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Evolution

Ancient and recent patterns of geographic speciation in the oyster mushroom *Pleurotus* revealed by phylogenetic analysis of ribosomal DNA sequences

(biogeography/species concept/vicariance)

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ABSTRACT **Evidence from molecular systematic studies suggests that many mushroom species may be quite ancient. Gene phylogenies were developed to examine the relationship between reproductive isolation, genetic divergence, and biogeography in oyster mushrooms (*Pleurotus*). Sequence data were obtained for two regions of DNA from populations belonging to eight intersterility groups (biological species). Phylogenetic analysis of sequences from the 5′ portion of the nuclear encoded large subunit rDNA demonstrates an ancient origin for four intersterility groups of broad geographic distribution (worldwide), with a more recent radiation of several intersterility groups that are restricted to the Northern Hemisphere. An expanded analysis using sequence data from the more variable rDNA internal transcribed spacer region also reveals a phylogenetically based pattern of genetic divergence associated with allopatric speciation among populations from different continents in the Northern Hemisphere. The ability of rDNA sequences to resolve phylogenetic relationships among geographically isolated populations within intersterility groups illustrates the importance of biogeography for understanding speciation in *Pleurotus*. Patterns of geographic distribution among intersterility groups suggest that several species lineages evolved quite early, with recently evolved groups restricted to the Northern Hemisphere and older lineages occurring throughout the world. Based on phylogenetic evidence, analysis of historical biogeography using area cladograms shows that multiple dispersal and vicariance events are responsible for patterns of speciation observed.**

Speciation in many mushroom groups is often associated with tremendous levels of genetic divergence that suggest an ancient origin for some species (1). Within many mushroom species, clear patterns of morphological and genetic divergence among geographically distinct populations also suggest an allopatric mode for speciation (2). The combined study of phylogeny and biogeography provides a framework for understanding the relationship among different components of evolution at the species level, including geographic variation, genetic isolation mechanisms, and morphological evolution (3). The recent development of rigorous molecular phylogenetic approaches has made it possible to reexamine many classic questions regarding the importance of biogeography as a primary factor involved in speciation.

The oyster mushroom *Pleurotus ostreatus* and its related species are among the more conspicuous fungi causing wood decay in terrestrial ecosystems worldwide and are widely collected and cultivated as edible fungi. Mating compatibility studies have demonstrated the existence of discrete intersterility groups (biological species) in *Pleurotus*, many of which are broadly distributed over one or more continents (4). Saprobic basidiomycetes such as *Pleurotus* present an excellent system for analysis of speciation in fungi. Many species may be grown and fruited in pure culture, permitting analysis of their mating relationships and other features. Phylogenetic analyses using molecular sequence data particularly show much promise for resolving phylogenetic relationships and understanding speciation for many problematic species complexes in basidiomycetes (4–6).

In this study we analyzed (*i*) mating compatibility relationships among collections from different parts of the world that reveal at least eight intersterility groups in *Pleurotus* and (*ii*) gene phylogenies for two different regions of the nuclear rDNA locus representing 38 individuals.* Molecular phylogenetic analysis reveals two groups of species in *Pleurotus*, with one ancient group of broad worldwide distribution and a second group of species evolving much more recently within the Northern Hemisphere. These results demonstrate the utility of rDNA phylogenies for understanding patterns of evolution and speciation in basidiomycete fungi within a biogeographical context.

MATERIALS AND METHODS

Cultures and Mating Compatibility Studies. The fungal strains used in this study are listed in Table 1 along with other data on their geographic origin and intersterility group. All strains were grown and stored on YPSS/2 agar (14). Tester strains for determining mating compatibility relationships representing five intersterility groups were available from previous studies (4, 15). Spore prints were obtained from additional strains by fruiting them on sterilized rye grain substrate in the laboratory. Patterns of mating compatibility among single-spore isolates originating from the same parent (intrastock crosses) were used to determine mating system. Interstock pairings among single-basidiospore isolates from different parent strains were used to determine the limits of additional intersterility groups. Formation of clamp connections in positive matings was used as the primary criterion for mating compatibility (15).

DNA Amplification and Sequencing. Cultures of each isolate were grown in liquid YPSS/2 medium, and DNA minipreparations were prepared using CTAB extraction buffer described by Zolan and Pukkila (16). PCR amplification was performed using reagents and primers described in Vilgalys and Hester (17) and White *et al.* (19). Primers 5.8SR and LR7 were used to amplify a 1.7- to 1.8-kb fragment of rDNA including the internal transcribed spacer 2 (ITS-2) region and the 5′ half of the large subunit (LSU) RNA coding region, and

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Abbreviations: indel, insertion/deletion; ITS, internal transcribed spacer; LSU, large subunit; CI, consistency index; RI, retention index.

*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U04058–U04160).

Table 1. *Pleurotus* strains studied, with information concerning their intersterility groups, geographic origin, and other data

Intersterility group	Strain,* geographic origin, other cultures†	Comments
I	261, Virginia; 403, Arizona; 850, California; 330, Czechoslovakia; 331, Czechoslovakia; 334, Germany; 1743, Japan (= TMI 30054); 1742, Japan (= TMI 30055)	Includes *P. ostreatus* (with several varieties), presently known only from North America and northern Eurasia. Deciduous hardwood logs are the preferred substrates for this group.
II	263, Virginia; 352, North Carolina; 700, British Columbia; 479, Germany; 480, Germany; 481, Sweden; 1745, Japan (= TMI 30632); 1748, Japan (= TMI 30058)	Known in Europe as *Pleurotus pulmonarius*, widely distributed across Eurasia and North America. Depending on where they are found, group II populations occur on coniferous or deciduous logs.
III	399, Maryland; 765, Montana	Group III (*Pleurotus populinus*) is known only from North America, where it is mainly restricted to aspen species (*Populus* spp.), on which it is sometimes a weak pathogen (7).
IV	383, England; 1166, Germany (= ATCC 38547); 1030, Czechoslovakia (= ATCC 42045); 1738, Czechoslovakia (= TMI 30141); 1736, Czechoslovakia (= TMI 30142)	Collected in Europe as *Pleurotus cornucopiae*, but also as a morphologically distinct variant *Pleurotus citrinopileatus* in eastern Asia. Found on a variety of deciduous hardwood substrates.
V	1847, Mexico; V, 1830, Malaysia (= ATCC 38137); V, 1829, Malaysia (= ATCC 38138)	Pantropical, occurring in warm regions all over the world. Numerous species have been described in this group based on morphology, including *Pleurotus djamor, Pleurotus flabellatus, Pleurotus salmoneostramineus*, and *Pleurotus salmonicolor* (8, 9). Angiosperm wood is the preferred substrate.
VI	625, Europe (= VT 1477, Penn State MW 85); 643, Europe (= VT 458, CBS 130.21); 1832, Czechoslovakia (= ATCC 36047); 1821, India (= ATCC 52666); 1822, India (= ATCC 62885)	Represents two morphologically unique species, *Pleurotus eryngii* (with several varieties) from Europe and the Middle East and *Pleurotus fossulatus* from Afghanistan. The former species is restricted to the root stocks of shrubby *Eryngium* species on which it is known to be a weak parasite. Substrate preference of *P. fossulatus* is not known.
VII	418, Louisiana; 420, Indiana; 478, Mexico	Occurs throughout the world on angiosperm wood where it is most commonly identified as *Pleurotus cystidiosus* (10, 11). Geographic variants of this group are known from Taiwan as *Pleurotus abalonus* (12) and in Mexico as *Pleurotus smithii* (13).
VIII	691, Pennsylvania (= Penn State MW84); 764, North Carolina	Growing on angiosperms in Northern and Southern Hemispheres (North America, Europe, and New Zealand), referable to the morphological species *Pleurotus dryinus*.

*Duke Culture Collection.

†Other culture collections; ATCC, American Type Culture Collection, Rockville, MD; TMI, Tottori Mycological Institute, Tottori, Japan; CBS, Centraalbureau von Schimmelcultures, Baarn, Netherlands; VT, Virginia Tech Mycological Culture Collection, Blacksburg, VA; Penn State Fungal Culture Collection, State College, PA.

the amplified DNA was purified using Magic Prep columns (Promega). DNA sequencing was performed using a cycle sequencing reagent kit (GIBCO) with [γ-^{33}P]ATP (NEN). Approximately 980 bases were sequenced for 28 strains from the 5′ portion of the LSU rDNA using primers LR0R, LR5, LR16, and LR21. The ITS-2 region (341 bases) was sequenced from the same templates using primers LR15, LR1, and 5.8SR. The ITS-1 region (250 bases) was separately amplified and sequenced using primers ITS1 and ITS4.

Molecular Evolution and Phylogenetic Analysis. Sequences were aligned using CLUSTALV (20) and later adjusted by eye using the SeqApp editor (21) to produce separate LSU RNA and ITS data sets (copies of the original data sets used for all analyses are available from R.V. by mail or via e-mail at fungi@acpub.duke.edu). Levels of molecular sequence divergence in each data set were compared by calculating pairwise estimates of nucleotide substitution rates by the two-parameter method of Kimura (22) using PHYLIP (23). Relative rate differences between the two data sets were compared by linear regression of paired distance values from each distance matrix. Phylogenetic relationships were estimated for each data set using PAUP (24). Maximum parsimony trees were identified using the heuristic search option in PAUP using only informative characters with at least 30 random input orders of sequences. Support for phylogenetic groupings was assessed by bootstrap analysis using 200 replicate data sets (sampled from informative characters) with random addition of sequences during each heuristic search. Evolutionary patterns of mating behavior and biogeography were examined using MACCLADE (25).

RESULTS

Mating Compatibility and Intersterility Groups. As in previous studies with other *Pleurotus* species, mating reactions among single-basidiospore isolates from individual parental strains were consistent with a bifactorial mating incompatibility system in every case (26). Patterns of mating compatibility among interstock pairings grouped the strains from this study into eight intersterility groups (biological species). Tester strains for each group have been deposited with the American Type Culture Collection.

Molecular Evolution and Phylogeny. Sequences for 27 strains from the 5′-most portion of the LSU rDNA were easily alignable. Within the LSU data set, several small regions of uncertain alignment due to short insertions/deletions (indels) were excluded from subsequent analyses. By contrast, alignment of all sequences was more problematic for the ITS rDNA region. Indels and other regions of uncertain nucleotide sequence homology were more prevalent within the ITS-1 and ITS-2 regions, such that it was not possible to align all of the most divergent taxa. Positions lacking indels for the ITS rDNA

region were used to calculate pairwise distance values among all the taxa for comparison with the LSU rDNA data set. Overall levels of nucleotide substitution were higher for the ITS data set (range = 0–0.41) than for the LSU sequences (range = 0–0.05). A linear regression of pairwise distances calculated for ITS versus LSU data sets showed a positive linear relationship ($P < 0.001$), with a slope of 7.65, which provides an estimate of the difference in substitution rates between the two regions (Fig. 1).

Maximum parsimony analysis of the LSU rDNA data with all eight intersterility groups yielded two most parsimonious trees of 94 steps each, each with a consistency index (CI) = 0.77 and retention index (RI) = 0.88 (Fig. 2; only one tree shown). Because of the lack of a satisfactory outgroup, the resulting trees were provisionally rooted along their longest internal branch by midpoint rooting. Both trees had identical topologies except for a reversal in branching order between intersterility groups VII and VIII. Bootstrap replication frequencies show most internal branches to be well supported by the LSU data, with significant support for branches leading to intersterility groups V, IV, VII, and VIII. Relationships among four remaining groups (I, II, III, and VI) were only partly resolved by LSU data, although parsimony analysis and bootstrap values strongly support their separate grouping (96%) from the remaining taxa. The relatively shallow branching of these four mating groups indicated by the phylogram in Fig. 1 suggests that they may be of recent origin. Levels of nucleotide substitution within this "northern hemisphere" group are much lower (<0.01) relative to that observed between the other mating groups, which are believed to be of more ancient origin.

Because of difficulties aligning ITS sequences across all mating groups, phylogenetic analysis of the ITS data was restricted to a larger sampling of strains with diverse geographic origin belonging within the northern hemisphere groups (I, II, III, VI) along with an outgroup (VII) for rooting purposes. Alignments for this set of sequences were not problematic and resulted in a data set with only several single-base indels, which were included in the phylogenetic analysis to provide maximum resolution of phylogenetic relationships. Maximum parsimony analysis of the ITS data yielded a single tree with a length of 179 steps, with a CI = 0.89 and RI = 0.94 (Fig. 3). Phylogenetic relationships among all 10 population samples were completely resolved using the ITS data, with high bootstrap values providing support for

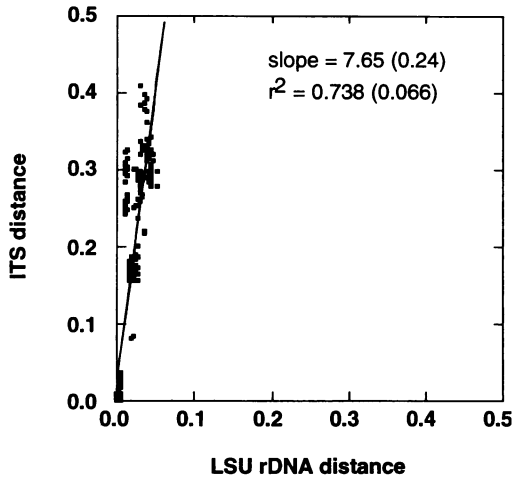

FIG. 1. Higher rates of substitution observed within ITS versus LSU rDNA sequence data from *Pleurotus* species. The plot shows linear regression of pairwise substitution estimates and standard deviations (in parentheses) for ITS and LSU data sets [estimates based on the two-parameter model of Kimura as implemented by PHYLIP (23)].

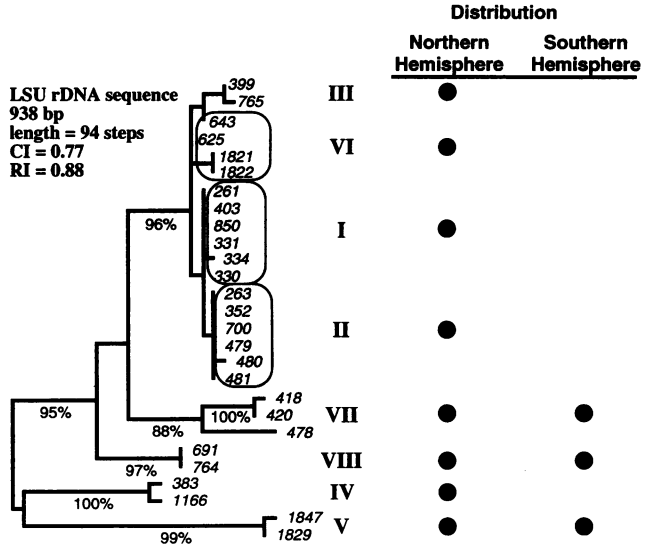

FIG. 2. Phylogenetic relationships among eight intersterility groups in *Pleurotus* based on LSU rDNA sequences. The unrooted tree shown here is based on maximum parsimony analysis using sequence data from the 5′ portion of the LSU RNA coding region from 28 individuals. Significant bootstrap replication frequencies (above 50%) are indicated, with data on intersterility groups (roman numerals) and their geographic distribution.

several internal branches as well as nearly all terminal clades. Two major divisions can be recognized from the ITS phylogram, with one branch giving rise to several geographic populations of intersterility group II and another well-supported lineage giving rise to groups I, VI, and III. Within this second lineage, intersterility groups VI and III are inferred to have evolved from within intersterility group I.

DISCUSSION

Intersterility Groups. Many studies have employed mating criteria to delimit intersterility groups in *Pleurotus* (4, 7–10, 15, 27–30). Together with other recent studies that used common reference strains with ours, this study has been able to identify at least eight intersterility groups of *Pleurotus* (Table 1). Several intersterility groups are represented by populations from several different continents, and additional groups remain to be identified. By employing a standard

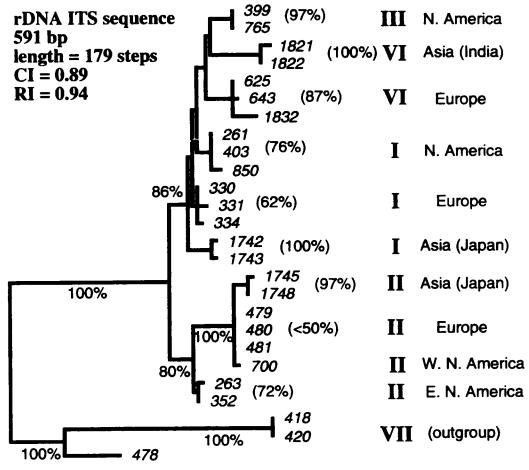

FIG. 3. Maximum parsimony tree for 26 ITS sequences representing 10 populations belonging to four intersterility groups (I, II, III, VI) in *Pleurotus*. The tree is rooted using three sequences from intersterility group VII. Bootstrap replication frequencies and geographic origin are also indicated.

series of tester strains for each group, mating tests provide a convenient method for detecting new groups. Since completing the molecular portion of this study at least three additional intersterility groups have subsequently been identified (unpublished results).

Because of their ephemeral fruiting patterns, the ranges and distributions of most mushroom species are poorly known. Based on what is currently known about their distributions, different intersterility groups in *Pleurotus* may be grouped according to whether they are primarily distributed in the Northern Hemisphere or more broadly across both hemispheres (Fig. 2). A summary of our current knowledge about the distribution of these groups is presented in Table 1.

Biological and Phylogenetic Species. Mating criteria are widely employed by basidiomycete systematists (27), who regularly employ biological species concepts for species circumscription (2). One important observation from mating tests in basidiomycetes is that intersterility barriers between most species are prezygotic and nearly always absolute. Although few examples are known of partial intersterility or hybridization in basidiomycetes, genetic analyses of infrequent crosses between reproductively isolated groups in the *Heterobasidion annosum* complex have shown that intersterility may be controlled by as few as one or two loci (28, 29). Because mating criteria are so easily testable in saprobic fungi, mushrooms may represent the best possible example of a group of organisms where a biological species concept is broadly applicable. Biological species concepts have always been controversial, however, since species grouping based on mating may not always accurately reflect evolutionary relationships (7, 8, 18).

The availability of a completely resolved phylogeny at the population level provides a unique opportunity to examine the phylogenetic basis for patterns of mating behavior and vicariance in *Pleurotus*. A cladogram summarizing patterns of biogeography and mating relationships shows that most lineages resolved by molecular phylogenetic analysis (groups II, III, V, IV, VII, and VIII) are also congruent with species groupings based on a biological species concept (Figs. 2–4). Within the more recent northern hemisphere group, however, phylogenetic analysis shows two intersterility groups to be paraphyletic, with different geographic populations of intersterility group I giving rise to populations of group VI, which itself is paraphyletic with respect to mating group III (Figs. 3 and 4). As pointed out by Cracraft (8), many broadly distributed biological species are often found to be paraphyletic and thus may not always represent unique evolutionary units. This may also be the case in *Pleurotus* as well. However, resolution of these paraphyletic relationships is not strongly supported by either bootstrap analysis or parsimony (an alternative tree in which all intersterility groups are monophyletic is only three steps longer than that shown in Fig. 3). Based on the possibility that some intersterility groups may in fact be paraphyletic, we and others have proposed that phylogenetically based species concepts (in which monophyletic geographic populations could represent species groups) should be more desirable for developing species concepts in mushrooms and other fungi (2). The largely concordant groupings based on molecular phylogeny with groupings based on intersterility, however, nevertheless still also provide a strong phylogenetic basis for continued recognition of biological species as fundamental taxonomic units (9).

Biogeography and Speciation. Cladistic analysis of LSU and ITS sequences provides a completely resolved phylogeny for intersterility groups in *Pleurotus*, with robust support for most internal and terminal clades. Most significantly, the 7-fold higher rate of substitution in the ITS sequences than in the LSU sequences provided sufficient variation for resolving phylogenetic relationships among geographically isolated

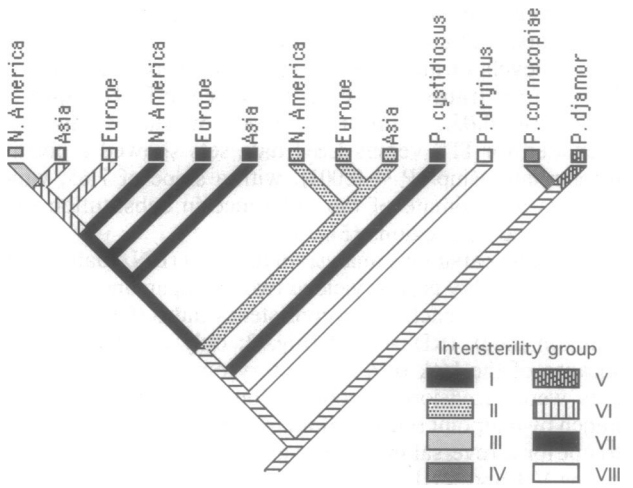

FIG. 4. Summary of phylogenetic relationships in *Pleurotus* showing patterns of mating behavior and geographic vicariance associated with speciation.

populations within several intersterility groups. This resolution enables phylogeographic analyses of vicariance and speciation. The high levels of bootstrap support for near-terminal branches leading to geographically defined populations within each intersterility group provide strong support for the view that speciation in these fungi is associated with geographic isolation. Numerous studies with other mushroom groups also demonstrate clear patterns of genetic divergence among geographically isolated populations that are still capable of crossing (1, 2, 10). Congruence between patterns of rDNA evolution with mating behavior and biogeography suggests that processes that might otherwise confound analysis of organismal phylogeny (e.g., paralogy, interspecific gene flow) are not likely to be a significant factor for analysis of speciation at the population levels sampled in this study.

The phylogenetic relationships among different populations also provide a framework for understanding the biogeographic history of speciation in *Pleurotus*. Geographic distribution patterns show the earlier evolving species groups (IV, V, VII, VIII) to be broadly distributed across Northern and Southern Hemispheres, while the more recently evolved groups (I, II, III, VI) appear to be largely restricted within the Northern Hemisphere. Two possible historical explanations are proposed to account for this distribution. One explanation, based on continental drift theory, is that the early species groups were already present before the breakup of the Pangean continent 200 million years ago, with subsequent "recent" speciation events occurring in the Northern Hemisphere with the breakup of Laurasia (100 million years ago). This scenario, however, predicts an extremely ancient origin for *Pleurotus* and other groups of basidiomycetes that would be predicted to share similar patterns of gross vicariance across the Northern and Southern Hemispheres. Under this hypothesis, origins of higher fungal taxa would probably need to be dated back to the Cambrian or even earlier. An alternative hypothesis that does not require such drastically ancient origins is based on intermittent but regular dispersal of species over broad distances to new areas. Under such a diffusion model, geographic distributions of species would be correlated with ages since each species diverged. Older species lineages would therefore be predicted to have broader distributions, while recent species groups would have more narrow distributions.

Phylogenetic evidence also provides a framework for examining patterns of speciation based on vicariance biogeography. Within the Northern Hemisphere, at least two and

possibly three separate North American–Eurasian vicariance events (along with dispersal) are required to account for the phylogenetic hypothesis presented in Fig. 4. Two contrasting biogeographic histories are necessary to explain phylogenetic history in intersterility groups I and II, which differ in their interpretation of relationships among European, North American, and Asian populations. The evolution of two biogeographically diverse intersterility groups from within group I also suggests evidence for several recent species radiations that have occurred within the Northern Hemisphere, possibly associated with Pleistocene glaciation.

Because the meager fossil record for fungi provides little data on the origin and dispersal of species, molecular phylogenetic studies may be the only means for reconstructing evolutionary history and for testing biogeographic hypotheses. The ability of rDNA sequence data to provide complete phylogenetic resolution at the population level provides a window through which mycologists might view the ancient and recent origins of fungal species within a biogeographic context.

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