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# Hybridization among cryptic species of the cellar fungus *Coniophora puteana* (Basidiomycota)

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**Abstract**

**In this study we have analysed the genetic variation and phylogeography in a global sample of the cellar fungus *Coniophora puteana*, which is an important destroyer of wooden materials indoor. Multilocus genealogies of three DNA regions (beta tubulin, nrDNA ITS and translation elongation factor 1α) revealed the occurrence of three cryptic species (PS1–3) in the morphotaxon *C. puteana*. One of the lineages (PS3) is apparently restricted to North America while the other two (PS1–2) have wider distributions on multiple continents. Interspecific hybridization has happened between two of the lineages (PS1 and PS3) in North America. In three dikaryotic isolates, two highly divergent beta tubulin alleles coexisted, one derived from PS1 and one from PS3. Furthermore, one isolate included a recombinant ITS sequence, where ITS1 resembled the ITS1 version of PS3 while ITS2 was identical to a frequent PS1 ITS2 version. This pattern must be due to hybridization succeeded by intralocus recombination in ITS. The results further indicated that introgression has happened between subgroups appearing in PS1. We hypothesize that the observed reticulate evolution is due to previous allopatric separation followed by more recent reoccurrence in sympatry, where barriers to gene flow have not yet evolved. A complex phylogeographical structure is observed in the morphotaxon *C. puteana* caused by (i) cryptic speciation; (ii) the interplay between natural migration and distribution patterns and probably more recent human mediated dispersal events; and (iii) hybridization and introgression.**

*Keywords*: cellar fungus, *Coniophora puteana*, cryptic speciation, hybridization, introgression, phylogenetic species recognition

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## Introduction

Fungi often have complex and unpredictable population structures and phylogeographical patterns due to their highly variable life history characteristics (Anderson & Kohn 1998). For example, some fungi are clonally spread, others sexually, while others disperse both with meio- and mitospores during their life cycle. Human activities may also influence the population structure of fungi, for example by human-mediated long-distance dispersal events (e.g. Brasier & Buck 2001). The occurrence of unknown intersterility barriers and cryptic speciation has been demonstrated to be very common phenomena in fungi, adding an extra layer of complexity to the analysis of population structure in fungi. Recent studies contain numerous examples of fungal morphospecies wherein reproductively and genetically isolated subgroups (cryptic species) occur, often in sympatry (e.g. Koufopanou & Taylor 1997; O'Donnell *et al*. 2000; Dettman *et al*. 2003a; Nilsson *et al*. 2003; Kauserud *et al*. 2006). Taylor *et al*. (2000) have advocated analyses of multiple unlinked genes as a criterion to identify phylogenetic species within fungi. In such 'phylogenetic species recognition', transition

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from concordance to conflict between independent gene tree topologies determines the limits of species.

Another factor that may obscure population histories is reticulate evolution caused by hybridization or introgression. Hybridization has long been known as a common phenomenon in plants, but is recognized much less widely in fungi (reviewed by Brasier 2000; Schardl & Craven 2003). However, in recent years evidence has accumulated that interspecific hybrids may occur in all fungal phyla. In Ascomycota, rare interspecific hybrids have been detected of the two Dutch elm disease pathogens *Ophiostoma ulmi* and *O. novo-ulmi* (Brasier *et al*. 1998). A form of parasexual hybridization has been shown to occur frequently between the asexual grass endophytes of *Neotyphidium* and their sexual relatives of *Epichlöe* (Tsai *et al*. 1994). In Basidiomycota, rare interspecific hybrids have been detected in the genera *Heterobasidion* (Garbelotto *et al*. 2004) and *Flammulina* (Hughes & Petersen 2001). Hybridization may lead to limited introgression of genetic material between lineages, as in *O. novo-ulmi* (Brasier *et al*. 1998), or to the establishment of more stable hybrids, as has happened in the rust fungi *Melampsora* (Newcombe *et al*. 2000).

In this study, a global sample of the cellar fungus *Coniophora puteana* (Schum. Fr.) P. Karsten is analysed genetically using multilocus sequencing. *Coniophora puteana* is characterized by its production of thin 'paint-like' brownish fruit bodies on decaying wood. The fruit bodies of *C. puteana* can be variable in colour and thickness but, according to Ginns (1982), much of this variation is just a reflection of the age of the fruit body. As its popular name indicates, *C. puteana* often occurs indoors and, together with the dry rot fungus *Serpula lacrymans*, it is the most severe fungal destroyer of indoor wooden constructions. Due to its importance as a wood destroyer, *C. puteana* has been used for nearly 70 years as a test fungus for wood preservatives in Europe. In nature, *C. puteana* is distributed worldwide on various types of dead wood, mainly coniferous (Ginns 1982). Different varieties within *C. puteana* have earlier been described based on morphological characteristics. However, in his monograph, Ginns (1982) accepted only the two varieties, var. *puteana* and var. *incrustata*, of which the latter only included a single collection from South Africa. Var. *puteana* is described as a cosmopolitan occurring on a variety of tree species, especially conifers (Ginns 1982). Employing mating experiments, multiple breeding units have been observed in *C. puteana* (Ainsworth 1987; Ainsworth & Rayner 1990), indicating that intrinsic barriers to gene flow exist within the morphotaxon.

The aims of the present study were to (i) explore the population structure of *C. puteana*; (ii) reveal whether cryptic species occur; and (iii) analyse phylogeographical patterns in a global sample of the fungus. For these purposes, we used a multilocus DNA sequencing approach, including three independent DNA loci (beta tubulin, translation elongation factor 1α and nrDNA ITS).

## Materials and methods

### *Material*

A total of 69 isolates of *Coniophora puteana* were included in this study (see Table 1). Fifty-one isolates were dikaryotic (each cell including two haploid nuclei), two monokaryotic (each cell including one haploid nuclei) and 16 had an unknown ploidy level (dikaryotic mycelia of *C. puteana* rarely produce clamps in culture and it can therefore be difficult to assess whether isolates have a monokaryotic or dikaryotic state). One isolate of the sister taxon *C. olivacea* (from West Australia) was used as an outgroup in the phylogenetic analyses.

### *Molecular analyses*

DNA was extracted from all isolates using the 2% CTAB miniprep method described by Murray & Thompson (1980) with minor modifications: DNA was resuspended in 100 μL $dsH_2O$ at the final step of extraction, and DNA templates were diluted 50 fold before PCR amplification. Three different DNA regions were PCR amplified from all isolates: the internal transcribed spacer (ITS) nrDNA region and parts of the beta tubulin (*tub*) and translation elongation factor (*tef*) 1α regions. PCR amplification was accomplished using the primers ITS4 and ITS5 (White *et al*. 1990) for the ITS region, primers B36F and B12R (Thon & Royse 1999) for the *tub* region, and primers EF595F and EF1160R for the *tef* region (Kauserud & Schumacher 2001). The *tub* and *tef* sequences included two partial and one complete exon and two introns (inferred by comparisons with data of previously published sequences of homologous genes of other fungi). PCR was performed in 30 μL reactions containing 17.5 μL 50 × diluted template DNA and 12.3 μL reaction mix (final concentrations: 4 × 250 mM dNTPs, 0.625 mM of each primer, 2 mM $MgCl_2$ and 1 unit DyNazyme™ II DNA polymerase [Finnzymes Oy, Espoo, Finland] on a Biometra thermocycler). The PCR amplification program was as follows: 4 min at 94 °C, followed by 37 cycles of 30 s at 94 °C, 35 s at 54 °C, 72 °C for 40 s, and a final extension step at 72 °C for 10 min before storage at 4 °C. PCR products were sequenced in both directions by MWG-biotech (Ebersberg, Germany) following their procedures. All sequence chromatograms were controlled manually and sequence alignments established in the program BIOEDIT (Hall 1999) by manual adjustments. As shown in Table 1, 75.3% of the sequences had one or more heterozygous sites (e.g. C/T = Y).

In order to obtain haplotype phases of all sequences derived from dikaryotic isolates, and thus avoid composite heterozygous sequences obscuring the phylogenies, all sequences having two or more heterozygous sites ($n$ = 34) were cloned. In order to minimize the problem with recombinant sequences (cf. Popp & Oxelman 2001), new

**Table 1** Isolates included in the study and their geographical origin and substrate. Information about which lineage (PS1–PS3) the isolates belong to, the ploidy level of the isolates (n + n = dikaryons, n = monokaryon) and the number of heterozygous sequence positions observed in the various sequenced DNA regions, are provided

| | | No. of heterozygous sites | | | | | |
|---|---|---|---|---|---|---|---|
| Isolate | Group | ITS | *tub* | *tef* | Ploidy | Origin | Substrate |
| *C. olivacea* (#69) | Outgroup | 0 | 0 | 0 | — | Australia | *Eucalyptus marginata* |
| MUCL 44825 | PS1 | 0 | 0 | 1 | n + n | — | — |
| MUCL 30484 | PS1 | 2 | 0 | 0 | n + n | Belgium | House |
| MUCL 30543 | PS1 | 0 | 0 | 0 | — | Belgium | House |
| MUCL 30566 | PS1 | 0 | 7 | 1 | n + n | Belgium | House |
| MUCL 30744 | PS1 | 1 | 0 | 1 | n + n | Belgium | Forest, *Salix* sp. |
| MUCL 31020 | PS1 | 1 | 8 | 1 | n + n | Belgium | Church |
| MUCL 31046 | PS1 | 3 | 1 | 1 | n + n | Belgium | House |
| MUCL 31052 | PS1 | 2 | 0 | 0 | n + n | Belgium | Meadow |
| MUCL 34768 | PS1 | 2 | 1 | 0 | n + n | Belgium | House |
| MUCL 35230 | PS1 | 0 | 6 | 0 | n + n | Belgium | House |
| MUCL 39473 | PS1 | 1 | 6 | 1 | n + n | Belgium | — |
| MUCL 39475 | PS1 | 1 | 0 | 0 | n + n | Belgium | — |
| MUCL 39476 | PS1 | 1 | 0 | 1 | n + n | Belgium | — |
| MUCL 43730 | PS1 | 0 | 8 | 1 | n + n | Belgium | House |
| DAOM 21055 | PS1 | 0 | 10 | 0 | n + n | Canada: Brit. Col. | *Picea canadensis* |
| DAOM 17535* | PS1 | 0 | 0 | 0 | — | Canada: Manitoba | *Picea glauca* |
| DAOM 137697 | PS1 | 0 | 0 | 0 | — | Canada: Ontario | — |
| DAOM 145623 | PS1 | 0 | 0 | 0 | — | Canada: Ontario | Wood cabin |
| DAOM 137404 | PS1 | 0 | 0 | 0 | — | Canada: Quebec | House |
| DAOM 147445 | PS1 | 0 | 0 | 0 | — | Canada: Th. Bay | *Pinus* sp. |
| MUCL 1000 | PS1 | 3 | 0 | 1 | n + n | Germany | — |
| MUCL 11662 | PS1 | 1 | 0 | 1 | n + n | Germany | — |
| P 1 | PS1 | 2 | 0 | 1 | n + n | Germany | — |
| P 152 | PS1 | 2 | 0 | 1 | n + n | Germany | — |
| P 155 | PS1 | 0 | 1 | 1 | n + n | Germany | — |
| P 167 | PS1 | 2 | 0 | 1 | n + n | Germany | Forest |
| P 168 | PS1 | 1 | 0 | 0 | n + n | Germany | Forest |
| P 169 | PS1 | 0 | 1 | 0 | n + n | Germany | Forest |
| P 221 | PS1 | 2 | 0 | 1 | n + n | Germany | Forest |
| P 222 | PS1 | 0 | 1 | 0 | n + n | Germany | Forest |
| P 233 | PS1 | 4 | 1 | 0 | n + n | Germany | Building |
| P 234 | PS1 | 1 | 0 | 1 | n + n | Germany | Building |
| MUCL 28146 | PS1 | 1 | 0 | 1 | n + n | Netherlands | — |
| 45–654 | PS1 | 0 | 0 | 0 | — | Norway | Stump, Picea abies |
| 52–1094/8 | PS1 | 0 | 7 | 0 | n + n | Norway | Forest, Pinus sylvestris |
| 59–1913/4 | PS1 | 0 | 0 | 1 | n + n | Norway | Forest, Pinus sylvestris |
| 76–77/2 | PS1 | 0 | 0 | 0 | — | Norway | *Betula* sp. |
| 82–97/3† | PS1 | 0 | 1 | 0 | n + n | Norway | Pinus sylvestris |
| DAOM 196038 | PS1 | 1 | 1 | 2 | n + n | Russia: Irkutsk | *Larix siberica* |
| 178/3 | PS1 | 0 | 0 | 0 | n | Sweden | — |
| 180PS† | PS1 | 1 | 0 | 0 | n + n | Sweden | — |
| P 159 | PS1 | 1 | 1 | 0 | n + n | Sweden | Sawn timber |
| P 160 | PS1 | 0 | 0 | 0 | n | Sweden | — |
| 8 | PS1 | 0 | 0 | 0 | — | USA | Conifer |
| FP 100258* | PS1 | 0 | 0 | 0 | — | USA: Colorado | *Picea engelmanni* |
| FP 104403* | PS1 | 0 | 1 | 10 | n + n | USA: Maryland | Southern pine board |
| RLG-5410 | PS1 | 2 | 0 | 0 | n + n | USA: New York | *Pinus strobus* |
| FP 105938 | PS1 | 0 | 11 | 8 | n + n | USA: S. Dakota | *Pinus ponderosus* |
| MD 189 | PS1 | 3 | 0 | 1 | n + n | USA: Wisconsin | Southern yellow pine |
| S 31 | PS1 | 3 | 0 | 0 | n + n | USA: Wisconsin | *Quercus* sp. |
| DAOM 137908‡ | PS1 | 0 | 8 | 0 | n + n | USA: New York | Hardwood |
| NBRC 6275 | PS1 | 2 | 0 | 1 | n + n | Japan | — |

**Table 1** *Continued*

| Isolate | Group | No. of heterozygous sites | | | Ploidy | Origin | Substrate |
|---|---|---|---|---|---|---|---|
| | | ITS | *tub* | *tef* | | | |
| MUCL 30793 | PS2 | 0 | 0 | 0 | — | — | — |
| MUCL 30396 | PS2 | 0 | 0 | 0 | — | Belgium | Forest |
| DAOM 216046 | PS2 | 0 | 0 | 2 | n + n | Canada: Brit. Col. | — |
| DAOM 138703 | PS2 | 0 | 0 | 1 | n + n | Canada: Brit. Col. | — |
| DAOM 194147 | PS2 | 0 | 5 | 3 | n + n | Canada: Brit. Col. | *P. menziesii* pole |
| CCBAS 524 | PS2 | 0 | 0 | 0 | — | Czech: Brno | — |
| BamEbw-109 | PS2 | 0 | 0 | 0 | — | Germany | — |
| DAOM 52883* | PS2 | 0 | 0 | 0 | n + n | India: Uttar Prad. | *Pinus excelsa* |
| 81 | PS2 | 0 | 0 | 0 | — | New Zealand | Podocarpus spicatus |
| MUCL 20565 | SP2 | 0 | 0 | 0 | — | UK | Pole, *Cupressus* sp. |
| DAOM 137693 | PS3 | 0 | 0 | 0 | n + n | Canada: Ontario | *Prunus* sp. |
| DAOM 31271*§ | PS3 | 1 | 17 | 0 | n + n | Canada: Ontario | *Betula lutea* |
| # 309 | PS3 | 0 | 5 | 0 | n + n | Canada: Quebec | *Abies balsamea* |
| DAOM 198027§ | PS3 | 1 | 17 | 0 | n + n | Canada: Quebec | Hardwood |
| DAOM 102773§ | PS3 | 1 | 16 | 1 | n + n | Canada: Quebec | *Picea mariana* |
| FP 94445 | PS3 | 0 | 0 | 1 | n + n | USA: Main | On roots |
| DAOM 137779 | PS3 | 0 | 6 | 0 | n + n | USA: New York | *Fagus* sp. |

* = isolates/specimens inspected and designated to *Coniophora puteana* var. *puteana* by Ginns (1982).
† = dikaryon established from polyspore culture (i.e. inbred isolate).
‡ = hybrid isolate including a recombinant ITS sequence (see text).
§ = hybrid isolates including a composite tub sequence (see text).

PCR reactions employing the high fidelity enzyme Phusion™ (Finnzymes) were performed according to the manufacturer's directions. All PCR products were purified with QIAquick PCR Purification Kit (QiaGen) according to the manufacturers' instructions. Fragments were cloned with the TOPO TA Cloning ® kit (Invitrogen) using blue/white screening according to the manufacturer's manual. Positive colonies were subjected to direct PCR with the M13R primer using the same PCR conditions as described above, except that the annealing temperatures were increased by two degrees. The number of colonies sequenced from each isolate varied from three to eight, depending on the level of recombinant sequences obtained. The cloned sequences were compared with the original heterozygous sequence and two haplophase sequences were obtained from each heterozygous sequence, thus representing the two different nuclei in the dikaryotic isolates.

Sequences have been deposited in GenBank under the accession nos: AM293005–AM293074 (ITS), AM293103–AM293172 (*tef*), AM293183–AM293252 (*tub*) (original data sets including heterozygous sequences) and AM293075–AM293102 (ITS), AM293173–AM293182 (*tef*), AM293253–AM293282 (*tub*) (additional haplophase sequences obtained through cloning of heterozygous sequences possessing two or more heterozygous sites).

### *Alignments and phylogenetic inferences*

Primarily, three sequence alignments were established for the ITS, *tef* and *tub* sequences obtained through direct sequencing of PCR products obtained from the 69 isolates. The ITS data set consisted of 1139 characters, of which 76 characters were polymorphic. A few ITS sequences (see below) included a 430 bp insert in the ITS1 regions that was removed from further analyses. The *tef* and *tub* data sets included 547 and 423 characters, of which 53 and 41 were variable, respectively. The three alignments were also combined in a concatenated data set, thus including three partitions. Most systematists consider data partitions to be combinable if and only if they are not strongly incongruent with one another (cf. Huelsenbeck *et al*. 1996), which was not the case with our data. Partition homogeneity tests (Farris *et al*. 1994), as implemented in PAUP* version 4.02b (Swofford 1999) failed to detect significant incongruence between the three partitions (employing 10 replicated heuristic searches and otherwise default settings).

After cloning and re-sequencing isolates possessing two or more heterozygous sites, three corresponding haplophase data sets were established, each alignment including 136 sequences. The original sequences including only a single heterozygous site were separated into two haplotypes in the haplophase data sets. For example, in a DNA sequence

**Table 2** DNA divergence between the phylogenetic species PS1–PS3 in the tree analysed DNA loci measured as number of segregating sites, fixed and shared polymorphic sites, average number of nucleotide differences (k) and average number of nucleotide substitutions per site between populations (π). Hybrid sequences were discarded from the calculations. Numbers in brackets indicate proportions in percent

| | No. of segregating sites | Fixed differences | Polym. 1* Monom. 2 | Polym. 2† Monom. 1 | Shared differences | *k* | π |
|---|---|---|---|---|---|---|---|
| ITS | | | | | | | |
| PS1–PS2 | 38 | 1 (2.6) | 31 (81.6) | 5 (13.2) | 1 (2.6) | 4.1 | 0.006 |
| PS1–PS3 | 43 | 12 (27.9) | 28 (65.1) | 3 (6.9) | 0 | 16.1 | 0.028 |
| PS2–PS3 | 22 | 13 (59.1) | 6 (27.3) | 3 (13.6) | 0 | 14.6 | 0.026 |
| *tub* | | | | | | | |
| PS1–PS2 | 42 | 2 (4.8) | 25 (59.5) | 12 (28.6) | 3 (7.1) | 9.3 | 0.022 |
| PS1–PS3 | 50 | 11 (22.0) | 27 (54.0) | 10 (20.0) | 2 (4.0) | 19.1 | 0.045 |
| PS2–PS3 | 38 | 12 (31.6) | 14 (36.8) | 11 (28.9) | 1 (2.6) | 21.4 | 0.051 |
| *tef* | | | | | | | |
| PS1–PS2 | 43 | 17 (39.5) | 12 (27.9) | 12 (27.9) | 2 (4.7) | 25.8 | 0.047 |
| PS1–PS3 | 39 | 24 (61.5) | 13 (33.3) | 1 (2.6) | 1 (2.6) | 30.1 | 0.055 |
| PS2–PS3 | 45 | 30 (66.7) | 13 (28.9) | 1 (2.2) | 1 (2.2) | 33.6 | 0.061 |

* = Number of sites polymorphic in the first group compared and monomorphic in the second group.
† = Number of sites polymorphic in the second group compared and monomorphic in the first group.

('genotype') containing a 'Y' (= C/T), the two resulting haplotypes included either a 'C' or a 'T'. Sequences obtained from dikaryons without any heterozygous sites were also represented twice in the haplophase data sets. In those cases where the nucleic states of isolates were unknown and no heterozygous sites appeared in the sequences (otherwise indicating a heterozygous state), isolates were treated as dikaryons and represented twice in the haplophase data sets (this is because *C. puteana* isolates most often appear as dikaryons in culture collections).

Maximum parsimony (MP) analyses were performed in PAUP* version 4.02b (Swofford 1999), treating characters as unordered with equal weights and gaps as unknown. The heuristic search option, with the tree bisection–reconnection (TBR) branch swapping algorithm and the random addition sequence option with 100 replicates to find multiple islands, was employed for all searches for most parsimonious tree(s). All other settings were default. Bootstrap support for branching topologies (Felsenstein 1985) was determined with the same parameter settings using 1000 search replicates. No branch swapping was used on the concatenated data set and the ITS haplophase data set (due to limited computational power).

Estimates of molecular variation (nucleotide diversity per site and average number of nucleotide differences) and shared, fixed and unique number of mutations between lineages were calculated in DNASP 4.0 (Rozas *et al*. 2003).

## Results

Phylogenetic analyses of the initial ITS, *tef* and *tub* data sets revealed that three subgroups appeared in *Coniophora puteana* across the three gene trees, apparently reflecting three cryptic species. In Fig. 1, a phylogenetic tree derived from a concatenated data set is shown, where the different subgroups are depicted in different colours. The three groups are hereafter referred to as phylogenetic species 1–3 (PS1–3), extending the naming convention of Dettman *et al*. (2003a). Based on the current material, it seems as PS3 is restricted to North America while PS1 and PS2 have wider distributions on different continents. All three species occur in North America (Fig. 2) and PS1 and PS2 occur in sympatry in Europe as well. Within PS1, three subgroups appeared as well, two of them restricted to North America (Fig. 1). A low proportion of shared polymorphisms were observed among lineages when hybrid isolates (see below) were not taken into consideration (Table 2).

A high proportion of the isolates (75.3%) included one or more heterozygous sequence positions, which is expected in dikaryotic isolates of an outcrossing (recombining) fungus including intraspecific genetic variation. However, some of the sequences were extraordinarily heterozygous, including up to 17 heterozygous sites. This indicated that some of the dikaryotic isolates included highly divergent alleles (haplotypes). In order to separate the divergent alleles co-occurring in the heterozygous dikaryons, sequences including two or more heterozygous sites were cloned. In this way, three new haplophase data sets including 136 sequences were obtained. Most sequence variation, measured as nucleotide diversity (π), appeared in the *tef* data set (π = 0.024), and less in *tub* (π = 0.018) and ITS (π = 0.008).

As shown in Fig. 3, phylogenetic analyses of the three haplophase data sets largely divided the sequences into the same three main groups as in the previous analyses (the haplotypes are coloured according to the placement of isolates in Fig. 1). However, the phylogenetic analyses of

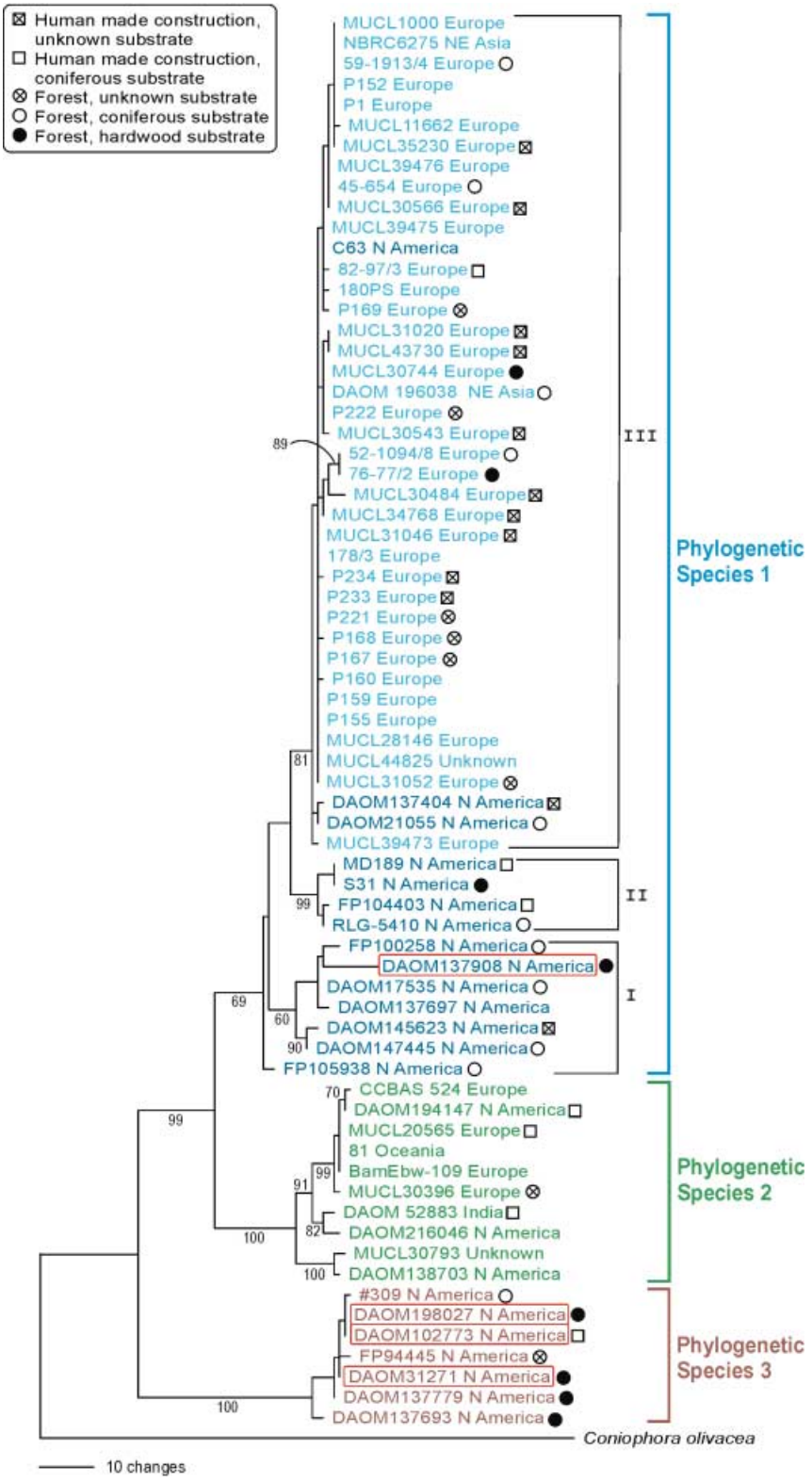


**Fig. 1** A phylogeny obtained using maximum parsimony analysis of the concatenated data set, including *tef*, *tub* and ITS sequences from 69 isolates of *Coniophora puteana*. One isolate of *C. olivaceae* was used as outgroup. Bootstrap support values (1000 replicates) are given below branches. The different symbols (circles or squares) indicate what type of substrate the isolates were obtained from (soft- or hardwood) and whether the isolates were derived from a natural environment (forest) or human made constructions. The 69 isolates of *C. puteana* grouped into three clades, depicted in different colours, representing the phylogenetic species (PS) 1–3 (see text). Dark blue colour is used for North American PS1 isolates. In PS1, three subgroups (I–III) appeared as well, two of them (I–II) with a strictly North American distribution. The red frames indicate interspecific hybrid isolates (see text).

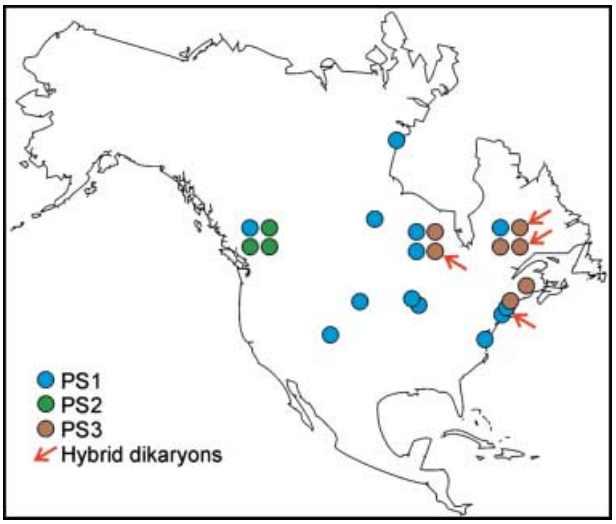


**Fig. 2** Approximate geographical distribution of the analysed North American isolates of *Coniophora puteana*. The three phylogenetic species appearing in *C. puteana* (PS1–3) are depicted in the same colours as in Fig. 1. Based on the included isolates, PS2 and 3 seem to have northwestern and northeastern distributions on the North American continent, respectively, while PS1 is more widespread. The red arrows pinpoint the four interspecific hybrid isolates (see text), all appearing in the region where PS1 and PS3 have overlapping distributions.

the haplotype data sets revealed that interspecific hybridization has happened between PS1 and PS3 in North America. In the *tub* phylogeny (Fig. 3c), three dikaryotic North American isolates (DAOM 102773, DAOM 31271 and DAOM 198027) included one typical PS3 allele in addition to one PS1 allele, resulting in highly heterozygous composite sequences with 16, 17 and 17 heterozygous sites, respectively (labelled in red in Figs 1 and 3c). Furthermore, the ITS sequence obtained from the North American isolate, DAOM 137908, represented a recombinant sequence between PS1 and PS3 (cf. Figure 3b). The ITS1 part resembled the ITS1 sequence of PS3 (although a few unique mutations also appeared), while the ITS2 part was identical with a frequent PS1 ITS2 haplotype (Fig. 4). A 430 bp insert occurred in the ITS1 region of the PS3 isolates, resulting in a highly elongated ITS region in this group (1139 bp). This very characteristic insert also occurred in the recombinant sequence of DAOM 137908. Thus, the recombinant sequence must have originated through a former hybridization event between PS1 and PS3, succeeded by intralocus recombination between the two divergent ITS sequences. Through cloning, only a single recombinant ITS sequence was obtained from DAOM 137908, indicating that the new recombinant sequence has been homogenized across the nrDNA repeats and that the isolate is not a F1 hybrid. The occurrence of a few unique mutations in the ITS1 region of DAOM 137908 (Fig. 4) also suggests that this hybrid is not a result of a very recent hybridization event. As shown in Fig. 2, PS1 and PS3 appear in sympatry in the northeastern part of North America, enabling hybridization to occur in this region.

The analyses of the concatenated data set (Fig. 1) indicated that three different subgroups appeared in PS1, two of them with a North American affiliation. The *tef* haplophase data set supported the existence of North American subgroups in PS1 (Fig. 3a). In addition to the above mentioned interspecific hybridization between PS1 and PS3, the two mentioned phylogenies indicated that introgression has happened between subgroups appearing in PS1. In the *tef* phylogeny, two dikaryotic isolates (FP104403 and FP105938) included two divergent alleles derived from the two North American subgroups (see Fig. 3a), resulting in composite sequences including 10 and eight heterozygous sites, respectively. Likewise, in the *tub* phylogeny, several largely North American alleles occurred in European dikaryotic isolates, and alleles with a distinct European affiliation were detected in American dikaryotic isolates, again leading to highly heterozygous sequences.

## Discussion

Using multilocus phylogenetic species recognition (PSR), three separate lineages (PS1–3) were uncovered among isolates ascribed to the cellar fungus *Coniophora puteana*, which is recognized as one of the most severe fungal destroyers of indoor wooden materials (Ginns 1982). Species delimitation by the PSR approach rests on the concordance of multiple gene genealogies from independent loci. In this context, a species is a group of organisms all of whose genes coalesce more recently with each other than with those of any organism outside the group. Although some reticulate evolution has happened between PS1 and PS3, somewhat obscuring the phylogenetic relationships (see below), the three lineages are consistently separated across the tree loci analysed (cf. Figures 1 and 3). When the hybrid isolates were not taken into consideration, a very low proportion of shared polymorphisms appeared among the three lineages (cf. Table 2), which also indicates a long lasting separation not compatible with a hypothesis of a recent split between the lineages. Employing the PSR approach, a number of recent studies have demonstrated that cryptic speciation is a very common phenomenon in fungi (e.g. Koufopanou *et al*. 1997; O'Donnell *et al*. 2000; Dettman *et al*. 2003a; Geml *et al*. 2006; Kauserud *et al*. 2006).

It seems that genetic isolation often precedes morphological differentiation in fungi, which could be explained by the fact that rather few morphological characters, largely only those associated with the fruit body, are available to distinguish between fungal taxa. It is worth noting that five specimens/isolates inspected by Ginns (1982) and classified as *C. puteana* var. *puteana* were included in our study. These five isolates distributed into the three cryptic species (cf.

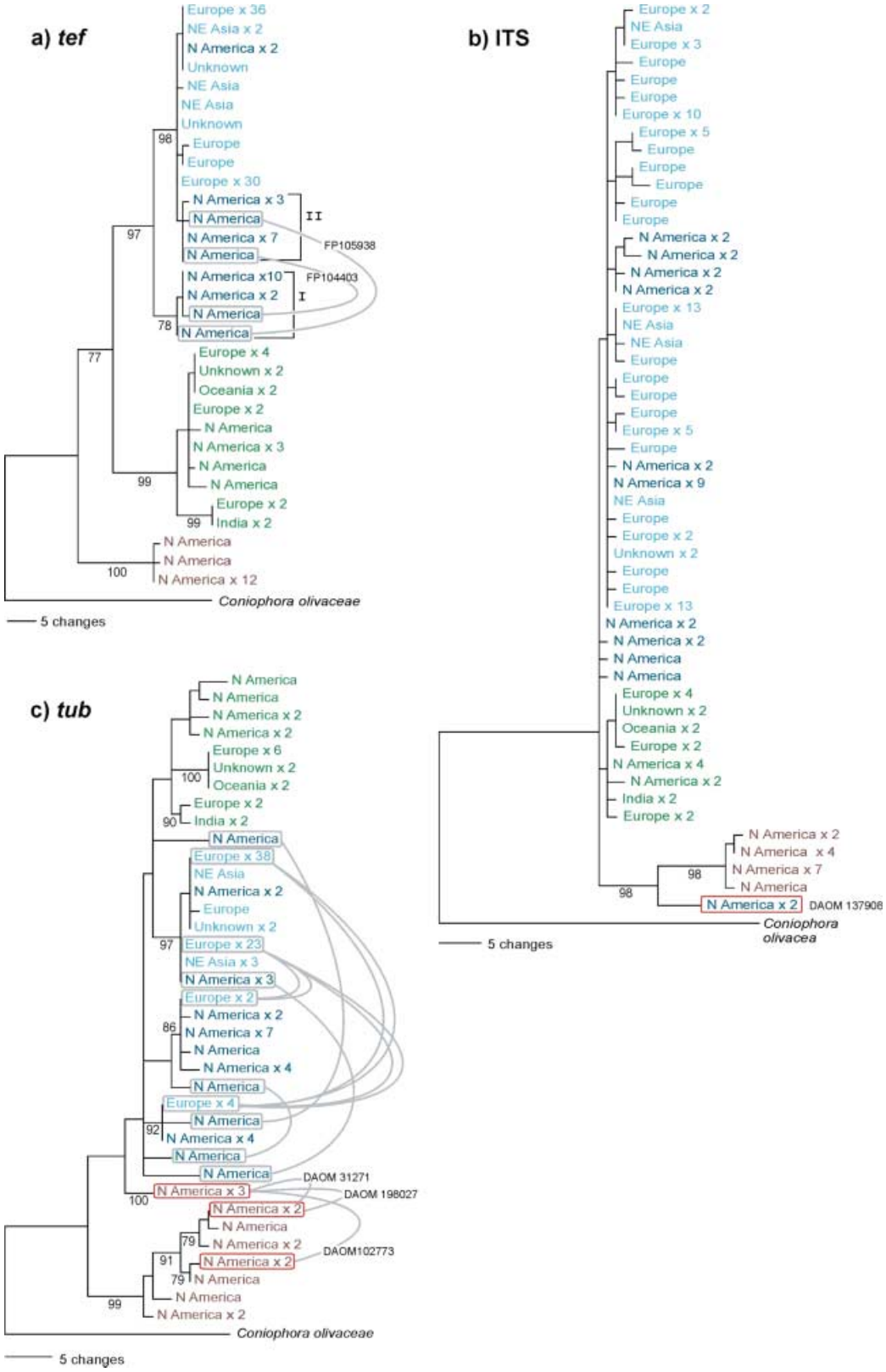


**Fig. 3** Phylogenies obtained through separate analyses (maximum parsimony) of the (a) *tef*; (b) ITS; and (c) *tub* haplophase data sets, each including 136 sequences of *Coniophora puteana*. One isolate of *C. olivaceae* was used as outgroup. Bootstrap support values (1000 replicates) are given below branches. The three phylogenetic species (PS1–3) are depicted in similar colours as in Figs 1 and 2. Identical sequences from the same geographical region are represented by a single sequence (and the numbers given behind reflect the number of sequences). Grey lines reflect dikaryotic isolates including two highly divergent haplotypes. Admixed isolates/sequences mentioned in the text are pinpointed by their isolate codes. Red frames mark interspecific hybrids. In the *tef* phylogeny, two North American subgroups referred to in the text are marked as I and II.

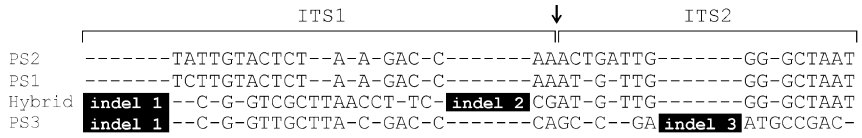


**Fig. 4** Alignment including variable sites appearing in the nrDNA ITS region. The alignment includes one sequence representing each phylogenetic species (PS1–3) in addition to the recombinant (hybrid) sequence from isolate DAOM 137908. The ITS1 part is similar to the ITS1 sequence of PS3 (although a few unique mutations also appear), while the ITS2 part is identical with a frequent PS1 ITS2 haplotype. The alignment demonstrates that an intralocus recombination event has happened between ITS1 and 2 (adjacent to the 5.8S region) leading to the recombinant hybrid sequence. Three lineage specific indels appear (indel 1 = 430 bp, indel 2 = 40 bp, indel 3 = 25 bp).

Table 1), which underlines that taxonomic discrimination based solely on morphological characteristics can be an intractable task in fungal taxonomy, even for an expert. In such cases, morphological diagnostic characters can possibly be more easily distinguished a *posteriori*, guided by the multilocus phylogenies. Our observation of three cryptic species in *C. puteana* corresponds fully with earlier documentation of three breeding units in *C. puteana* (Ainsworth 1987). Ainsworth observed one widespread breeding unit, one represented by an isolate from UK and one restricted to Eastern USA, apparently corresponding to PS1–PS3. Subsequently, one additional breeding unit was discovered (Ainsworth & Rayner 1990), but a fourth lineage is not recognized among our isolates.

Our data indicate that the *C. puteana* species complex originated in North America. Overall, most genetic variation appeared in this region, and the PS3 group, clustering basally in the phylogenies, is apparently endemic to North America. North American isolates also clustered basically in the PS1 group, corroborating the hypothesis that the species complex originated in this region. However, some reservations, arising from our limited sample size, must be voiced at this point. Northeastern Asia and Beringia, demonstrated to be important regions in other fungal taxa (e.g. Nilsson *et al*. 2003; Geml *et al*. 2006), are especially poorly represented in our sample. With the exception of the *tub* data set, limited genetic variation appeared in the PS1 group outside North America, indicating that the PS1 group has experienced a recent range expansion to Asia and Europe from North America. The results correspond well with a scenario where *C. puteana* reached Europe during the last few thousand years, after the last Weichselian glaciation.

In all the analyses, PS2 turned up as an independent monophyletic group not interfering with PS1 and PS3. This indicates that intersterility barriers probably have evolved between PS2 and the two other lineages. PS1 and PS2 occur in sympatry both in Europe and North America (Fig. 2), supporting the view that mating barriers have developed between these two lineages. On the other hand, it seems as PS2 and PS3 largely occur in allopatry in North America (cf. Figure 2) and we are therefore not yet able to conclude whether there exist fully developed intersterility barriers between PS2 and PS3. However, the mating experiments carried out by Ainsworth (1987) indicate that interesterility barriers have developed.

The observation of a recombinant ITS sequence in one isolate and *tub* alleles from both PS1 and PS3 shared in three other dikaryotic isolates clearly demonstrate that interspecific hybridization has happened between the two cryptic species PS1 and PS3 in North America. Generally, there is a greater possibility for hybridization when fungi spread beyond their original geographical ranges because fungi from the same geographical area often exhibit stronger pre- or postzygotic barriers to interspecific hybridization (cf. Korhonen *et al*. 1992; Dettman *et al*. 2003b). We hypothesize that PS1 and PS3 have had a separate evolutionary history in allopatry, possibly due to Pleistocene glaciations, succeeded by a more recent reintroduction in sympatry in northeastern North America enabling reticulate evolution.

Hybridization is a highly common phenomenon in plants, but only a handful of examples yet exist from the fungal kingdom, and to our knowledge, only a few examples from Basidomycota. Similar to the DAOM 137908 isolate of *C. puteana*, a recombinant ITS sequence was also observed in *Flammulina*, taken as evidence for an interspecific hybridization event (Hughes & Petersen 2001). The recombinant *Flammulina* sequence combined in the very same way ITS1 and ITS2 types from the two parent species (*F. velutipes* and *F. rossica*). We obtained only a single recombinant ITS variant through cloning from DAOM 137908, indicating that the novel ITS type has been homogenized across the nrDNA repeats, as was also found to be the case with the *Flammulina* sequence (Hughes & Petersen 2001). In hybrids between the rust fungi *Melampsora medusae* and *M. occidentalis*, isolates (dikaryons) included either a single or both parental ITS variants (Newcombe *et al*. 2000). Similarly, in *Trichaptum abietinum*, two highly divergent allelic nrDNA types were observed in the same populations (and sometimes in the same dikaryons), also taken as support for an earlier hybridization event (Kauserud & Schumacher 2003). In the *Gibberella fujikuroi* complex of *Fusarium*, which is a haploid ascomycete, two highly divergent non-orthologous ITS2 sequences appeared in the same isolates (at the intragenomic level), reflecting either an ancient interspecific hybridization or a gene duplication event (O'Donnell & Cigelnik 1997). Thus, in this case, no homogenization of the

different ITS repeats has happened, which indicates that there might be different outcomes concerning ITS homogenization succeeding a hybridization event. Such different evolutionary outcomes have also been observed in plant hybrids (e.g. Wendel *et al*. 1995).

In another study, it was shown that fungal hybrids might appear at very low frequencies (Brasier *et al*. 1998). After examining more than 11 000 isolates of *Ophiostoma ulmi* and *O. novo-ulmi*, nine hybrid isolates were detected between these two closely related taxa (eight of them from the same bark sample). However, although the *Ophiostoma* hybrids are rare, they might function as an important genetic bridge between the two species (Brasier *et al*. 1998). Different types of evidence indicated that these hybrids were unfit and probably transient. Thus, one cause why fungal hybrids rarely are detected could be due to low hybrid survival. Closely related fungi with the potential to hybridize are often adapted to different substrates with different essential qualities. Hybrids might therefore experience lower fitness and survival because the mixed genotypes are not well adapted to either of the two substrates. For example, saprotrophic species may either colonize hardwood or softwood trees and the hybrid may become a poor decayer and weak competitor on both types of substrate. In the *C. puteana* complex, 67% of the PS3 isolates (with known substrate affiliation) were derived from hardwood trees, while 82% of the North American PS1 isolates were derived from softwood substrates (cf. Table 1). Thus, it seems that a partial substrate differentiation has happened among these two lineages. In spite of the putative niche divergence, the hybrid DAOM 137908 (derived from a hardwood log) produced a vigorous fruit body. In his monograph, Ginns (1982) classified one of the other hybrids (DAOM 31271) as a typical *C. puteana* specimen, also indicating normal development and fruit body formation. In culture, the DAOM 137908 isolate showed very crippled growth that could indicate reduced fitness. However, crippled growth is not necessarily a good indication of fitness in a natural environment. Obviously, more experimental data must be assembled to draw conclusions on this topic.

Our data also indicate that introgression has happened between subgroups within PS1. This was most clearly demonstrated in the *tef* phylogeny, where two highly divergent alleles (both derived from two North American subgroups) co-occurred in two North American dikaryons (cf. Figure 3a). The *tub* phylogeny further indicates that introgression has happened between European and North American isolates; typical American alleles were present in European dikaryons and vice versa. This implies that recent long-distance dispersal events have taken place. It is documented that *C. puteana*, together with other wood-decay fungi, caused huge rot problems on sailing vessels in the 18th century (Ramsbottom 1937) and it might have dispersed worldwide in this way. Introgression between well-adapted ecotypes may lead to reinforcement of reproductive isolation if the resulting hybrids have reduced fitness. This phenomenon has been observed in *Heterobasidion annosum*, where sympatric S and F group pairings of isolates were 24% interfertile, whereas allopatric pairings were about 72% interfertile (Korhonen *et al*. 1992). The same relationship was observed among sympatric and allopatric isolates of *Neurospora crassa* (Dettman *et al*. 2003b). It seems obvious that reinforcement is not happening in PS1 but rather that the subgroups merge together.

From our data we can conclude that a complex phylogeographical structure is observed in the morphotaxon *C. puteana* caused by (i) cryptic speciation; (ii) the interplay between natural migration and distribution patterns and probably more recent human-mediated dispersal events; and (iii) hybridization and introgression. Our study supports the view that cryptic speciation is a highly common phenomenon in fungi and that interspecific hybridization potentially is also an important evolutionary mechanism among fungi.

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