

Phylogenetic and Biogeographic Relationships of Eastern Asian and Eastern North American Disjunct *Suillus* Species (Fungi) as Inferred from Nuclear Ribosomal RNA ITS Sequences

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Species of *Suillus* produce fleshy, pored mushrooms. They are important symbiotic (ectomycorrhizal) partners of many coniferous trees. The genus includes several putative eastern Asian and eastern North American disjunct species, i.e., the *S. americanus*-*S. sibiricus* and *S. decipiens*-*S. spraguei* complexes. Phylogenetic relationships among the groups were determined to further understand the biogeographic pattern. Analyses were based on 40 sequences of the ITS region of the nuclear ribosomal RNA tandem repeats, representing 18 distinct species/populations. Our phylogenetic analyses suggested that: (1) Chinese and United States' (U.S.) *S. spraguei* plus *S. decipiens* form a strongly supported monophyletic group, with North American *S. decipiens* and Chinese *S. spraguei* being sister taxa; (2) *S. americanus*, Asian and U.S. *S. sibiricus*, plus *S. umbonatus* form a clade supported by a high bootstrap value; and (3) little ITS sequence divergence exists within the latter group compared to the *S. decipiens*-*S. spraguei* clade. Phylogenetic patterns revealed by this study imply a close phylogenetic relationship between eastern Asian and eastern North American disjunct population/species of *Suillus*. These fungi display relatively high host fidelity (at least to the host subgenus level), suggesting potential coevolutionary/comigratory trends. © 2000 Academic Press

Key Words: *Suillus*; *Pinus*; phylogeny; biogeography; eastern Asian–eastern North American disjunct; host specificity.

INTRODUCTION

One of the intriguing and understudied questions in the biogeography of macrofungi is the relationship be-

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tween putative eastern Asian and eastern North American disjunct species. Wu and Mueller (1997) discussed the north temperate disjunct distributions of some macrofungi based on morphology and herbarium specimen records. They concluded that (1) the similarity between the mycota of eastern Asia and eastern North America is relatively high as compared to that observed between other regions in the north temperate zone; and (2) disjunct distributions of macrofungi are usually only seen at species or lower taxonomic levels. Results from this and other similar studies (i.e., Redhead, 1989; Hongo and Yokoyama, 1978; Wolfe and Bougher, 1993), however, are greatly influenced by the authors' species concepts and interpretations of morphological characters. Thus, phylogenetic analyses of putative disjunct fungal species are necessary to rigorously test the eastern Asian–eastern North American affinity hypothesis.

Studies on phylogeny and its implications for understanding the biogeography of macrofungi have been progressing significantly in recent years due to advancements in DNA sequencing and phylogenetic analysis methods (e.g., Hughes *et al.*, 1998; Kretzer *et al.*, 1996; Vilgalys and Sun, 1994). However, phylogenetic examinations of fungal taxa sharing putative eastern Asian and eastern North American disjunct distribution patterns have not been published.

The phylogenetic study by Berbee and Taylor (1993) implied multiple independent emergence of ectomycorrhizae. This, however, does not exclude the possibility that relationships between certain fungi and certain plants have developed and persisted through time. For example, Kretzer *et al.* (1996) showed that *Suillus* species associated with pines are more closely related to each other than to those associated with *Larix*. Further phylogenetic studies incorporating fungus and pine species are needed to better understand host specificity, mycorrhizal evolution, and biogeographic patterns.

Species of *Suillus* provide ideal material to gain a better understanding of the phylogenetic relationships between eastern Asian and eastern North American disjunct taxa and their implications for biogeography and host specificity. The genus *Suillus* is widely distributed in northern temperate regions and its species are often a dominant ectomycorrhizal partner with species classified within the Pinaceae, as well as a few deciduous species (Singer, 1986; Smith and Thiers, 1964). Various distribution patterns have been observed for *Suillus* species, including regional endemic, eastern Asian–North American disjunct, and circum-boreal distributions. Host specificity of *Suillus* species ranges from being restricted to a single host species to being associated with both coniferous and deciduous trees (Singer, 1986). Finally, a recent phylogenetic study that included a good sampling of *Suillus* species (Kretzer *et al.*, 1996) provided an essential phylogenetic framework for biogeographical analyses as well as an indication of the utility of the internal transcribed spacer (ITS) sequences of nuclear ribosomal RNA to infer phylogenetic relationships at the species level in this genus.

Our ingroup taxa are the *Suillus americanus*–*S. sibiricus* and *S. decipiens*–*S. spraguei* species complexes. *S. americanus* (Peck) Snell in Slipp and Snell [= *Boletus americanus* Peck, 1888] is reported only from eastern North America, but it shares a mosaic of morphological characters with *S. sibiricus* (Singer) Singer [= *Ixocomus sibiricus* Singer, 1938] (Slipp and Snell, 1944). The latter has been reported to have a wider geographic range, including western North America (Smith and Thiers, 1964), China (Ying and Zang, 1994), Nepal (Kretzer *et al.*, 1996), the Altai Mountains (type locality; Singer, 1938), and other parts of Europe (as *S. sibiricus* ssp. *helveticus* Singer, 1951). *S. umbonatus* Dick and Snell is another published name that has been confused with the western U.S. *S. sibiricus* (see Smith and Thiers, 1964, pp. 83–84). *S. spraguei* (Berk. & Curt.) Kuntze [= *S. pictus* (Peck) Smith & Thiers] reportedly displays an eastern Asian [China, Korea, and Japan] and eastern North American disjunct distribution (Hongo and Yokoyama, 1978). Collections from both sides of the distribution range share remarkable morphological similarities. *S. decipiens* (Berk. & Curt.) Kuntze is the sister taxon to *S. spraguei* (Kretzer *et al.*, 1996) and is reported only from the southeastern United States.

The objective of this study is threefold: (1) determine the phylogenetic relationships among eastern Asian and eastern North American disjunct *Suillus* species, namely *S. americanus*, *S. sibiricus*, and *S. spraguei*; (2) reassess eastern Asian and eastern North American floristic affinities in the light of *Suillus* biogeography and phylogeny; and (3) extend the understanding of *Suillus* host specificity and biogeographical patterns by comparing *Suillus* and *Pinus* phylogenies.

MATERIALS AND METHODS

Material Studied

Sequences of the ITS region (ITS1–5.8S–ITS2) were obtained from *Suillus* specimens gathered by either field collecting or herbarium loans, or downloaded directly from GenBank. Scientific name, locality, depositary, and GenBank accession number of all *Suillus* specimens with DNA data used in the analyses are listed in Table 1. The ingroup taxa were represented by multiple collections from various localities. The sister taxa to each of the target species and to the ingroup as a whole were chosen based on the published phylogeny by Kretzer *et al.* (1996). *S. cavipes* and *S. asiaticus* were selected as outgroup taxa. All specimens collected within this project were deposited at both the Field Museum of Natural History, Chicago, and the Institute of Microbiology, Chinese Academy of Sciences, Beijing.

Gene sequences of chloroplast ribulose-1,5-bisphosphate carboxylase large subunit (*rbcL*) of relevant *Pinus* species and outgroup taxa were downloaded from the GenBank database, including those of *P. armandii* [AB019804], *P. cembra* [AB019795], *P. densiflora* [AB019814], *P. hwangshanensis* [AB019812], *P. koraiensis* [AB019797], *P. kwangtungensis* [AB019802], *P. massoniana* [AB019815], *P. monticola* [AB019799], *P. nigra* [AB019817], *P. parviflora* [AB019800], *P. pinaster* [AB019818], *P. pumila* [AB019796], *P. strobus* [AB019798], *P. sylvestris* [AB019809], *P. taeda* [AF119177], *P. yunnanensis* [AB019816], *Abies numidica* [AB019827], *Larix decidua* [AB019826], and *Picea abies* [AB019825]. All but one were generated by Wang *et al.* (1999).

DNA Extraction, Amplification, and Sequencing

DNA was extracted from either air-dried herbarium specimens or tissue of fresh basidiomata preserved in a fixative solution [20% DMSO, 250 mM EDTA, and saturated NaCl; Steve Rehner, University of Puerto Rico, personal communication]. Fixed material was transported back to the laboratory and stored at 4°C. Prior to DNA extraction, the sample was removed from the fixative solution, blot-dried with a piece of tissue paper, and then placed in a 80°C freezer overnight before grinding.

Puregene kit “DNA isolation from filamentous fungi” (Gentra Systems, Inc.) and CTAB methods (Gardes and Bruns, 1993) were used to isolate DNA. In general, Puregene methods worked better for fixed fresh materials, while the CTAB method provided better genomic DNA from dried herbarium specimens. Genomic DNA was usually diluted between 10^{-1} and 10^{-3} before amplification.

A fragment including ITS1, 5.8S, and ITS2 of the nuclear ribosomal RNA repeat unit was amplified using the polymerase chain reaction (PCR) with primers ITS1–ITS4 (White *et al.*, 1990). For a few dried herbar-

ium specimens, primer pairs ITS5–ITS2 and ITS3–ITS4S were used to separately amplify the ITS1 and ITS2 regions (White *et al.*, 1990; Kretzer *et al.*, 1996). Each of the final mixture of the PCR solutions contained 5 μ l 10 \times reaction buffer (25 mM MgCl₂, 100 mM Tris, 500 mM KCl, pH 8.3), 5 μ l of dNTPs (equal mixture of the four deoxyribonucleotide triphosphates), 0.5 μ l *Taq* polymerase, 2.5 μ l each of the two primers, and 1 to 5 μ l DNA template, and was filled to 50 μ l with distilled sterile water. PCR thermal cycling conditions were initial denaturation at 94°C for 1.5 min, 35 cycles of 15 s at 94°C, 15 s at 50°C, and 30 s at 72°C, followed by extension at 72°C for 3 min and then a soak at 4°C. PCR products were visualized with TAE buffered 1.5% low-melt agarose gel. In a few cases, in order to produce an appropriate amount of DNA fragments, the bands on agarose gel were taken out, melted in TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) at 65°C, diluted 3 \times 10² times, and reamplified using the same protocol. DNA amplification products were purified with BIO 101 GeneClean III kit (BIO 101, Inc.).

Sequencing reactions were performed with the same primers used for the symmetric amplifications and were either purified using CentriSep columns (samples sequenced with FS; Princeton Separations, Princeton, NJ) or ethanol-precipitated according to the manufacturer's directions (samples sequenced with dRhodamine). Reactions were then electrophoresed on an ABI 377 automated sequencer (Perkin-Elmer Applied Biosystems, Inc.). Gels were tracked using the ABI Prism Sequencing 2.1.1 program and analyzed with Sequencing Analysis 3.3 (Perkin-Elmer Applied Biosystems, Inc.).

Alignment and Phylogenetic Analyses

The ITS sequences of *Suillus* were assembled and aligned with Sequencher 3.0 (Gene Codes Co.) and exported in a NEXUS format. The *rbcL* sequences of *Pinus* were also aligned using Sequencher. All phylogenetic analyses were performed with PAUP* 4.0b1a (Swofford, 1998). Sequence regions in which nucleotides could be aligned in different ways due to the presence of indels were delimited as ambiguously aligned regions (Lutzoni *et al.*, 2000). Constant sites were excluded from all analyses. The final alignment of *Suillus* sequences was deposited in TreeBASE.

Most-parsimonious trees were generated by running the heuristic search with tree-bisection-reconnection (TBR) as the branch-swapping algorithm, gaps as the 5th character state, 1000 random-addition sequence replications, and branches collapsed if maximum branch length was zero. Under these settings, three maximum-parsimony analysis schemes were performed for *Suillus* data set: (1) changes among character states equally weighted and ambiguous regions excluded (MPE); (2) changes among character states unequally weighted using a symmetric step matrix and ambiguous regions excluded (MPU); and (3) changes

among character states unequally weighted, and ambiguous regions recoded and incorporated into analysis (MPUR). In MPU and MPUR, the costs included in the step matrix for the nonambiguously aligned sites were estimated by counting each type of change using the character status option of the "Data" menu of PAUP*, calculating their relative frequencies, and converting them to costs using the negative natural logarithm (Felsenstein, 1981; Wheeler, 1990). In MPUR, ambiguous regions were first excluded from the sequence data matrix; each region was then recoded as a single character with character states corresponding to different sequences; changes among character states were then weighted by developing and implementing step matrices following Lutzoni *et al.* (2000). For *Pinus* and the regional representative *Suillus* data sets, only MPU was performed. Clade stability of most-parsimonious trees was examined by 1000 bootstrap replicates (Felsenstein, 1985) using the same parameters implemented in the initial heuristic search. One random addition sequence replicate was performed for each bootstrap data set.

Ambiguously aligned regions were excluded from the maximum-likelihood analysis (ML). Base frequencies, transition/transversion ratio, and number of rate categories to account for site to site variation were estimated using one of the most-parsimonious trees generated in MPU analysis. The Hasegawa-Kishino-Yano model (Hasegawa *et al.*, 1985) was selected and parameters were optimized using a likelihood-ratio test (Huelsenbeck and Crandall, 1997). The heuristic search of 1000 replicates was performed with random-addition sequences, TBR, and branches collapsed if maximum branch length was zero. Clade stability was estimated by 1000 bootstrap replicates after setting base frequencies, transition/transversion ratio, and number of rate categories identical to those in the initial maximum-likelihood analysis.

Morphology and Distribution Studies

Morphological examinations of ingroup taxa were carried out on both fresh and herbarium specimens following standard methods and terminology (Smith and Thiers, 1964). Up to 30 basidiospores and 10 cystidia were measured per specimen to obtain descriptive statistical data. Distribution ranges of fungi were outlined based on our own field records and previously published data.

RESULTS

Suillus Molecular Data

DNA sequences. Sequences of ITS1, 5.8S, and ITS2 of the nuclear ribosomal RNA tandem repeats were obtained for a total of 40 collections (Table 1). The final alignment included 608 sites. Nine ambiguously

TABLE 1

List of *Suillus* Specimens Studied, Their Geographic Origin, Collection Data, DNA Data Source, and GenBank Accession Number

Scientific name (code)	Location	Collection No.	Herbarium	Sequence source	GenBank Accession No.
<i>Suillus amabilis</i> (U.S.A.)	U.S.A., Washington	N-5387	F	W ^a	AF166499
<i>S. americanus</i> (U.S.A.-MI)	U.S.A., Michigan	TDB-581	MICH	K ^b	L54103
<i>S. americanus</i> (U.S.A.-NC1)	U.S.A., North Carolina	1008-NC	TENN	W	AF166500
<i>S. americanus</i> (U.S.A.-NC2)	U.S.A., North Carolina	Am-1	TENN	W	AF166501
<i>S. americanus</i> (U.S.A.-NY)	U.S.A., New York	TJB-7683	CORT	W	AF166502
<i>S. americanus</i> (U.S.A.-OH)	U.S.A., Ohio	MAV-5625	F	W	AF166503
<i>S. asiaticus</i> (China)	China, Jilin	QXW-2408	F, HMAS	W	AF166504
<i>S. asiaticus</i> (Finland)	Finland	JV-4850F	MICH	K	L54090
<i>S. cavipes</i> (China-JL1)	China, Jilin	QXW-2328	F, HMAS	W	AF166505
<i>S. cavipes</i> (China-JL2)	China, Jilin	QXW-2406	F, HMAS	W	AF166506
<i>S. cavipes</i> (China-JL3)	China, Jilin	QXW-2066	F, HMAS	W	AF166507
<i>S. cavipes</i> (Switzerland)	Switzerland	HDT-31407	SFSU	K	L54085
<i>S. cavipes</i> (U.S.A.-ID)	U.S.A., Idaho	WJS-618	SFSU	K	L54119
<i>S. cavipes</i> (U.S.A.-MI)	U.S.A., Michigan	TDB-646	TDB	K	L54105
<i>S. decipiens</i> (U.S.A.-TX1)	U.S.A., Texas	DG-1451	MICH	W	AF166508
<i>S. decipiens</i> (U.S.A.-TX2)	U.S.A., Texas	GMM-6168	F	W	AF166509
<i>S. decipiens</i> (U.S.A.-TX3)	U.S.A., Texas	DPL-5724	F	W	AF166510
<i>S. luteus</i> (Germany)	Germany	HB-348	HB	K	L54083
<i>S. luteus</i> (U.S.A.-MI1)	U.S.A., Michigan	LBG-17	F	W	AF166511
<i>S. luteus</i> (U.S.A.-MI2)	U.S.A., Michigan	TDB-824	TDB	K	L54110
<i>S. luteus</i> (U.S.A.-MI3)	U.S.A., Michigan	TDB-571	MICH	K	L54100
<i>S. punctipes</i> (U.S.A.)	U.S.A., Minnesota	TDB-265	TDB	K	L54098
<i>S. sibiricus</i> (China-GS)	China, Gansu	HMAS-66061	HMAS	W	AF166512
<i>S. sibiricus</i> (China-JL1)	China, Jilin	QXW-2092	F, HMAS	W	AF166513
<i>S. sibiricus</i> (China-JL2)	China, Jilin	GMM-5702	F, HMAS	W	AF166514
<i>S. sibiricus</i> (China-YN)	China, Yunnan	QXW-2567	F, HMAS	W	AF166515
<i>S. sibiricus</i> (Nepal)	Nepal	VC-1040	VC	K	L54117
<i>S. sibiricus</i> (U.S.A.-AZ)	U.S.A., Arizona	HDT-53720	SFSU	W	AF166516
<i>S. sibiricus</i> (U.S.A.-NM)	U.S.A., New Mexico	HDT-54723	SFSU	W	AF166517
<i>S. spraguei</i> (China-JL1)	China, Jilin	GMM-5703	F, HMAS	W	AF166518
<i>S. spraguei</i> (China-JL2)	China, Jilin	QXW-2362	F, HMAS	W	AF166519
<i>S. spraguei</i> (China-JL3)	China, Jilin	QXW-2409	F, HMAS	W	AF166520
<i>S. spraguei</i> (China-YN1)	China, Yunnan	QXW-2415	F, HMAS	W	AF166521
<i>S. spraguei</i> (China-YN2)	China, Yunnan	QXW-2435	F, HMAS	W	AF166522
<i>S. spraguei</i> (China-YN3)	China, Yunnan	QXW-2566	F, HMAS	W	AF166523
<i>S. spraguei</i> (U.S.A.-MI)	U.S.A., Michigan	TDB-638	MICH	K	M91617
<i>S. spraguei</i> (U.S.A.-NY1)	U.S.A., New York	Hodge-32	CORT	W	AF166524
<i>S. spraguei</i> (U.S.A.-NY2)	U.S.A., New York	TJB-6228	CORT	W	AF166525
<i>S. umbonatus</i> (U.S.A.-CA)	U.S.A., California	TDB-978	TDB	K	L54115
<i>S. umbonatus</i> (U.S.A.-ID)	U.S.A., Idaho	F1068943	F	W	AF166526

Note. CORT, New York State University, College at Cortland. F, Field Museum of Natural History, Chicago. HB, H. Besl, University of Regensburg, Germany. HMAS, Herbarium of Mycology, Academia Sinica. MICH, University of Michigan Herbarium. NY, New York Botanical Garden. SFSU, San Francisco State University. TDB, T.D. Bruns, University of California, Berkeley. TENN, University of Tennessee Herbarium. VC, Van Cotter, Cyanamid Forschung GmbH, Germany.

^a This study.

^b Kretzer *et al.*, 1996.

aligned regions were identified, covering 119 sites. One of them was not recoded because of a substantial number of indels. The remaining 8 were recoded, forming eight additional characters in MPUR, all of which were parsimony-informative. Of the 489 nonambiguously aligned sites, 92 were variable.

Maximum parsimony—equally weighted (MPE). There was a total of 70 parsimony-informative sites in this analysis. Six equally most-parsimonious trees

were found (tree length = 158 steps, consistency index [CI] = 0.72, retention index [RI] = 0.92, rescaled consistency index [RC] = 0.67). Differences among the six most-parsimonious trees were all nested within the *S. decipiens*-*S. spraguei* clade. These six trees are part of one-tree island that was hit 1000 times. All most-parsimonious trees reveal a well-supported *S. americanus*-*S. sibiricus*-*S. umbonatus* clade, although the Asian *S. sibiricus* is not resolved (Fig. 1).

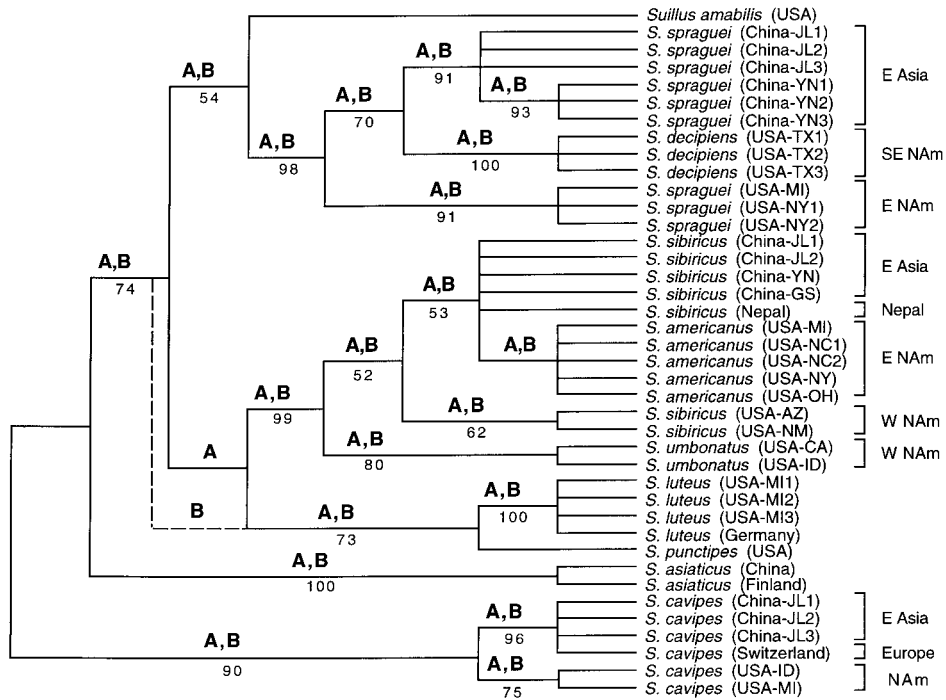


FIG. 1. Strict consensus of the 6 most-parsimonious trees generated by MPE. Terminal taxa are individual collections as listed in Table 1. Numbers below each internode are the percentage of 1000 bootstrap replicates supporting that binary partition (value ≤ 50 not shown). Letters above each internode indicate that the particular clade was revealed by the following analysis schemes: **A**, Strict consensus of 6 equally most-parsimonious trees generated by MPU. **B**, Strict consensus of 519 equally most-parsimonious trees generated by MPUR. E Asia, eastern Asia; SE NAm, southeastern North America; E NAm, eastern North America; and W NAm, western North America.

Maximum parsimony—unequally weighted (MPU). There was a total of 70 parsimony-informative sites in this analysis. The step matrix implemented to weight character state changes consisted of $A \leftrightarrow C = 2.00$; $A \leftrightarrow G = 2.23$; $A \leftrightarrow T = 2.11$; $A \leftrightarrow \text{gap} = 4.94$; $C \leftrightarrow G = 2.30$; $C \leftrightarrow T = 0.97$; $C \leftrightarrow \text{gap} = 3.33$; $G \leftrightarrow T = 2.38$; $G \leftrightarrow \text{gap} = 4.94$; $T \leftrightarrow \text{gap} = 4.25$. This analysis generated six equally most-parsimonious trees (tree length = 264.41 steps, CI = 0.72, RI = 0.92, RC = 0.67), all of which are identical to those generated by MPE. Bootstrap support for each node is similar to those found in MPE.

In general, both MPE and MPU analyses generated a well-resolved strict consensus tree topology that is congruent with the geographic distributions of the collections in the same species (Fig. 1). For example, all collections of *S. spraguei* from China are monophyletic and all collections of *S. americanus* are monophyletic. Eastern U.S. *S. americanus*, Asian and western U.S. *S. sibiricus*, and western U.S. *S. umbonatus* form a monophyletic clade with strong bootstrap support. However, the relationship between *S. americanus* and Asian *S. sibiricus* is not well resolved, and the *S. americanus* clade, although consistent in all most-parsimonious trees, had virtually no bootstrap support. *S. decipiens*, Chinese *S. spraguei*, and U.S. *S. spraguei* originate from a common ancestor in all phylogenetic analyses,

with the first two being sister groups, and accordingly, Chinese and U.S. *S. spraguei* being paraphyletic.

Maximum parsimony—unequally weighted and ambiguous regions recoded (MPUR). There was a total of 78 parsimony-informative characters in this analysis. The heuristic search generated 519 equally most-parsimonious trees (tree length = 401.49 steps, CI = 0.73, RI = 0.92, RC = 0.68). The strict consensus of the 519 equally parsimonious trees is in agreement with both MPE and MPU (Fig. 1), except that MPUR places the *S. luteus*–*S. punctipes* clade basal to both the *S. amabilis*–*S. decipiens*–*S. spraguei* clade and the *S. sibiricus*–*S. americanus*–*S. umbonatus* clade, instead of being a sister group only to the latter. The same pattern was revealed by the ML analysis (Fig. 2). In addition, minor discrepancies show in a few subterminal branches in the most-parsimonious trees. Compared to MPE and MPU methods, MPUR resulted in many more equally parsimonious trees, but this was caused by choosing to collapse branches with minimum length equal to zero.

Maximum-likelihood analysis (ML). Estimated base frequencies were A, 0.13761; C, 0.36873; G, 0.15707; T, 0.33659. Estimated transition/transversion ratio was 1.86527. No rate heterogeneity among sites was detected (identical likelihood for one or two rate catego-

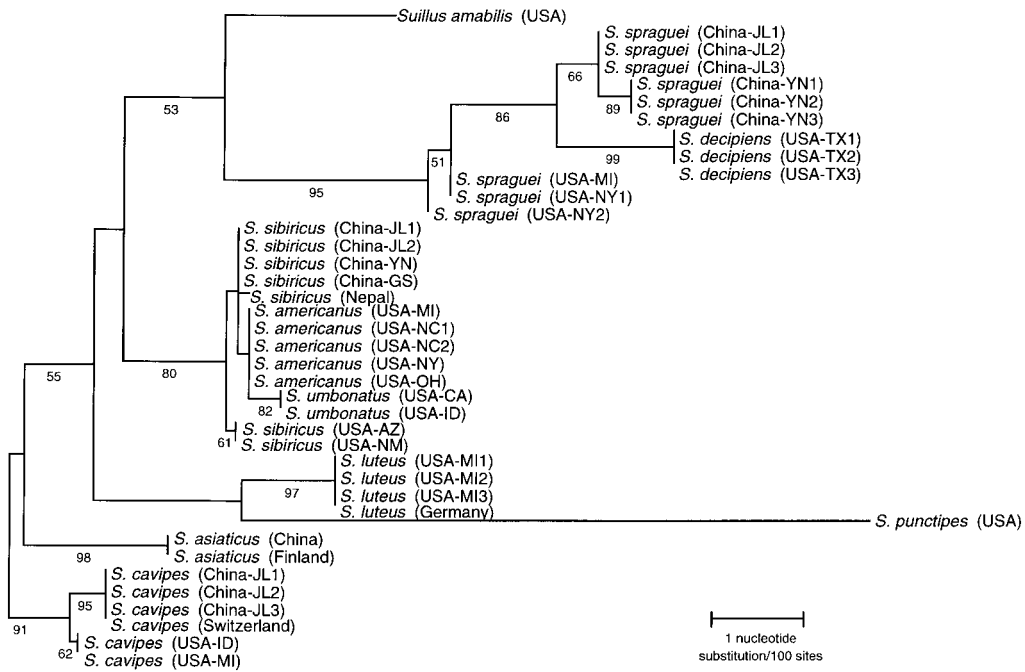


FIG. 2. Most likely tree generated by the ML search ($-\ln$ likelihood = 700.60235). Numbers below each internode are the percentage of 1000 bootstrap replicates supporting that binary partition (value ≤ 50 not shown).

ries) and, therefore, all sites were assumed to evolve at the same rate. Heuristic search using these parameters generated one best tree [$-\ln = 700.60235$]. Topology, branch lengths, and bootstrap values are shown in Fig. 2. This topology was hit 1000 times.

The most likely tree is similar to the most-parsimonious trees in outgroup positions and branching patterns, but differs somewhat in topology within the *S. decipiens*–*S. spraguei* and the *S. americanus*–*S. sibiricus*–*S. umbonatus* clades. As seen in the most-parsimonious trees, the *S. decipiens*–*S. spraguei* clade is strongly supported by bootstrap value (95%). Within this clade, the U.S. *S. spraguei* is shown to be paraphyletic, but this hypothesis is weakly supported by bootstrap (51%). Chinese *S. spraguei* and U.S. *S. decipiens* are sister taxa and form a well-supported monophyletic group (86%). Compared to the *S. americanus*–*S. sibiricus*–*S. umbonatus* group, substantial genetic divergence has occurred, resulting in a well-supported phylogenetic structure. For example, ITS provided several genetic markers to distinguish northern and southern Chinese populations of *S. spraguei*. This is not the case for the Asian *S. sibiricus* population.

S. americanus, *S. sibiricus*, and *S. umbonatus* form a well-supported monophyletic group (80%). However, except for the U.S. *S. sibiricus* clade, there is no well-supported phylogenetic structure within this group. In the ML analysis, the position of *S. umbonatus* is unresolved within the *S. americanus*–*S. umbonatus* monophyletic group, and the latter is unresolved with regard

to Asian *S. sibiricus*. Topological discrepancies between the MP and ML analyses within this clade can be explained by the position of *S. umbonatus* (Fig. 2). Both ML, the method that includes the lowest number of variable characters but is least sensitive to branch-length attraction, and MPUR, the method that includes the largest number of parsimony-informative characters, supported the *S. luteus*–*S. punctipes* group to be sister to the two main ingroup clades. Finally, in all four analytical schemes, the relationships among members of the outgroup taxa, i.e., *S. asiaticus* and *S. cavipes*, are stable.

Alternate Hypothetical Phylogenetic Relationships of *Suillus*

Two alternate phylogenetic relationships were explored: (1) Chinese and U.S. *S. spraguei* are monophyletic; (2) Asian and U.S. *S. sibiricus* are monophyletic, excluding *S. americanus*. This was done by constraining one monophyletic node at a time and performing maximum-parsimony analyses using PAUP*. Unequally weighted maximum-parsimonious analyses with the constraint of monophyly of Chinese and U.S. *S. spraguei* generated two equally most-parsimonious trees [tree length = 266.79 steps, CI = 0.71, RI = 0.92, RC = 0.65]. With the constraint of monophyly of Asian and U.S. *S. sibiricus*, MPU generated six equally most-parsimonious trees [tree length = 265.08 steps, CI = 0.72, RI = 0.92, RC = 0.66]. In both cases, the tree lengths are slightly greater than the MPU tree [tree length = 264.41 steps]. To test if the tree length dif-

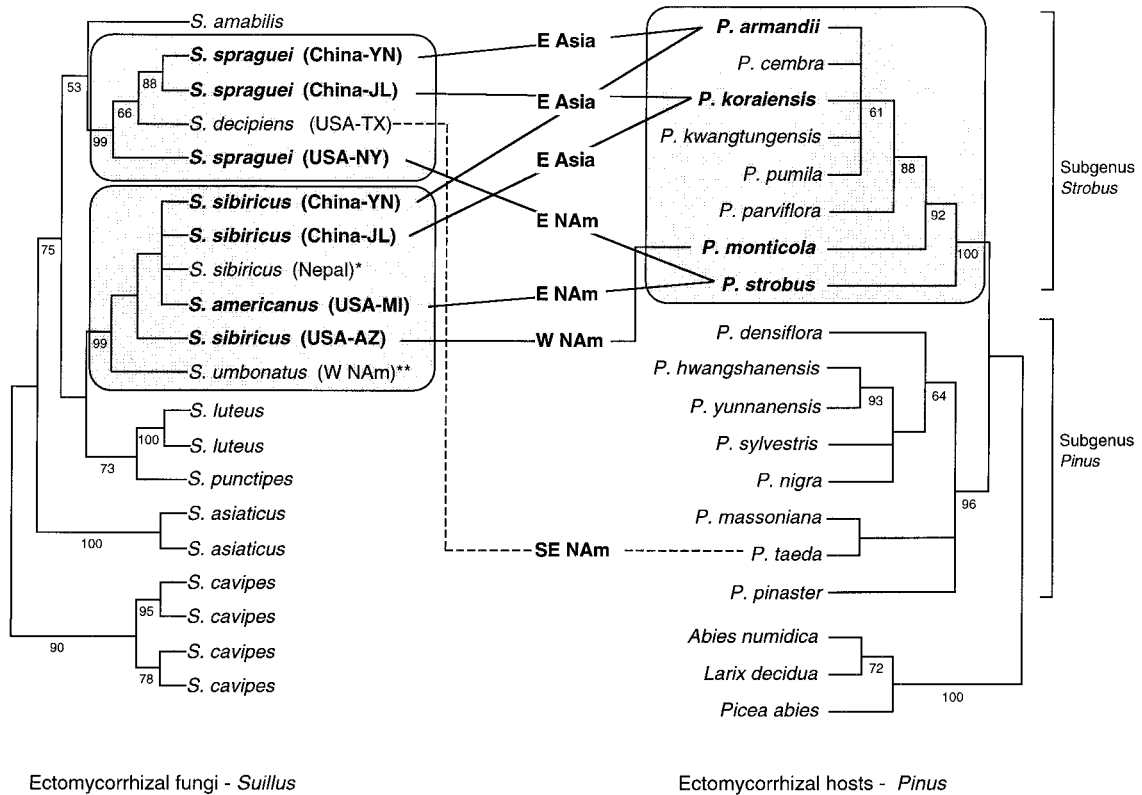


FIG. 3. The single most-parsimonious tree of regional representative *Suillus* and strict consensus of the four most parsimonious trees of *Pinus*, both generated by MPU, showing ingroup *Suillus* biogeographic patterns and associations with *Pinus*. Numbers below each internode are the percentage of 1000 bootstrap replicates supporting that binary partition (value ≤ 50 not shown). Lines between *Suillus* and *Pinus* species indicate observed mycorrhizal associations and their distribution. Shaded areas encompass concerned monophyletic groups. See Fig. 1 for area abbreviations. **Suillus sibiricus* is associated with *P. sibirica* (Subgenus *Strobos*) in type locality (Singer, 1938). ***Suillus umbonatus* is reportedly associated with *P. contorta* (Subgenus *Pinus*; Smith and Thiers, 1964). Chloroplast *rbcl* sequences of *P. sibirica* and *P. contorta* were not available for analysis.

ference between the constrained MPU tree and the unconstrained MPU tree is due to sampling error, a tree length null distribution was developed. This consisted of bootstrapping the sequence data set 1000 times, performing a heuristic search on each of them, saving one tree at each replicate, obtaining tree length for all 1000 trees, and calculating frequencies of tree lengths. If more than 95% of the tree lengths in the null distribution are smaller than the constrained MPU tree length, then the null hypothesis that the constrained trees are due to sampling error is rejected. We observed that only 4 and 3% of the tree lengths in the null distribution are shorter than the constrained MPU trees for monophyly of Chinese and U.S. *S. spraguei* and that of Asian and U.S. *S. sibiricus*, respectively. Therefore, we cannot reject the null hypothesis that the difference in tree length between the MPU tree and the tested monophylies is due to sampling error. Thus, the monophyly of Chinese and U.S. *S. spraguei* and that of Asian and U.S. *S. sibiricus* cannot be rejected based on this test.

Comparison of Suillus and Pinus Phylogenies

To illustrate and compare *Suillus* and *Pinus* phylogenies, regional representatives of *Suillus* species were selected and analyzed using the MPU method. There was a total of 64 parsimony-informative sites in this analysis. The step matrix consisted of $A \leftrightarrow C = 2.04$; $A \leftrightarrow G = 2.29$; $A \leftrightarrow T = 2.09$; $A \leftrightarrow \text{gap} = 4.93$; $C \leftrightarrow G = 2.36$; $C \leftrightarrow T = 0.92$; $C \leftrightarrow \text{gap} = 3.32$; $G \leftrightarrow T = 2.36$; $G \leftrightarrow \text{gap} = 4.93$; $T \leftrightarrow \text{gap} = 4.24$. This analysis generated one most-parsimonious tree (tree length = 258.17 steps, CI = 0.73, RI = 0.83, RC = 0.61; Fig. 3). The tree topologies resemble that of the most-parsimonious trees generated by MPU (Fig. 1).

The final alignment of chloroplast *rbcl* sequences of *Pinus* species and outgroup taxa consisted of 1331 sites, out of which 42 are parsimony-informative. There is no indel observed. MPU analysis was performed by implementing a step matrix consisting of $A \leftrightarrow C = 2.69$; $A \leftrightarrow G = 1.20$; $A \leftrightarrow T = 2.33$; $C \leftrightarrow G = 2.69$; $C \leftrightarrow T = 0.96$; $G \leftrightarrow T = 2.56$. This analysis generated four equally most-parsimonious trees (tree

TABLE 2

Morphological Characteristics and Hosts of *Suillus americanus*, *S. sibiricus*, and *S. umbonatus*

Name:	<i>S. americanus</i>	<i>S. sibiricus</i>	<i>S. sibiricus</i>	<i>S. sibiricus</i>	<i>S. umbonatus</i>
(Area):	(E NAm)	(Altai Mtn.)	(China)	(W NAm)	(W NAm)
No. of collections:	7	1, lectotype	5	2	1
Pileus surface:	Bright yellow	Olive-yellow Sulfur-olive	Bright yellow Mustard yellow	Olive-yellow	Olive-yellow
Margin squamules:	Orange to reddish	Yellowish	Yellowish, orange to reddish	Brownish	Brownish
Annulus:	Absent	Present	Absent or present	Absent or present	Present
Stipe diameter:	3–10 mm	8–15 mm	3–10 (12) mm	7–15 mm	4–8 (12) mm
Pleurocystidia:	38–60 × 7–11 μm	50–60 × 4–11 μm	48–56 × 5–10 μm	40–70 × 6–9 μm	28–32 × 5–8 μm
Basidia:	17–22 × 5–7 μm	26–35 × 5–7 μm	22–30 × 6–7 μm	22–28 × 5–7 μm	24–28 × 5–7 μm
Basidiospore Range:	7–11 × 3–4.8 μm	8.5–11.5 × 3–4.8 μm	7.8–11 × 3–4.8 μm	7–12 × 3.2–4.8 μm	8–10 × 3.6–4.8 μm
Mean L; W; Q:	9.5; 3.7; 2.5	10.2; 4.1; 2.5	9.5; 3.9; 2.5	9.8; 4.1; 2.4	9.3; 4.2; 2.1
Host species:	<i>Pinus strobus</i>	<i>P. sibirica</i>	<i>P. armandii</i> & <i>P. koraiensis</i>	<i>P. monticola</i>	<i>P. contorta</i>

Note. L, length; W, width; Q, length/width.

length = 163.47 steps, CI = 0.89, RI = 0.93, RC = 0.82; Fig. 3). The tree topologies are, in most part, similar to that of the most-parsimonious tree based on multigene combined sequences in Wang *et al.* (1999).

DISCUSSION

Phylogeny of *Suillus* Species

Phylogenetic relationships among all included species as resolved by this study are largely in agreement with Kretzer *et al.* (1996). There are significant similarities among tree topologies generated with the different phylogenetic analysis methods in this study. These can be summarized as follows. (1) Chinese and U.S. *S. spraguei* and *S. decipiens* were derived from a most recent common ancestor. The clade is strongly supported by bootstrap (98 and 95% for MPE and ML, respectively). Within this group, the degree of divergence is high, and four subgroups are recognizable, i.e., *S. decipiens*, U.S. *S. spraguei*, China-Yunnan *S. spraguei*, and China-Jilin *S. spraguei*. (2) *S. americanus*, Asian and U.S. *S. sibiricus*, and *S. umbonatus* formed a clade supported by high bootstrap values (99 and 80% for MPE and ML, respectively). While *S. umbonatus* and U.S. *S. sibiricus* can be separated from the rest of the group, the phylogenetic relationships among the subgroups inferred from different methods are not always consistent and are not supported by bootstrap analyses. Overall, little divergence is detected in this group. (3) In all analyses, European and Asian *S. cavipes* populations are closer to each other than to the North American ones.

S. americanus appears to have a closer relationship with Chinese *S. sibiricus* than with U.S. *S. sibiricus*. However, the relationships among species in this clade are not well resolved, which mirrors the situation when

taxonomists try to circumscribe these taxa based on morphology. The genetic divergences within the circumboreal species *S. cavipes* are somewhere between that seen between the disjunct populations of species in the *S. americanus*–*S. sibiricus* and the *S. decipiens*–*S. spraguei* clades. Variations in pileus color among allopatric populations of *S. cavipes* are evident, but all *S. cavipes* share at least one distinctive morphological character, hollow stipe.

The species differentiation used in all previous studies were based mostly on morphological characteristics. Due to the abundance of published descriptions of these species, only key characters are summarized, along with their mycorrhizal associations, in Table 2 and Table 3. Morphological characters alone are often found to be confusing in separating species and subspecies of macrofungi, especially when those characters supposed to be diagnostic are variable. This is particularly true for *Suillus* species because many of them have similar micromorphological characters. Phylogenetic inference derived from DNA sequences often lead to further scrutiny of morphological characters. We have not yet found any consistent morphological characters that can be used to distinguish Chinese and U.S. *S. spraguei*. Thus, divergence following geographic separation from a common ancestor by *S. decipiens* and by Asian *S. spraguei* took different tracks, with *S. decipiens* changing in morphology, ITS sequence, and subgenus of host, while the changes in Asian *S. spraguei* were restricted to sequence divergence.

Evolution of Ectomycorrhizal Associations

Recent studies on the phylogeny of *Pinus* have made it possible to access phylogenetic groups for the *Pinus* species associated with our ingroup *Suillus* species

TABLE 3
Morphological Characteristics and Hosts of *Suillus decipiens* and *S. spraguei*

Name: (Area): No. of collections:	<i>S. decipiens</i> (SE NAM) 2	<i>S. spraguei</i> (E NAM) 8	<i>S. spraguei</i> (China-JL) 3	<i>S. spraguei</i> (China-YN) 4
Pileus size:	4–7 cm diameter	3–11 cm diameter	4–11 cm diameter	4–11 cm diameter
Color of pileus:	Cinnamon buff	Red	Red	Red
Context color change	Brownish yellow after cutting	Reddish after cutting	Reddish after cutting	Reddish after cutting
Stipe size:	4–7 × 1–1.5 cm	4–12 × 1–2.5 cm	4–11 × 1–2 cm	3–11 × 1–2 cm
Basidiospores:				
Range:	8.4–12 × 3.6–4.2 μm	7.1–11.6 × 2.8–4.3 μm	8.5–10.8 × 2.8–4.0 μm	8.3–10.8 × 2.9–4.1 μm
Mean L; W; Q:	10.1; 3.9; 2.8	9.3; 3.5; 2.6	9.5; 3.4; 2.8	9.4; 3.3; 2.8
Host:	<i>Pinus palustris</i> , <i>P. taeda</i>	<i>P. strobus</i>	<i>P. koraiensis</i>	<i>P. armandii</i>

Note. JL, Jilin Province; YN, Yunnan Province; L, length; W, width; Q, length/width.

(Liston *et al.*, 1999; Krupkin *et al.*, 1996; Price *et al.*, 1998; Wang and Szmidt, 1993; Wang *et al.*, 1999). Phylogenies based on both chloroplast and ribosomal DNA data support two discrete lineages in *Pinus*, i.e., subgenus *Pinus* and subgenus *Strobus*. Using *rbcl* sequence data generated mostly by Wang *et al.* (1999), our analysis shows that pine species that are associated with disjunct *Suillus* species (i.e., *S. spraguei*, *S. americanus*, and *S. sibiricus*) are part of a monophyletic group with *P. strobus* being basal to the Asian species (Fig. 3).

In eastern North America, *S. spraguei* and *S. americanus* appear to be associated only with *P. strobus*, an ancestral lineage within the subgenus *Strobus*, while their Chinese disjunct counterparts appear restricted to *P. koraiensis* in the northeast and *P. armandii* in the south, two more recently derived *Pinus* lineages within the subgenus *Strobus* (Fig. 3). The pattern of associations of *S. spraguei*, *S. americanus*, and *S. sibiricus* with the subgenus *Strobus* supports a certain degree of cospeciation and vertical transfer occurring in ectomycorrhizal associations. Pines, on the other hand, do not show high levels of specificity with mycorrhizal fungi, and a pine species is usually associated with an assemblage of macrofungal species.

The symbiosis between fungi and pines has a long evolutionary history. The *Pinus* fossil record indicates that ancestral species of subgenus *Pinus* appeared in the Early Cretaceous (ca. 125–130 Ma), and that subgenus *Strobus* was well established by the Paleocene (ca. 60 Ma) (Axelrod, 1986). A hypothesis based on analyses of rDNA sequence data suggested that ectomycorrhizal fungi also occurred during the Early Cretaceous (Berbee and Taylor, 1993). Recently, LePage *et al.* (1997) estimated that a well-formed fossil ectomycorrhiza that might be the association of *Pinus* and *Suillus* may date from the Middle Eocene (ca. 50 Ma). If this estimate is correct, the symbiotic relationship between species of *Suillus* and *Pinus* was established at the latest when direct migration from North Amer-

ica to Asia was possible via the Bering connection. For ectomycorrhizal fungi, comigrating with plants to new areas through short-distance spore dispersal and symbiotic connections may be an important means for expanding into new areas as mycorrhizal symbiosis is required for both plants and fungi to adapt to new and/or harsh environments (Malloch *et al.*, 1980; Pirozynski, 1980).

Phylogenetic studies on *Suillus* suggest that species of *Suillus* have shifted host species several times during its evolution (this study; Fischer *et al.*, 1997; Kretzer *et al.*, 1996). For example, *S. decipiens*, which is associated with subgenus *Pinus*, is nested within a clade of species all restricted to subgenus *Strobus* (Fig. 3). This is not surprising as strict host specificity would be disadvantageous for survival in changing environments and for migrating to new areas.

Eastern Asian and Eastern North American Disjuncts

This study demonstrates that a close phylogenetic relationship exists between eastern Asian and eastern North American disjunct population/species of *Suillus*. It also suggests that studies based only on morphological characters provide an incomplete and oversimplified picture of distribution patterns and associated historical events. Similar results were seen in Chinese ginseng (*Panax ginseng*) and American ginseng (*Panax quinquefolius*) (Wen and Zimmer, 1996) as well as that of morphological pairs in *Campsis* (Wen and Jansen, 1995). Different attributes of fungi, e.g., morphology, sexual compatibility, molecular composition, and ecology, may have different responses to natural selection and environmental pressures and, thus, are not always well coordinated (Petersen and Hughes, 1999). Within the *Suillus* species included in this study, ITS sequence data imply two phylogenetic lineages connecting eastern Asia and eastern North America. This close relationship is congruent with patterns displayed in recently studied flowering plant genera such as *Aralia*

(Wen *et al.*, 1998), *Nyssa* (Wen and Stuessy, 1993), and *Panax* (Wen and Zimmer, 1996).

The observed low level of genetic divergence between the disjunct *Suillus* species does not contradict the hypothesis of a relatively recent separation of formerly broadly distributed taxa through vicariance-promoted extinctions. A lack of fossil records for these fungi makes testing this hypothesis difficult. Circumstantial evidence for a recent isolation explanation was provided by Axelrod (1986) as he proposed that species in *Pinus* subgenus *Strobus* were once widely distributed and that the current discontinuous distribution patterns are the results of extinction due to pleistocene glaciation, mountain rising, and environmental changes. The symbiosis of *Suillus* and *Pinus* and the observed host specificity suggest that factors similar to those affecting the distribution of pines also influenced the current distribution of *Suillus* species.

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