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A molecular phylogeny of thermophilic fungi

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ABSTRACT

Sequences from 86 fungal genomes and from the two outgroup genomes *Arabidopsis thaliana* and *Drosophila melanogaster* were analyzed to construct a robust molecular phylogeny of thermophilic fungi, which are potentially rich sources of industrial enzymes. To provide experimental reference points, growth characteristics of 22 reported thermophilic or thermotolerant fungi, together with eight mesophilic species, were examined at four temperatures: 22 °C, 34 °C, 45 °C, and 55 °C. Based on the relative growth performances, species with a faster growth rate at 45 °C than at 34 °C were classified as thermophilic, and species with better or equally good growth at 34 °C compared to 45 °C as thermotolerant. We examined the phylogenetic relationships of a diverse range of fungi, including thermophilic and thermotolerant species, using concatenated amino acid sequences of marker genes *mcm7*, *rpb1*, and *rpb2* obtained from genome sequencing projects. To further elucidate the phylogenetic relationships in the thermophile-rich orders Sordariales and Eurotiales, we used nucleotide sequences from the nuclear ribosomal small subunit (SSU), the 5.8S gene with internal transcribed spacers 1 and 2 (ITS 1 and 2), and the ribosomal large subunit (LSU) to include additional species for analysis. These phylogenetic analyses clarified the position of several thermophilic taxa. Thus, *Myriococcum thermophilum* and *Scytalidium thermophilum* fall into the Sordariales as members of the Chaetomiaceae, *Thermomyces lanuginosus* belongs to the Eurotiales, *Malbranchea cinnamomea* is a member of the Onygenales, and *Calcarisporiella thermophila* is assigned to the basal fungi close to the Mucorales. The mesophilic alkalophile *Acremonium alcalophilum* clusters with *Verticillium albo-atrum* and *Verticillium dahliae*, placing them in the recently established order Glomerellales. Taken together, these data indicate that the known thermophilic fungi are limited to the Sordariales, Eurotiales, and Onygenales in the Ascomycota and the Mucorales with possibly an additional order harbouring *C. thermophila* in the basal fungi. No supporting evidence was found for thermophilic species belonging to the Basidiomycota.

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Abbreviations: OTU, operational taxonomic unit; SSU, rDNA (18S) from the small ribosomal subunit; LSU, rDNA (28S) from the large ribosomal subunit; ITS, internal transcribed spacer; BSS, bootstrap support

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Introduction

Thermophilic organisms can be classified as those organisms with an optimal growth temperature between 45 °C and 80 °C, hyperthermophiles are those with an optimum growth temperature above 80 °C, and mesophiles are those that grow optimally below 45 °C (Stetter et al. 1990; Madigan & Orent 1999; Taylor & Vaisman 2010). Thermophily is common in bacteria and Archaea, whereas hyperthermophiles are mainly confined to the Archaea. Only a small fraction of the estimated 600 000 fungi (Mora et al. 2011) is considered to be thermophilic and no fungus has been described as hyperthermophilic. Most reported thermophilic fungi have been placed into the *Sordariales*, *Eurotiales*, and *Mucorales* (Berka et al. 2011). However, Straatsma et al. (1994) described the existence of two thermophilic isolates of *Basidiomycota*. Furthermore, *Myriococcum thermophilum* is listed by National Center for Biotechnology Information (NCBI) taxonomy as a mitosporic basidiomycete and by Index Fungorum as an agaricomycete.

The temperature preferences of thermophilic fungi have been defined in different ways. According to Cooney & Emerson (1964), fungi growing with a minimum temperature of 20 °C or higher and a maximum temperature of growth above 50 °C are thermophilic, whereas fungi growing below 20 °C and up to about 50 °C are regarded as thermotolerants. They set the upper limit of growth for mesophiles at 40 °C (Cooney & Emerson 1964). On the other hand, Crisan (1964) and Maheshwari et al. (2000) proposed to classify fungi as thermophilic if their optimal growth temperature is above 40 °C or 45 °C, respectively.

Fungi are the main decomposers of lignocellulosic biomass in terrestrial ecosystems and the enzymes they secrete to break down lignocellulose may be useful in industrial processes such as bleaching in the pulp and paper industry, bioremediation of polluted soils, clean-up of wastewater effluents, and the production of second and third generation biofuels (Wesenberg et al. 2003; Gianfreda & Rao 2004; Sigoillot et al. 2005; Turner et al. 2007). Thermophilic fungi are of special interest for biomass conversion applications since they are potential sources of thermostable enzymes. The advantages of biomass conversion at high temperatures include higher reaction rates, enhanced mass transfer, lowered substrate viscosity, and reduced risk of contamination (Haki & Rakshit 2003; Viikari et al. 2007). At least some thermophilic fungi possess cellulose-degrading capacities that are higher than those of mesophilic reference species (Tansey 1971; Berka et al. 2011). The screening of thermophilic fungi and other thermophilic organisms for improved enzyme varieties may contribute to lowering the costs of enzyme preparations (Banerjee et al. 2010).

The nomenclature and taxonomic classification of thermophilic fungi is in a state of disarray, often leading to misidentifications and confusion (Mouchacca 1997, 2000a). New approaches that use molecular markers combined with efforts to establish a natural classification system that is based solely on monophyletic groups (Doolittle 1999; Voigt & Kirk 2011) have appreciably improved fungal taxonomy. The most dramatic changes affect the basal fungal lineages, but new taxonomic entities have also been introduced in the *Basidiomycota* and *Ascomycota* (Hibbett et al. 2007). The rapidly

increasing number of available sequenced genomes has changed the way phylogenetic analyses are conducted. Studies based solely on the analysis of a single marker locus are being replaced by multilocus and phylogenomic studies that can produce well-resolved trees with high support values for the majority of nodes.

The aim of this study is to produce a robust phylogenetic framework for thermophilic fungi. A further goal is to identify the fungal orders harbouring thermophilic species and to resolve the evolutionary relationships among the thermophilic and nonthermophilic species within these orders. A detailed knowledge of the phylogenetic distribution of thermophilic fungi would provide insights into the evolution of thermophily in fungi and help to identify closely-related mesophiles for comparative studies to reveal the molecular mechanisms underlying the ability to grow at high temperature. The phylogenetic analyses are complemented by experimental growth-temperature relationships for fungal species reported to be thermophilic. Using the criterion that a thermophilic fungus is one that grows faster at 45 °C than at 34 °C, our phylogenetic analyses suggest that the known thermophilic fungi belong to the orders *Sordariales*, *Eurotiales*, *Mucorales*, and *Onygenales*. Moreover phylogenetic reconstructions enabled us to correct the placement of six thermophilic species.

Materials and methods

Growth at different temperatures

Growth performance of 30 fungal strains was examined: 22 have been mentioned in the literature as thermophilic or thermotolerant (Maheshwari et al. 2000; Mouchacca 2000a) and the remaining species can be regarded as mesophilic. Cultures were grown on mycobroth agar plates adjusted to pH 5.5, a condition suitable for all but one of the tested strains, *Acremonium alcalophilum*, which was grown instead on malt extract agar plates at pH 9.0. The agar plates were inoculated in the centre with 2 µl of a spore solution containing either 500 or 10 000 spores per µl, or with a 6 mm diameter agar plug cut out from the edge of an actively growing culture. Cultures were grown at 22 °C, 34 °C, 45 °C, and 55 °C until differential growth was clearly visible, which took from 1 d (*Rhizomucor miehei*) to 32 d (*Pleurotus ostreatus*), but in most cases took fewer than 3 d. At this time relative growth performance was recorded for each strain by estimating the relative surface area of the agar plates covered with fungal mycelium at the different temperatures. Thus, a simple ranking from strongest to weakest (or absent) growth was obtained for each strain.

Data mining

For the protein-coding gene analysis we selected a set of publicly available fungal genomes representing a wide taxonomic spectrum of the fungi, from basal lineages to the *Ascomycota* and *Basidiomycota*, to which we added ten presumed thermophilic and three mesophilic genomes sequenced recently by the Genozymes research project (<http://>

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