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Archaeorhizomyces borealis sp. nov. and a sequence-based classification of related soil fungal species

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ABSTRACT

The class Archaeorhizomycetes (Taphrinomycotina, Ascomycota) was introduced to accommodate an ancient lineage of soil-inhabiting fungi found in association with plant roots. Based on environmental sequencing data Archaeorhizomycetes may comprise a significant proportion of the total fungal community in soils. Yet the only species described and cultivated in this class is *Archaeorhizomyces finlayi*. In this paper, we describe a second species from a pure culture, *Archaeorhizomyces borealis* NS99-600^{T} (=CBS138755^{ExT}) based on morphological, physiological, and multi-locus molecular characterization. *Archaeorhizomyces borealis* was isolated from a root tip of a *Pinus sylvestris* seedling grown in a forest nursery in Lithuania. Analysis of Archaeorhizomycete species from environmental samples shows that it has a Eurasian distribution and is the most commonly observed species. *Archaeorhizomyces borealis* shows slow growth in culture and forms yellowish creamy colonies, characteristics that distinguish *A. borealis* from its closest relative *A. finlayi*. Here we also propose a sequence-based taxonomic classification of Archaeorhizomycetes and predict that approximately 500 species in this class remain to be isolated and described.

Introduction

The genus *Archaeorhizomyces* and class Archaeorhizomycetes (Taphrinomycotina, Ascomycota) was introduced to characterize cryptically reproducing, slow-growing, ubiquitous soil fungi commonly associated with plant roots (Rosling *et al.* 2011). Until recently their presence was only known from environmental DNA sequences (Schadt *et al.* 2003; Porter *et al.* 2008), but isolation of Archaeorhizomycete cultures allowed the placement of the class in the fungal tree of life. A phylogenetic analysis of a combined gene dataset showed that Archaeorhizomycetes belong to the poorly-known subphylum Taphrinomycotina composed of five morphologically distinct classes, which represent a broad phylogenetic and ecological diversity (Sugiyama *et al.* 2006; Lui *et al.* 2009; Schoch *et al.* 2009). Although life cycle, ecology, and evolution of Archaeorhizomycetes remain largely unknown, the presence of relatively high species diversity (more than 250 operational taxonomic units (OTUs) predicted) was hypothesized to be the result of adaptation to specific ecosystems and habitats (Rosling *et al.* 2013). For example, the three most commonly recovered *Archaeorhizomyces* OTUs were found to share a characteristic ecological niche with ectomycorrhizal fungi though they were never observed to form distinctive ectomycorrhizal

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structures in the field or in the laboratory microcosm systems in which inoculated seedlings remained healthy-looking for months, suggesting that Archaeorhizomycetes is at least non-pathogenic (Rosling et al. 2011).

Fungal community studies employing environmental DNA suggest that Archaeorhizomycetes constitute a significant component in the rhizosphere soil, comprising up to one third of the total fungal community (Porter et al. 2008) but with a great seasonal variation (Schadt et al. 2003). Presently, 101 studies of environmental fungal communities have detected and submitted sequences that can be assigned to Archaeorhizomycetes to GenBank, nevertheless Archaeorhizomycetes have been rarely cultured and their reproductive structures are still uncertain (Grelet et al. 2010; Rosling et al. 2011). The type species, *Archaeorhizomyces finlayi*, is characterized by slow growth and thin filamentous hyphae 1–2 μm in diameter which are extensively coated by extracellular material, often intertwined into cord-like structures, and form numerous swellings which may function as resistant chlamydospores (Rosling et al. 2011). *Archaeorhizomyces finlayi* occurs throughout the northern hemisphere and is commonly associated with roots of different tree species, suggesting that it is probably dependent on the hosts for nutrition though the specific trophic mode of nutrition remains largely unknown (Rosling et al. 2011).

Here we formally describe a second species within Archaeorhizomycetes designated as *Archaeorhizomyces borealis* sp. nov. based on morphological, physiological, and molecular characterization. *Archaeorhizomyces borealis* exhibits slower growth than *A. finlayi*, produces relatively higher numbers of swellings and shows preferential association with roots of conifer trees grown throughout Eurasia.

Materials and methods

Isolation of the culture

Isolation of fungal culture of *Archaeorhizomyces borealis* strain NS99-600 from a root tip of *Pinus sylvestris* seedling was done as described by Menkis et al. (2005). Briefly, individual root tips were excised from root systems and assessed for mycorrhizal colonization using a dissection microscope. Then, root tips were surface sterilized in 33 % hydrogen peroxide for between 15 and 60 s, rinsed three times in sterile deionized water, dried for a few seconds on a sterile filter paper, plated onto modified (half strength of glucose) Melin Norkrans medium (MMN) (Marx 1969) in Petri dishes, and incubated at room temperature (ca. 21 °C) in the dark. Petri dishes were checked regularly and emerging mycelia were immediately transferred onto fresh MMN agar medium. Days after plating were recorded for all transfers.

Colony morphology and microscopy characterization

In order to reveal differences between cultured Archaeorhizomycetes – *Archaeorhizomyces borealis* and *Archaeorhizomyces finlayi*, a culture characterization was carried out by testing the physiological growth responses of the cultures to the different components in the MMN medium by addition of 0.5 % Malt Extract (ME, Thermo Fisher Scientific, Waltham, USA), 0.5 % yeast extract (YE, Thermo Fisher Scientific, Waltham, USA), 0.001 % plant hormones (Pyruvic acid, 5-Amino Valeic acid, and Butyric acid, Sigma–Aldrich, St. Louis, USA), 0.5 % glucose (Sigma–Aldrich, St. Louis, USA) and 0.5 % cellulose (Sigma–Aldrich, St. Louis, USA) as different sources of nutrients. In particular, plant hormones have been shown to promote growth in slowly growing lichen-forming fungi (Wang et al. 2010). One strain of *A. borealis* (NS99-600^T) and three of *A. finlayi* (UP613-Ny9^T, UP606-Ny10 and UP605-Ny14) obtained from three-week-old liquid MMN cultures, were macerated with a sterile plastic pestle in 500 mL of sterile distilled water and 20 μL of homogenate was placed in the middle of each 6-cm diameter Petri plate as inoculum. All plates were inoculated in triplicate on the same day and incubated at room temperature and in darkness for four months. In addition, growth and culture characterization of *A. borealis* was carried out on MMN medium supplemented with 0.1 % β-cyclodextrin (Sigma–Aldrich, St. Louis, USA) as in a preliminary BiOLOG Fungi Identification Test Panel FF MicroPlate (BiOLOG, Hayward, USA) assay *A. borealis* was able to utilize β-cyclodextrin. To estimate growth rate of fungal mycelia, single point inoculated cultures of *A. borealis* strain NS99-600 were established on MMN medium in triplicate. Colony measurements were calculated by averaging two perpendicular measurements of the diameter and are presented as an average of three colonies.

Morphology and anatomy of *A. borealis* strain NS99-600 was studied using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) utilizing a Zeiss Supra 35-VP electron microscope equipped with EDAX Genesis 4000 sensor (Zeiss, Oberkochen, Germany). In addition, light microscopy was carried out using a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) in order to visualize cell walls, septa and nuclei by staining mycelia with calcoflour-white and/or propidium iodide. Sample preparation for light microscopy, SEM, and TEM was done following protocols described by Rosling et al. (2011).

Molecular identification and phylogenetic analyses

After isolation of DNA, the molecular identification of strain NS99-600 was carried out by amplification of the internal transcribed spacer of ribosomal RNA (ITS rRNA) using primers ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990), followed by sequencing and BLAST analysis as described by Menkis et al. (2005). In addition to ITS rRNA, five other genetic markers (18S rRNA, 28S rRNA, *RPB1*, *RPB2*, and *TEF1*) were sequenced from NS99-600 for phylogenetic analyses as described by James et al. (2006). The GenBank accession numbers for sequences other than *Archaeorhizomyces borealis* NS99-600 can be found in Rosling et al. (2011). The sequences of *A. borealis* NS99-600 are available from the GenBank under accession numbers KF993708 (18S-ITS-28S rRNA), KF993709 (*RPB1*), KF993710 (*RPB2*), and 7KF993711 (*TEF1*). All DNA sequences except the ITS1 and ITS2 spacer regions of the rRNA were concatenated into a single supermatrix using the same taxa as in Rosling et al. (2011). The Maximum likelihood and Bayesian phylogenetic approaches were performed in RAxML 8.0.9 (Stamatakis 2006) with the GTR+GAMMA model and MrBayes v3.1.2 with a GTR+INVGAMMA model with four