

Identification of a new order of root-colonising fungi in the *Entorrhizomycota*: *Talbotiomyceales* ord. nov. on eudicotyledons

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Abstract: The recently described fungal phylum *Entorrhizomycota* was established solely for the genus *Entorrhiza*, species of which cause root-galls in *Cyperaceae* and *Juncaceae*. *Talbotiomyces calosporus* (*incertae sedis*) shares morphological characteristics and an ecological niche with species of *Entorrhiza*. We investigated the higher classification of *T. calosporus* to determine whether it belongs in *Entorrhizomycota*. Ribosomal DNA sequences showed *Talbotiomyces* to be a close relative of *Entorrhiza* and both taxa form a highly supported monophyletic group. Based on molecular phylogenetic analyses and in congruence with existing morphological and ecological data, *Entorrhiza* and *Talbotiomyces* represent a deep dichotomy within the *Entorrhizomycota*. While species of *Entorrhiza* are characterised by dolipores and occur on monocotyledons, members of *Talbotiomyces* are characterised by simple pores and are associated with eudicotyledons. This expands the host range of the recently described *Entorrhizomycota* from *Poales* to other angiosperms. Higher taxa, namely *Talbotiomyceales* ord. nov. and *Talbotiomycetaceae* fam. nov., are proposed here to accommodate *Talbotiomyces*.

Key words:

Entorrhiza
fungal evolution
molecular phylogeny
root galls
Talbotiomyces

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INTRODUCTION

Entorrhiza consists of species that form galls on roots of two monocotyledon sister families, *Cyperaceae* and *Juncaceae*. This enigmatic genus had an unstable and unresolved systematic position within the kingdom *Fungi*. Traditionally, it was considered a smut genus and classified in *Tilletiaceae* (Zundel 1953). In the modern classification of smut fungi, based on ultrastructure and molecular phylogeny, *Entorrhiza* was accommodated in the distinct family, order, subclass and class, *Entorrhizaceae*, *Entorrhizales*, *Entorrhizomycetidae* and *Entorrhizomycetes*, within the *Ustilaginomycotina* (Bauer *et al.* 1997, Begerow *et al.* 1998, 2007). Bauer *et al.* (2015) demonstrated that *Entorrhiza* could not be assigned to any of the main fungal lineages and consequently established the novel phylum *Entorrhizomycota*. However, it remains unclear if *Entorrhizomycota* belongs to the subkingdom *Dikarya*, which comprises the phyla *Ascomycota* and *Basidiomycota*, or represents a sister group (Bauer *et al.* 2015).

Entorrhizomycota currently comprises a single genus, *Entorrhiza*, with 14 species that cause galls in the roots of *Cyperaceae* and *Juncaceae* (Fineran 1978, Vánky 1994,

2012, 2013, Denchev & Denchev 2012). Due to the different age estimates of the *Entorrhizomycota* versus those of their host groups, Bauer *et al.* (2015) hypothesized that known entorrhizomycotan species and their respective host range only reflect a very small portion of a much larger, yet undiscovered diversity. Species of *Entorrhiza* regularly form septate, clampless hyphae, which are coiled in living host cells. During spore formation, hyphae terminate with globose cells, which detach from the hyphae and become thick-walled teliospores (Fineran 1980, Deml & Oberwinkler 1981, Bauer *et al.* 2015). *Talbotiomyces*, a genus morphologically similar to *Entorrhiza*, was established to accommodate *Entorrhiza calospora*, a species causing galls on roots of some South African members of the eudicotyledonous order *Caryophyllales* (Vánky *et al.* 2007). Given the similar morphology and ecology of *Talbotiomyces calosporus* to those of *Entorrhiza* species, it is possible that *Talbotiomyces* might be an unrecognized member of the *Entorrhizomycota*. To test this hypothesis, we analysed the nuclear rDNA of an isotype of *T. calosporus* collected in 1943, with five species of *Entorrhiza*, and representatives of *Dikarya* and *Glomeromycota*.

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MATERIALS AND METHODS

Material

Specimen examined: Talbotiomyces calosporus: **South Africa**: Gauteng: Pretoria, Brummeria, near Murray Farm, on *Limeum viscosum* (syn. *L. glomeratum*), 30 Jan. 1943, J.J.O. Pazzi (BRIP: HUV 587 – isotype from PREM 33770 – holotype).

DNA isolation, PCR, and sequencing

Total genomic DNA was extracted using the InnuPREP Plant DNA Kit (Analytik Jena, Jena) according to the manufacturer's instructions. The small subunit (SSU) rDNA region was amplified with various primer combinations from the sets NS1 to NS8 (White *et al.* 1990) and NS17 to NS24 (Gargas & Taylor 1992). PCRs were performed using Phusion High-Fidelity DNA Polymerase (Finnzymes Oy, Vantaa, Finland), following the protocol recommended by the manufacturer with annealing temperatures of approximately 5 °C above the mean primer melting temperatures, and 30 cycles. The 5' end of the large subunit (LSU) of the nuclear rDNA was amplified with the primer combination LR0R (R. Vilgalys Lab., <http://biology.duke.edu/fungi/mycolab/primers.htm>) and LR6 (Vilgalys & Hester 1990). PCR mixtures consisted of 5 µL of PCR reaction buffer (10x), 0.75 mM of MgCl₂ (50 mM), 14.5 µL of water, 1 µL of dNTP mix (5 mM), 0.50 µL of each primer (25 pmol/µL), 0.25 µL of MangoTaq DNA polymerase (Bioline, Luckenwalde) (2 U/µL), and undiluted 2.5 µL template DNA. PCRs were performed with the following thermal profile: 10 cycles of 30 s at 94 °C, 45 s at 60 °C (-1 °C per cycle), and 75 s at 72 °C, followed by 26 cycles of 30 s at 94 °C, 45 s at 50 °C and 75 s at 72 °C, with a final extension of 7 min at 72 °C. PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland, OH), and diluted (1:20) for further analysis. Cycle sequencing of SSU and LSU rDNA was accomplished using a 1:6 diluted BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the amplification primers. Sequencing was performed on an ABI Prism 3130xl Genetic Analyser (Applied Biosystems). Forward and reverse sequence chromatograms were checked for accuracy and edited using Sequencher v. 4.1 (Gene Codes Corporation, Ann Arbor, MI). The newly generated DNA sequences of *Talbotiomyces calosporus* have been deposited in GenBank under the accession numbers KJ666129 (SSU) and KP413056 (LSU).

Molecular phylogenetic analyses

To determine the phylogenetic position of *Talbotiomyces*, we assembled datasets containing rDNA SSU and LSU sequences. Both datasets included a sequence of the isotype of *T. calosporus*, all *Entorrhiza* sequences currently available in GenBank and two representatives of each dikaryan subphylum and the *Glomeromycota* (Fig. 1). The chytrids *Gromochytrium mamkaevae* and *Olpidium brassicae* were used as outgroup taxa. For GenBank accession numbers and full datasets see TreeBASE submission ID 17604. Alignments were performed separately with MAFFT v. 7.147b (Katoh & Standley 2013) using the E-INS-i option (Katoh *et al.* 2005). Initial

alignments were further modified with Gblocks v. 0.91b (Castresana 2000) to remove ambiguously aligned regions using standard parameters with one exception: 'allowed gap positions with half'. The final SSU alignment had a length of 1498 bp and the LSU alignment had a length 514 bp. For further analyses, both alignments were analysed separately as well as in a concatenated dataset. Maximum likelihood (ML) phylogenetic analyses and rapid bootstrapping with 1000 replicates under the GTRCAT model were performed using RAxML v. 8.0.17 (Stamatakis 2014). Additionally, posterior probabilities for nodal support were determined in a Bayesian phylogenetic MCMC search with MrBayes v. 3.2.2 (Ronquist *et al.* 2012) using the GTR+G model. Each search comprised two runs of four chains, each for 5 × 10⁶ generations. Posterior probabilities were sampled every 100 generations with the first 2.5 × 10⁶ generations being discarded as burn-in. The genetic divergences (uncorrected p-distance) between *Talbotiomyces* and *Entorrhiza* were calculated based on full-length sequence alignments using Mesquite v. 2.75 (Maddison & Maddison 2011).

RESULTS

Phylogenetic placement of *Talbotiomyces*

The results of our molecular phylogenetic analyses of rDNA sequences of *Talbotiomyces calosporus*, five species of *Entorrhiza*, and two representatives of each dikaryan subphylum and *Glomeromycota* are shown in Fig. 1. In phylogenetic analyses of individual SSU and LSU data (not shown) as well as the combined dataset (Fig. 1), *Entorrhiza* and *Talbotiomyces* formed a strongly supported monophyletic group. The genetic distance between *T. calosporus* and the closest related species was 77 bp or 5.0 % to *E. fineranae* (18S) and 57 bp or 9.0 % to *E. aschersoniana* (LSU). In the phylogenetic tree derived from a concatenated dataset, the *Entorrhiza/Talbotiomyces* clade was placed as a sister to the *Dikarya*, supported by 99 % ML bootstrap and 1.00 Bayesian posterior probability (Fig. 1).

TAXONOMY

Our results indicate that *Talbotiomyces calosporus* belongs to *Entorrhizomycota*. Molecular phylogenetic analyses revealed a dichotomy between *Entorrhiza* and *Talbotiomyces*, and together with ultrastructural and host range information, it justifies a new classification at a higher taxonomical level. We assume a much larger potential diversity of *Talbotiomyces*, and propose to accommodate *Talbotiomyces* in a new family and order within the class *Entorrhizomycetes*:

Talbotiomycetales K. Riess, R. Bauer, R. Kellner, Kemler, Piątek, Vánky & Begerow, **ord. nov.**
Mycobank MB810797

Diagnosis: Members of the *Entorrhizomycetes* Begerow *et al.* 2007 having simple pores.

Type: *Talbotiomyces* Vánky *et al.* 2007.

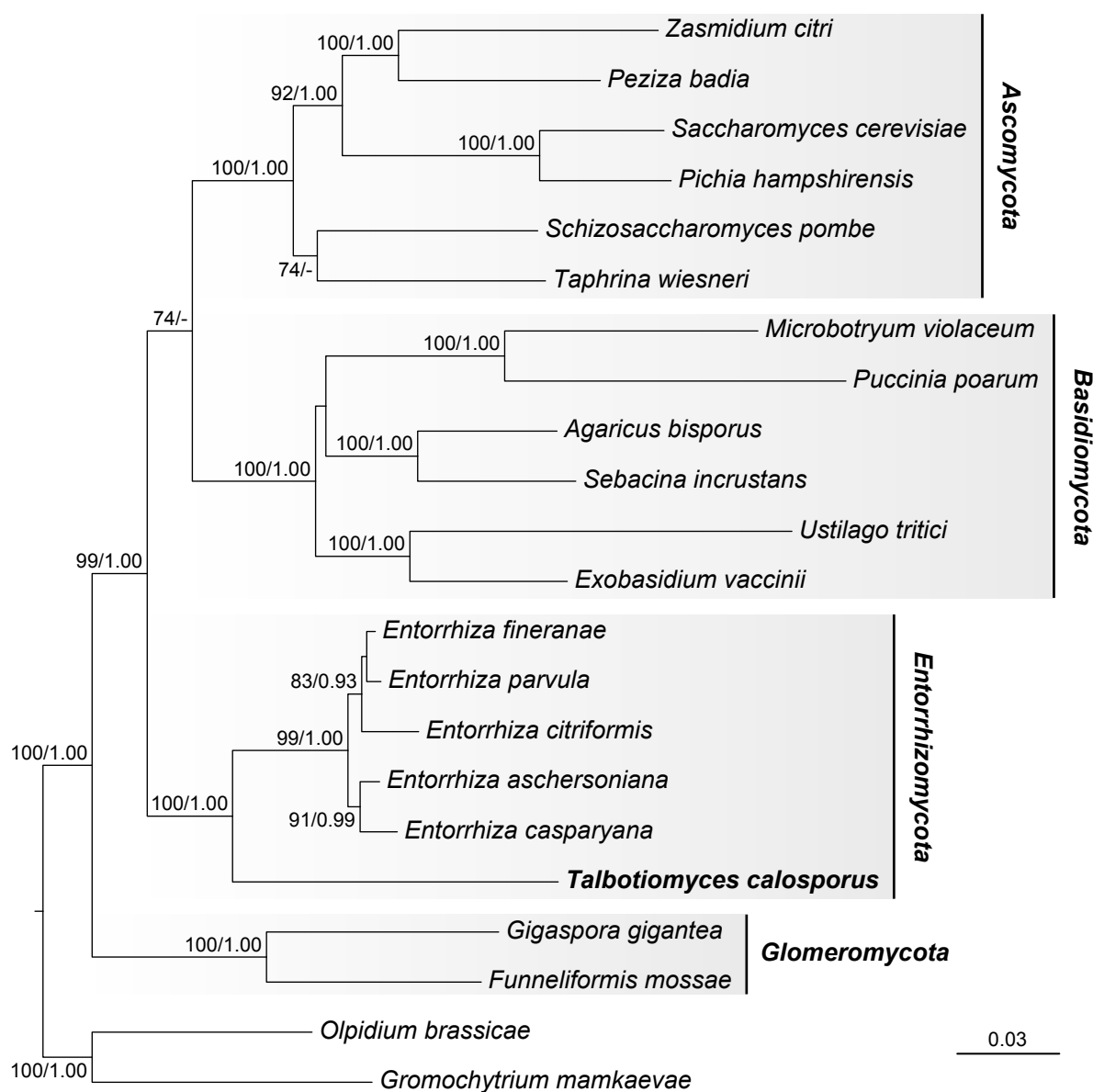


Fig. 1. Phylogenetic placement of *Talbotiomyces calosporus*. Best ML tree topology derived from a combined dataset of SSU and LSU rDNA sequences of *Talbotiomyces*, *Entorrhiza*, and representatives of Ascomycota, Basidiomycota, and Glomeromycota. Branch support is given as ML bootstrap percentage (≥ 70) / Bayesian posterior probability (≥ 0.90). The tree was rooted with the chytrids *Gromochytrium mamkaevae* and *Olpidium brassicae*.

Talbotiomycetaceae K. Riess, R. Bauer, R. Kellner, Kemler, Piątek, Vánky & Begerow, **fam. nov.**
Mycobank MB810798

Diagnosis: Members of the *Talbotiomycetales* occurring on eudicotyledons.

Type: *Talbotiomyces* Vánky *et al.* 2007.

DISCUSSION

Entorrhiza/*Talbotiomyces* relationship

Talbotiomyces calosporus was recovered in a monophyletic group with species of *Entorrhiza*, with strong phylogenetic support from the SSU and LSU regions of rDNA (Fig. 1).

The phylogenetic relationship between *Talbotiomyces* and *Entorrhiza* is additionally well supported by morphological and ecological characters of both genera. Both cause galls on plant roots and regularly have septate and clampless hyphae, which form coils in living host cells. *Entorrhiza* and *Talbotiomyces* also have hyphae that terminate with globose cells that detach from the hyphae and become thick-walled teliospores (Talbot 1956, Fineran 1980, Deml & Oberwinkler 1981, Bauer *et al.* 1997, 2001, 2015, Vánky *et al.* 2007). However, the septal pore architecture and host range of *Entorrhiza* differs from *Talbotiomyces*. *Entorrhiza* is characterised by dolipores and colonizes monocotyledons (*Cyperaceae* and *Juncaceae*) (Bauer *et al.* 1997, 2001). In contrast, *T. calosporus* possesses simple pores with rounded pore lips and occurs on members of the dicotyledonous order *Caryophyllales* (Vánky *et al.* 2007). Therefore, multiple lines

of evidence indicate that *Entorrhizomycota* should be split into two orders: *Entorrhizales* and *Talbotiomycesales*, the latter name is introduced in this study. The large genetic distance between *Talbotiomyces* and *Entorrhiza* indicates an early divergence of the *Entorrhizomycota* into two lineages. The inclusion of *Talbotiomyces* into *Entorrhizomycota*, however, did not resolve the phylogenetic position of this recently established fungal phylum (Bauer et al. 2015).

Coevolution and presumed host spectrum

Specimens of *Entorrhiza* and *Talbotiomyces* are rarely collected and their evolutionary relationships are still poorly understood. *Entorrhiza* species are associated with roots of *Cyperaceae* and *Juncaceae* worldwide, although mostly reported from temperate or high mountain regions (Vánky 2012). *Talbotiomyces calosporus* is only known from a few localities in subtropical South Africa on *Limeum viscosum* (*Molluginaceae*) and *Trianthema pentandra* (*Aizoaceae*) (Talbot 1956). Furthermore, Vánky et al. (2007) reported an additional host plant, *Portulaca oleracea* (*Portulacaceae*), apparently based on the material preserved in HUV 21397. That specimen is annotated by K. Vánky: "I was unable to find any spores of *Entorrhiza calospora* type". The *T. calosporus*/*P. oleracea* fungus/host combination needs to be confirmed. It is unclear whether *T. calosporus* is a polyphagous species that associates with host plants from two or three different families, or represents a suite of closely related cryptic or pseudo-cryptic species, each restricted to one host genus or species, as in other biotrophic plant pathogens (e.g. Bauer et al. 2008, Lutz et al. 2008, Göker et al. 2009, Ploch et al. 2011, Beenken et al. 2012, Liu and Hambleton 2013, Piątek et al. 2012, 2013, Vasighzadeh et al. 2014, Voglmayr et al. 2014, McTaggart et al. 2015). Due to the different age estimates of the *Entorrhizomycota* and their hosts, Bauer et al. (2015) hypothesized that the known species and their respective host range reflects only a small part of a larger diversity. The inclusion of *Talbotiomyces* in *Entorrhizomycota* extends the host spectrum of the phylum from *Cyperaceae*/*Juncaceae* (*Poales*) to the eudicotyledons. A large gap remains between the age estimates of *Entorrhizomycota* (roughly 600 Mya; Bauer et al. 2015) and their hosts (roughly 150 Mya; Wikström et al. 2001). The possibility that the host spectrum of *Entorrhizomycota* is even broader, comprising also early-diverged land plants, such as ferns and mosses. Due to the lack of above-ground symptoms of infection, infected host plants of *Entorrhizomycota* are difficult to detect in nature, and such evolutionarily ancient interactions have not been discovered so far. Additionally, some members of the *Entorrhizal*/*Talbotiomyces* clade may not cause symptoms or may occur as endophytes in roots. The next step will be to develop specific primers for *Entorrhizomycota* to test whether species of this fascinating group of root-colonizing fungi also may occur as endophytes in plant roots, in analogy to *Sebacinales* (Weiß et al. 2011, Garnica et al. 2013).

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