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The *Paxillus involutus* (*Boletales*, *Paxillaceae*) complex in Europe: Genetic diversity and morphological description of the new species *Paxillus cuprinus*, typification of *P. involutus* s.s., and synthesis of species boundaries

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ABSTRACT

Paxillus involutus is a model species for ecological or physiological studies of ectomycorrhizal agaricomycetes. Three to six groups or species linked to it have been ecologically and morphologically distinguished. Phylogenetic studies have revealed the existence of four species in Europe: *Paxillus ammoniavirescens*, *Paxillus obscurisporus*, *P. involutus*, and a fourth as yet not described species. We studied 47 collections from 24 French and Italian locations, supplemented with GenBank data, in order to genetically and taxonomically delineate these species. Phylogenetic analyses of three nuclear DNA regions (rDNA internal transcribed spacer (ITS), *tef*1-α, and *gpd*) confirmed the four European species. Morphology, culture, and ecology features allowed us to delineate species boundaries and to describe the fourth species we named *Paxillus cuprinus* since it turns coppery with age. As there is no existing original herbarium specimen for *P. involutus*, one of our collections was chosen as the epitype. The low genetic diversity found in *P. cuprinus* correlates with stable morphological traits (basidiome colour, ovoid–amygdaliform spores with an apical constriction) and with ecological preferences (association with *Betulaceae* in open and temperate areas). In contrast, *P. ammoniavirescens* is characterized by a high genetic diversity and a high variation of its morphological and ecological features.

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Introduction

Members of the genus *Paxillus* (*Agaricomycota*, *Boletales*, *Paxillaceae*) are ectomycorrhizal fungi associated with various woody host plant species including hardwood and coniferous trees. They are commonly encountered in a wide range of ecosystems and habitats through the northern hemisphere.

Paxillus is a taxon of importance for academic research, as shows a bibliographic search done in early 2013 through the international database ISI Web of Science (http://www.isiknowledge.com/) among the scientific literature dealing with fungi. The item *Paxillus* was recorded in the title of 152 scientific publications and in the topic of at least 690 different published works originating from about 60 different countries. The species *Paxillus involutus* accounted for most of these records. It is in the top four of ectomycorrhizal species used as models for scientific studies that have significantly contributed to advances in the knowledge of ectomycorrhizal symbiosis functioning. The genomes of *P. involutus* and *Paxillus rubicundulus* strains have been entirely sequenced (Martin *et al.* 2011; Martin & Bonito 2012) underlining scientists' keen interest in the genus. Regarding human health, fatal outcomes due to immune haemolysis (Winkelmann *et al.* 1986; Anthowiak *et al.* 2003) have been reported after ingestion of *P. involutus*. However, assessments whether *P. involutus* and other closely related species are harmful or not vary depending on different observers' experiences in different countries. Differences in 'toxicity' assessments can depend on the speciation pattern. After all, there is no type reference sequence for *P. involutus* and the concept of this species is based on Batsch's icon (1786).

In Europe, the genus *Paxillus* is divided into two groups: the *P. involutus* complex and *P. rubicundulus*. The latter is mainly encountered in wetland habitats and exclusively associated with alder trees (*Alnus* spp., *Betulaceae*) (Orton 1969) whereas species of the *P. involutus* complex are found in more diverse habitats and associated with several tree species. Thus, host specificity is a useful trait to distinguish *P. rubicundulus* but species recognition within the *P. involutus* complex remains very difficult. Mycologists have long suspected that the name *P. involutus* encompasses different species (Fries 1985; Hahn & Agerer 1999; Jarosch & Bresinsky 1999; Bresinsky 2006). Thus, mating tests performed with Swedish isolates revealed three intersterility groups (Fries 1985). Although these groups were not correlated with clear-cut morphological characters, ecological traits distinguished them: group 1 was found in forest habitats whereas groups 2 and 3 were found in city parks and gardens (Fries 1985). On the basis of a morphological study, Hahn & Agerer (1999) recognized four European species in the *P. involutus* complex: *P. involutus*, *Paxillus validus*, *Paxillus albidulus*, and *Paxillus obscurisporus*. Finally, a phylogenetic study based on five genes unambiguously revealed four genetic lineages within the *P. involutus* complex (Hedh *et al.* 2008). More recently, Vellinga *et al.* (2012) revisited the *P. involutus* complex with a larger number of samples originating from a broader geographic range, including Europe and North America, and also found the same four genetic lineages. Two of these lineages corresponded to the described morphological species *P. obscurisporus* and *Paxillus ammoniavirescens* (syn. *P. validus*), a third one was assigned to *P. involutus* s.s. (Hedh *et al.* 2008), and the fourth lineage, referred to as PS IV and as *P. involutus* II by Hedh *et al.* (2008) and Vellinga *et al.* (2012) respectively, remains a phylospecies without an established correspondence with any described morphospecies. All these species can be encountered in highly diverse environments and locations and may thus be included in molecular environmental studies. Therefore it appears useful to remove any ambiguity regarding them.

Exploration of France led us to observe a large and unusual variation among *Paxillus* specimens and to suspect the presence of the not yet described phylospecies reported by Hedh *et al.* (2008) and Vellinga *et al.* (2012). This paper aims to describe the species corresponding to PS IV/*P. involutus* II and to study various aspects (genetic, morphological, and ecological) of the diversity of the *P. involutus* complex as exhaustively as possible, based on our collections and on data from the literature, in order to establish a framework to delineate these *Paxillus* species. The objectives were (1) to assign specimens to lineages and to study genetic variation within and among the lineages in order to determine a phylogenetic cut-off for species delimitation (2) to identify key differentiating morphological features between species (3) to reveal the ecological preferences of each species.

Materials and methods

Collections and ecological features

From 2008 to 2012, we obtained 47 collections of *Paxillus* basidiomes that matched with the macroscopic characteristics of the *Paxillus involutus* complex, from 23 locations in France and one in Italy (Table 1). After morphological investigations, the collected specimens were dried, except for a small amount of fresh tissues that was frozen at −20 °C. During the collecting step, an environment type was assigned to each collection. Environment was defined as 'closed' (forest) or 'open', in natural conditions (forest edge, roadside in forest, river bank) and in urban areas (public lawn, city park including garden and car park) (Table 1). Available environmental data from the literature (Table 2) were also taken into account and considered as 'closed' environments (forest) or 'open' environments (park, lawn, pasture). As much as possible, potential ectomycorrhizal host plants present in the surroundings of the collecting places were recorded (Table 1).

All French voucher specimens are available at the Laboratoire Evolution et Diversité Biologique, Université Paul Sabatier Toulouse 3 and the Italian collection is in the Torino (TO) herbarium.

Isolates and culture conditions

Pure mycelial cultures were obtained from basidiome fragments placed on solid malt extract peptone (MP) medium [50 mg L^{-1} $CaCl_2$, 25 mg L^{-1} NaCl, 500 mg L^{-1} KH_2PO_4, 250 mg L^{-1} $(NH_4)_2HPO_4$, 150 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 1 mg L^{-1} $FeCl_3 \cdot 6H_2O$, 1 g L^{-1} casein peptone (ThermoFisher, Illkirch, France), 5 g L^{-1} malt extract (Merck, Darmstadt, Germany),