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Multiple gene genealogies and species recognition in the ectomycorrhizal fungus *Paxillus involutus*

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ABSTRACT

Paxillus involutus (basidiomycetes, *Boletales*) is a common ectomycorrhizal fungus in the Northern Hemisphere. The fungus displays significant variation in phenotypic characters related to morphology, physiology, and ecology. Previous studies have shown that *P. involutus* contains several intersterility groups and morphological species. In this study, we have used concordance of multiple gene genealogies to identify genetically isolated species of *P. involutus*. Fragments from five protein coding genes in 50 isolates of *P. involutus* collected from different hosts and environments in Europe and one location in Canada were analysed using phylogenetic methods. Concordance of the five gene genealogies showed that *P. involutus* comprises at least four distinct phylogenetic lineages: phylogenetic species I (with nine isolates), II (33 isolates), III (three isolates), and IV (five isolates). The branches separating the four species were long and well supported compared with the species internodes. A low level of shared polymorphisms was observed among the four lineages indicating a long time since the genetic isolation began. Three of the phylospecies corresponded to earlier identified morphological species: I to *P. obscurosporus*, II to *P. involutus* s. str., and III to *P. validus*. The phylogenetic species had an overlapping geographical distribution. Species I and II differed partly in habitat and host preferences.

Introduction

Paxillus involutus is one of the most well-studied ectomycorrhizal (ECM) fungi. The fungus is widespread in the Northern hemisphere and forms ectomycorrhizal with numerous coniferous and deciduous tree species (Wallander & Söderström 1999). The unusually broad host range, relatively fast-growing mycelium, and rapid colonization of roots can explain why *P. involutus* is one of the most commonly used ECM fungi in laboratory experiments. Between 1996 and 2007, more than 300 research articles containing information on *P. involutus* were published (ISI Web of Science). For example, studies of this species have contributed to our understanding of the mechanisms of nutrient assimilation, carbon transfer, weathering, and heavy metal tolerance by ECM fungi. *P. involutus* is also an important model for molecular studies of the ECM symbiosis, and it was recently approved for genome sequencing by the US Department of Energy (DOE) and the Joint Genome Institute (JGI).

Several studies have reported a large variability in the morphology, physiology, and ecology between different isolates of *P. involutus* (e.g. Gafur *et al.* 2004; Laiho 1970). The large phenotypic variation raises the question whether *P. involutus* consists of several, genetically isolated species. Based on mating-type tests of specimen collections from the surroundings of Uppsala, Sweden, Fries (1985) reported the existence of three intercompatibility groups (biological species) in what was considered to be *P. involutus*. Sporocarps from the first group were mainly found in coniferous and deciduous forests (forest group), while those from the second and third groups

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were mainly found in park and garden areas (park groups). However, no distinct morphological differences were identified between sporocarps of these groups. Accordingly, it was suggested that the intersterility groups represented three cryptic species of *P. involutus* (Fries 1985). More recently, Hahn & Agerer (1999) made an extensive morphological and anatomical study of strains assigned to *P. involutus* collected in Europe and North America. Based on these data, it was proposed that the *P. involutus* species group should be divided into six morphological species: *P. involutus s. str.*, *P. validus*, *P. obscurosporus*, *P. vernalis*, *P. albidulus*, and *P. rubicundulus* (syn. *P. filamentosus*).

The division of *P. involutus* into several species has also been indicated by phylogenetic analyses of DNA sequence data generated from the nuclear encoded ITS region (Jarosch & Bresinsky 1999; Le Quéré *et al.* 2004). However, the support values for several clades were weak. Furthermore, data from a single locus cannot be used for delineating genetically isolated species (Avise & Wollenberg 1997). Taylor *et al.* (2000) have promoted the use of multiple gene genealogies to recognize boundaries of such lineages, referred to as phylogenetic species (PS). According to this approach, trees of multiple genes have the same topology due to fixations of previously polymorphic loci following genetic isolation. Conflicts among independent gene topologies can be caused by recombination between individuals within a species, and transition from concordance to conflict determines the limits of species (Taylor *et al.* 2000). This approach has been used to identify genetically isolated lineages and species in a number of different fungi including basidiomycetes (Johannesson & Stenlid 2003; Kauserud *et al.* 2006). Many of these studies have shown that the PS approach often recognizes additional genetically isolated species that have not been defined using mating tests (biological species) or phenotypic characters (morphological species) (Taylor *et al.* 2000; Taylor *et al.* 2006).

In this study, we have used concordance of multiple gene genealogies to identify genetically isolated species in *P. involutus*. Fragments from five protein coding genes in 50 strains of the *P. involutus s. lat.* group collected from different hosts and environments in Europe and one location in Canada were analysed.

Materials and methods

Isolates

The study included 50 strains of what was assumed to be *Paxillus involutus* collected in Sweden, several other countries in Europe and one from Canada (Table 1). In addition, two specimens of *P. filamentosus* (syn. *P. rubicundulus*) and herbarium specimens of *P. obscurosporus*, *P. rubicundulus* and *P. validus* were included. Isolates included in this study obtained as mycelium (Table 1) are kept in a culture collection at the Department of Microbial Ecology, Lund University.

PCR and DNA sequencing

DNA was extracted from fruit bodies (dried or frozen) or cultures using the E.Z.N.A.® Fungal DNA Kit (Omega Bio-Tek). Regions from five nuclear, protein-encoding genes previously isolated from the *Paxillus involutus* strain ATCC 200175 were amplified: actin (*actA*, GenBank accession number AY586027, 725 bp encompassing 482 bp exons and 243 bp introns), β-tubulin (*β-tubA*, AY586022, 569 bp encompassing 316 bp exons and 253 bp introns), glucose 6-phosphate isomerase (*gpiA*, AY585998, 526 bp encompassing 424 bp exons and 102 bp introns), small GTPase protein (*rabA*, AY585950, 801 bp encompassing 485 bp exons and 316 bp introns), and hydrophobin A (*hydA*, DQ646583, 463 bp encompassing 355 bp exons and 108 bp introns) (Le Quéré *et al.* 2006; Rajashekar *et al.* 2007). In addition, the ITS region encompassing ITS1, ITS2 and 5.8S rRNA (AY585913, 555 bp) was analysed.

The genes were PCR amplified using the primers in Table 2. PCR was performed in 25 μl reactions containing 19.4 μl dH_20, 0.1 μl Easy-A polymerase (5 U/μl; Stratagene), 2.5 μl 10× Easy-A buffer, 0.5 μl dNTPs (10 mM each), 0.5 μl of each primer (10 μM) and 1.5 μl of genomic DNA. Templates that did not amplify with the Easy-A enzyme, were amplified using the AmpliTaq Gold DNA polymerase (Applied Biosystems). The 20 μl reactions contained 13.3 μl dH_20, 2 μl 10× PCR buffer, 2.4 μl $MgCl_2$ (25 mM), 0.4 μl dNTPs (10 μM each), 0.08 μl Taq polymerase (5 U/μl), 0.4 μl of each primer (10 μM) and 1.0 μl genomic DNA. PCR and cycle sequencing reactions were performed in a GeneAmp PCR system 9700 thermocycler. Cycling conditions for Easy-A polymerase were as follows: 2 min at 95 °C followed by 30 cycles of 95 °C for 40 s, 54–60 °C for 30 s and 72 °C for 1.5 min, and a final 7 min extension step at 72 °C. Cycling conditions for Taq polymerase were as follows: 7 min at 94 °C followed by 31 cycles of 94 °C for 30 s, 54–60 °C (depending on gene) for 30 s and 72 °C for 2 min and 30 s, and then finally a 7 min extension step at 72 °C. Amplification products were electrophoresed in a 1 % agarose gel with ethidium bromide. PCR products were purified with isopropanol and used as templates for sequencing.

The amplification products were used as starting material for DNA sequencing using the Big Dye Terminator kit (Applied Biosystems) and template-specific primers (Table 2). A standard cycling protocol was followed (Applied Biosystems). The products were purified by ethanol precipitation and finally loaded onto an ABI 3100 DNA sequencer (Applied Biosystems). Overlapping sequences were aligned and trimmed using the Sequencher 3.1.1b4 program (Gene Codes Corporation). In the majority of the amplicons, both strands were sequenced.

Phylogenetic analysis

The sequences were aligned using MUSCLE (version 3.6) (Edgar 2004). The ends were manually trimmed and ambiguous sites were removed using JalView (Clamp *et al.* 2004). Initial analysis showed that the gaps in the alignment were phylogenetically un-informative and they were removed from the analyses. Maximum parsimony (MP) and statistical-parsimony genealogies were constructed using PAUP (Swofford 2002) and TCS v. 1.21 (Clement *et al.* 2000), respectively. Bayesian analyses were performed using MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003). The evolutionary model was selected by using the program MrModeltest v. 2.2 (Nylander JAA, 2004. MrModeltest v2. Program distributed by the author, Evolutionary Biology