_w = Watterson's theta, π = average pairwise nucleotide diversity.

analysis supported the four lineages above as clades (FIG. 1), and backed up by BPP, strongly supported these clades (NAA, NAB, SW, and EUR) as four species within a species complex, demonstrating the high resolution of coalescent methods when gene trees are incongruent. The conclusions of the phylogenetic and coalescent analyses were further supported by analysis of sequence polymorphism in DnaSP (TABLES II, III); particularly compelling was the observation of no shared haplotypes between any of these four clades.

The addition of a population genetics perspective augments species delimitation by highlighting gene flow vs. genetic distinctiveness of the focal groups. Comparison of the number of polymorphic sites and number of fixed differences between NAB and the other clades support the high posterior probability values assigned to nodes in the coalescent species tree. The number of fixed differences and shared polymorphisms (TABLE II) confirm the relatively close relationship between NAA and the European clade seen in the gene trees and taken in its entirety, the genetic, coalescent, and population genetic analyses help us to interpret *F. pinicola* as a species complex.

Our genetic analyses suggest that *F. pinicola* sensu lato is a species complex comprised of at least four

well-supported phylogenetic species, three in North America and one in Europe. These findings coincide with and help explain the results of the mating study performed by Irene Mounce (Mounce and McCrae 1938). However, she declined to designate two species in North America due to the compatibility of their Group A with their European isolates and only partial incompatibility of their Group B with their European isolates. Mounce isolate 1264 (Mounce and Macrae 1938) is available from the Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre (CBS 221.39), and Genbank sequences for both *RPB2* and ITS place it in NAA (SUPPLEMENTARY FIG. 4). This isolate links North American clade A with Mounce Group A, and because both studies found two groups with overlapping distribution in North America, it is likely that North American clade B from our study corresponds to Group B from their earlier study. We suspect that the Southwest clade recovered in our study was not sampled or studied by Mounce. Mounce and McCrae tested only two isolates from the geographic range covered by the Southwest clade: one from South Dakota and one from Arizona. Their pairings placed these isolates into Mounce Group A and Group B, respectively, and the results of single-spore mating tests

of these two isolates were similar to other members of their respective groups. Our results suggest that the Southwest clade is sympatric with NAA and NAB in this region, so it is reasonable to suppose that Mounce's isolates from this region belonged to the latter clades. It is also possible that members of the SW clade are compatible with NAA and NAB but are kept genetically distinct by other pre- or post-zygotic barriers.

Significance of host species.—Members of North American clades A and B were found on both hardwoods and softwoods. Similarly, Mounce group A and group B each included isolates from hardwoods and softwoods, requiring mating tests to segregate them. The long held hypothesis (Fries 1821, Saccardo 1888) that fruiting bodies found on species of *Populus*, exhibiting white or gray color and lacking the red belt, a character typically associated with *F. pinicola* sensu lato, indicates a variety or even a separate species, as *F. marginatus*, was not supported by genetic evidence in our study. Specimens spanning the range of color types common to *F. pinicola* sensu lato (Gilbertson and Ryarden 1986) were found on both hardwoods and softwoods and were represented in both North American clades A and B and the Southwest clade.

Taxonomic implications.—Our data suggest that the European clade, which has a geographic range confined to Eurasia, is *F. pinicola* (Sw) P. Karst. North America is home to three phylogenetic species, designated in this investigation as NAA, NAB, and SW. North American clade B numbers among its members *F. ochracea* (Ryarden and Stokland 2008), represented by two collections in our study, the isotype (Stokland-223) and another specimen collected by Ryarden (48800). Based on previous single-spore culture studies of Mounce and Macrae (1938) and applying phylogenetic and population-genetic species concepts, we propose that *F. pinicola* sensu stricto is restricted to Europe, that North American clade B is *F. ochracea*, and that two new species are represented by North American clade A and the Southwest clade, which remain to be named. Additional morphological and anatomical work is needed to propose characters that are effective in separating the four species in the *F. pinicola* complex.

Evaluation of reinforcement.—Reinforcement accords individuals who mate within their own species a fitness advantage (Servedio and Noor 2003). Sibling species in allopatry likely would not face selection pressure to diverge reproductively, and development of reproductive isolating mechanisms could occur gradually through mutation and genetic drift (Turelli et al.

2001). Reproductive isolation was found to be significantly stronger in sympatry than in allopatry for other Homobasidiomycota (Le Gac and Giraud 2008), suggesting selective pressure for the evolution of pre-mating isolation mechanisms.

Presence of pre-mating isolating mechanisms might be what Mounce and McCrae (1938) observed in the two groups of *F. pinicola* they studied in North America that had wide geographic overlap and were almost completely incompatible with each other. This could suggest that NAA and NAB have existed in sympatry for an extended period, developing extensive pre-zygotic isolation barriers between them due to selective pressure. It has been suggested that only species with a strong pre-mating isolation due to sexual partner recognition can coexist as discrete species in sympatry (Le Gac and Giraud 2008).

Implications for speciation.—Though we have not carried out detailed historical, phylogeographic analyses, our data do allow some initial hypotheses concerning the history of speciation events in the *F. pinicola* complex. Because the divergence between NAB and the other three clades is the oldest and deepest and was found in North America, as was the greatest genetic diversity overall, we propose North America as the origin of the complex. The EUR clade is not particularly variable genetically (see also Hogberg et al. 1999) and is most closely related to NAA. Perhaps during the radiation of the abundant and widespread NAA clade, one lineage adapted to more southerly climates, giving rise to Southwest clade, while another lineage continued north and migrated to Europe, perhaps via Eurasia and the Beringian Land Bridge. Both NAA and NAB occur sympatrically in the Alaskan region of Beringia. Two specimens from eastern Russia (HK 15879 and HK 12485) were not resolved within but were basal to the EUR clade in the ITS tree (unfortunately, all three genes could not be sequenced for these specimens), while all collections from western Russia belong to the EUR clade. These observations do not provide strong evidence but are consistent with our migration scenario.

The coalescent approach provided a highly resolved species delimitation within the *F. pinicola* complex. By analyzing samples spanning a wide geographic and host range together with these sensitive statistical methods, our data suggests that host jumps have not been a major driver of speciation in *F. pinicola*. In contrast, the fact that only two of the four species display broad sympatry suggests that historical migration has played a key role in speciation. Other, as yet unknown, ecological factors are also likely to be important, particularly in the case of the widely overlapping NAA and NAB pair. Intensive sampling with advanced

statistical-genetic analyses is likely to open new perspectives on fungal speciation and biodiversity.

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