

Molecular phylogeny, biogeography and speciation of the mushroom species *Pleurotus cystidiosus* and allied taxa

Georgios I. Zervakis,¹ Jean-Marc Moncalvo² and Rytas Vilgalys³

Correspondence

Georgios I. Zervakis

zervakis@kal.forthnet.gr

¹National Agricultural Research Foundation, Institute of Kalamata, Lakonikis 87, 24100 Kalamata, Greece

²Centre for Biodiversity and Conservation Biology, Royal Ontario Museum and Department of Botany, University of Toronto, 100 Queen's Park, Toronto, Ontario, Canada M5S 2C6

³Department of Biology, Duke University, Durham, North Carolina, NC 27708, USA

Members of the mushroom genus *Pleurotus* form a heterogeneous group of edible species of high commercial importance. Subgenus *Coremiopleurotus* includes taxa that produce synnemata fructifications (anamorphic state). Several species, subspecies and varieties have been described in *Coremiopleurotus*. These taxa are discriminated by minute morphological differences and correspond to *Pleurotus cystidiosus sensu lato*. A worldwide geographical sampling of *Coremiopleurotus* taxa and nucleotide sequence data from the internal transcribed spacer of the nuclear rRNA genes (ITS) were used to produce a molecular phylogeny for the group. Also conducted were new interfertility studies, and a summary of the mating data currently available in the literature is provided. Both ITS phylogeny and mating data supported the distinction between *Pleurotus australis* (a species apparently endemic to New Zealand and Australia) and *P. cystidiosus sensu lato*. Within *P. cystidiosus sensu lato*, ITS phylogeny showed a deep split between Old and New World isolates and clearly distinguished four distinct clades that strongly corresponded to the geographical origin of the strains. In the Old World, one clade is composed of isolates from Europe and Africa, and one clade is composed of isolates from Asia (including collections from Hawaii). In the New World, one clade is restricted to isolates from Mexico, and one clade includes all the authors' North America isolates, one collection from Japan and one collection from South Africa. Mating data revealed a high level of interfertility among strains of *P. cystidiosus sensu lato*, except that isolates from Mexico were nearly fully intersterile with the other collections. Nucleotide sequence divergence in the ITS1–5.8S rDNA–ITS2 regions among intercompatible *P. cystidiosus* collections was very high (0–6.9%) in comparison to that reported in other biological species of basidiomycetes (0–3%), indicating significant genetic divergence between geographically isolated populations of the *P. cystidiosus* group. The phylogenetic species concept, as well as molecular, mating and geographical evidence, was used to recognize five species in the subgenus *Coremiopleurotus*: *P. australis* (in New Zealand and Australia), *Pleurotus abalonus* (in Asia and Hawaii), *Pleurotus fuscusquamulosus* (in Africa and Europe), *Pleurotus smithii* (in Mexico) and *Pleurotus cystidiosus sensu stricto* (in North America). However, geographical boundaries between these species are not strict, as rare events of long distance dispersal have occurred.

Received 28 July 2003

Revised 12 November 2003

Accepted 14 November 2003

INTRODUCTION

Members of the mushroom genus *Pleurotus* (Jacq. Fr.) P. Kumm. (Basidiomycotina, Pleurotaceae) form a heterogeneous group of edible species of high commercial importance. A morphologically distinct infrageneric grouping

consists of taxa which share the common character of producing arthrospores from asexual fructifications on basidiomata and/or in mycelial cultures.

Species which produce synnemata fructifications (i.e. white synnematal columns topped with a black mucous mass of hyaline arthrospores) are taxonomically arranged in the subgenus *Coremiopleurotus* Hilber. The type species of this subgenus is *Pleurotus cystidiosus* O. K. Miller, described

The GenBank accession numbers for the sequences reported in this article are AY315758–AY315810.

from North America, and its anamorphic state is assigned to the hyphomycete genus *Antromyces* Pat. & Trabut (Miller, 1969; Pollack & Miller, 1976). *P. cystidiosus sensu lato* has a worldwide distribution with a marked preference for warmer climatic zones and grows on a large variety of angiospermous hosts (Zervakis, 1998). Other taxa of the subgenus include *Pleurotus abalonus* Han *et al.* (1974) and *Pleurotus cystidiosus* var. *formosensis* Moncalvo (1995) both described as endemics from Taiwan, *Pleurotus smithii* Guzman (1975) from Latin America and *Pleurotus fuscusquamulosus* Reid & Eicker (Reid *et al.*, 1998) from South Africa. Morphological differences among members of this group are few and rather minute; therefore, their taxonomic assignment has been in the past an issue of debate. For example, significant ambiguities were noted concerning the exact status of *P. abalonus* (Jong & Peng, 1975; Neda & Furukawa, 1987), the relationships between *P. cystidiosus* and *P. smithii* (Guzman *et al.*, 1991; Vilgalys & Sun, 1994) and the position of *P. cystidiosus* var. *formosensis* and *P. fuscusquamulosus* within the subgenus (Moncalvo, 1995; Reid *et al.*, 1998). Especially with regard to the latter, it is noteworthy that it has been classified as a distinct species despite having complete mating inter-compatibility with *P. cystidiosus* strains (Reid *et al.*, 1998; Zervakis, 1998).

Initial studies of the systematics of *Coremiopleurotus* and allied taxa focused primarily on fruit-body and culture morphology (Guzman *et al.*, 1991; Han *et al.*, 1974; Jong & Peng, 1975; Miller, 1969; Petersen *et al.*, 1997; Segedin *et al.*, 1995). Mating compatibility studies were later introduced as an adjunct to morphological studies (Hilber, 1982; Moore, 1985; Zervakis, 1998), followed by molecular studies (Gonzalez & Labarère, 2000; Iraçabal *et al.*, 1995; Vilgalys & Sun, 1994; Vilgalys *et al.*, 1996). Since morphological characters alone are often inadequate for resolving the systematics and evolutionary relationships, molecular phylogenetic data should be useful for establishing a reliable taxonomic scheme for *Pleurotus* taxa.

Two other *Pleurotus* species reported to produce arthroconidia are *Pleurotus australis* Cooke & Massee in Cooke (1892) and *Pleurotus purpureoolivaceus* Segedin *et al.* (1995), which are both geographically restricted to Australia and New Zealand. *P. australis* produces darkly pigmented arthroconidia forming a black turf on mycelium or basidiomata (Petersen *et al.*, 1997; Zervakis, 1998), while *P. purpureoolivaceus* forms sessile spherical conidiomata on stipe surfaces and on associated basal mycelium (Segedin *et al.*, 1995). Both species are also intersterile with *P. cystidiosus*, although very low mating intercompatibility was reported between few isolates of *P. cystidiosus* and *P. australis* (Zervakis, 1998). Based on synnemata production, these species may also belong in subgenus *Coremiopleurotus*. Though early studies of molecular phylogeny did not preclude a close relationship between *P. australis* and other *Coremiopleurotus* species (Vilgalys *et al.*, 1996; and unpublished data), recent analyses of large-subunit ribosomal rDNA sequences have shown that *P. purpureoolivaceus* is not closely related to the

P. cystidiosus group or to other *Pleurotus* species (Thorn *et al.*, 2000).

Study of rDNA sequences has provided valuable insights for several basidiomycete groups of evolutionary relationships and speciation in conjunction with biogeography (Hibbett *et al.*, 1997; Hughes *et al.*, 1999; Isikhuemhen *et al.*, 2000; Moncalvo *et al.*, 2000, 2002; Vilgalys & Sun, 1994). Within the rDNA locus, the ITS region has been particularly useful for analysis of closely related species in many genera, including cultivated mushroom species (Carbone & Kohn, 1993; Hallenberg *et al.*, 1996; Harrington & Potter, 1997; Hibbett *et al.*, 1995; Mitchell & Bresinsky, 1999; Moncalvo *et al.*, 1995a, b; Pringle *et al.*, 2000; Rehner & Uecker, 1994). In this study, we investigated patterns of molecular evolution for the ITS region from a global sample of 41 collections including *P. australis*, *P. cystidiosus*, *P. abalonus*, *P. smithii* and *P. fuscusquamulosus*. Phylogenetic analysis was used to infer relationships and genetic diversity within this species complex, to define evolutionary paths in accordance with biogeography and to provide insight about speciation processes for geographically isolated populations of the group.

METHODS

Strains studied. The material used for ITS sequencing consisted of 41 *Pleurotus* dikaryotic cultures assigned in the following taxa: *P. abalonus* Han *et al.*; *P. cystidiosus* O. K. Miller; *P. cystidiosus* var. *formosensis* Moncalvo; *P. fuscusquamulosus* Reid & Eicker; *P. smithii* Guzman; *P. australis* Cooke & Massee. Details of the identity of each strain appear in Table 1.

Mating compatibility studies. Mating experiments were performed to provide additional mating compatibility data to those published earlier (Zervakis, 1998) using monokaryotic strains of *P. cystidiosus* (D 1834, D 2222, D 419, D 420, D 667) and *P. australis* 87/017. These were tested against previously identified incompatibility (intersterility) groups examined by Zervakis (1998). *In vitro* fructification of dikaryotic strains, single-spore isolation and mating tests were performed as described previously (Zervakis & Balis, 1996). Pairings were performed in 90 mm Petri dishes with CYM. Agar plugs (3 mm in diameter) from 12 monokaryotic isolates per *Pleurotus* dikaryon were placed in pairs, about 15 mm apart, in all possible combinations. Then, they were left till resultant colonies overgrew the space between the inocula and developed a contact zone (usually after 3–6 days). However, pairings were not interpreted until 1–2 weeks after inoculation, in order to have an established nuclear migration and a well-formed confrontation zone. Additional pairings were carried out when these self-crosses failed to reveal all four mating types for a given dikaryon. For pairings among homokaryons from different dikaryons, two tester strains were selected out of each one of the four incompatibility groups from every dikaryon, and then were paired in all combinations and in the same way as in the case of the self-crosses. Pairings were scored as compatible if clamp-connections could be observed on the hyphae, both in the contact zone and away from it, under the microscope (400×). Mating results were interpreted as positive if clamp-connections could be microscopically observed on the contact zone of a pairing. The values given represent the percentage of successful matings (over the total of the matings performed) between the monokaryotic tester-strains of each dikaryon selected. For interpopulation mating experiments, the mean of all individual results among strains from the two populations was calculated (Zervakis, 1998).

DNA isolation, ITS amplification and sequencing. Mycelia were grown on malt extract agar or potato dextrose agar, harvested using a spatula, transferred into Eppendorf tubes, freeze-dried and ground into powder with a pestle (Kontes Pellet Pestle; Fisher cat. no. K749520-0000). DNA isolation used SDS as lysis buffer (3 % SDS, 1 % 2-mercaptoethanol, 50 mM EDTA, 50 mM Tris/HCl pH 7.2) and phenol/chloroform/isoamyl alcohol (25:24:1) as extractant. The DNA was precipitated with 0.1 vol. of 3 M sodium acetate and 0.6 vol. of isopropanol, washed with 70 % ethanol and

Table 1. Dikaryotic *Pleurotus* strains used in ITS sequencing; taxa names are as provided from the acquisition sources unless otherwise indicated

Strain no. and acquisition source*	Taxon	Geographical origin
LGAM P50	<i>P. cystidiosus</i>	Greece
ATCC 28598	<i>P. cystidiosus</i>	South Africa
UP 174†	<i>P. fuscosquamulosus</i>	South Africa
CBS 61580	<i>P. cystidiosus</i>	India
ZA 472	<i>P. cystidiosus</i>	Thailand
DSM 5335	<i>P. cystidiosus</i>	Thailand
DSM 5340	<i>P. cystidiosus</i>	Thailand
FCUP 661	<i>P. cystidiosus</i>	The Philippines
FCUP 761	<i>P. cystidiosus</i>	The Philippines
ATCC 28787	<i>P. cystidiosus</i>	Taiwan
ATCC 28785	<i>P. cystidiosus</i>	Taiwan
D 2221	<i>P. cystidiosus</i>	Taiwan
D 2222	<i>P. cystidiosus</i>	Taiwan
ASIK 3	<i>P. cystidiosus</i>	Taiwan
CBS 80391	<i>P. cystidiosus</i>	China
PSUMCC 609	<i>P. cystidiosus</i>	Korea
ASIK 2	<i>P. cystidiosus</i>	Japan
ASIK 1	<i>P. abalonus</i>	Unknown origin
LGM 39	<i>P. abalonus</i>	Japan
IFO 30607	<i>P. cystidiosus</i>	Japan
IFO 31074	<i>P. cystidiosus</i>	Japan
IFO 31075	<i>P. cystidiosus</i>	Japan
VT 2476	<i>P. cystidiosus</i>	Hawaii
D 1833	<i>P. cystidiosus</i>	Hawaii
D 1834	<i>P. cystidiosus</i>	Hawaii
CBS 29735	<i>P. cystidiosus</i>	USA, Louisiana
D 412	<i>P. cystidiosus</i>	USA
D 413	<i>P. cystidiosus</i>	USA
D 414	<i>P. cystidiosus</i>	USA
D 417	<i>P. cystidiosus</i>	USA
D 419	<i>P. cystidiosus</i>	USA
D 420	<i>P. cystidiosus</i>	USA
D 667	<i>P. cystidiosus</i>	USA
VT 1780	<i>P. cystidiosus</i>	USA
FP 1683	<i>P. cystidiosus</i>	USA, Indiana
ATCC 34678	<i>P. cystidiosus</i>	Unknown origin
IE 74	<i>P. smithii</i>	Mexico
ATCC 46391	<i>P. smithii</i>	Mexico
CBS 68082	<i>P. smithii</i>	Mexico
VT 1953	<i>P. australis</i>	Australia
ICMP 11571	<i>P. australis</i>	New Zealand

resuspended in water. Amplification of the ITS region of the rRNA gene followed Vilgalys & Hester (1990) using primers ITS1 and ITS4 (White *et al.*, 1990). PCR products were visualized in agarose gels stained with ethidium bromide. Unincorporated primers and dNTPs were removed by centrifugation through a Millipore filter (cat. no. UFC3LTKNB) prior to sequencing. Both strands of the amplified region were sequenced using a dye terminator cycle sequencing kit from Perkin Elmer (cat. no. 402122), following the manufacturer's instructions. Sequencing primers were ITS1, 5-8S, 5-8SR and ITS4. Oligonucleotide sequences for primers 5-8S and 5-8SR were given in Vilgalys & Hester (1990). The sequencing reactions were run on an ABI 373 automated sequencer. Resulting chromatograms were assembled and edited with the program SEQUENCHER 3.0 (Gene Codes Corporation). The sequences have been deposited in GenBank (accession nos AY315758–AY315810).

Cloning. The PCR product of strain ATCC 46391 was ligated into TA3 PCR Vector and cloned using the TA Cloning Kit (Invitrogen cat. no. 45-0046), following the manufacturer's protocol. Recombinant plasmids were identified by colour selection after growth on Luria–Bertani plates containing X-Gal. Plasmid minipreps were performed, and plasmid DNAs were diluted 100-fold for PCR amplification of the ITS insert with primers ITS1 and ITS4. The resulting PCR products were sequenced as described above.

Cladistic analyses. Cladistic analyses were conducted in PAUP* (Swofford, 1998). Nucleotide sequences were aligned by eye and gaps were introduced to optimize sequence similarities. Single gaps that aligned unambiguously were treated as a fifth character state. Larger indels that aligned unambiguously were coded as single gaps in order to score them as single evolutionary events. Gap regions with ambiguous alignment were excluded from the analyses. The characters were unordered and weighted equally.

Phylogenetic analyses used maximum-parsimony as the optimality criterion. Heuristic searches used 100 replicates of random addition sequence with tree-bisection–reconnection (TBR) branch-swapping. The following other options in PAUP* were set as follows: starting trees obtained via stepwise addition, one tree held at each step during stepwise addition, MULPARS option in effect, steepest descent option not in effect, MAXTREES setting unlimited and branches having maximum length zero allowed to collapse to yield polytomies. Branch robustness was evaluated by three different methods: (1) bootstrapping (Felsenstein, 1978), (2) jackknifing (Farris *et al.*, 1996) and (3) decay indices (Bremer, 1994). The bootstrap and jackknife methods used 100

*ASIK – Agricultural Sciences Institute, Suweon, Korea; ATCC – American Type Culture Collection, Manassas, VA, USA; CBS – Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; CFMR – Center for Forest Mycology Research, Madison, USA; D – Mycology Laboratory, Duke University, USA; DSM – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; FCUP – University of The Philippines, The Philippines; ICMP – International Collection of Microorganisms from Plants, Auckland, New Zealand; IE – Instituto de Ecología, Xalapa, Mexico; IFO – Institute for Fermentation, Osaka, Japan; LGAM – Laboratory of General and Agricultural Microbiology, Agricultural Université of Athens, Greece; LGM – Laboratoire de Génétique Moléculaire, Université de Bordeaux II – INRA, France; PSUMCC – Pennsylvania State University Culture Collection, USA; UP – University of Pretoria, South Africa; VT – Department of Biology, VPI & SU, Blacksburg, USA; ZA – Instituto de Botanica, Sao Paulo, Brazil.

†Originally deposited as *P. cystidiosus*, but in the course of this study it was assigned the name *P. fuscosquamulosus* (Reid *et al.*, 1998).

replicates of heuristic searches with the same settings as above, except that in each replicate MAXTREES was set to 100 and one replication of random addition sequence was performed. In the jackknife analysis, 50 % of the characters were deleted in each replicate. Calculations of decay values are computationally intensive; therefore, we have approximated these values only for the major clades by constraining the monophyly of the clade under scrutiny in heuristic searches set to keep only trees not compatible with the constraint, using random addition sequence (25 replicates) with MULPARS off. This search strategy is expected to produce higher decay values than more computationally intensive searches, but can still provide reliable indicators of branch robustness. Two strains of *P. australis* were used for rooting the *P. cystidiosus* clade.

RESULTS

PCR amplification, sequencing and sequence alignment

PCR products produced with primers ITS1 and ITS4 were visualized as a single band in agarose gels stained with ethidium bromide. The size of the PCR fragments was about 700 bp in length for all taxa. Some PCR products yielded partly readable sequence chromatograms (see below) that could not be automatically assembled together in the program SEQUENCHER. These chromatograms were characterized by a more or less sudden overlapping of sequence peaks starting at certain given positions in the sequence (see below). However, it was still possible to edit these chromatograms manually and reconstruct a nearly complete sequence for each strain (regions of ambiguities were removed from the analyses). The 41 sequences were aligned in 664 positions, of which 38 were ambiguous to align and were excluded from the analysis. Of the remaining 626 included characters, 505 were constant, 14 variable characters were parsimony-uninformative and 107 variable characters were parsimony-informative.

Intra-collection ITS sequence heterogeneity

For several strains, direct sequencing of the PCR fragment produced only partly readable sequence chromatograms. We

determined that the sequencing problems always occurred in corresponding positions 119–122 or 598–600 in the nucleotide sequence alignment of the 41 taxa. In the dikaryotic culture ATCC 46391, problems in direct sequencing occurred when the ITS1, ITS4, 5·8S or 5·8SR primer was used as sequencing primer. To investigate this problem, we cloned the PCR product of that strain and sequenced nine individual clones. Seven different sequences were recovered. Nucleotide sequence variation between these seven alleles is shown in Fig. 1. The partial alignment depicted in Fig. 1 shows that indels (insertion/deletion events) are differentially distributed between ITS alleles at positions 119–122 and 598–600 in the alignment. In both regions, indels result from insertion/deletion of a cytosine. We attributed these indels to be responsible for shifts and subsequent overlapping sequence chromatograms produced by direct sequencing of the PCR product of strain ATCC 46391 (and other isolates; data not shown). Other variations between the seven clones were located in positions 64 (A/G polymorphism), 118 (G/C polymorphism), 134 (C/T polymorphism), 220 (T/A polymorphism) and 539 (A/G polymorphism) in the corresponding nucleotide sequence alignment. Variations at positions 134, 220 and 539 in the alignment were unique among all the ITS alleles sampled here, and are possibly PCR-cloning errors.

Intra-collection nucleotide sequence variation was also observed between two single spore isolates derived from strain JMT94/174 (D 2221 and D 2222). Variable bases were located at positions 26 (G/– polymorphism), 166 (T/C polymorphism) and 294 (A/G polymorphism).

Inter-collection ITS sequence variation

Nucleotide sequence variation among the collections examined (excluding *P. australis*) was calculated from the data matrix. Within the ITS1 region (which was aligned in 269 positions), 63 of 269 aligned positions (23·4 %) varied among collections, and 53 of these were phylogenetically informative. For ITS2, 42 of 215 aligned positions (19·5 %)

Position in the sequence alignment						
	11111	1	2	5	566	
6	11122	3	2	3	900	
4	78901	4	0	9	901	
*	* *	*	*	*	*	
clone 8	G [...]	CGC–C [...]	T [...]	C [...]	A [...]	CCT
clones 1,2	G [...]	CGC–C [...]	T [...]	C [...]	A [...]	C–T
clone 3	A [...]	CCCCC [...]	C [...]	C [...]	G [...]	CCT
clone 4	A [...]	CGC–C [...]	T [...]	C [...]	A [...]	C–T
clone 5	G [...]	CCCCC [...]	T [...]	T [...]	A [...]	CCT
clones 6,9	G [...]	CCCCC [...]	T [...]	C [...]	A [...]	CCT
clone 7	G [...]	CGC–C [...]	T [...]	C [...]	A [...]	CCT

Fig. 1. Nucleotide sequence variation in the ITS region of *P. smithii* among nine clones obtained from a PCR product from the dikaryotic strain ATCC 46391. Asterisks indicate variable positions.

were variable, with 38 phylogenetically informative sites. A single substitution (A/G transition) was observed within the 5-8S RNA gene (157 bp in length) that is located between the ITS1 and ITS2 spacers. No variation was observed in the flanking 18S and 25S rDNA regions.

Cladistic analyses

Replicate searches using heuristic search algorithms with random addition sequences always found the same tree-island consisting of 387 equally parsimonious trees with length 144. All these trees had a high consistency index (0.9236) which indicated a low level of homoplasy in the data matrix. A strict consensus of all most-parsimonious trees resolved 12 clades that were supported by 50 % or higher bootstrap values (Fig. 2). Eight of these clades were also retained by jackknife-resampling with a statistical support higher than 50 %. Branches with higher bootstrap or jackknife values generally also have higher decay indices (Fig. 2). Six clades had bootstrap support values higher than 97 % (and > 94 % with jackknife). Bootstrapping generally gave slightly higher statistical support than jackknifing, but both statistics were strongly correlated ($R=0.98$; data not shown).

Fig. 2 shows support for monophyly of the *P. cystidiosus* group relative to *P. australis* (100 % bootstrap support). The deep splits among ITS lineages in *P. cystidiosus* correspond well with a separation between Old and New World regions. The ITS phylogeny resolves the *P. cystidiosus* complex into four major clades that also strongly (but not fully) correspond to the geographical origin of the strains. The first clade includes all the North American taxa sampled in this study, one isolate from Japan and one isolate from South Africa (100 % bootstrap support). In this clade, the South African isolate is weakly separated from the other taxa (63 % bootstrap support), whereas the Japanese isolate is indistinguishable from the North American collections. The sister group (78 % bootstrap support) of this clade is composed of three *P. smithii* isolates from Mexico (100 % bootstrap support). A third clade (99 % bootstrap support) is composed of all the Asian isolates sampled (except strain IFO 30607 from Japan) and also includes three strains collected in Hawaii. The sister group (97 % bootstrap support) of this Asia-Pacific clade is composed of one European and one South African isolate, which cluster together with 100 % bootstrap support.

Mating compatibility

Results from mating compatibility experiments that were conducted in this work and in an earlier study (Zervakis, 1998) are summarized and illustrated in Fig. 3. Inter-compatibility values of 0–100 % were calculated among the *P. cystidiosus* isolates. Nearly complete intersterility was observed between *P. australis* and members of the *P. cystidiosus* complex, and complete intersterility was found between the Mexican isolates assignable to *P. smithii* and all the other isolates.

DISCUSSION

Phylogenetic analyses reveal at least five major lineages within the subgenus *Coremiopleurotus*, which are strongly correlated with geographical provenance. This finding suggests that biogeography has played a much stronger role in determining evolutionary units in these fungi than recognized previously. Until recently, the *P. cystidiosus* species complex was generally characterized as an ill-defined group based only on morphology and compatibility studies.

ITS phylogeny distinguishes *P. australis* from other *Coremiopleurotus* taxa examined (Fig. 2). However, morphological evidence (particularly the formation of conidia in culture) suggests an evolutionary relationship with the *P. cystidiosus* group (Petersen *et al.*, 1997; Zervakis, 1998). Based on molecular phylogenetic studies, *P. australis* and *P. cystidiosus* may represent sister groups within the subgenus *Coremiopleurotus* (Vilgalys *et al.*, 1996; and unpublished data). Vicariance could be responsible for the phylogenetic divergence between *P. australis* and the *P. cystidiosus*/*P. smithii* group, since the former species is only reported from Australia and New Zealand, while the latter group of species does not occur in Australasia.

Biogeography and speciation

Results from this study demonstrate strong patterns of geographical subdivision in the genetic structure of the *P. cystidiosus* complex, but occasional long distance dispersal also seems to occur. Phylogenetic analysis of ITS sequences divides the *P. cystidiosus* complex into four well supported lineages that also correspond largely with geographical groupings (North America, Mexico, Europe–Africa and Asia–Pacific). Two exceptions were found to this general pattern, however, involving one isolate from Japan and one isolate from South Africa, which each clustered with collections from North America. The simplest explanation for this pattern of relationship is infrequent, but recent, gene flow involving North American germplasm. This finding is in agreement with the pattern of population structure of another broadly distributed mushroom, *Schizophyllum commune* (James *et al.*, 1999, 2001). The vectors responsible for long distance dispersal in fungi are still poorly known, but recent human-mediated dispersal has been invoked (Coetzee *et al.*, 2000; Kerrigan *et al.*, 1995).

Intercontinental distributions of mushroom fungi are governed in part by gene flow via long-distance spore dispersal, human-mediated transfer of substrates and/or commercial mushroom spawn, and vicariance events. Although oceans are an effective barrier to gene exchange and long distance spore dispersal is not an effective means of gene flow between continents (Hughes *et al.*, 1999; Kerrigan *et al.*, 1995; Lickey *et al.*, 2002; Petersen, 1995; Shen *et al.*, 2002; Vilgalys, 1986), common incompatibility alleles have been detected in intercontinental populations of *P. cystidiosus*, i.e. in populations from the USA and Taiwan (Moore, 1985; Zervakis, 1998). Species of the subgenus *Coremiopleurotus*

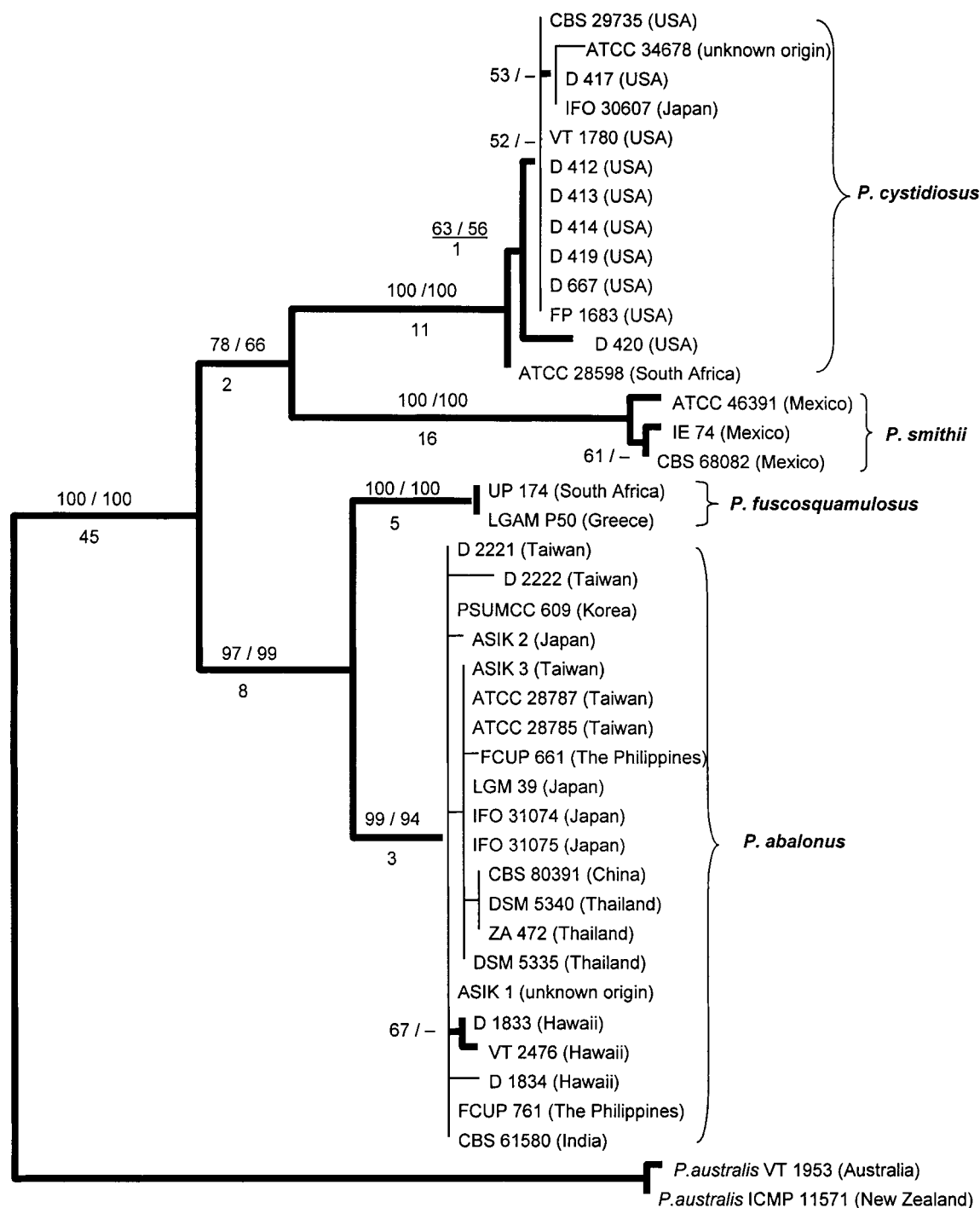


Fig. 2. One of the 387 equally parsimonious trees found by parsimony analysis in PAUP*. Branches in bold are also present in the strict consensus tree. Tree length = 144. Consistency index = 0.9236. Retention index = 0.9828. Values above branches are bootstrap/jackknife supports and values below branches are decay indices. The geographical origin of each strain is given in parentheses.

produce basidiospores with a particular physiology of dormancy, which can withstand adverse environmental conditions over long distances and germinate only at relatively high temperatures and in the presence of suitable substrates (Lahouvaris *et al.*, 1995).

The intercontinental distribution of *P. cystidiosus* and the genetic divergence among isolates from Eurasia, Africa and North America is indicative of dispersal before the formation of geographical barriers, through regular and progressive spread of populations to new regions along land routes.

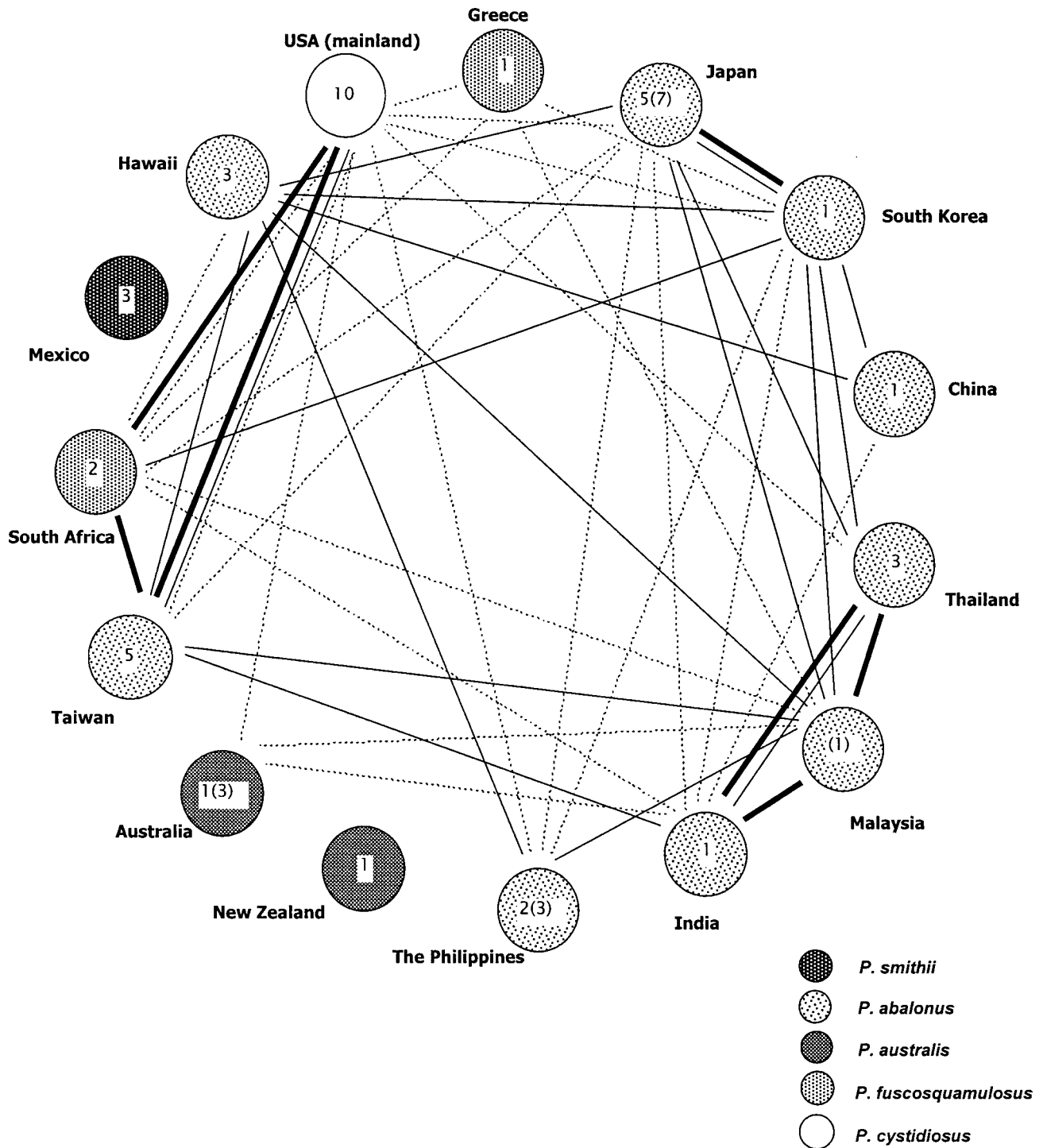


Fig. 3. Diagram summarizing the outcome of mating compatibility experiments between geographically distant populations of *P. australis* (Australia and New Zealand), *P. smithii* (Mexico) and *P. cystidiosus sensu lato* (all other origins). Thick lines, 100 % genetic compatibility or presence of common incompatibility alleles; thin lines, > 50 % intercompatibility; dotted lines, < 50 % intercompatibility; absence of lines connecting two populations is indicative of interincompatibility between them; numbers within circles denote number of examined strains per geographical origin, numbers in parentheses within circles denote number of additional strains examined in a previous study (Zervakis, 1998). In cases where more than one line is connecting two particular populations, different types of results were obtained in the matings between individual strains.

During the Pleistocene, the migration route between Asia and North America was the Bering land bridge which allowed transfer of biological material during the glaciation period (Graham, 1993). Such assumptions are supported by the observed sequence similarity between Asiatic and Hawaiian strains, in conjunction with successful mating results (relatively high genetic affinity has been also observed among Japanese and Korean vs USA collections). Disjunct patterns of distribution for eastern Asia and eastern North America have been noted for a large number of living organisms and are fairly common among plants and mushroom fungi (Futuyma, 1986; Redhead, 1988; Wu & Mueller, 1997). Another possible pathway could have been the Atlantic land bridge which allowed exchange of genes between Europe and North America until the late Cretaceous to early Tertiary (Tiffney, 1985; Wolfe, 1975).

Morphologically differentiated forms of *P. cystidiosus* occurring in eastern Asia, i.e. *P. abalonus* and *P. cystidiosus* var. *formosensis*, maintain the ability to interbreed and populations from near-by origins demonstrate partial intercompatibility. These cases of partially isolated populations are usually interpreted as instances of secondary contact between populations that differentiated in allopatry, but they did not achieve full species status. At such a zone each of several loci usually exhibits a cline in allele frequency (G. I. Zervakis, unpublished allozyme data) indicative of introgressive hybridization. In this particular fungal group, there is no apparent ecological component of reproductive isolation, which is usually an effective barrier to gene exchange, as occurs in other *Pleurotus* species demonstrating host specialization (Duncan, 1972; Zervakis *et al.*, 2001). In such cases of partial reproductive isolation, the biological species concept can not be applied. In addition, the existence of the anamorph of the fungus in most regions where the teleomorph has been recorded implies that asexual reproduction is occurring. Therefore, sexual compatibility processes are not as essential as in other *Pleurotus* groups.

In contrast, allopatric speciation is evident for distant populations that already show molecular differences as well as low (or no) *in vitro* mating intercompatibility (i.e. North American with most Asiatic populations, Greek and South African mainly with North American strains). In these cases isolating mechanisms are in effect and the process of divergence seems to be continuous, proceeding both before and after speciation (although genetic differentiation can become more pronounced after reproductive isolation is obtained). Biogeographical patterns of variation within such genetically related compatibility groups show that geographically isolated populations gradually accumulate genetic differences as detected by DNA sequencing.

These observations for *P. cystidiosus* are in accordance with the outcome of previous studies indicating that geographical separation plays an important role in the evolution and speciation in basidiomycete genera such as *Pleurotus* (Vilgalys & Sun, 1994), *Flammulina* (Hughes *et al.*, 1999) or *Omphalotus* (Hughes & Petersen, 1998). Furthermore, allopatric

isolates of *Armillaria mellea sensu stricto* were found to be highly divergent indicating that populations originating from different geographically distant areas (i.e. Europe, Asia, eastern and western North America) are genetically isolated (Coetzee *et al.*, 2000).

ITS divergence, phylogeny and mating intercompatibility

DNA sequence divergence within the ITS region is not necessarily associated with the degree of mating intercompatibility (Hughes & Petersen, 1998; Liou, 2000). This is also well illustrated by North American isolates of *P. cystidiosus*, which are genetically closer to *P. smithii* from Mexico than they are to other intercompatible Asian *P. cystidiosus* isolates (Fig. 2). Overall nucleotide sequence divergence in the ITS + 5.8S rDNA region among intercompatible *P. cystidiosus* collections was high (0–6.9%) in comparison to that reported in other biological species of basidiomycetes (0–3%; Anderson & Stasovski, 1992; Isikhuemhen *et al.*, 2000; James *et al.*, 2001; Lickey *et al.*, 2002). The latter observation indicates that significant genetic divergence has occurred between geographically isolated populations of the *P. cystidiosus* group. In at least one instance (*P. smithii*), geographical isolation has been accompanied with development of intersterility. The intermediate position of *P. smithii* in the ITS phylogeny made intercompatible populations of *P. cystidiosus sensu lato* paraphyletic (Fig. 2).

Many broadly distributed biological species are often found to be paraphyletic and hence they may not always represent unique evolutionary units (Cracraft, 1990). The morphological, mating and ITS data gathered from *P. cystidiosus* are a snapshot in the natural history of this fungus. Past events may be reconstructed, but forthcoming events are unpredictable. We speculate that the present biological and geographical patterns observed in *P. cystidiosus* indicate that the species may have undergone recent evolutionary bottlenecks. This hypothesis best explains the shape of the ITS phylogenetic tree in Fig. 2 which is characterized by four long branches (indicating long divergence time between four genetically distinct populations) terminated by very short branches (indicating low divergence within each of the four populations). Based on this scenario, *P. cystidiosus* may have once been composed of a broadly distributed, interbreeding population that has been geographically fragmented into at least four smaller groups restricted to Africa, Asia, North and Central America. These geographically and genetically isolated populations have accumulated several unique mutations, which result in the four long branches in Fig. 2. Short terminal branches (Fig. 2) indicate that each population is rather homogeneous, suggesting gene flow within each population (or low divergence time). In at least one instance, one population had accumulated sufficient genetic divergence from the other populations to develop intersterility barriers and become morphologically distinct (*P. smithii*). The presence of one isolate from Japan and one isolate from South Africa in a clade mostly composed of collections from North America may indicate that

populations of *P. cystidiosus* could be presently expanding with occasional events of long distance dispersal.

Other evolutionary scenarios are also possible. For instance, under the allopatric model of speciation, if the three populations of *P. cystidiosus* (Fig. 2) remain largely isolated their level of genetic differentiation will increase, and these populations may eventually develop intersterility barriers with each other, hence 'speciate'. In that case, application of the phylogenetic species concept instead of the biological species concept is still appropriate, because this would recognize the distinct clades as independent evolutionary units.

Monophyletic groups and species concepts in *Coremiopileurotus*

Evidence for strong phylogeographical structure provides an opportunity to re-examine current species concepts as they have been applied within the subgenus *Coremiopileurotus*. At least three broad kinds of species concept may be applied to out-crossing basidiomycetes: the morphological species concept (MSC), the biological species concept (BSC) and the phylogenetic species concept (PSC) (Clemençon, 1977; Davis, 1996; Hennig, 1966; Mayr, 1942; Smith, 1968).

Based on the MSC, mycologists have described at least six (e.g. *P. cystidiosus*, *P. abalonus*, *P. smithii*, *P. fuscusquamulosus*, *Pleurotus gemellarii*, *P. australis*; see Zervakis 1998) morphological taxa in *Coremiopileurotus* that could represent species based solely on morphological evidence. These include several varieties of *P. cystidiosus* that have been described based on colour, basidiospore and cystidia characteristics.

Mating compatibility evidence reveals three intersterility groups that represent species based on the BSC: *P. australis*, *P. cystidiosus sensu lato* and *P. smithii* (Zervakis, 1998). Although these groups are nearly intersterile, low percentages of successful matings were detected between a few strains of *P. australis* and *P. cystidiosus* (Zervakis, 1998), and *P. smithii* and *P. cystidiosus* (Reid *et al.*, 1998). Other cases of partial cross-species compatibility are known in other basidiomycetes including *Pleurotus* (Petersen & Ridley, 1996). Because mating compatibility is known to be a pleiomorphic character, and thus may not be a good indicator of recent genetic relationship, an increasing number of systematists have also suggested that the BSC is insufficient for determining species limits in fungi, instead favouring a species concept based on phylogenetic principles.

Phylogenetic analyses revealed five major clades supported by ITS sequence data (Fig. 2). Intercompatible but geographically distinct populations of *P. cystidiosus sensu lato* from North American, African, European and Asian collections are paraphyletic with respect to *P. smithii*. Accommodation of these populations into a single species would therefore violate the PSC. However, because of their strong geographical and genetic subdivision based on rDNA evidence, these clades are most likely to represent the primary units of evolution recognized under the PSC (Vilgalys &

Sun, 1994). Because mating compatibility groups (BSC) may not always be monophyletic, the PSC provides a more consistent criterion for species delimitation that also considers the biogeographical context under which species evolve. Based on conservation value, Hibbett & Donoghue (1996) have also argued convincingly that recognition of phylogenetic species is most likely to preserve the maximum amount of both phylogenetic and genetic diversity within a species concept. Based on these arguments, we also chose to recognize all five ITS lineages (Fig. 2) as phylogenetic species within the subgenus *Coremiopileurotus*. These are discussed in turn below.

***Pleurotus australis*.** Based on multiple criteria, *P. australis* appears to be a valid species. Excellent morphological descriptions are available from Segedin *et al.* (1995) and Bougher & Syme (1998) (as *Pleurotus ostreatus*). Mating evidence also demonstrates that *P. australis* is nearly intersterile with all other *Pleuroti* (Petersen *et al.*, 1997; Zervakis, 1998; Fig. 3). The known geographical distribution of *P. australis* (Australia and New Zealand) is also distinct from all other members of the subgenus *Coremiopileurotus*. Phylogenetic evidence suggests that it is distinct from all other ITS lineages in *Coremiopileurotus* (Fig. 2) and from other intersterility groups within *Pleurotus* (Vilgalys *et al.*, 1996; and unpublished data).

***Pleurotus smithii*.** *P. smithii* is a good species that fits the criteria of the MSC, BSC and PSC. It is distinguishable by the morphology of both its basidiocarp and anamorphic state (Guzman *et al.*, 1991; Stalpers *et al.*, 1991). It is intersterile with all the other *Pleurotus* taxa for which we have conducted mating tests (Fig. 3; Zervakis, 1998), although it presented low intercompatibility with a single North American strain (17.5% compatibility; Reid *et al.*, 1998). In addition, it represents a distinct evolutionary unit based on ITS sequence evidence (Fig. 2).

Distribution of *P. smithii* is restricted within the neotropical zone and could be characterized as amphitropical extending over a large contiguous area in Latin America (from Mexico to Argentina; Capelari, 1999; Guzman *et al.*, 1980, 1991; Rodriguez-Hernandez & Camino-Vilaro, 1990; Spinedi, 1995). This type of distribution has been observed for other groups of organisms as well (Raven, 1963).

P. smithii appears in the phylogram (Fig. 2) to be a sister group to the North American *P. cystidiosus* population. It is generally accepted that far fewer South American groups of organisms entered North America than vice versa (Brown & Gibson, 1983). Therefore, it is possible that the South American populations originated from their North American counterparts. This could have occurred before the Pliocene when the Central American land bridge became complete, or by dispersal through the Central American islands. Possibly, during a period of glaciation in North America, *P. cystidiosus* may have migrated southwards and became established in warmer regions. The subsequent glacial retreat could then

have left behind isolated daughter populations which further progressed to form a distinct species through selection forces and genetic drift.

***Pleurotus cystidiosus*.** *P. cystidiosus* was described from North America (Miller, 1969). Continental USA isolates of this taxon are monophyletic (Fig. 2) and morphologically rather homogeneous. We therefore restrict our concept of *P. cystidiosus sensu stricto* to these collections that are genetically closer to continental North American isolates than they are to other collections. However, as defined, *P. cystidiosus* does not represent an intersterility group.

ITS phylogeny indicates at least two occurrences of long distance dispersal of *P. cystidiosus* from North America. The species was also found in Japan and South Africa. The Japanese isolate IFO 30607 clusters among the North American isolates and is fully intercompatible with them (Zervakis, 1998); therefore, this dispersal seems recent. The South African strain ATCC 28598 was separated from the core *P. cystidiosus* clade (Fig. 2), which suggests earlier dispersal and genetic divergence by allopatry.

***Pleurotus abalonus*.** *P. abalonus* was described from Taiwan and was the first representative of the *P. cystidiosus* complex to acquire a species-level taxonomic assignment on the basis of morphological differences (Han *et al.*, 1974). Typical *P. abalonus* differs from *P. cystidiosus* in having thick-walled, yellow-brown cheilocystidia (instead of thin-walled and hyaline) and a dark-grey to dirty-brown colour of pileus (Han *et al.*, 1974; Stalpers *et al.*, 1991). However, subsequent compatibility investigations (Hilber, 1982; Zervakis, 1998) demonstrated high percentages of successful matings (>50%) between Asian collections assigned to *P. abalonus* and *P. cystidiosus*. These two taxa were also not distinguished by isozyme and RFLP data (Zervakis *et al.*, 1994; Iraçabal *et al.*, 1995).

The present study shows that Asia-Pacific strains referred to as *P. abalonus*, *P. cystidiosus* or *P. cystidiosus* var. *formosensis* are not phylogenetically distinct from each other. However, they form a phylogenetically distinct group from other *Coremiopleurotus* taxa. The low level of nucleotide sequence variation within this Asian-Pacific clade is indicative of a rather homogeneous gene pool among the areas sampled. This observation correlates with mating results that suggest ongoing gene flow in that region. Pairings performed between isolates originating from the Asian-Pacific area were successful in most cases (16 out of 22 matings produced over 50% compatibility percentages), and four common incompatibility factors were detected (South Korea vs Japan, Thailand vs India, India vs Malaysia and Malaysia vs Thailand) (Zervakis, 1998). In contrast, lower levels of intercompatibility were found with members of other clades.

We therefore propose to accommodate in *P. abalonus* all collections of *Coremiopleurotus* that are phylogenetically closer to Taiwanese *P. abalonus* than they are to isolates that

cluster in other clades. In other words, we define *P. abalonus* as a phylogenetic species that is composed of the bulk (if not the totality) of *Coremiopleurotus* in the Asia-Pacific region. As defined, *P. abalonus* is not fully intersterile with *P. cystidiosus* and *P. fuscusquamulosus*, and includes morphologically diverse varieties that correspond to the typical *P. abalonus* form or more closely resemble *P. cystidiosus* or *P. cystidiosus* var. *formosensis*.

***Pleurotus fuscusquamulosus*.** In the course of this study, the South African strain UP 174 has been identified as a distinct species, *P. fuscusquamulosus*, mainly on the basis of cystidial characters and mating data (Reid *et al.*, 1998). Reid *et al.* (1998) noted that the first criterion is of limited practical value because '[cystidia] can be difficult to detect in microscope preparations and may vary from absent to abundant in different basidiomes of the same species, possibly as a result of the stage of maturity of the latter'. Their species description was therefore essentially based on mating studies, which showed interincompatibility in pairings with two strains from the USA and Taiwan (both assigned to *P. cystidiosus*) and one *P. smithii* isolate.

In the ITS phylogeny, UP 174 and the Greek strain LGAM P50 form a distinct clade, which is sister to the Asia-Pacific lineage. The two strains have similar anatomical characteristics and identical ITS sequences. The Greek strain LGAM P50 was the first of two strains isolated from Greece; no other specimens of the subgenus *Coremiopleurotus* have been found elsewhere in Europe (Zervakis *et al.*, 1992). This finding suggests a recent extension of distribution range of an African population towards Europe. The report by Patouillard (1897) of the occurrence of the *Antromycopsis* imperfect state in Algeria could provide the evidence of on-going gene flow between Mediterranean Europe and South Africa through the tropical zone (e.g. Burundi), where the presence of the fungus has already been reported (Buyck, 1994).

On the other hand, UP 174 mating results showed intercompatibility with strains of Eurasia-Pacific origin and with the ATCC 28598 isolate from South Africa (Zervakis, 1998). Overall, the different phylogenetic placement of the two interfertile isolates from South Africa (Fig. 2), together with the high percentage of positive matings of ATCC 28598 with strains from the USA (in contrast to the intersterility of UP 174 with American strains), could indicate a relatively recent dispersal of ATCC 28598 from North America to South Africa.

In conclusion, although this analysis is based on nucleotide sequences from a single nuclear locus (ITS), the general agreement between the ITS phylogeny and biogeography, and in at least one instance with mating patterns (i.e. the genetic isolation of *P. smithii*), demonstrates that this molecular phylogeny is a reasonable estimate of the organismal phylogeny within the *P. cystidiosus* species complex. The study of additional genes, however, is still needed to fully understand speciation processes in this group of fungi.

ACKNOWLEDGEMENTS

This work was largely supported through grants by the US National Science Foundation to R. V. and J. M. M. We would like to thank two anonymous reviewers for their constructive comments, and Drs Orson K. Miller, Jr and Peter Buchanan for providing *Pleurotus* strains. G. Z. is particularly grateful for the facilities provided by the host laboratory during his stay at Duke University.

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