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Recent Advances in Molecular Systematics of the Genus *Pleurotus*

Rytas Vilgalys, Jean-Marc Moncalvo, Shian-Ren Liou, and Miguel Volovsek
Department of Botany, Duke University, Durham, NC 27708 USA. e-mail: fungi@acpub.duke.edu

ABSTRACT: Mating compatibility studies and molecular phylogenetic analyses provide a useful framework for understanding species concepts and taxonomy of the oyster mushroom *Pleurotus*. At least fifteen intersterility groups have been identified, with each group associated with one or more morphological species. Phylogenetic analyses based on the ribosomal RNA large subunit coding region and its associated ITS spacer regions provide strong support for several taxonomic groupings in *Pleurotus*. The importance of careful strain identification based on mating tests and other criteria is stressed.

1 INTRODUCTION

Species of oyster mushrooms in the genus *Pleurotus* occur commonly as wood-decomposers in forests throughout the world. In many countries, edible *Pleurotus* species are collected and cultivated as choice edibles (Chang and Miles 1989, Chang and Miles 1991) and have been the subject of many taxonomic and genetic studies. *Pleurotus* has always been attractive to mushroom growers because of the ease by which most species can be cultivated. With over 20 recognized species and varieties cultivated throughout the world (Buchanan 1993), *Pleurotus* is also one of the most diverse groups of cultivated fungi. Evolutionary relationships of *Pleurotus* species are not well understood, and many taxonomic problems exist in this group. In this paper, we summarize some recent results of mating compatibility studies and molecular systematics aimed at clarifying species concepts and higher level taxonomic relationships in *Pleurotus*.

2 MATERIALS AND METHODS

2.1 *Mating compatibility studies*

Methods for assessing mating systems and incompatibility have been described (Vilgalys *et al.* 1993, Vilgalys and Sun 1994). Single-spore isolates were obtained from field collections or laboratory grown fruit bodies. Mating compatibility was assessed by pairing single-spore isolates from the same parent (intrastock crosses) to determine mating system. Interstock crosses were performed among single-basidiospore isolates from different parent strains to determine intersterility groups (biological species). Formation of clamp connections in positive matings was used as the primary criterion for mating compatibility (Eger 1978).

2.2 *DNA amplification, sequencing and phylogenetic analyses*

The following strains were included for molecular systematic studies: *P. ostreatus* (Group I), D261 (USA), D331 (Czech Republic); *P. pulmonarius* (Group II), D263 (USA), D479 (Germany); *P. sajor-caju* (Group II), D257 (commercial strain), D1824 (=ATCC 32772), D1825 (=ATCC 32771); *P. populinus* (Group III), D399, D765 (both USA); *P. cornucopiae* (Group IV), D383 (England), D1166 (Germany); *P. djamor* complex (Group V), D1847 (Mexico), D1829 (Malaysia), RV95/920 (Papua New Guinea); *P. calyptratus* (Group V), D1839 (Czech Republic); *P. eryngii* complex (Group VI), D643 (cultivated strain), D1821 (as *P. fossulatus*, Afghanistan); *P. cystidiosus* complex (Group VII), D418 (USA), D420 (USA), D478 (Mexico); *P. levis* (Group VIII, previously as *P. dryinus* in Vilgalys and Sun 1994), D691, D764 (both USA); *P. tuberregium* (Group X) D2199, RV95/486 (both Papua New Guinea); *P. "agaves"* complex (Group XI), D2321 (Mexico); P. "brazil" (Group XIII), D327 (Brazil); *P. australis* (Group XIV), D2332, RV95/568 (both New Zealand); *P. purpureoolivaceus* (Group XV), D2342, RV95/486 (both New Zealand); *Hohenbeuhelia* ca. *niger*, D596 (USA); *Resupinatus* ca. *silvanus*, D2000 (USA). All of the strains and other collection data are deposited in the Duke Mycology Laboratory Culture Collection.

Procedures for DNA extraction, PCR amplification, and manual sequencing were described previously (Vilgalys and Sun 1994). Several sequences were also obtained using an ABI 373 automated sequencer with dye-primer chemistries (Perkin-Elmer Corporation, Foster City, CA). DNA sequences were obtained for two portions of the nuclear-encoded ribosomal DNA corresponding to about 900 bases from the large-subunit

(LSU) RNA coding region, and its corresponding internal transcribed spacer (ITS) regions (total approximately 650 bases including the entire 5.8S RNA coding region). Sequences were aligned and their phylogenetic relationships estimated using PAUP*(Swofford, in preparation) as described in Vilgalys and Sun (1994). The sequence data matrices used for this paper are available by e-mail from the senior author.

3 RESULTS AND DISCUSSION

3.1 *Intersterility groups (biological species) in Pleurotus*

Mating studies have been used for over 25 years to delineate species in *Pleurotus* (Anderson *et al.* 1973, Hilber 1982). Today at least 15 intersterility groups are recognized in *Pleurotus* (Table 1). All fifteen groups have a tetrapolar mating system governed by multiple alleles similar to that described for *P. ostreatus* (Eger 1978).

Table 1. Intersterility groups in *Pleurotus*. List of currently known intersterility groups (biological species) in *Pleurotus* along with data on their distributions.

Group[1]	Species name[2]	North America	Europe	Asia	South America	Africa	Austral-asia
I	*P. ostreatus*	•	•	•			
II	*P. pulmonarius*	•	•	•			•
III	*P. populinus*	•					
IV	*P. cornucopiae*		•	•			
V	*P. djamor*	•	•	•	•	•	•
VI	*P. eryngii*		•	•			
VII	*P. cystidiosus*	•	•	•		•	•
VIII	*P. levis*	•					
IX	*P. dryinus*	•	•				
X	*P. tuberregium*			•		•	•
XI	*P. "agaves"*	•					
XII	*P. "abieticola"*			•			
XIII	*P. "brazil"*				•		
XIV	*P. australis*						•
XV	*P. purpureo-olivaceus*					•	

[1]Intersterility groups defined on the basis of mating compatibility tests; [2]Species names given here are those most commonly associated with each group. Depending on geographic region, other taxon names may sometimes also be applicable. Distribution data are based on both published and published reports, and should therefore be considered to be incomplete or in need of further verification.

Genetic isolation due to intersterility is clearly one of the primary factors responsible for variation among species. As a result, mating compatibility studies have been very useful for establishing the limits of interbreeding populations and determining reliable morphological characters for species delimitation, especially within taxonomically difficult species complexes (Boidin 1986). Some well-known examples of species complexes include the *Armillaria mellea* complex (Anderson and Ullrich, 1979, Korhonen 1978) and *Collybia dryophila* group (Vilgalys and Miller 1983, Vilgalys and Miller 1987). Evidence from these and other studies suggests that most species of agarics including *Pleurotus* are rigorously intersterile and that natural hybridization is rare or absent, supporting the use of a biological species concept for recognizing species (Boidin 1986, Petersen 1995a, Petersen 1995b). However, exceptions to rigorous genetic isolation are known, and at least one collection of *P. pulmonarius* originating from New Zealand ("NZP", mating group II) is known to be at least partially mating-compatible with representatives of several other mating groups (Petersen and Ridley 1996).

Most intersterility groups are associated with at least one or more distinct morphological taxa. The definition of intersterility groups has clearly benefited *Pleurotus* systematics by providing insight about reliable taxonomic characters (Bresinsky *et al.* 1987, Hilber 1982, 1990). However, several intersterility groups are also characterized by complex patterns of morphological variation associated with geographic provenance. Examples of striking morphological variation associated with geographic distribution have been reported in *Pleurotus* , between North American and European populations of *P. ostreatus* (mating group I) (Vilgalys *et al.* 1993), eastern and western North American populations of *P. pulmonarius* (group II) (Vilgalys *et al.* 1993), European and Asian varieties of *P. cornucopiae* (group IV) (Ohira 1990), the *P. cystidiosus* complex (group VII), and a large number of variants in the *P. djamor* complex (group V) (Corner 1981, Petersen 1995a). Because geographic barriers may effectively prevent contact between widely separated populations, these biogeographic patterns of variation within genetically related mating groups strongly suggest that geography plays a major role in the evolution of *Pleurotus*.

3.2 *Molecular phylogenetic studies*

Considerable genetic diversity has been detected in *Pleurotus* using molecular and biochemical markers. Several studies of isozymes have demonstrated genetic variation consistent with known species boundaries (Kulkarni *et al.* 1987, May and Royse 1988, Zervakis and Labarere 1992).

More recent investigations have also demonstrated variation among species using restriction analysis of ribosomal DNA (Iracabal *et al.* 1995), mitochondrial DNA (Toyomasu *et al.* 1992), and total genomic DNA (Sagawa *et al.* 1992). Although the taxonomy of individual strains could not always be determined with certainty in these studies, all of them clearly demonstrate the utility of genetic markers for species delimitation.

Some of the most compelling evidence demonstrating genetic differentiation among intersterility groups comes from the application of phylogenetic approaches using sequence data. Molecular phylogenetic analysis of eight intersterility groups based on ribosomal RNA sequences divided the genus *Pleurotus* into several major groups that correspond with both mating behavior and geographic provenance (Vilgalys and Sun 1994). Building on that study, Moncalvo (1995) used sequence data to demonstrate a close phylogenetic relationship for a newly described taxon (*P. cystidiosus* var. *formosensis*) with other members of the *P. cystidiosus* complex belonging to intersterility group VII (Table 1). Subsequent mating compatibility studies with this strain have confirmed that it is indeed compatible with other group VII strains (Vilgalys, personal observation).

Neda and Nakai (1995) performed phylogenetic analyses using 18S RNA and ITS sequences from several *Pleurotus* spp. and obtained similar results to ours, confirming the utility of molecular systematic approaches. All of these initial studies focused largely on taxa from the northern hemisphere. A great deal of genetic variation still waits to be sampled from the southern hemisphere, especially the Australian, African and South American continents with their relatively older biota and unknown numbers of endemic taxa. For this study, we therefore expanded our analyses (Vilgalys and Sun 1994) to include members of several newly characterized intersterility groups from Australasia (*P. purpureoolivaceus, P. australis, P. tuberregium*), Mexico (*P.* "agaves"), and South America (*P.* "brazil").

3.3 *Molecular phylogeny of Pleurotus based on large-subunit RNA sequences*

Representative strains of 14 intersterility groups from Table 1 were chosen for phylogenetic analysis using a portion of the nuclear-encoded large subunit (LSU) RNA gene, which has been found to be useful for assessing relationships at the genus level in other agarics (Hibbett 1992). Two strains (one each) representing the pleurotoid agaric genera *Hohenbeuhelia* and *Resupinatus* (Tricholomataceae) were selected to use as outgroups for parsimony analysis. Although current taxonomic systems classify

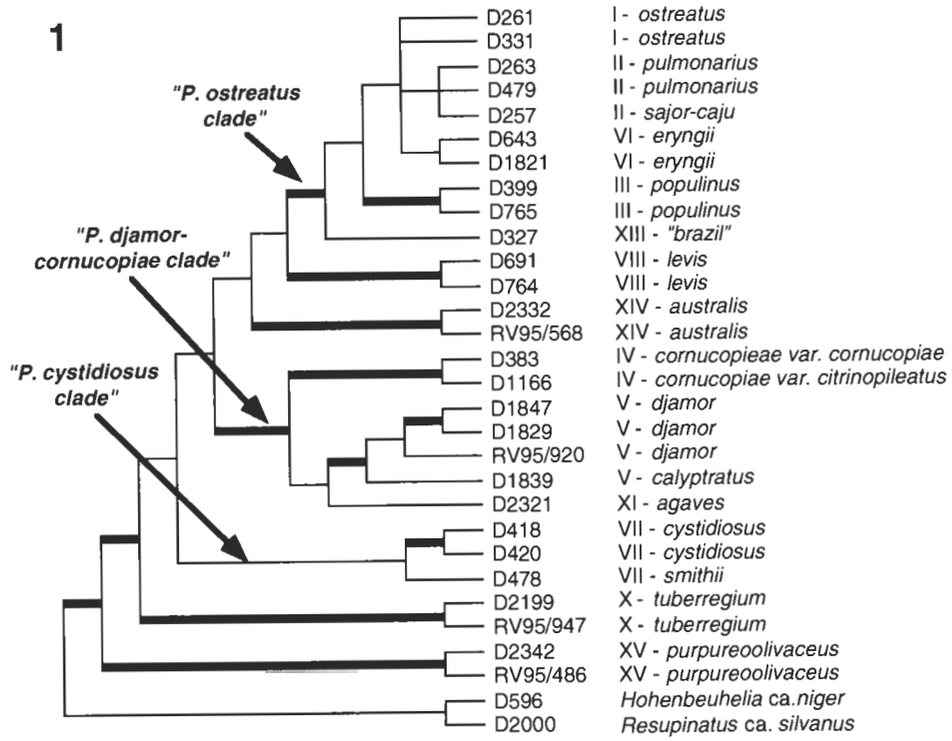
Pleurotus in the Polyporaceae (Singer 1986), more recent molecular studies support previous taxonomic systems that place it more closely with other agarics in the Tricholomataceae (Hibbett and Vilgalys 1993).

Phylogenetic analyses of LSU sequences yielded a well supported estimate of phylogenetic relationships in *Pleurotus* with excellent correspondence between trees based on rDNA and intersterility groups (Fig. 1). Sequences belonging within the same intersterility group were often placed into a single clade with a high level of support (indicated by bootstrap frequencies above 65%). Sequences from several strains nominally classified as different "species" but belonging to the same intersterility group were also usually placed into the same clade, including two varieties of *P. cornucopieae* (both Group IV), *P.* "*sajor-caju*" with other strains of *P. pulmonarius* (all Group II), *P. calyptratus* with other members of the *P. djamor* group (Group V), and *P. smithii* with other strains of *P. cystidiosus*. (all group VII). Above the species level, several major lineages comprising more than one intersterility group are also well supported, including a "*P. ostreatus* clade" (groups I, II, III, and VI) and a "*P. djamor-cornucopiae*" clade (groups IV, V, and XI).

3.4 *Phylogeny of the P. ostreatus clade based on ITS sequences*

The ITS regions of the rDNA locus evolve much faster than the LSU region (Vilgalys and Sun 1994). Although ITS regions are too divergent to obtain unambiguous alignment across all intersterility groups in *Pleurotus*, they are ideally suited for examining closely related groups such as the "*P. ostreatus-clade*" that are not well resolved by LSU data alone. A phylogenetic analysis using ITS sequences using *P. levis* (group VIII) as an outgroup provides evidence for at least two well-supported clades within the *P. ostreatus* clade (Fig. 2). One clade represents group II strains classified as *P. pulmonarius*, including two single-spore isolates of "*P. sajor-caju*" which also cross with other group II strains (Vilgalys, pers. obs.). The second clade includes representatives of several intersterility groups, including *P. ostreatus* (group I), *P. populinus* (group III), a newly discovered group from South America (P. "brazil", group XIII), and the *P. eryngii-fossulatus* complex (group VI). These phylogenetic relationships are also concordant with a previous analysis of ITS sequences using a larger geographic sampling of isolates for this group.

Several features are worth noting about the *P. ostreatus* clade. This group includes the type species of the genus (*P. ostreatus*) and thus could be recognized as a primary taxonomic subdivision in *Pleurotus*, either at the subgenus or sectional level. All of the members of this group are also



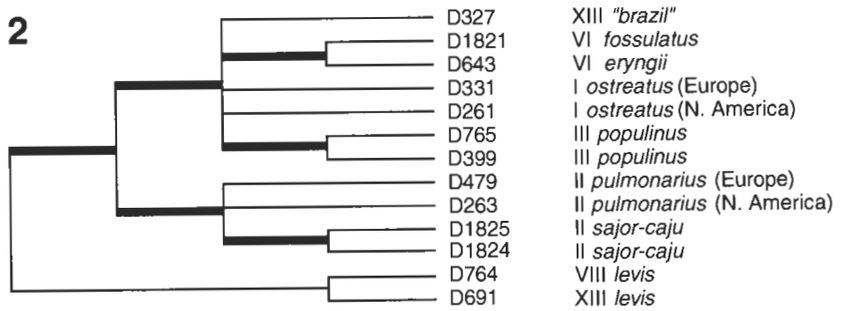


Figure 1. Phylogenetic relationships of 30 large-subunit ribosomal DNA sequences from different *Pleurotus* intersterility groups. A strict consensus tree is shown for 8 most-parsimonious trees (202 steps each) detected using the branch-and-bound algorithm of PAUP* (CI=.609, RI=.789). These analyses were based on a total of 942 aligned positions from the large-subunit RNA gene, with 104 characters being phylogenetically informative. Bold lines indicate groups supported by greater than 70% bootstrap frequencies (bootstrapping values based on 500 replicates).

Figure 2. Phylogenetic relationships of ITS sequences from representative strains belonging to the *Pleurotus ostreatus* complex. A strict consensus tree is shown for 6 most parsimonious trees (109 steps each) detected using the branch-and-bound algorithm of PAUP* (CI = .963, RI = .971). Total aligned lengths of ITS-1, 5.8S RNA, and ITS-2 sequences for these analyses were 720 bases with 94 bases being phylogenetically informative. Bold lines indicate groups supported by greater than 65% bootstrap frequencies (bootstrapping values based on 500 replicates).

monomitic, and are primarily distributed in the northern hemisphere. Phylogenetic analyses also show that at least one other intersterility group not included in this study (*P. abieticola*, group XII) also belongs in the *P. ostreatus* clade (Vilgalys, unpublished data). The *P. ostreatus* clade appears to have evolved relatively recently (Vilgalys and Sun 1994), possibly since the Pleistocene, which would explain why some sequences from the same intersterility group might not always nest as a monophyletic group (Fig. 2). A recent radiation of the *P. ostreatus* clade could also provide an explanation for the recent discovery of a group II strain from New Zealand (NZP) that is partially or fully compatible with other mating groups in the *P. ostreatus* clade, but intersterile with other groups (Petersen and Ridley 1996).

4 SOME CONCLUSIONS AND RECOMMENDATIONS

Combination of mating compatibility studies with morphological studies and molecular phylogenetic analyses is providing a framework for understanding patterns of speciation and systematics in *Pleurotus*. In most cases, phylogenetic studies have supported the recognition of intersterility groups as units of evolution (Vilgalys and Sun 1994). However, exceptions to rigorous intersterility between species in *Pleurotus* are also known to occur; instead of negating the utility of mating evidence for understanding species concepts (Petersen and Ridley 1996), evidence of partial compatibility and bridging isolates instead provide a valuable window for examining the evolutionary dynamics of reproductive isolation and speciation. Thus overall, intersterility barriers largely correspond with the development of independent evolutionary units, and provide a rational basis for recognizing taxonomic groups.

Because mating compatibility studies provide the most meaningful system for classification in *Pleurotus* (Table 1), we propose that mating criteria be universally adopted by oyster mushroom scientists for identification of cultivated and research strains. New intersterility groups should be identified after they have been cross tested against all of the other mating groups. Because numbering of intersterility groups has no formal taxonomic status, such a convention could serve as a useful approach for identifying mating groups until their taxonomic nomenclature becomes sorted out. This approach has been very beneficial for stabilizing taxonomy in other problematic groups of fungi, including the *Armillaria mellea* complex (Anderson and Ullrich 1979) and *Rhizoctonia solani* (Vilgalys and Cubeta 1994). Standardized numbering of intersterility groups would reduce confusion concerning the identity of groups from independent studies, and could eventually serve as one basis for stabilizing taxonomy. Useful guidelines for establishment of tester strains have been proposed (Petersen 1995a).

Frequent name changes are inevitable as new information about species comes to light (Buchanan, 1993), and researchers should always consult appropriate taxonomic authorities concerning the identity of their strains. It is especially important that collectors and growers make note of the geographic origin of wild-collected material used in cultivation as well as any other data that might be useful for identification. Because so much research on cultivated strains lacking a clear pedigree, it is important to determine the intersterility groupings of all strains used instead of relying on mistaken or invalid taxonomic determinations.

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