

Species recognition, geographic distribution and host-pathogen relationships: a case study in a group of lignicolous basidiomycetes, *Phellinus* s.l.

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Abstract: Morphological, phylogenetic (sequencing of the ribosomal ITS region) and, if applicable, biological (pairings of single-spore testers) species recognition have been used to resolve relationships among 69 collections belonging to the Hymenochaetales genera *Phellinus* s.str. and *Fomitiporia*. The isolates originate from a variety of host plants in Europe, North America and Asia. Separate application of recognition modes led to differing results concerning the number of species, geographic distribution and host range. Sole application of morphological criteria was of limited value, especially in taxa exhibiting a wide distribution, both in terms of geographic origin and ecological niche. Relationships of putatively conspecific collections originating from different continents preferably should be resolved by using an integrative approach. In this study, application of a strict morphological approach led to the recognition of seven species. When using molecular and pairing test data, at least 12 species were detectable. Two of them, *F. hesleri* and *F. polymorpha*, are described as new. The number of *Phellinus* s.str. and *Fomitiporia* species supposed to have Northern Hemispheric or cosmopolitan distribution, when morphological characters are applied for species recognition, has been reduced significantly. As firm tendencies within morphological species, genetic divergence was more distinct in uniparental than in biparental taxa. In the latter, a strong correlation was observed between phylogenetic and biological species recognition. Overall length of the ribosomal ITS region clearly separated *Phellinus* s.str. and *Fomitiporia* but was of limited value as a diagnostic tool at species level. The level of innerspecific morphological plasticity of fruit bodies differs widely between even closely related species, suggesting that morphological transitions occur quite

frequently in this fungal group. Considerable instability of the reproduction mode was evident in strains belonging to *Phellinus tremulae* and among closely related species of *Fomitiporia*.

Key words: biogeography, cryptic species, host range, Hymenochaetales, ITS phylogeny, pairing tests

INTRODUCTION

Species recognition and species concepts are intriguing subjects in fungal biology (for an overview, see Petersen and Hughes 1999). Speciation usually is understood as a dynamic evolutionary process affected by factors, including geographical distribution, host range and host specificity, genetic compatibility, hybridization events, horizontal gene transfer or gene duplication, that result in phenotypic expression. The diagnosis of species depends on the application of operational concepts such as morphological species recognition, biological species recognition and phylogenetic species recognition as defined by Taylor et al (2000), reserving the term “species concept” for theoretical approaches. A prerequisite to test for congruence among different recognition modes is the choice of a fungal group that meets the necessary requirements.

To study cryptic speciation and host-pathogen relationships, lignicolous fungi are well suited for two reasons: They usually are easy to cultivate under laboratory conditions, and single-spore isolates for mating studies can be obtained via germinating basidiospores. *Phellinus* s.l. Quél. represents the largest group in the Hymenochaetales, an order of homobasidiomycetes that contains saprotrophic and/or parasitic fungi living on a wide variety of wood. Recent studies have shown that *Phellinus* s.l. is polyphyletic and as a consequence it was split into a number of smaller monophyletic subunits, each designated as a separate genus (Fiasson and Niemelä 1984, Fischer 1996, Hansen and Knudsen 1997, Wagner and Fischer 2001, Wagner and Fischer 2002). *Phellinus* s.str. at present is limited to the *P. igniarius* Quél. group. Other important groups, comprising a considerable number of taxa, are *Fomitiporia* Murrill (*P. robustus* group), *Porodaedalea* Murrill (*P. pini* group) or *Fuscoporia* Murrill (*P. ferruginosus* group).

Treating *Phellinus* in a wider sense, more than 150

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species are recognized (Larsen and Cobb-Pouille 1990). Diversity of this group is best examined in Europe and North America, with 36 and 48 species presently acknowledged (Gilbertson and Ryvarden 1987; Ryvarden and Gilbertson 1994). Out of 23 species common to Europe and North America, 20 also have been documented in China (Zhao and Zhang 1992; Dai 1996, 1999) and at least eight of these 20 have been reported from different parts of the Southern Hemisphere (Cunningham 1965, David and Rajchenberg 1985, Larsen and Cobb-Pouille 1990).

With few exceptions, the type localities of species thought to be common to Europe, North America and Asia are in Europe. The original descriptions, based on morphological species recognition, have been applied to other geographic regions. However, one should be aware of a fundamental problem inherent in taxa exhibiting a wide distribution, both in terms of geographic origin and ecological niche: Relationships of putatively conspecific collections are more exactly resolved by the additional application of biological and/or phylogenetic species recognition. This integrative approach frequently leads to the detection of new species, for example, in *Armillaria mellea* s.l. (Korhonen et al 1978), *Flammulina velutipes* s.l. (Hughes et al 1999, Methven et al 2000) or *Heterobasidion annosum* s.l. (Korhonen and Niemelä 2000, Dai et al 2002). In *Phellinus* s.l., the use of morphological, biological and phylogenetic species recognition revealed undescribed taxa within *Phellinus* s.str. (Fischer 1995), *Porodaedalea* (Fischer 1994, 1996, 2000) or *Fomitiporia* (Fischer 2002).

The present study includes a number of species within *Phellinus* s.str. and *Fomitiporia* that are widely distributed and show different host preferences, as well as different patterns of reproduction, that is uniparental (homothallic) or biparental (heterothallic). In uniparental taxa, no gene flow is supposed to occur within a species, making reproductive isolation against other species complete. In biparental taxa, gene exchange is an essential step of the life cycle; at the same time, however, the taxon is isolated reproductively from others. Intermediate forms may occur during speciation events in biparental taxa, where gene flow and/or isolation are incomplete.

The taxa included in this study were: *Phellinus alni* (type locality: Russia), *P. cinereus* (Finland), *P. laevigatus* (Finland), *P. lundellii* (Finland) and *P. tremulae* (Russia), all belonging to the former *P. igniarius* group, and *Fomitiporia mediterranea* (Germany), *F. hartigii* (Germany), *F. punctata* (Sweden) and *F. robusta* (Finland), all belonging to the former *P. robustus* group. In addition, several collections have been included not clearly assignable to any of the above

taxa. For studies in the *P. igniarius* group, one strain of this species was added as a reference.

Based on several specimens each, originating from a variety of geographic regions and host plants, all taxa were characterized by the application of morphological and anatomical data (morphological species recognition) as well as molecular data, such as the overall size and phylogenetic analysis of sequences of the ribosomal ITS1-5.8S-ITS2 region (phylogenetic species recognition). Additional pairing tests of single-spore isolates were performed for the biparental taxa (biological species recognition).

In this way these questions should be addressed: (i) Application of different species recognition modes to *Phellinus* s.str. and *Fomitiporia* is expected to result in a differing number of species; to what degree do morphological, biological, and phylogenetic recognition correlate and which of these approaches is preferable? (ii) In the past, non-European collections of *Phellinus* s.l. usually have been identified on the basis of traditional characters; now, what are the exact relationships among such collections and well-defined European material? (iii) If there are cosmopolitan species within *Phellinus* s.l., does genetic divergence exist within these taxa? (iv) On the basis of the wide sampling available for this study, is it possible to provide new insights into host-pathogen relationships and speciation processes in lignicolous fungi?

MATERIALS AND METHODS

Fungal material and culturing.—Field data and GenBank accession numbers of the strains used are listed in TABLE I. Specimens are deposited at the herbarium of the University of Regensburg (REG). Mycelial cultures were grown on malt-extract medium (ME; 2% agar, 2% malt extract, 0.05% yeast extract) in daylight.

Comparative microscopy.—Sections of fruit bodies were placed on a slide in a drop of Melzer's reagent or lactophenol-cotton blue (Meixner 1975); examinations were at 500× or 1250× under phase contrast optics. Twenty observations were recorded for measurements of basidiospores and setae.

Pairing tests.—For isolation of single-spore isolates a section of the hymenium was attached to the inside of a Petri plate lid. Discharged spores dropped on the medium and were dispersed with Ringer's solution (NaCl, 0.225%; KCl, 0.01%; CaCl₂, 0.0045%; NaHCO₃, 0.005% in distilled water) or with sterile cultural filtrate (ME) of the corresponding strain. Plates with dispersed spores were incubated at 21 or 30 C in daylight.

Interstrain pairings were made using eight single-spore mycelia, which were paired in all possible combinations. Paired mycelia were incubated 4–5 wk before final examination (Fischer 1987, 1995, 2002). For interstrain pairings,

two different isolates per mating type were selected from each strain as testers; these were paired in all possible combinations.

DNA isolation and PCR amplification.—Whole-cell DNA was isolated from lyophilized cultures, as described by Lee and Taylor (1990). DNA pellets were air-dried and resuspended in 100 μ L TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA quantity and quality was examined on 1% agarose gels. Extracted DNA was diluted 1:500 in distilled water. The polymerase chain reaction (PCR) was used to amplify a portion of the nuclear encoded ribosomal DNA unit defined by the primer combination prITS1 and prITS4 (for primer sequences, see White et al 1990). The fragment spans the entire region of the internal transcribed spacers (i.e., ITS1 and ITS2, as well as the 5.8S gene).

The PCR reactions were set up in 100 μ L volumes and were overlaid with two drops of mineral oil. Hot-start PCR was applied throughout (d'Aquila et al 1991). Thirty-five cycles were performed on a Biometra TRIO-Thermoblock, using these parameters: 94 C denaturation step (1.5 min), 53 C annealing step (45 s), 72 C primer extension (1.5 min). A final incubation step at 72 C (7 min) was added after the final cycle. Three μ L of each PCR reaction were electrophoresed on 1% agarose gels. DNA molecular weight marker VI (Boehringer, Mannheim, Germany) was used as standard. The amplified products were purified with the QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instruction. DNA was suspended in 50 μ L Tris-HCl buffer (10 mM, pH 8.0).

Sequencing.—ITS sequences were generated for 69 strains listed in TABLE I (GenBank accession numbers AY340001–AY340069). Instead of mycelium derived from the fruit body, a single-spore isolate was used for strain 45/23, designated 45/23.3. Fragments were sequenced with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, California), using 2 μ L of pre-mix, 1 μ L of the primers (8 pmol of prITS1 and prITS4, respectively), and 3.5 μ L of the PCR products. The reactions were set up in 11 μ L volumes and were overlaid with one drop of mineral oil.

Sequences were generated in two directions and 25 amplification cycles were carried out, using these parameters: 96 C denaturation step (30 s), 59 C annealing step (15 s) for prITS1, 53 C annealing step (15 s) for prITS4, 60 C primer extension (4 min). DNA was precipitated by addition of 2 μ L of NaAc (3 M, pH 4.8) and 55 μ L of EtOH 100% and purified with 150 μ L of EtOH 70%. The DNA pellet was resuspended in 1:4 EDTA (50 mM, pH 8.0): formamide. The electrophoresis was done with an ABI 373A automated sequencer (Applied Biosystems).

Alignments and phylogenetic analyses.—This study involved two separate ITS datasets called the *Phellinus* s.str. dataset, containing 35 strains, and the *Fomitiporia* dataset, containing 34 strains. Both were analyzed independently. After processing the raw data with SeqEd (version 3.0), sequences were aligned using Clustal X version 1.64b (Thompson et al 1997). The final alignments were made by eye in the edition of PAUP* 4.0b10 (Swofford 2003). Alignment gaps

were treated as missing data, and all positions were included in the final alignments. The sequence alignments have been deposited in TreeBASE as submission No. SN 1528.

Heuristic searches using maximum parsimony (MP) and maximum likelihood (ML) were performed in PAUP* with 1000 replicates, using tree-bisection-reconnection (TBR) branch-swapping, random taxon addition sequences, MulTrees option in effect and zero length branches collapsed. All characters were unordered and had equal weight. MAXTREES was set to auto-increase in all analyses, except for the MP analysis of the *Fomitiporia* dataset, in which the maximal number of trees was 10 000. Bootstrap analyses (Felsenstein 1985) were performed under the same settings.

The best-fit maximum-likelihood model estimated using Modeltest 3.06 (Posada and Crandall 1998) was the general time-reversible model (GTR+G) on one of the shortest trees derived from the MP analyses. ML settings included assumed nucleotide frequencies A = 0.38080, C = 0.17840, G = 0.15390, T = 0.28690, six rate classes modeled on discrete gamma distribution, gamma shape parameter α = 1.6.

RESULTS

Identification of collections (morphological species recognition).—First-hand identification was carried out by morphological and anatomical means, substrate data were taken into account, as well, for all collections. The results obtained are those given in TABLE I. Species recognized were *Phellinus alni*, *P. cinereus*, *P. igniarius*, *P. laevigatus*, *P. lundellii*, *P. tremulae*, *Fomitiporia hartigii*, *F. mediterranea*, *F. punctata* and *F. robusta*. Information on host range and/or geographic distribution was necessary to distinguish collections of *P. alni* (deciduous trees not including *Salix*) and *P. cinereus* (mostly *Betula*) from those of *P. igniarius* (exclusively *Salix*) and to distinguish between *F. mediterranea* (mostly *Vitis*; Mediterranean region) and *F. punctata* (deciduous trees not including *Vitis*; central Europe).

A number of strains, all originating from North America and collected on *Salix hindsiana*, could not be assigned to a certain taxon. These strains, labeled "uncertain affinity" in TABLE I, were different from *F. punctata* in having hymenial setae; in addition, shape of fruit bodies was more variable than in *F. punctata*, ranging from resupinate to slightly effused-reflexed. Resupinate fruit bodies also may be formed by *F. robusta*, but setae are infrequent in the latter species, which occurs mostly on living oaks. *Phellinus texanus* also is related, but this species forms pileate fruit bodies; in addition, it is found exclusively on desert plants and has larger basidiospores (Gilbertson and Ryvarden 1987).

Sequence data of the ribosomal ITS1-5.8S-ITS2 region and interstrain pairings of single-spore testers (phyloge-

TABLE I. List of fungal taxa and strains

| Species (strain number, date, location, collector) | Substrate | GenBank number |
|---|---------------------------------------|----------------|
| <i>Phellinus alni</i> (Bond.) Parmasto: | | |
| 83-1022, 22.10.1983, Germany, IN ¹ | <i>Alnus incana</i> (L.) Moench | AY340036 |
| TW 162, 3.7.1995, Germany, TW | <i>Laburnum anagyroides</i> Med. | AY340035 |
| TW 322, 6.8.1995, Germany, TW | <i>Betula</i> L. | AY340041 |
| TN 3301, 5.10.1985, Finland, TN | <i>Betula</i> | AY340040 |
| 88-57, 7.5.1988, USA (WA), MF | <i>Alnus rubra</i> Bong. | AY340038 |
| 89-828a, 28.8.1989, Estonia, AB | <i>Alnus</i> Mill. | AY340037 |
| 94-1120, 20.11.1994, CSFR, MF | <i>Betula</i> | AY340042 |
| <i>Phellinus laevigatus</i> (Fr. : Karst.) Bourd. & Galz.: | | |
| 83-912, 12.9.1983, Germany, MF | <i>Betula pendula</i> Roth | AY340051 |
| 89-119, 9.11.1989, Germany, MF | <i>Betula</i> | AY340053 |
| TN 3260, 3.9.1985, Finland, TN | <i>Betula pubescens</i> Ehrh. | AY340055 |
| TN 5769, 5.7.1994, Finland, TN | <i>Betula</i> | AY340054 |
| Dai 2274, 7.8.1997, China, YCD | <i>Betula</i> | AY340052 |
| Dai 2930, 6.9.1998, China, YCD | <i>Betula</i> | AY340056 |
| <i>Phellinus cinereus</i> (Niemelä) Fischer: | | |
| 85-917/1, 17.9.1985, Germany, MF | <i>Betula pubescens</i> | AY340048 |
| 86-IX/1, IX-1986, Germany, MF | <i>Betula pubescens</i> | AY340044 |
| 89-822a, 22.8.1989, Estonia, AB | <i>Betula</i> | AY340047 |
| 89-826a, 26.8.1989, Estonia, AB | <i>Tilia cordata</i> Mill. | AY340046 |
| TN 5747, 26.4.1994, Finland, TN | <i>Betula</i> | AY340043 |
| TN 5770, 5.7.1994, Finland, TN | <i>Betula pubescens</i> | AY340050 |
| 92-926b1, 26.9.1992, USA (MI), MF | <i>Betula</i> | AY340045 |
| 92-927, 27.9.1992, USA (MI), MF | <i>Betula</i> | AY340049 |
| 96-1012, 12.10.1996, Canada, SA | <i>Betula</i> | AY340039 |
| <i>Phellinus tremulae</i> (Bond.) Bond. & Boriss.: | | |
| 83-526, 26.5.1983, Germany, IN | <i>Populus tremula</i> L. | AY340067 |
| 90-822, 22.8.1990, Germany, MF | <i>Populus tremula</i> | AY340066 |
| 89-826b, 26.8.1989, Estonia, AB | <i>Populus tremula</i> | AY340064 |
| 88-621/I, 21.6.1988, USA (UT), MF | <i>Populus tremuloides</i> Michx. | AY340068 |
| 88-621/II, 21.6.1988, USA (UT), MF | <i>Populus tremuloides</i> | AY340065 |
| Dai 2352, 11.8.1997, China, YCD | <i>Populus</i> L. | AY340063 |
| <i>Phellinus lundellii</i> Niemelä: | | |
| 86-1125, 25.11.1986, Germany, NL | <i>Betula</i> | AY340059 |
| TN 5760, 8.6.1994, Finland, TN | <i>Alnus incana</i> | AY340060 |
| Dai 2684, 16.9.1997, Finland, YCD | <i>Betula</i> | AY340058 |
| 92-926a, 26.9.1992, USA (MI), MF | <i>Acer</i> L. | AY340057 |
| 92-926b, 26.9.1992, USA (MI), MF | <i>Acer</i> | AY340062 |
| 95-430, 30.4.1995, Germany, MB | <i>Fraxinus excelsior</i> L. | AY340061 |
| <i>Phellinus igniarius</i> (L. : Fr.) Quél.: | | |
| 85-625, 25.6.1985, Germany, MF | <i>Salix caprea</i> L. | AY340069 |
| Strains identified as <i>Fomitiporia robusta</i> (P. Karst.) Fiasson & Niemelä: | | |
| 91-262, 26.2.1991, USA (CA), MF | <i>Cercis occidentalis</i> Torr. | AY340005 |
| 91-42/2, 2.4.1991, USA (CA), JA, MF | <i>Prunus dulcis</i> (Mill.) DA Webb. | AY340003 |
| 91-42/3, 2.4.1991, USA (CA), JA, MF | <i>Prunus dulcis</i> | AY340004 |
| 91-319/1, 19.3.1991, USA (CA), MF | <i>Pittosporum</i> Banks ex Solander | AY340006 |
| 91-319/2, 19.3.1991, USA (CA) | <i>Cupressus macrocarpa</i> Hartweg | AY340017 |
| 96-515, 15.5.1996, Germany, LK | <i>Fraxinus excelsior</i> | AY340007 |
| 89-828c, 28.8.1989, Estonia, AB | <i>Quercus</i> L. | AY340018 |

TABLE I. Continued

| Species (strain number, date, location, collector) | Substrate | GenBank number |
|---|------------------------------------|----------------|
| Strains identified as <i>Fomitiporia punctata</i> (P. Karst.) Murrill: | | |
| 85-74, 4.7.1985, Germany, MF | <i>Salix caprea</i> | AY340015 |
| 87-511, 11.5.1987, Germany, WP | <i>Rhamnus cathartica</i> L. | AY340016 |
| 89-826c, 26.8.1989, Estonia, AB | <i>Sorbus aucuparia</i> L. | AY340014 |
| Dai 2727, 5.10.1997, Finland, YCD | <i>Sorbus aucuparia</i> | AY340013 |
| 01-77/4, 7.7.2001, USA (TN), MF | dead standing hardwood | AY340026 |
| 01-712/2, 12.7.2001, USA (TN), MF | dead lying hardwood | AY340031 |
| 01-1028, 28.10.2001, CAN, SA | <i>Acer rubrum</i> L. | AY340032 |
| <i>Fomitiporia mediterranea</i> M. Fischer: | | |
| K9/9, II-2000, Germany, IM | <i>Vitis vinifera</i> (DC.) Beg. | AY340024 |
| 45/23.3, VIII-2001, Germany, MF | <i>Vitis vinifera</i> | AY340034 |
| 940417.3, IX-1994, Italy (Liguria), OH | <i>Olea europaea</i> L. | AY340027 |
| LM1, X-1998, Italy, LM | <i>Vitis vinifera</i> | AY340023 |
| LM2, X-1998, Italy, LM | <i>Vitis vinifera</i> | AY340022 |
| TW7.98a, VII-1998, Italy, TW | <i>Acer negundo</i> L. | AY340029 |
| TW7.98b, VII-1998, Italy, TW | <i>Lagerstroemia indica</i> L. | AY340030 |
| EK1, V-2001, Greece, EK | <i>Olea europaea</i> | AY340021 |
| EK2, V-2001, Greece, EK | <i>Actinidia chinensis</i> Planch. | AY340025 |
| GP1, 10.5.2001, Slovenia, GP | <i>Laurus nobilis</i> L. | AY340033 |
| Strains identified as <i>Fomitiporia hartigii</i> (Allesch. & Schnabl) Fiasson & Niemelä: | | |
| 84-811, 11.8.1984, Germany, MF | <i>Picea abies</i> (L.) Karst. | AY340012 |
| 88-58, 8.5.1988, USA (WA), MF | standing dead conifer | AY340019 |
| 01-1117, 17.11.2001, CAN, SA | <i>Tsuga canadensis</i> (L.) Carr. | AY340028 |
| 83-IXa, IX-1983, Japan, HP | <i>Abies firma</i> Siebold & Zucc. | AY340001 |
| 83-IXb, IX-1983, Japan, HP | <i>Abies firma</i> | AY340002 |
| Dai2148, 16.9.1995, China, YCD | <i>Abies</i> Mill. | AY340020 |
| Strains of uncertain affinity: | | |
| 91-42/I, 2.4.1991, USA (CA), JA, MF | <i>Salix hindsiana</i> Benth. | AY340008 |
| 91-42/II, 2.4.1991, USA (CA), JA, MF | <i>Salix hindsiana</i> | AY340009 |
| 91-42/III, 2.4.1991, USA (CA), JA, MF | <i>Salix hindsiana</i> | AY340010 |
| 91-42/V, 2.4.1991, USA (CA), JA, MF | <i>Salix hindsiana</i> | AY340011 |

¹JA James Adaskaveg, SA Serge Audet, MB Manfred Binder, AB Andreas Bresinsky, YCD Yu-Cheng Dai, MF Michael Fischer, OH Ottmar Holdenrieder, EK Elena Kalomira, LK Lothar Krieglsteiner, NL Norbert Luschka, IM Ingo Morgenstern, LM Laura Mugnai, TN Tuomo Niemelä, IN Ingo Nuss, WP Wolfgang Paulus, GP Gregor Podgornik, HP Hansjörg Prillinger, TW Tobias Wagner.

netic and biological species recognition).—Within *Phellinus* s.str., the overall size of the ITS region ranged between 636 bp (strains of *Phellinus laevigatus*) and 670 bp (strain 89-826a, *P. cinereus*); within *Fomitiporia*, size was between 702 bp (strain 01-712/2, morphologically identified as *F. punctata*) and 749 bp (strain 91-42/III, morphologically close to *F. punctata*). The size of the 5.8S rRNA gene was constantly 170 bp for all taxa; length variations of the overall size were due to several small indels in the ITS1 and ITS2 regions.

The mode of reproduction was biparental unifactorial in all taxa belonging to *Phellinus* s.str. and in *F. mediterranea*, while it was uniparental in all other taxa of *Fomitiporia*. No data are available for North American strains morphologically identified as *F.*

punctata. As an exception within *Phellinus* s.str., no pattern could be assigned to the strains of *P. tremulae* originating from the U.S.A.

The total length of the aligned *Phellinus* s.str. dataset is 674 characters; 21 positions are variable, and 75 positions are parsimony informative. *Phellinus igniarius* (strain 85-625, Germany) was used for rooting purpose. Parsimony analysis resulted in 180 equally most-parsimonious trees (127 steps, CI = 0.835, RI = 0.948). All MP trees were highly resolved and were identical to the tree obtained with ML ($-\ln L = 1789.20009$) using the GTR+G model of evolution (FIG. 1). Four main groups that were supported strongly by bootstrap values are assignable as *P. cinereus* (bootstrap = 91%), *P. laevigatus* (100%), *P. lundellii* (100%) and *P. tremulae* (100%). *Phellinus alni*

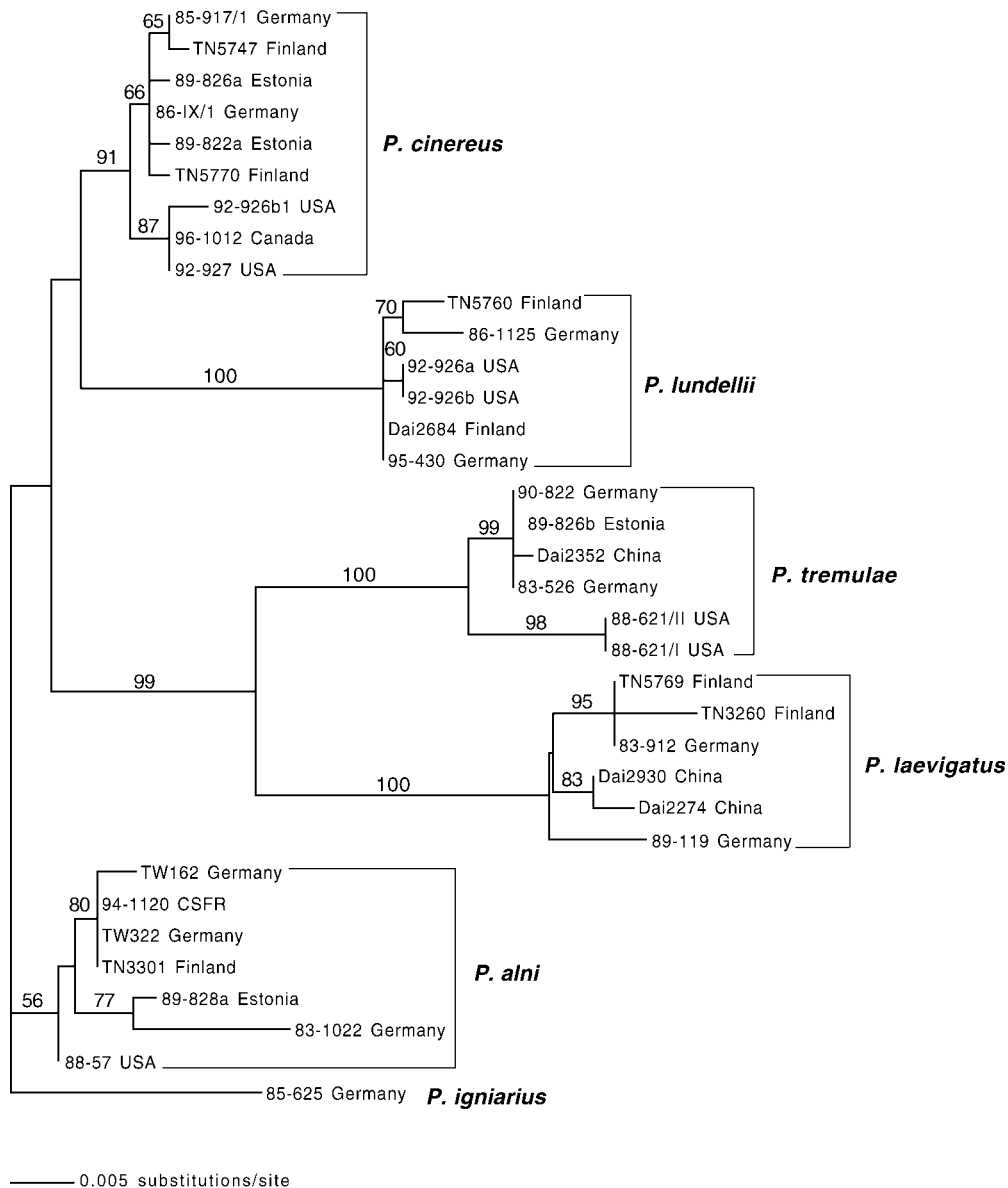


FIG. 1. Analysis of the *Phellinus* s.str. ITS dataset including 35 collections. The maximum likelihood tree (127 steps, $-\ln L = 1789.20009$) rooted with *P. igniarius* resolves five groups corresponding to recognized species. Bootstrap values greater than 50% are indicated above branches.

was supported weakly by 56%. *Phellinus lundellii* was the sister group of *P. cinereus*, and another pair was formed by *P. tremulae* and *P. laevigatus*. *Phellinus alni* has a basal position, next to the rooting group, *P. igniarius*.

Within groups, the North American collections were slightly divergent in *P. cinereus*, *P. tremulae* and *P. alni* while they were well integrated in *P. lundellii*. If available, such as in *P. tremulae* and *P. laevigatus*, no differentiation was noted between Chinese and European isolates.

Within *P. cinereus*, the North American strains, in addition to forming a separate subgroup (87%), also

are characterized by a shorter ITS region, with a size of 659–660 bp compared to 667–670 bp in European strains. Still, the pairing tests between single-spore testers showed that North American strains are mostly compatible with European strains. In spite of a wide variety of hosts and geographic origin, compatibility also was nearly complete within *P. lundellii* and *P. alni*. In the case of *P. lundellii*, the phylogenetic trees showed that North American strains are well integrated in the European strains, while considerable divergence, mainly caused by the position of strain 88-57 (U.S.A.), was evident in *P. alni*. Still, strains of *P. alni* were uniform with respect to the

overall size of the ITS region (657–658 bp); in *P. lundellii*, size of the ITS region was 653 bp for the European strains and 649 bp for the North American strains.

Phellinus tremulae was strongly supported as the sister group of *P. laevigatus* (bootstrap = 99%). Strains identified as *P. tremulae* form two subgroups, separating the North American collections (98% support) from European and Chinese collections (99% support). While compatibility is nearly complete within the subgroups, only negative pairings were obtained between the subgroups. The length of ITS sequences was 643 bp for all strains.

Some genetic divergence was observed in *P. laevigatus*, where collection 89-119 (Germany) is basal to the other strains, originating from Germany, Finland and Asia. One strain from China (Dai 2930) shows distinctly reduced compatibility with all other strains. Nevertheless, the sequence data place it within the other strains of *P. laevigatus*, next to another strain from China (Dai 2274), which was fully compatible with the European collections.

The aligned *Fomitiporia* dataset includes 800 positions, of which 75 are variable and 113 are parsimony informative. MP analysis revealed 10 000 equally most-parsimonious trees with a length of 259 steps (CI = 0.811, RI = 0.889) and tree topologies were largely congruent with the best tree (–lnL = 2771.13187) found in the ML analysis under the GTR+G model (FIG. 2), supporting seven lineage divergences. Four of the groups can be assigned to the accepted species, *F. punctata*, *F. robusta*, *F. mediterranea* and *F. hartigii*. Besides *F. mediterranea*, all *Fomitiporia* species in this study are uniparental and for this reason no additional data are provided by pairing tests.

MP and ML analyses support the presence of two distinct phylogenetic lineages in a morphologically conceptualized *F. punctata*. The *F. punctata* clade comprises collections from Europe and Canada (bootstrap = 100%), overall size of the ITS region is 716–720 bp. Strains 01-77/4 and 01-712/2, collected from hardwood trees in North Carolina and Tennessee respectively, are morphologically conspecific with *F. punctata* but form a well separated clade (bootstrap = 100%). Size of the ITS region of this group (described below as *F. hesleri*) was the smallest observed within *Fomitiporia*, with 702 bp and 704 bp. *Fomitiporia mediterranea* was separated from *F. punctata* in an earlier study (Fischer 2002) and here is supported 100%. The ITS sequences in this group range from 739 bp to 746 bp.

The *F. robusta* clade (bootstrap = 100%) includes only two strains, 88-828c (Estonia) and 95-515 (Germany). The European-based *F. robusta* is the sister

group of *F. punctata* from Europe and Asia, with 85% support. A group of pileate specimens collected on a variety of deciduous and in one case coniferous trees, is morphologically conspecific with *F. robusta*. However, together with the resupinate to effused-reflexed collections of uncertain morphological affinity (see TABLE I) it formed a separate clade (bootstrap = 100%), combined by a considerable genetic homogeneity. All strains of this group (described below as *F. polymorpha*) originate from the western U.S.A. The divergence between European and U.S.A. collections also was evident in the size of the ITS region: in *F. robusta*, it was 725–726 bp, whereas it was 746–749 bp in the newly discovered group.

Genetic divergence between collections from Europe/Asia on one side and collections from North America on the other side was noted in *F. hartigii*. Four strains, two from Japan and one each from Germany and China do not form a distinct group and are delimited from two strains from Canada and the U.S.A. Still, the size of the ITS region was more homogenous here, with 738–743 bp for European and Asian strains and 740–741 bp for North American strains.

Taxonomic conclusions.—Pairing tests of single-spore testers and the phylogenetic data as shown in FIG. 1 suggest the existence of two separate taxa within the morphological species *P. tremulae*. However, taxonomic consequences for *P. tremulae* will be provided in a future study, based on a larger number of strains.

As shown in FIG. 2, at least two groups of strains of *Fomitiporia* could not be classified as known taxa. The first group, comprising two strains from southeastern U.S.A., is morphologically identical to *F. punctata*, from Europe and China, but is well separated by the molecular data. The second group includes nine strains, all from the western U.S.A. These collections originally were recognized as *F. robusta* or as a taxon of uncertain affinity. Also, this group formed a well-separated extra clade. Therefore, a specific taxonomic status, as *F. hesleri* and *F. polymorpha* respectively, is proposed for these groups:

***Fomitiporia hesleri* M. Fischer, sp. nov.**

Basidiomata perennia, resupinata; superficies pororum luteobrunnea ad brunnea, pori circulares, 4–7 in quoque millimetro; systema hypharum dimiticum, omnia septa fibulis egentia; hyphae skeletales brunneae, 2.5–5.0 μm latae, hyphae generativae hyalinae, septatae, 2.5–4.0 μm latae; setae absunt; sporae subglobosae ad globosae, hyalinae, crassitunicatae, cyanophilicae et amyloideae, 6.5–7.5 \times 6–7 μm .

Holotypus 01-77/4 in REG, collectus a M. Fischer, in arbore frondifera in Foederatae Americae Septentrionalis Civitates, 2001.

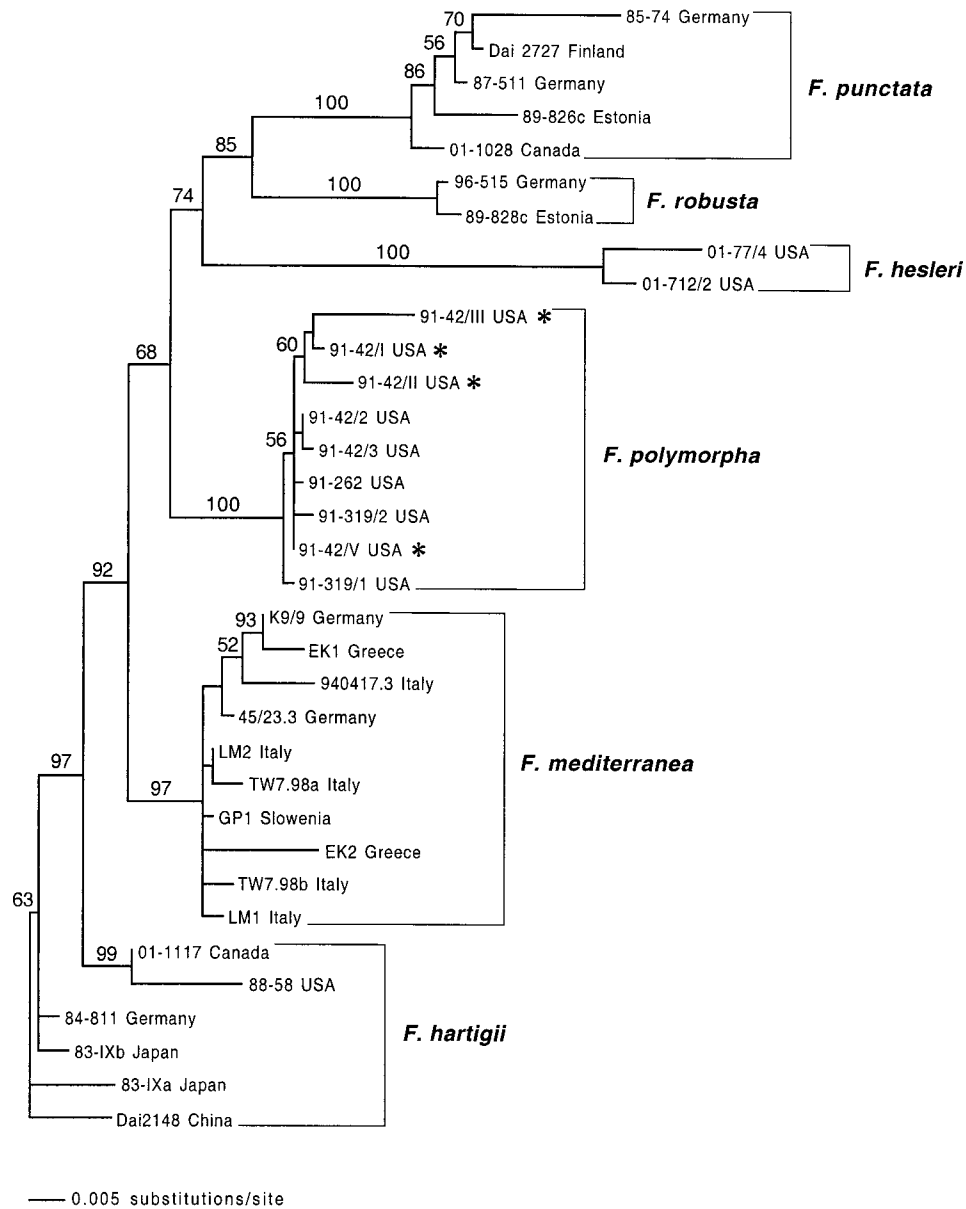


FIG. 2. Analysis of the *Fomitiporia* ITS dataset including 34 collections. The phylogenetic tree inferred using maximum likelihood (259 steps, $-\ln L = 2771.13187$) resolves *F. hesleri* and *F. polymorpha* as new groups in *Fomitiporia*. Collections that remained of uncertain affinity after using morphological characters for identification are followed by asterisks. Bootstrap values greater than 50% are indicated above branches.

Fruit bodies perennial, resupinate, inseparable, woody hard, light in weight when dry; up to 10 mm thick; sterile margin narrow, yellowish brown. Pore surface yellowish brown to pale brown; pores more or less circular, 4–7 per mm; dissepiments thin, entire. Context golden brown, corky, up to 3 mm thick; tubes concolorous with pore surface, up to 7 mm long, distinctly stratified. Hyphal system dimitic; septa without clamp connections; tissue darkening with KOH. Skeletal hyphae rust brown, rarely branched, aseptate, 2.5–4.0 μm wide; generative hyphae hyaline, thin-walled, frequently branched, simple sep-

tate, 2.0–3.5 μm wide; setae absent; spores subglobose to globose, hyaline, smooth, dextrinoid and cyanophilous, (6)6.5–7.5 \times (5.5)6–7(7.5) μm .

Fomitiporia polymorpha M. Fischer, sp. nov.

Basidiomata perennia, resupinata ad unguata; superficies pororum luteobrunnea ad brunnea, pori circulares, 4–7 in quoque millimetro; systema hypharum monomiticum vel dimiticum, omnia septa fibulis egentia; hyphae skeletales brunneae, 2.5–5.0 μm latae, hyphae generativae hyalinae, septatae, 2.0–3.5 μm latae; setae adsunt in hymenio, ventricosae, 18–57 \times 4–12 μm , brunneae; sporae ovoideae

vel subgloboae, hyalinae, crassitunicatae, cyanophilicae et amyloideae, $6.5\text{--}7.5 \times 5.5\text{--}6.5 \mu\text{m}$.

Holotypus 91-42/II in REG, collectus a M. Fischer et J. Adaskaveg, in *Salix hindsiana* in Foederatae Americae Septentrionalis Civitates, 1991.

Fruit bodies perennial, variable in shape, resupinate to effused-reflexed, sometimes pileate, inseparable, woody hard, up to $5 \times 5 \times 6 \text{ cm}$; upper surface in pileate specimens at first pale brown, becoming blackened and deeply rimose with age; margin rounded; pore surface pale brown to golden brown; pores circular, 4–7 per mm, dissepiments thick, entire. Context yellowish brown to rusty brown, up to 3 cm thick; tube layers stratified; tubes slightly darker than pore surface, up to 15 mm long. Hyphal system monomitic to dimitic; septa without clamp connections; tissue darkening with KOH. Skeletal hyphae golden brown, rarely branched, aseptate, $2.5\text{--}5.0 \mu\text{m}$ wide; generative hyphae hyaline, thin-walled, frequently branched, simple septate, $2.0\text{--}3.5 \mu\text{m}$ wide; setae existing in the hymenium, rare or absent in some specimens, slightly ventricose, $18\text{--}57 \times 4\text{--}12 \mu\text{m}$, pale brown to rusty brown; spores broadly ellipsoid to subglobose, hyaline, thick-walled, smooth, dextrinoid and cyanophilous, $(6.0)6.5\text{--}7.5(8.0) \times (5.0)5.5\text{--}6.5(7.0) \mu\text{m}$.

DISCUSSION

After an extended period of molecular and pairing test data studies, the vast majority of fungal taxa on species level still are defined by traditional characters, taken from morphology and anatomy (morphological species recognition). Additional application of biological and phylogenetic species recognition might lead to different results in terms of number, biogeography and, in case of lignicolous fungi, host-pathogen relationships of such taxa. While far-reaching results can be obtained by using morphological characters (for *Phellinus* s.l., best illustrated by Niemelä's studies in the 1970s), they are of limited value in cases of taxa exhibiting wide geographic distribution and host range. Using an integrative approach in the present paper, it was shown that, out of the morphological species *Phellinus igniarius*, *P. laevigatus*, *P. lundellii*, *P. tremulae*, *Fomitiporia punctata*, *F. hartigii* and *F. robusta*, at least three—*P. igniarius*, *F. punctata* and *F. robusta*—represent species complexes (TABLE II). While the number of species with cosmopolitan or Northern Hemispheric distribution has been reduced, several previously unknown species have been detected, which morphologically are hardly separable from their close relatives. These cryptic taxa, forming a system of vicariant species with their relatives, are detectable after the application of phylogenetic and/

or biological species recognition and are distinct by host preferences and geographic distribution.

A clear agreement between the morphological species recognition on one side and phylogenetic as well as biological species recognition on the other was observed only in few cases. For instance, in *P. lundellii* and, to a lesser extent, *P. laevigatus*, DNA data and pairing tests supported the conspecificity of strains originating from different geographic regions throughout the Northern Hemisphere. The majority of the morphological species, however, appeared less uniform, particularly so in *P. igniarius*, *Fomitiporia punctata* and *F. robusta* and less so in *P. tremulae* and *F. hartigii*. In these taxa, the sole application of the morphological species recognition often does not reflect the actual taxonomic state of the specimens investigated. *Phellinus cinereus* and *P. alni* have been considered subspecies or synonyms of *P. igniarius* until recently (see Gilbertson and Ryvarden 1987, Larsen and Cobb-Pouille 1990, Ryvarden and Gilbertson 1994), but they are, in fact, well-defined species (Niemelä 1975, Parmasto 1976, Fischer, 1995, Fischer and Binder 1995), clearly separated from the morphologically widely identical *P. igniarius* by DNA data and host range. With the data at hand, *P. igniarius* is a European species restricted to *Salix* while *P. alni*, occurring on a wide range of hardwood trees, and *P. cinereus*, nearly exclusively on *Betula*, are Northern Hemispheric taxa. In *P. tremulae*, molecular divergence among morphologically conspecific strains was supported by pairing tests, showing intersterility between European and North American collections, even though all collections were from *Populus*. It is interesting to note that unifactorial mating behavior was observed for European *P. tremulae* (Fischer 1987) whereas at least some North American collections seem to be bifactorial (Mallett and Myrholm 1995).

Fomitiporia punctata turns out to be a tangling species complex, in which genetic divergence occurred without an indication of perceptible morphological changes. For example, *F. mediterranea* collections until recently were identified as *F. punctata* using morphological characters (Cortesi et al 2000). Phylogenetic species recognition as well as the different mode of reproduction allow distinction among the species. Yet another lineage was found in *F. punctata*, which is described here as a new species, *F. hesleri*. A similar weakness becomes evident when morphological species recognition is applied to *F. robusta*. On the basis of the present data, the species seems restricted to Europe, where it occurs mostly on oak trees (Jahn 1963). North American counterparts, mostly classified as *F. robusta* by traditional means, were assigned to a new species, *F. polymorpha*. When compared with *F. robusta*, *F. polymorpha* is character-

TABLE II. Geographic distribution and host range of taxa of *Phellinus* s.str. and *Fomitiporia* as defined by

| Taxon: | Morphological species recognition | Phylogenetic species recognition | Biological species recognition |
|---------------------------------|--|--|---|
| <i>Phellinus ignitarius</i> | Northern Hemisphere; hardwood | Europe; <i>Salix</i> | Europe; <i>Salix</i> |
| <i>Phellinus alni</i> | see <i>P. ignitarius</i> | Northern Hemisphere; hardwood (not <i>Salix</i>) ¹ | Northern Hemisphere; hardwood (not <i>Salix</i>) ¹ |
| <i>Phellinus cinereus</i> | see <i>P. ignitarius</i> | Northern Hemisphere; mostly <i>Betula</i> | Northern Hemisphere; mostly <i>Betula</i> ¹ |
| <i>Phellinus larvigatus</i> | world wide; mostly <i>Betula</i> | Northern Hemisphere; mostly <i>Alnus</i> , <i>Betula</i> | Northern Hemisphere; mostly <i>Alnus</i> , <i>Betula</i> ² |
| <i>Phellinus lundellii</i> | Northern Hemisphere; mostly <i>Alnus</i> , <i>Betula</i> | Northern Hemisphere; mostly <i>Alnus</i> , <i>Betula</i> | Northern Hemisphere; mostly <i>Alnus</i> , <i>Betula</i> ¹ |
| <i>Phellinus tremulae</i> | Northern Hemisphere; <i>Populus</i> | Europe; <i>Populus</i> ³ | Europe; <i>Populus</i> ³ |
| <i>Fomitiporia punctata</i> | world wide; hardwood | Northern Hemisphere; hardwood | does not apply |
| <i>Fomitiporia hartigii</i> | Northern Hemisphere; conifers | world wide ⁴ ; mostly <i>Abies</i> , <i>Picea</i> | does not apply |
| <i>Fomitiporia hesleri</i> | see <i>F. punctata</i> | North America; hardwood | does not apply |
| <i>Fomitiporia mediterranea</i> | see <i>F. punctata</i> | Europe; mostly <i>Vitis</i> | Europe; mostly <i>Vitis</i> |
| <i>Fomitiporia polymorpha</i> | no data | North America; mostly hardwood | does not apply |
| <i>Fomitiporia robusta</i> | world wide; mostly <i>Quercus</i> | Europe; mostly <i>Quercus</i> | does not apply |

¹ Compatibility not complete between strains from Europe and North America.

² Distinctly reduced compatibility of one strain from Asia.

³ North American strains form a separate group (not included here).

⁴ Collections from North America are of uncertain affinity.

ized by a much broader host range, including conifers and a distinct variability in the shape of fruit bodies. North American isolates also were found to be genetically divergent in *F. hartigii*, forming a separated group from the European and Asian collections in all phylogenetic analyses. Members of both geographic groups occur on conifers, and additional data are necessary to come to a taxonomic conclusion here.

As a tendency, the genetic divergence of morphological species was more distinct in uniparental taxa while biparental taxa came out as genetically more uniform. No outbreeding events occur in uniparental taxa, but they are favored by a biparental mode of reproduction connected with a multiple allelism of the mating-type factors. The biparental taxa enclosed in this study all are unifactorial (except for *P. tremulae* from U.S.A., for which no distinct pattern could be resolved), exhibiting a multiple allelism of the mating-type factor, *A*; so, genes, at least theoretically, may be dispersed over the whole range of distribution. In promoting gene flow, outbreeding tends to reduce the speciation rate and so may explain the wide geographic distribution of fungal species. In fact, biparental species such as *P. cinereus* or *P. lundellii* have been shown as widely distributed in this study, but essentially the same is true for uniparental species such as *F. hartigii* or *F. punctata*.

In biparental taxa, a strong correlation was observed between biological and phylogenetic species recognition. In *P. alni* and *P. cinereus*, a slightly reduced compatibility between European and North American collections was reflected by the ITS data, resulting in two subgroups for both taxa. In *P. tremulae*, the subgroup formed by the North American isolates was intersterile with the strains collected in Europe and Asia. An exception was noted only for *Phellinus laevigatus*, where intersterility of one strain (Dai 2930, China) precedes a distinct differentiation of the molecular characters. In this way it might be speculated that some taxa will be discernable only after using biological species recognition while they remain undetected in phylogenetic trees based on ITS sequence data.

In several recent studies, results of pairing tests were found to be in accordance with molecular data and so are thought to provide a useful tool in defining species, especially in lignicolous fungi, which are relatively easy to culture (Anderson and Ullrich 1979, Boidin 1986, Anderson and Stasovski 1992, Fischer 1995, Methven et al 2000). Still, they are of limited value in homothallic species (Parmasto 1985) and provide little if any indication of phylogeny.

It has been speculated (Fiasson and Niemelae 1984, Fischer 1996, Wagner and Fischer 2001) that

taxa belonging to *Fomitiporia* are younger phylogenetically than those belonging to *Phellinus* s.str. In this way, an expansion of the ITS region might be correlated with a more derived character of certain groups within *Phellinus* s.l. This idea would be in accordance with several characters taken from anatomy, nuclear behavior and reproduction (Fischer 1996, Wagner and Fischer 2001, 2002).

While *Phellinus* s.str. and *Fomitiporia* can be distinguished readily by the overall size of the ITS region, its use as a diagnostic tool in separating species is of limited value. On one side, length variations in the morphological species *F. punctata* and *F. robusta* are clearly linked to the existence of different lineages within these taxa; they also indicate a different geographic origin of strains in *P. cinereus*. On the other side, however, length of the ITS region combines the otherwise distinct species *P. igniarius* and *P. tremulae* (both 643 bp long) as well as *F. hartigii* (738–743 bp) and *F. mediterranea* (739–746 bp).

It was shown in this study that morphological plasticity may differ widely among closely related species. For instance, the shape of fruit bodies is strictly resupinate in *F. punctata* and *F. mediterranea* while it ranges from resupinate to effused-reflexed and pileate in *F. polymorpha*. The possible phylogenetic tendencies concerning fruit body morphology in polypores (i.e., morphological transformation from resupinate to pileate or vice versa) have been discussed at length (Parmasto 1985, Ryvardeen 1991). Collections belonging to *F. polymorpha*, representing several types of fruit bodies, clearly show that morphological transitions may occur frequently and, possibly, in both directions.

Another striking phenomenon is the instability of the mode of reproduction within the morphological species *P. tremulae* and within *Fomitiporia*. While a uniparental pattern is prevalent in the latter group, one species, *F. mediterranea*, is biparental unifactorial. Perhaps there is only a slight difference between uniparental and biparental in basidiomycetous fungi. For example, biparental isolates have been converted to uniparental ones in *Schizophyllum commune* (Parag 1962, Raper et al 1965) and *Coprinus lagopus* (Day 1963). Biparental unifactorial, biparental bifactorial and uniparental taxa have been detected within the *Sistotrema brinkmannii* complex (Lemke 1969, Hallenberg 1984). Usually the shift between reproductive modes has been explained as a reduction, leading from the biparental to the uniparental state. In the case under study here, however, this idea hardly can be confirmed by any of the data available.

All in all, the data in this study demonstrate that the systematic tools available nowadays should allow for a precise refinement of fungal taxa at the species

level in terms of biogeography, reproduction and, if applicable, host range. Such taxa, defined by using different species recognition modes side-by-side, are more likely to reflect the actual taxonomic state of its individual members. While the number of species showing a wide distribution has been reduced in this study, several previously unknown taxa were revealed. Appropriate studies in other fungal groups, especially in geographic areas not taken into account in the original description, most likely will result in a further increase of species not easily separable from their relatives by traditional means.

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