



The origin and diversification of the Entorrhizales: deep evolutionary roots but recent speciation with a phylogenetic and phenotypic split between associates of the Cyperaceae and Juncaceae

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Abstract

Fungi belonging to the Entorrhizales (Entorrhizomycota) comprise biotrophic pathogens associated with roots of the Cyperaceae and Juncaceae plant species. They are nearly globally distributed but rarely studied due to a hidden lifestyle without causing visible effects on host plants. Therefore, the evolutionary origin and phylogenetic relationships of the group are still poorly understood and it is not known whether species diversification was the result of co-evolution with their hosts or the result of host jumps. To infer hypotheses about the evolutionary history of the Entorrhizales, divergence times were estimated and plant-fungal tanglegrams calculated. Relaxed molecular clock analyses suggest that the Entorrhizomycota originated around the Neoproterozoic-Palaeozoic and diverged during the Late Cretaceous-Paleogene into the extant orders Entorrhizales and Talbotiomyetales. The split of the major lineages within the Entorrhizales took place in the Eocene, somewhat later than the divergence of the host families Cyperaceae and Juncaceae. Topology- and distance-based co-phylogenetic analyses of the fungi and their hosts revealed a large number of co-speciation and lineage sorting events in early fungal speciation, which resulted in a phylogenetic split corresponding to species infecting Cyperaceae or Juncaceae. Given that this split is congruent with spore differences, *Entorrhiza* s. str. is emended for species infecting hosts in the Cyperaceae, and a new genus *Juncorrhiza* is described for species restricted to hosts in the Juncaceae. Additionally, three new species are described: *Entorrhiza fuirenae*, *Juncorrhiza maritima* and *J. oxycarpi*.

Keywords Age estimation · Co-phylogeny · Cyperaceae · *Entorrhiza* · Entorrhizomycota · Fungi · Juncaceae

Introduction

Obligate biotrophic associations offer a unique interface to evaluate hypotheses on co-speciation and host-shift

speciation events. Co-speciation is the process by which the speciation of biotrophic organisms parallels that of their hosts (Refrégier et al. 2008) and as a consequence, the biotrophic organisms' phylogeny should mirror their hosts'

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phylogeny (Escudero 2015). By contrast, host-shift speciation occurs when a population of biotrophic organisms acquires a new host and subsequently undergoes speciation (de Vienne et al. 2013), which decreases phylogenetic congruence (Roy 2001). In true co-evolution, the congruence between the fungus and host plant phylogenies should also include their synchronous divergence in geological time (de Vienne et al. 2013). However, recent studies have questioned the importance of co-evolution as an important driver of fungal diversification and have revealed, for example, that host jumps have shaped species diversification in rust fungi (McTaggart et al. 2016), *Cyttaria* spp. (Peterson et al. 2010) and downy mildews (Choi and Thines 2015). The functioning of co-evolution as a major speciation driver was also questioned for associations between insects and plants (Suchan and Alvarez 2015).

The phylum Entorrhizomycota is a lineage of dikaryotic, obligate, root-associated fungi that currently contains two orders, the Entorrhizales and the Talbotiomyceales (Bauer et al. 2015; Riess et al. 2015). The Entorrhizomycota was thought to have originated around 600 million years ago (Mya), based on the hypothesis that the Entorrhizomycota was sister to the remaining Dikarya; however, this estimate was not supported by dating based on molecular clock (Bauer et al. 2015). Recently, Zhao et al. (2017) estimated the stem age of the Entorrhizomycota as 530 Mya using two species of *Entorrhiza* C.A. Weber [*E. aschersoniana* (Magnus) Lagerh. and *E. parvula* Vánky] sequenced by Bauer et al. (2015). A similar estimation of the stem age of the phylum was obtained by Tedersoo et al. (2018). However, the ages of the species diversifications within the order Entorrhizales are still unknown.

The Talbotiomyceales contains only one species, *Talbotiomyces calosporus* (P.H.B. Talbot) Vánky, R. Bauer & Begerow on *Limeum viscosum* (J. Gay) Fenzl (Limeaceae) and *Zaleyia pentandra* (L.) C. Jeffrey (syn. *Trianthema pentandra* L.) (Aizoaceae) in South Africa (Vánky et al. 2007; Riess et al. 2015), whereas the Entorrhizales contains 14 species assigned to one genus *Entorrhiza* (Weber 1884), typified by *Entorrhiza cypericola* (Magnus) C.A. Weber (Vánky 2002). *Entorrhiza* species are associated with host plants belonging to the Cyperaceae or Juncaceae and occur in temperate and mountain regions over almost all the world (Vánky 2012). All species are characterised by obligate biotrophy, a hypogeous lifestyle and the production of spores inside the living root cells (Fig. 1); the spores are released from dead and decaying host tissues (Vánky 2012; Bauer et al. 2015). The only visible symptoms of host plant infection are swellings (galls) of the roots, occurring usually in late summer and autumn, whereas the aboveground parts of infected plants are indistinguishable from those of healthy plants (Fineran 1983; Fineran and Fineran 1992).

Plants infected with *Entorrhiza* species show no symptoms of infection on aboveground organs, and the collection of specimens requires excavation of whole plants. Furthermore, soil moisture seems to play a crucial role in the degree and distribution of the infection (Fineran 1983). Despite these difficulties, *Entorrhiza* species were found on all continents except Antarctica (Vánky 2012). Currently, a total of 14 species are recognised, based on morphology, with some known only from the type localities. Most *Entorrhiza* species are considered specific to a host species; these include *Entorrhiza casparyanella* Vánky on *Juncus gregiflorus* L.A.S. Johnson, *Entorrhiza citrififormis* Vánky & McKenzie on *Isolepis reticularis* Colenso, *Entorrhiza cypericola* on *Pycreus flavescens* (L.) P. Beauv. ex Rchb., *Entorrhiza globoidea* Vánky on *Isolepis cernua* var. *setiformis* (Benth.) Muasya, *Entorrhiza guttiformis* M. Piepenbr. & S.R. Wang on *Carex parva* Nees, *Entorrhiza parvula* on *Eleocharis parvula* (Roem. & Schult.) Link ex Bluff, Nees & Schauer, *Entorrhiza raunkiaeriana* Ferd. & Winge on *Isolepis fluitans* (L.) R. Br., *Entorrhiza seminarii* J. Walker on *Isolepis inundata* R. Br. and *Entorrhiza tenuis* (Denchev & H.D. Shin) Denchev, Vánky & T. Denchev on *Juncus tenuis* Willd. Other species are considered generalists able to infect different species of one genus [*Entorrhiza aschersoniana* or *Entorrhiza casparyana* (Magnus) Lagerh. on *Juncus* spp.], or different genera within one family [*Entorrhiza fineraniae* Vánky and *Entorrhiza scirpicola* (Correns) Sacc. & P. Syd. on *Eleocharis* R. Br. and *Isolepis* R. Br.], or even species belonging to different families [*Entorrhiza caricicola* Ferd. & Winge on hosts in the Cyperaceae (*Carex* L., *Eleocharis*) and Juncaceae (*Juncus* L.)] (Vánky 2012). Host specificity has never been experimentally tested by either pathogenicity experiments or molecular methods. It is unknown whether *Entorrhiza* species are specific to a host species (similar to other biotrophic pathogens), and this knowledge may provide a better understanding of the evolutionary drivers of the parasitic lifestyle in this genus.

The Cyperaceae contains about 5500 species and the Juncaceae about 400 species. These plant species are globally distributed, but they occur mostly in temperate and/or montane regions, often in damp habitats. The Cyperaceae and Juncaceae are sister families and together with the basal, neotropical Thurniaceae, they constitute the so-called cyperids that are placed within the core Poales (Muasya et al. 2009; Givnish et al. 2010). The origin of the Cyperaceae and Juncaceae is estimated to be during the Late Cretaceous (e.g. Janssen and Bremer 2004; Bouchenak-Khelladi et al. 2014). The huge difference of approximately 500 million years between the assumed origin of the Entorrhizomycota and that of their host plants points at an enigmatic evolutionary history of this fungal lineage (Oberwinkler 2012; Bauer et al. 2015).

In this study, the following questions were addressed to understand the evolutionary history of the Entorrhizales and the events that shaped their host associations: (1) What is the

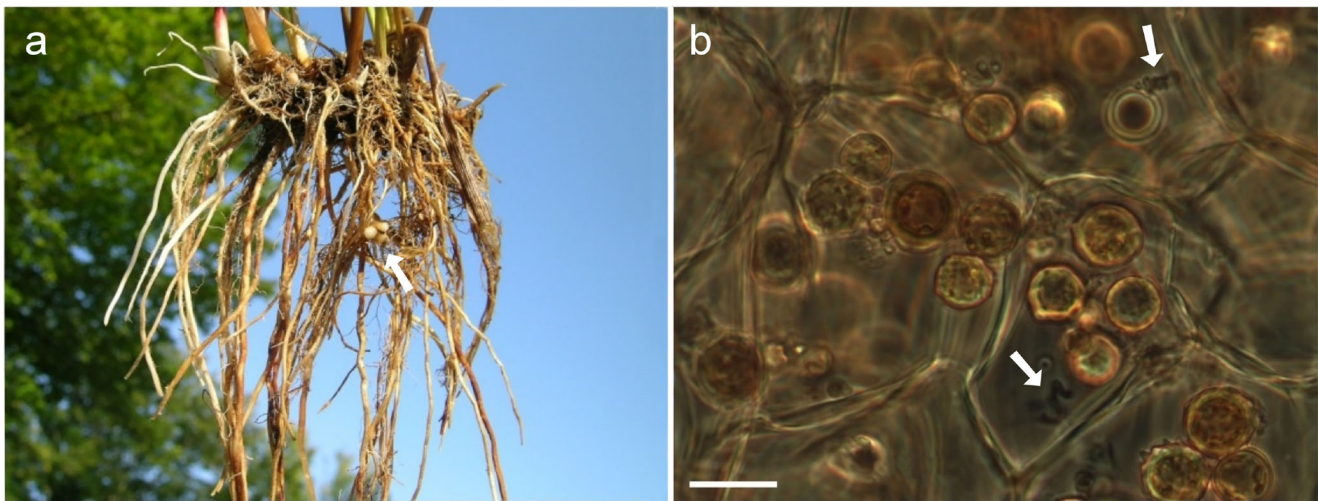


Fig. 1 A root galls and spores of *Juncorrhiza casparyana* associated with *Juncus articulatus*: **a** plant roots with galls (arrowed); **b** coiled hyphae (arrowed) and spores in root cells. Scale bar = 20 μ m

divergence time (origin and diversification) of the Entorrhizales? (2) Is there phylogenetic congruence between evolutionary patterns of species of the Entorrhizales and their host plant species? (3) Is there congruence between phenotypic characters and phylogenetic lineages? A fossil-calibrated molecular clock with three gene regions was used to estimate the divergence time of the Entorrhizomycota from other fungi. Subsequently, the diversification ages within the Entorrhizales were calculated from secondary calibrations based on rDNA sequences. To answer the second and third questions, rDNA regions and morphological data of 26 *Entorrhiza* specimens from four different continents, as well as sequences of two loci of 13 different host plant species were analysed.

Material and methods

Fungal and plant sampling

This study is based on analyses of DNA sequences and morphology of 26 herbarium specimens of *Entorrhiza* spp. collected in Africa, Australasia, Europe and South America, including 22 specimens freshly collected and/or sequenced for this study. The DNA sequences of four remaining *Entorrhiza* specimens and *Talbotiomyces calosporus* were taken from the GenBank nucleotide sequence database (www.ncbi.nlm.nih.gov). The specimens are deposited in the herbaria BRIP, HUV (Herbarium Ustilaginales Vánky, now deposited in BRIP), KR-M, KRAM F, PDD and TUB (Table 1). Prior to sequencing, specimens were assigned to morphological species delineated by Vánky (2012, see Table 1, second column). The host plant of the specimen of *Entorrhiza fineraniae* from Bolivia (HUV 21683), originally identified as *Eleocharis* sp., was determined as *Eleocharis geniculata* (L.) Roem. & Schult. using *rbcL* sequence (not deposited in GenBank).

For co-phylogenetic analyses, the DNA sequences for the host plants of *Entorrhiza* species were downloaded from GenBank, or, if not available, new sequences were generated in this study from plant material assembled with the respective *Entorrhiza* specimens (Table 2).

Morphological analyses

Sori and spore characteristics of *Entorrhiza* specimens used for molecular analyses were studied using dried herbarium material. All specimens were examined by light microscopy (LM), and specimens HUV 18060 and KRAM F-56780 (that served as holotypes for newly described species) were additionally analysed using scanning electron microscopy (SEM). For LM, small pieces of sori were mounted in lactic acid, heated to boiling point, cooled and then examined under a Nikon Eclipse 80i light microscope or Leica DM2500 light microscope. LM micrographs were taken with a Nikon DS-Fi1 camera or Leica DFC550 camera. Spore measurements were adjusted to the nearest 0.5 μ m. For SEM, spores taken directly from dried herbarium samples were dusted onto carbon tabs and fixed to an aluminium stub with double-sided transparent tape. The stubs were sputter-coated with carbon using a Cressington sputter-coater and viewed under a Hitachi S-4700 scanning electron microscope, with a working distance of about 12 mm.

DNA isolation, PCR and sequencing

Fresh root galls of *Entorrhiza* specimens were rinsed extensively in tap water and then in distilled water. One to two root galls containing spores of *Entorrhiza* or small amounts of dried leaf material (0.5 cm²) from host plants were deep frozen in liquid nitrogen and pulverised to a fine powder. Total fungal and plant genomic DNA was extracted using the InnuPREP

Table 1 List of species and specimens of the order Entorrhizales used for morphological and/or phylogenetic analyses

Species—current identification	Species identification as delineated by Vánky (2012)	Host plant	Sample origin	Collector(s)	Voucher	GenBank accession no(s). ITS + LSU	Sequence reference
<i>Entorrhiza citriformis</i>	<i>Entorrhiza citriformis</i>	<i>Isolepis reticularis</i>	New Zealand	E.H.C. McKenzie & K. Vánky	HUV 19051 (isotype)	KP413065	This study
<i>Entorrhiza citriformis</i>	<i>Entorrhiza citriformis</i>	<i>Isolepis reticularis</i>	New Zealand	E.H.C. McKenzie, C. Vánky & K. Vánky	PDD 70949 (holotype)	DQ645525/DQ645527	Matheny et al. (2006)
<i>Entorrhiza citriformis</i>	<i>Entorrhiza fineraniae</i>	<i>Isolepis inundata</i>	New Zealand	E.H.C. McKenzie & K. Vánky	HUV 19091	KP413068	This study
<i>Entorrhiza cypericola</i>	<i>Entorrhiza cypericola</i>	<i>Pycnus flavescens</i>	Romania	K. Vánky	HUV 592	KP413066	This study
<i>Entorrhiza cypericola</i>	<i>Entorrhiza cypericola</i>	<i>Pycnus flavescens</i>	Romania	K. Vánky	HUV 590	KP413067	This study
<i>Entorrhiza fineraniae</i>	<i>Entorrhiza fineraniae</i>	<i>Eleocharis geniculata</i>	Bolivia	R.G. Shivas, M.D.E. Shivas, A. McTaggart, W. Arce, C. Vánky & K. Vánky	HUV 21683	KP413069	This study
<i>Entorrhiza fuirenae</i>	N/A	<i>Fuirena ciliaris</i>	Australia	T. Vánky, K. Vánky & R.G. Shivas	HUV 21857 (holotype)	KP413071	This study
<i>Entorrhiza globoidea</i>	<i>Entorrhiza globoidea</i>	<i>Isolepis cernua</i>	Australia	C. Vánky & K. Vánky	HUV 19052 (isotype)	KP413070	This study
<i>Entorrhiza parvula</i>	<i>Entorrhiza parvula</i>	<i>Eleocharis parvula</i>	France	M. Lutz & M. Piątek	TUB 021488	KM359780	Bauer et al. (2015)
<i>Juncorrhiza aschersoniana</i>	<i>Entorrhiza aschersoniana</i>	<i>Juncus bufonius</i>	Poland	J. Piątek & M. Piątek	KRAM F-56779	KP413059	This study
<i>Juncorrhiza aschersoniana</i>	<i>Entorrhiza aschersoniana</i>	<i>Juncus bufonius</i>	Poland	J. Piątek & M. Piątek	KRAM F-56778	KM359781	Bauer et al. (2015)
<i>Juncorrhiza aschersoniana</i>	<i>Entorrhiza aschersoniana</i>	<i>Juncus bufonius</i>	Poland	M. Piątek	KRAM F-56777	KP413061	This study
<i>Juncorrhiza casparyana</i>	<i>Entorrhiza casparyana</i>	<i>Juncus alpinoarticulatus</i>	Italy	M. Lutz	KR-M-0041617	KP413075	This study
<i>Juncorrhiza casparyana</i>	<i>Entorrhiza casparyana</i>	<i>Juncus alpinoarticulatus</i>	Italy	M. Lutz	KR-M-0041618	KP413074	This study
<i>Juncorrhiza casparyana</i>	<i>Entorrhiza casparyana</i>	<i>Juncus articulatus</i>	Germany	R. Bauer	KR-M-0041621	FJ641906	Bauer et al. (2015)
<i>Juncorrhiza casparyana</i>	<i>Entorrhiza casparyana</i>	<i>Juncus articulatus</i>	Germany	R. Bauer	KR-M-0041619	KP413072	This study
<i>Juncorrhiza casparyana</i>	<i>Entorrhiza casparyana</i>	<i>Juncus articulatus</i>	Germany	R. Bauer	KR-M-0041620	KP413073	This study
<i>Juncorrhiza casparyana</i>	<i>Entorrhiza casparyana</i>	<i>Juncus articulatus</i>	Poland	J. Piątek & M. Piątek	KRAM F-56782	KP413057	This study
<i>Juncorrhiza casparyana</i>	<i>Entorrhiza casparyana</i>	<i>Juncus articulatus</i>	Poland	J. Piątek & M. Piątek	KRAM F-56784	KP413060	This study
<i>Juncorrhiza casparyana</i>	<i>Entorrhiza casparyana</i>	<i>Juncus articulatus</i>	Poland	J. Piątek & M. Piątek	KRAM F-56783	KP413063	This study
<i>Juncorrhiza casparyana</i>	<i>Entorrhiza casparyana</i>	<i>Juncus articulatus</i>	Poland	M. Piątek	KRAM F-57377	KP413080	This study
<i>Juncorrhiza maritima</i>	<i>Entorrhiza casparyana</i>	<i>Juncus ranarius</i>	Poland	J. Piątek & M. Piątek	KRAM F-56780 (holotype)	KP413062	This study

Table 1 (continued)

Species—current identification	Species identification as delineated by Vánky (2012)	Host plant	Sample origin	Collector(s)	Voucher	GenBank accession no(s). ITS + LSU	Sequence reference
<i>Juncorrhiza oxycarpi</i>	<i>Entorrhiza casparyana</i>	<i>Juncus oxycarpus</i>	South Africa	C. Vánky & K. Vánky	HUV 18060 (holotype)	KP413064	This study
<i>Juncorrhiza tenuis</i>	<i>Entorrhiza tenuis</i>	<i>Juncus tenuis</i>	Poland	M. Piątek	KRAM F-57376	KP413076	This study
<i>Juncorrhiza tenuis</i>	<i>Entorrhiza tenuis</i>	<i>Juncus tenuis</i>	Poland	M. Piątek	KRAM F-57375	KP413081	This study
<i>Juncorrhiza tenuis</i>	<i>Entorrhiza tenuis</i>	<i>Juncus tenuis</i>	Slovakia	J. Piątek & M. Piątek	KRAM F-56785	KP413082	This study
<i>Talbotiomyces calosporus</i>	<i>Talbotiomyces calosporus</i>	<i>Limeum viscosum</i>	South Africa	F.F. Pazzi	HUV 587 (isotype)	KP413056	Riess et al. (2015)

Accession numbers of sequences generated in this study are set in bold

Abbreviations of herbaria: *HUV*, Herbarium Ustilaginales Vánky, Brisbane, Australia; *KRAM*, Mycological Herbarium of the Staatliches Museum für Naturkunde Karlsruhe, Germany; *KRAM F*, Mycological Herbarium of the W. Szafer Institute of Botany, Polish Academy of Sciences, Kraków, Poland; *PDD*, New Zealand Fungal and Plant Disease Collection, Auckland, New Zealand; *TUB*, Herbarium of the University of Tübingen, Germany

Plant DNA Kit (Analytik Jena, Jena, Germany) according to the manufacturer's instructions.

The internal transcribed spacer region, including the 5.8S (ITS), and the D1/D2 LSU (LSU) of the fungal nuclear rDNA were amplified using the primer combination ITS1F (Gardes and Bruns 1993) and NL4 (White et al. 1990). In cases of negative or weak amplifications, PCRs

were repeated with the primer sets ITS1F/ITS4 (White et al. 1990) for ITS and LR0R/LR6 (Vilgalys and Hester 1990) for LSU. The host plant ITS region was amplified with the primers ITS1/ITS4, or if this combination failed, the PCR was repeated using the plant specific primer ITS2-S2F (Chen et al. 2010) and ITS4. The chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase large

Table 2 List of species of the order Entorrhizales, host plant species and their sequences used for co-phylogenetic analyses

Fungal species	GenBank accession no. ITS + LSU	Host plant species	GenBank accession no. ITS	GenBank accession no. <i>rbcL</i>
<i>Entorrhiza citriformis</i>	KP413068	<i>Isolepis reticularis</i>	DQ385585	N/A
<i>Entorrhiza cypericola</i>	KP413067	<i>Pycneus flavescens</i>	KF150598	Y13005
<i>Entorrhiza fineraniae</i>	KP413069	<i>Eleocharis geniculata</i>	AF190590	AM999823
<i>Entorrhiza fuirenae</i>	KP413071	<i>Fuirena ciliaris</i>	AB373099	JX644697
<i>Entorrhiza globoidea</i>	KP413070	<i>Isolepis cernua</i>	DQ385576	HM850076
<i>Entorrhiza parvula</i>	KM359780	<i>Eleocharis parvula</i>	AF190583	KT324231
<i>Juncorrhiza aschersoniana</i>	KP413061	<i>Juncus bufonius</i>	JQ811751	HM850085
<i>Juncorrhiza casparyana</i>	KP413057	<i>Juncus articulatus</i>	AY727819	AY395543
<i>Juncorrhiza maritima</i>	KP413062	<i>Juncus ranarius</i>	KT324229	KT324230
<i>Juncorrhiza oxycarpi</i>	KP413064	<i>Juncus oxycarpus</i>	N/A	AY216631
<i>Juncorrhiza tenuis</i>	KP413076	<i>Juncus tenuis</i>	JQ811752	HM850091

Accession numbers of sequences generated in this study are set in bold

subunit gene (*rbcL*) was amplified with the primer combinations *rbcL*-1F (Fay et al. 1997) and *rbcL*-1500R (Olmstead et al. 1992) or *rbcLa*-F (Levin et al. 2003) and *rbcLa*-R (Kress et al. 2009). All PCRs were performed with MangoTaq™ DNA Polymerase (Bioline, Luckenwalde, Germany), following the PCR protocol for DNA as described in Riess et al. (2013).

PCR products were cleaned and cycle sequenced as described in Riess et al. (2013) using the PCR primers as well as the primers 5.8S-R (Vilgalys and Hester 1990) for the fungal ITS and *rbcL*-674R (Olmstead et al. 1992), *rbcL*-724R, and *rbcL*-636F (Fay et al. 1997) for *rbcL*. Sequence chromatograms were assembled and manually edited using Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, MI, USA). All newly generated DNA sequences were deposited in the GenBank nucleotide sequence database under the accession numbers KP413057–KP413076 and KP413080–KP413082 for *Entorrhiza* spp. (Table 1) and KT324229–KT324231 for the host plants (Table 2).

Sequence identification, alignments and phylogenetic reconstructions

Fungal DNA sequences generated from plant root galls were compared against *Entorrhiza* sequences available in GenBank using BLAST (Altschul et al. 1997). Phylogenetic relationships and divergence time estimations of the Entorrhizomycota were inferred from two assembled datasets. Dataset 1, including 18S + 28S + *rpb1* domains B-C sequences, was composed of the sampling from Riess et al. (2016) and three representative species of the order Entorrhizales (Fig. 2; Supplementary material Fig. S1). Dataset 2 included full-length ITS + LSU sequences of all analysed *Entorrhiza* specimens and a specimen of *Talbotiomyces calosporus* (Fig. 3). Additionally, for co-phylogenetic analyses, dataset 3 was assembled which included concatenated ITS + LSU sequences of all analysed *Entorrhiza* species (only concatenated ITS + LSU from one specimen per species was used) and concatenated ITS + *rbcL* sequences of their host plant species (Fig. 4). For a detailed information about the specimen sampling, see Tables 1 and 2. Multiple sequence alignments of datasets 1, 2 and 3 are deposited in TreeBASE under submission ID S21273.

For each DNA region, nucleotide sequences were aligned separately with MAFFT 7.147b (Katoh and Standley 2013) using the E-INS-i option (Katoh et al. 2005). Alignments of datasets 1 and 2 were subsequently modified with Gblocks 0.91b (Castresana 2000) by removing ambiguously aligned and divergent regions using standard program parameters with one exception: ‘Allowed Gap Positions With Half’. The final alignment lengths (percentage of original nucleotide positions) were 3903 bp (54%) for dataset 1 and

1570 bp (35%) for dataset 2. For co-mapping analyses (dataset 3), both full alignments of *Entorrhiza* species (1623 bp) and host plants (1858 bp) were concatenated prior to further processing. Phylogenetic relationships were estimated in maximum likelihood (ML) analyses with combined rapid bootstrapping under the GTRCAT model from 1000 runs with RAxML 8.0.17 (Stamatakis 2014). Additional posterior probabilities for nodal support were determined in a Bayesian phylogenetic MCMC search using MrBayes 3.2.2 (Ronquist and Huelsenbeck 2003), under the general time-reversible model with gamma-distributed rate variation (GTR+G). Each search comprised two runs of four chains each, for 10×10^6 generations sampled every 100 generations with the first 2.5×10^6 generations discarded as burn-in. For co-phylogenetic analyses (dataset 3), topologies were mid-point rooted.

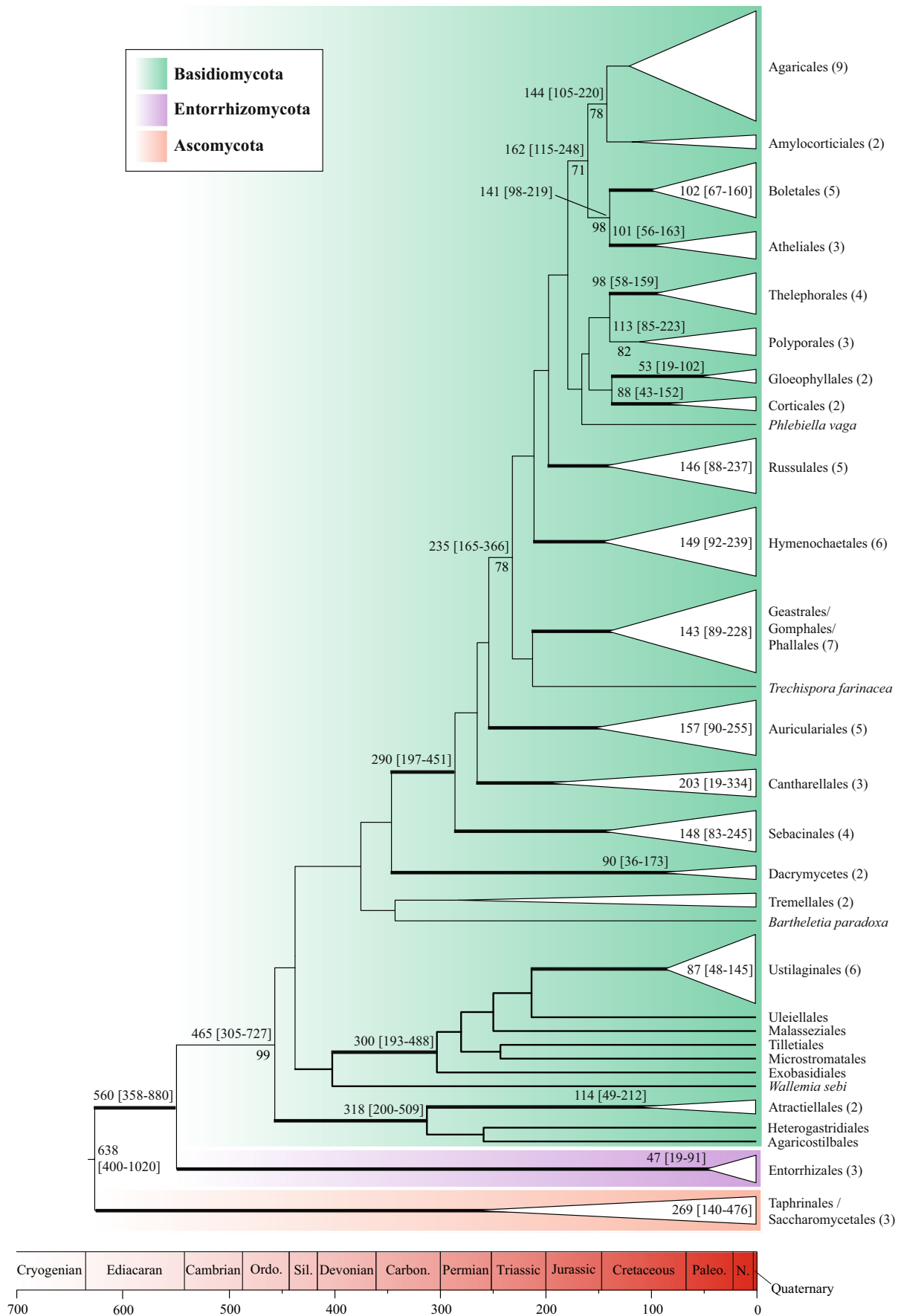
Divergence time estimations

The age of the phylum Entorrhizomycota was estimated using a MCMC-based time estimation in BEAST 1.8.1 (Drummond et al. 2012) of dataset 1, with the same fossil calibrations and parameters previously published by Riess et al. (2016). Subsequently, the age estimation of the split between *Entorrhiza fineraniae* and *E. parvula* from fossil-calibrated dataset 1 was used as a secondary calibration point in dataset 2. The 95% highest density probability range was taken as a normal prior, and the starting tree was calibrated with the mean age estimation, as proposed by Forest (2009).

Co-phylogenetic topology-based and distance-based approaches

One currently unanswered question is whether some species of the Entorrhizales (e.g. *Entorrhiza aschersoniana* or *E. casparyana*), which have been reported from multiple hosts (Vánky 2012), are polyphagous or whether they represent species complexes composed of distinct species, with each fungal species specific to a single host plant species or a narrow group of closely related host species. The host plants (type hosts) reported in original descriptions of species

Fig. 2 Collapsed chronogram for Basidiomycota and Entorrhizomycota evolution. The tree topology represents the consensus of trees inferred with BEAST from combined 18S + 28S + *rpb1* domains B-C sequences from 83 Basidiomycota species, three Entorrhizomycota species and three Ascomycota species as outgroup. Alignment length = 3903. The age estimation mean is followed by the 95% highest density probability range in square brackets. Numbers on branches represent bootstrap values obtained from 1000 replicates (values ≥ 70), maximum support of 100 is encoded with bold lines. For full dataset, see Supplementary material (Fig. S1). Abbreviations: Ordo., Ordovician; Sil., Silurian; Carbon., Carboniferous; Paleo., Paleogene; N., Neogene



of the Entorrhizales were used for the co-phylogenetic analysis to represent species of the Entorrhizales in their strict sense. In the case of *Entorrhiza casparyana* and *E. fineraniae*, the DNA sequences from specimens on the type host were not available. For these two fungal species, their corresponding host plant species, *Juncus articulatus* and *Eleocharis geniculata*, respectively, were set as the unique associated host plant species (Table 2).

The Entorrhizales-host plant associations were illustrated with a tanglegram based on dataset 3 and generated using TreeMap 3b1243 (Page 1994; Charleston 2011). The significance of the correlations between topologies was calculated by mapping the dependent phylogeny of *Entorrhiza* onto the independent host phylogeny using Jungles (Charleston 1998), as incorporated in TreeMap. In a distance-based approach, we used the AxParafit method (Legendre et al. 2002; Stamatakis et al. 2007), as implemented in Copycat 2.03 (Meier-Kolthoff et al. 2007), to compare patristic distances between *Entorrhiza* and their corresponding host phylogenies. The significance of individual fungal-plant links and the global fit was tested with

9999 permutations. The TreeMap cost model (Charleston and Robertson 2002) with default settings was used to score the numbers of evolutionary events (co-speciation, duplication, host shift and lineage sorting).

Results

Divergence time estimations of and within the Entorrhizomycota

Divergence time estimates revealed a stem age of the Entorrhizomycota around 560 Mya (358–880 Mya), i.e., divergence in the Neoproterozoic-Palaeozoic (Fig. 2; Supplementary material Fig. S1). Within the Entorrhizomycota, the orders Entorrhizales and Talbotiomycetales may have diverged during the Late Cretaceous-Paleogene—50 Mya (40–74 Mya) (Fig. 3). Within the Entorrhizales, two major lineages may have diverged in the Eocene—42 Mya (40–50 Mya) (without

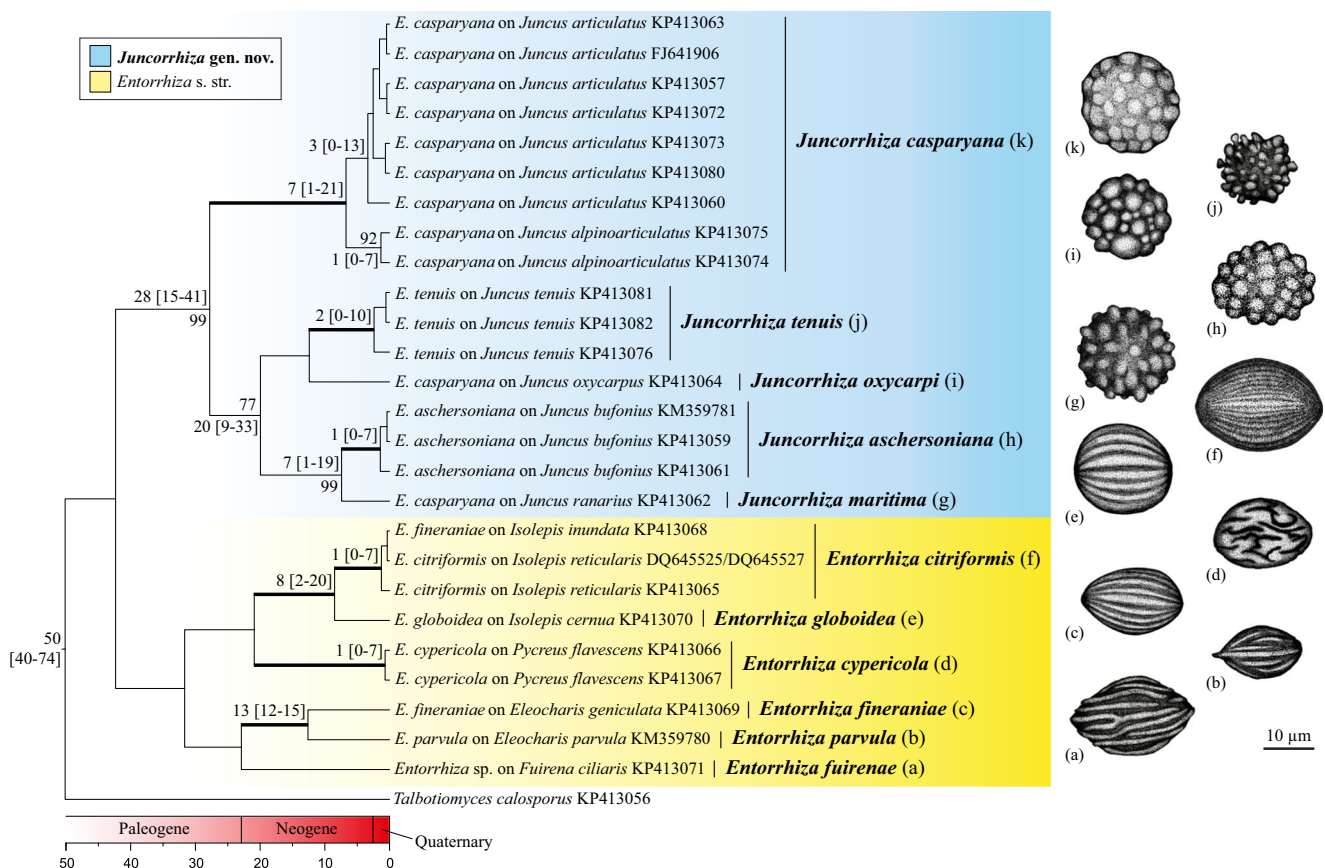


Fig. 3 Chronogram for Entorrhizales evolution. The tree topology represents the consensus of trees inferred with BEAST from ITS + LSU sequences from 26 specimens of the Entorrhizales with *Talbotiomyces calosporus* (Talbotiomycetales) as outgroup. Alignment length = 1570 bp. The age estimation mean is followed by the 95% highest density

probability range in square brackets. Numbers on the branches represent bootstrap values obtained from 1000 replicates (values ≥ 70); the maximum support of 100 is encoded with bold lines. Currently accepted species names are written in bold on the right side. Spore morphology is illustrated (a–k). Scale bar = 10 μm

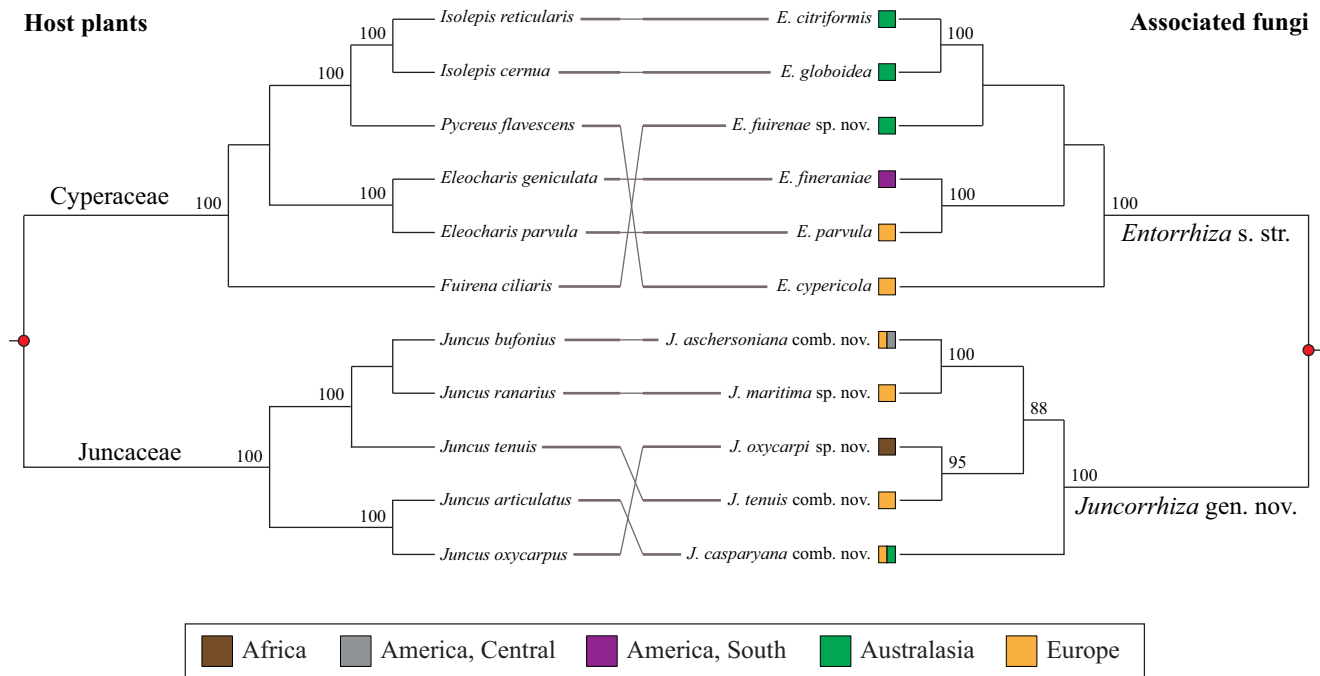


Fig. 4 Tanglegram between host plant (left) and fungal (right) phylogenies reconstructed from the ITS + *rbcL* and ITS + LSU sequences, respectively. Nodal support is given as maximum likelihood bootstrap (\geq statistical support) and a major Entorrhizales radiation took place during the Oligocene-Miocene (approx. 5–30 Mya) (Fig. 3).

Phylogenetic relationships in the Entorrhizales

The phylogenetic analyses of sequences obtained from plant root galls (dataset 2) revealed two main clades within *Entorrhiza*. One clade contained sequences of all specimens associated with Cyperaceae, including the type species *E. cypericola*, with no significant support value. The other clade contained sequences of all specimens occurring on Juncaceae, with significant statistical support (Fig. 3). The six morphologically defined species [*Entorrhiza aschersoniana*, *E. cypericola*, *E. globoidea*, *E. parvula*, *E. tenuis* and *Entorrhiza* sp. on *Fuirena ciliaris* (L.) Roxb.] were resolved as monophyletic and specific to one host species, and they were supported with significant bootstrap values. The sequences from specimens of *Entorrhiza casparyana* (sensu Vánky 2012) clustered in four different clades, according to their respective host plant species, of which sequences from specimens on *Juncus alpinoarticulatus* Chaix and *J. articulatus* L., respectively, were sister (showing divergence of 0.1% or 2 bp in ITS + LSU sequences, accession nos. KP413060 vs. KP413075) while sequences of specimens on *Juncus oxycarpus* Kunth and *Juncus ranarius* Songeon & E.P. Perrier formed separate clades. The sequences from specimens of *Entorrhiza fineraniae* on *Eleocharis geniculata* and *Isolepis inundata* were placed separately. The sequence from the specimen on *Eleocharis geniculata* formed

70). Red dots indicate statistically supported co-divergence events established by co-phylogenetic analyses in TreeMap

a separate lineage, while the sequence from the specimen on *Isolepis inundata* grouped with sequences from the holotype and isotype specimens of *Entorrhiza citriformis* on *Isolepis reticularis*. Within the clade that contained species associated with Cyperaceae, the sister group relationships of *Entorrhiza fineraniae* and *E. parvula*, and *E. citriformis* and *E. globoidea*, respectively, were supported by bootstrap values of 100. *Entorrhiza* sp. on *Fuirena ciliaris* was sister to the *E. fineraniae*–*E. parvula* clade, but without statistical support. In the second main clade that included species occurring on Juncaceae, *Entorrhiza casparyana* on *Juncus alpinoarticulatus* and *J. articulatus* was statistically supported as a sister taxon to the remaining species (Fig. 3).

Morphological features in the Entorrhizales

In all *Entorrhiza* specimens that were examined, the sori produced irregularly shaped galls at the tips of the roots. The galls contained spores produced inside host root cells. The spore shape and ornamentation were similar within associated host plant families and, with some exceptions, all specimens could be assigned to known species based on spore morphology.

In the *Entorrhiza* specimens on hosts in the Cyperaceae, the spores were irregularly longitudinally ridged to cerebriform and ellipsoidal in *Entorrhiza cypericola* or were uniformly longitudinally ridged and citriform, globoid, citriform to elongate, long ellipsoidal and oval to ellipsoidal in *E. citriformis*, *E. globoidea*, *E. fineraniae*, *E. parvula* and *Entorrhiza* sp. on *Fuirena ciliaris*, respectively (Fig. 3). The spore sizes of known species were roughly congruent with the



Fig. 5 Macroscopic symptoms of the infection of *Fuirena ciliaris* roots by *Entorrhiza fuirenae* (arrows). Scale bar = approx. 1 cm

sizes given by Vánky (2012), while the spore sizes of *Entorrhiza* sp. on *Fuirena ciliaris* are given below in the description of the new species *Entorrhiza fuirenae*.

In the *Entorrhiza* specimens on hosts in the Juncaceae, the spores were verrucose-tuberculate, and subglobose to broadly ellipsoidal in *Entorrhiza aschersoniana*, verrucose-tuberculate and globose to slightly subglobose in *E. casparyana* (sensu Vánky 2012) on *Juncus alpinoarticulatus*, *J. articulatus*, *J. oxycarpus* or *J. ranarius* and verrucose-tuberculate and globose to slightly subglobose in *E. tenuis* (Fig. 3). The specimens of *E. casparyana* on different host plants differed by spore sizes. The spore sizes of known species were roughly congruent with the sizes given by Vánky (2012), while the spore sizes of specimens on

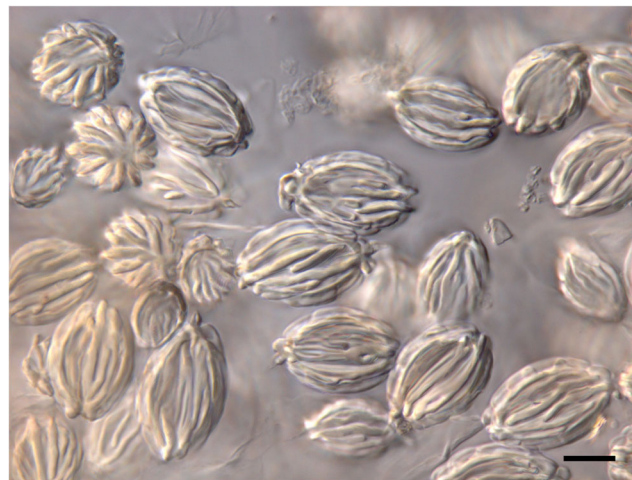


Fig. 6 Spores of *Entorrhiza fuirenae* (holotype) seen by light microscopy. Scale bar = 10 μ m

J. oxycarpus and *J. ranarius* are given below, in the descriptions of the new species *Juncorrhiza oxycarpi* and *Juncorrhiza maritima*, respectively.

Co-phylogenetic analyses between the Entorrhizales and host plants

Evaluation of the co-divergence events on the tanglegram using dataset 3 in TreeMap revealed that the host plant and fungal trees did not match exactly (Fig. 4). The test of the significance of the correlation coefficient between pairs of associated leaves in plants and fungal phylogenies identified only deep nodes as statistically supported ($z = 0.836$, $p = 0.000$). Furthermore, no global significance ($p = 0.335$) was found between the fungal and host topologies using TreeMap. Distance-based analyses using patristic distances confirmed these results of TreeMap and exhibited no significance in the AxParafit global test (3.970, $p = 0.264$). Testing of a co-phylogenetic signal of individual links between *Entorrhiza* and host plant species using the methods described before revealed no significant links (data not shown). Mapping the parasite onto the host phylogeny gave a least-cost tree that comprised 12 co-speciation events, 8 duplication events and 13 lineage sorting events.

Taxonomy

The phylogenetic split of *Entorrhiza* species into two main clades supported by molecular phylogenetic analyses, spore morphology and biology (host plant preferences at the family level) foster a rearrangement of the generic boundaries within Entorrhizales. *Entorrhiza* s. str. is emended and restricted to species infecting hosts in the Cyperaceae, and a new genus is established to accommodate species infecting hosts in the



Fig. 7 Macroscopic symptoms of the infection of *Juncus ranarius* roots by *Juncorrhiza maritima* (arrows). Scale bar = 5 mm

Juncaceae. The corresponding new combinations are substantiated. Additionally, three new species are described for specimens that exhibit isolated positions in the phylogenetic analyses and possess unique combinations of morphological characters and host plant preferences.

Entorrhiza C.A. Weber, Bot. Zeitung (Berlin) 42: 378 (1884) *emend.* K. Riess & Piątek

Description: Members of the Entorrhizaceae R. Bauer & Oberw. (in Bauer et al. 1997). Sori as galls in the roots of hosts in the Cyperaceae, having intracellular hyphal coils and intracellular spores developing in living cells. Spores with longitudinally ridged or, rarely, cerebriform ornamentation. Type species: *Entorrhiza cypericola* (Magnus) C.A. Weber.

Comments: Spore germination of species in *Entorrhiza* s. str. is not known as all successful germination experiments conducted to date have used the species *Entorrhiza aschersoniana* and *E. casparyana* that infect hosts in the Juncaceae (Weber 1884; Fineran 1982; Bauer et al. 2015), which we exclude from *Entorrhiza* and transfer to a new genus.

Entorrhiza fuirenae R.G. Shivas, Vánky, Piątek & K. Riess, **sp. nov.** Figs. 3, 5 and 6

Mycobank MB821930

Etymology: Referring to the host plant genus, *Fuirena*.

Type: Australia, Northern Territory: between Darwin and Batchelor, Chinner Road, close to Lake Bennett, 12° 58' 16" S, 131° 09' 54" E, alt. ca. 60 m a.s.l., on *Fuirena ciliaris* (Cyperaceae), 24 April 2011, *leg.* T. Vánky, K. Vánky & R.G. Shivas (holotype: HUV 21857, isotype: BRIP 54476; type sequence, including the ITS and LSU, is available in GenBank: KP413071).

Description: Associated with *Fuirena ciliaris*. Sori forming galls at the tips of the roots, galls cylindrical to irregularly fusiform, pale brown, 3–10 × 1–2 mm, filled with a pale yellowish brown, agglutinated spore mass. Spores intracellular, elongate-ellipsoidal to lemon-shaped, with a papilla at one end, 19–30 × 12–18 μm (including ornamentation) ($n = 30/1$), subhyaline to pale yellow; wall two-layered, inner layer 1–1.5 μm thick at sides and up to 5 μm at the apex, outer layer with coarse irregular parallel longitudinal ridges, 1–3 μm wide, convergent at the apex; 10–15 ridges around the equator, 4–7 ridges in side view.

Distribution and ecology: *Entorrhiza fuirenae* is currently known only from the type locality in northern Australia. The fungus was found on plants growing at an ephemeral pond formed at the end of the wet season.

Comments: This species is morphologically similar to *Entorrhiza citrififormis* and *E. fineraniae*. *Entorrhiza citrififormis* has wider spores (17–23 μm) and more ridges (22–30) on the equatorial circumference of the spores (Vánky 2012) than *E. fuirenae*. *Entorrhiza fineraniae* also has more ridges (14–24) on the equatorial circumference of the spores (Vánky 2012) than *E. fuirenae*. *Entorrhiza citrififormis*, *E. fineraniae* and *E. fuirenae* are phylogenetically distinct and not directly related to each other.

Juncorrhiza K. Riess & Piątek, **gen. nov.**

Mycobank MB821931

Etymology: Referring to the occurrence in the roots of host plants in the Juncaceae.

Description: Members of the Entorrhizaceae R. Bauer & Oberw. (in Bauer et al. 1997). Sori as galls in the roots of hosts in the Juncaceae, having intracellular hyphal coils and intracellular spores developing in living cells. Spores with verrucose-tuberculate ornamentation. Spore germination internal via cruciform septation that leads to four internal cells, each cell producing external germination hyphae with apically developing falcate sporidia. Type species: *Juncorrhiza aschersoniana* (Magnus) K. Riess & Piątek.

Juncorrhiza aschersoniana (Magnus) K. Riess & Piątek, **comb. nov.**

Mycobank MB821932

Basionym: *Schinzia aschersoniana* Magnus, Ber. Deutsch. Bot. Ges. 6: 103 (1888).

Synonyms: *Entorrhiza aschersoniana* (Magnus) Lagerh., Hedwigia 27: 262 (1888); *Melanotaenium aschersonianum* (Magnus) Thirum. & M.D. Whitehead, Am. J. Bot. 55: 184 (1968).

Juncorrhiza casparyana (Magnus) K. Riess, M. Lutz & Piątek, **comb. nov.**

Basionym: *Schinzia casparyana* Magnus, Ber. Deutsch. Bot. Ges. 6: 103 (1888).

Synonyms: *Entorrhiza casparyana* (Magnus) Lagerh., Hedwigia 27: 262 (1888); *Melanotaenium casparyanum*

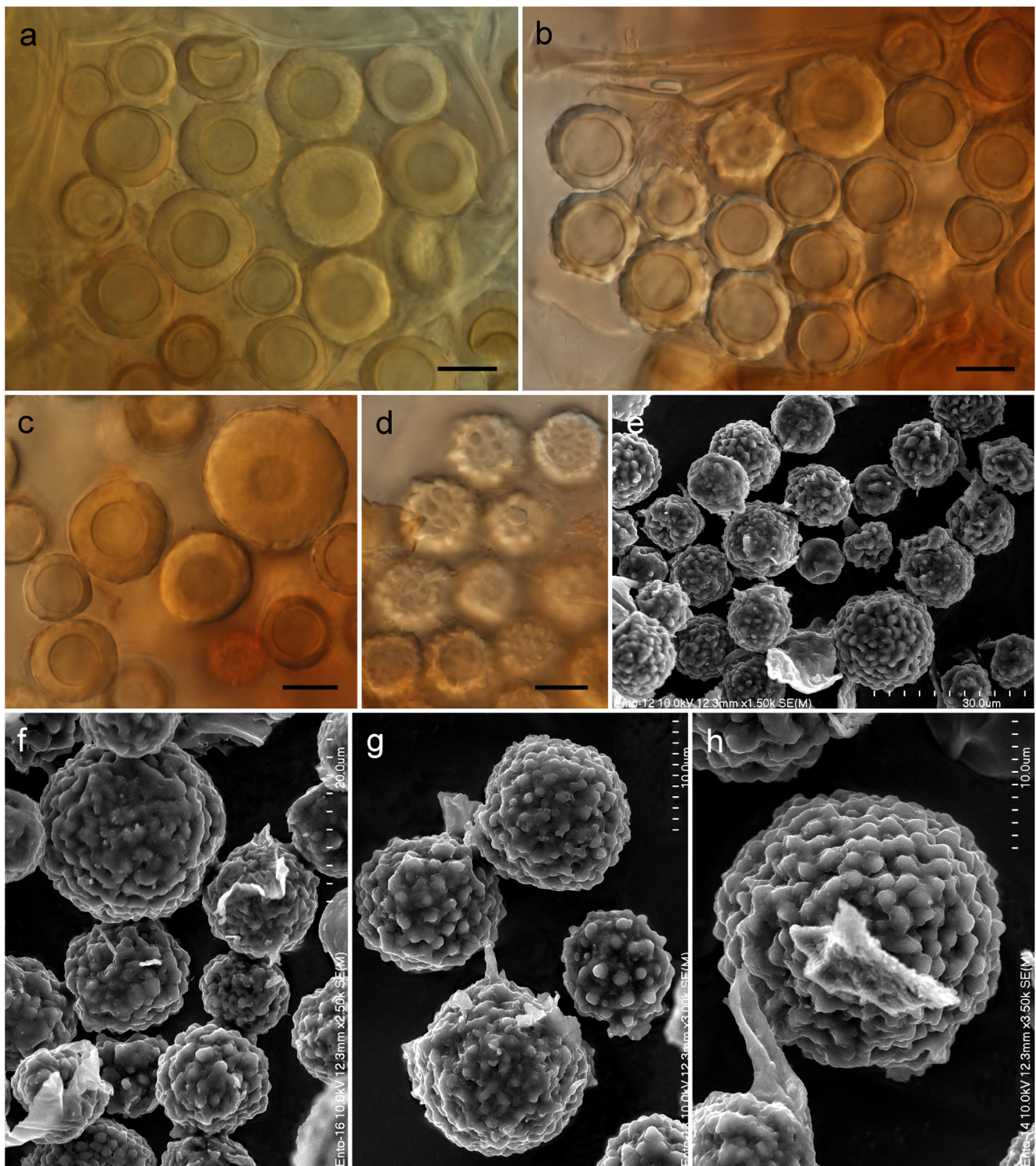


Fig. 8 *Juncorrhiza maritima* (holotype) on *Juncus ranarius*: **a–b** spores in host cells seen by light microscopy; **c–d** spores seen by light microscopy, median and superficial views; **e–h** spores seen by scanning electron microscopy. Scale bars: **e** 30 µm, **f** 20 µm, **a–d, g–h** 10 µm

(Magnus) Thirum. & M.D. Whitehead, Am. J. Bot. 55: 185 (1968); *Entorrhiza digitata* Lagerh., Hedwigia 27: 264 (1888); *Schinzia digitata* (Lagerh.) Magnus, Jahresber. Naturf. Ges. Graubündens, N.S. 34: 7 (1891);

Melanotaenium digitatum (Magnus) Thirum. & M.D. Whitehead, Am. J. Bot. 55: 185 (1968).

Comments: The specimens on *Juncus alpinoarticulatus* and *Juncus articulatus* are assigned to *Juncorrhiza*



Fig. 9 Macroscopic symptoms of the infection of *Juncus oxycarpus* (a, healthy plant) roots by *Juncorrhiza oxycarpi* (b). Scale bars: a approx. 1 cm, b = 1 mm

caspariana, although they are separated genetically and may represent two different species. However, as the sequences of *Juncorrhiza caspariana* on the type host (*Juncus tenageia*) are not available, this complex remains unresolved.

Juncorrhiza caspariana (Vánky) K. Riess & Piątek, **comb. nov.**

Mycobank MB821934

Basionym: *Entorrhiza caspariana* Vánky, Mycotaxon 68: 342 (1998).

Comments: Spore morphology and the host plant species (*Juncus gregiflorus*; see Vánky 2012) support this transfer, although sequence data are not available for this species.

Juncorrhiza maritima Piątek & K. Riess, **sp. nov.** Figs. 3, 7 and 8

Mycobank MB821935

Etymology: Referring to the occurrence of the species near the sea.

Type: Poland, Pomerania Province: near Rewa, ca. 31 km N of Gdańsk, 54° 37' 26" N, 18° 30' 34" E, alt. 0 m a.s.l., on *Juncus ranarius* (Juncaceae), 27 July 2006, leg. J. Piątek & M. Piątek (holotype: KRAM F-56780; type sequence, including the ITS and LSU, is available in GenBank: KP413062).

Description: Associated with *Juncus ranarius*. Sori forming galls at the tips of fine roots, galls cylindrical to irregularly fusiform, rarely bifurcated, moderately dark brown, except for the white colour of the gall tips, 2–7 × 1–2 mm, filled with a pale yellowish brown, agglutinated mass of spores. Spores intracellular, globose or rarely slightly subglobose, extremely variable in size, (10–)10.5–30 × 10–28.5(–30) μm (including ornamentation) ($n = 30/1$), pale yellow to yellow, rarely golden yellow; wall two-layered, extremely variable in thickness, 1.5–9.0(–10.0) μm thick (including very thin inner layer, ca. 0.5 μm thick), spore profile undulate to tuberculate, spore surface verrucose to tuberculate as seen by LM, tuberculate as seen by SEM.

Distribution and ecology: The only known collection of *Juncorrhiza maritima* is from plants growing close to the shoreline, periodically saturated by sea water, in a halophyte grassland community (*Juncetum gerardii*). The host plant *Juncus ranarius* was in the past commonly considered synonymous with *Juncus bufonius* L., but it is a distinct species growing in the coastal and inland saline environments in Eurasia, North Africa and North America (Cope and Stace 1978). *Juncorrhiza maritima* is currently known only from the type locality in Poland, but the reports of *Juncorrhiza aschersoniana* on *Juncus ranarius* (Vánky 2012) and *Juncorrhiza caspariana* on *Juncus bufonius* (Fineran 1978; Vánky and McKenzie 2002; Vánky 2012) may refer to this new species.

Comments: This species is morphologically close to *Juncorrhiza caspariana*, which differs in having smaller spores with thinner spore walls (spores (12–)13.5–23(–28) μm in diam., spore walls (1–)2–4(–10) μm thick, Vánky 2012). The high variability of spore sizes is also characteristic of *Juncorrhiza maritima*. Phylogenetically, this species is not related to *Juncorrhiza caspariana*, but to *J. aschersoniana*, which has smaller spores that are ovoid in shape, and possess

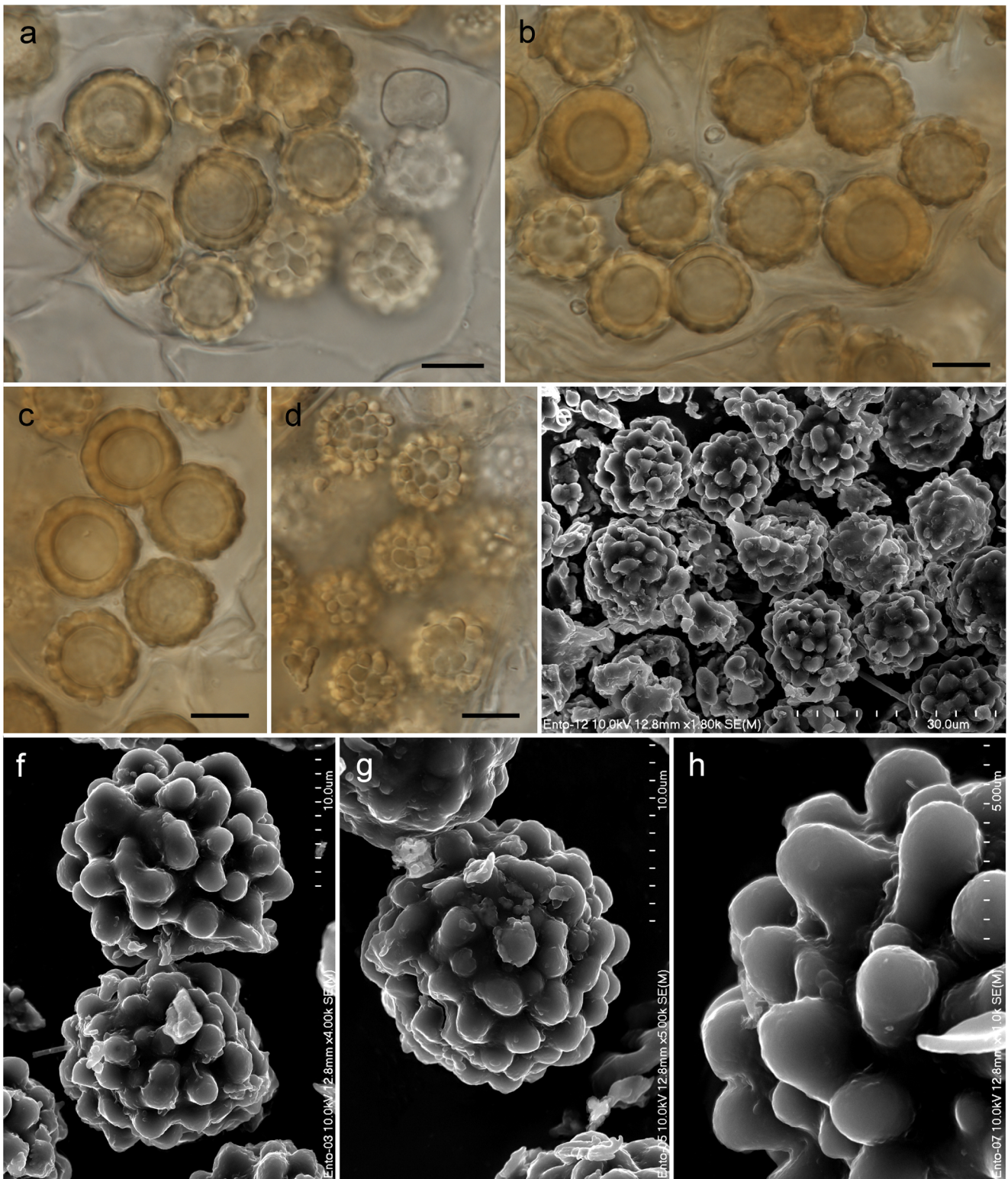


Fig. 10 *Juncorrhiza oxycarpi* (holotype) on *Juncus oxycarpus*: **a–b** spores in the living host cells seen by light microscopy; **c–d** spores seen by light microscopy, median and superficial views; **e–g** spores seen by

scanning electron microscopy; **h** spore wall ornamentation seen by scanning electron microscopy. *Scale bars: e* 30 μm , *a–d, f–g* 10 μm , *h* 5 μm

thinner spore walls [spores ovoid to subglobose, (12.5–)14.5–21.5(–25.5) \times (11–)12.5–17(–21.5) μm , spore walls 0.5–2.5 μm thick, Vánky 2012].

Juncorrhiza oxycarpi Piątek & K. Riess, **sp. nov.** Figs. 3, 9 and 10

Mycobank MB821936

Etymology: Referring to the host plant species, *Juncus oxycarpus*.

Type: South Africa, Orange Free State: Sterkfontain Dam Nature Reserve, 28° 24' 32" S, 29° 02' 13" E, alt. ca. 1820 m a.s.l., on *Juncus oxycarpus* (Juncaceae), 31 Dec. 1996, leg. C. Vánky & K. Vánky (holotype: HUV 18060, isotype: KRAM F-57733; type sequence, including the ITS and LSU, is available in GenBank: KP413064).

Description: Associated with *Juncus oxycarpus*. Sori forming galls at the tips of roots, galls cylindrical, sometimes bifurcated, brown, up to 5 mm long, 1 mm wide, filled with a pale brown, agglutinated mass of spores. Spores intracellular, globose or very rarely slightly subglobose, uniform in size, 14–21(–22.5) × (13–)13.5–20.5(–22) μm (including ornamentation) ($n = 30/1$), hyaline (when young), pale yellow to yellow, rarely golden yellow; wall two-layered, moderately variable in thickness, (1.5–)2.0–6.0(–6.5) μm thick (including a very thin inner layer, ca. 0.5 μm thick), spore profile undulate, spore surface tuberculate as seen by LM, verrucose to tuberculate as seen by SEM.

Distribution and ecology: The only known collection of *Juncorrhiza oxycarpi* is from South Africa, and was previously assigned to *J. casparyana* (Vánky et al. 2011). The host plant is an African endemic, occurring mostly in the eastern part of the continent (Juffe 2010). Assuming that *Juncorrhiza oxycarpi* is host species-specific, it could be considered an African endemic species. The ecological details of the South African location are unknown.

Comments: This specimen was originally assigned to *Entorrhiza casparyana* by Vánky (2012) but represents a distinct species. *Juncorrhiza oxycarpi* is morphologically similar to *Juncorrhiza tenuis*, except that the spore size range in *J. tenuis* is greater, due to a lower limit of the minimum spore diameter (11.5–20 × 10.5–20 μm, Denchev et al. 2007). Phylogenetically, *Juncorrhiza oxycarpi* and *J. tenuis* are sister species that are separated by a considerable genetic distance: 4.6% or 28 bp in ITS. This is a larger genetic distance than occurs between the morphologically distinct sister species *Juncorrhiza aschersoniana* and *J. maritima* (1.4% or 10 bp in ITS). Thus, *Juncorrhiza oxycarpi* is a distinct cryptic species, probably confined to *Juncus oxycarpus*, while *Juncorrhiza tenuis* apparently is restricted to *Juncus tenuis*.

Juncorrhiza tenuis (Denchev & H.D. Shin) K. Riess & Piątek, **comb. nov.**

MycoBank MB821937

Basionym: *Entorrhiza casparyana* var. *tenuis* Denchev & H.D. Shin, in Denchev, Shin & Kim, Mycotaxon 100: 74 (2007).

Synonym: *Entorrhiza tenuis* (Denchev & H.D. Shin) Denchev, Vánky & T. Denchev, Mycotaxon 121: 222 (2013) [2012].

Discussion

On the origin of the Entorrhizomycota

The age estimations conducted in this study indicate a relatively ancient origin of the phylum Entorrhizomycota and are similar to age estimations obtained by Zhao et al. (2017) and Tedersoo et al. (2018) for this fungal phylum. The most recent common ancestor of Entorrhizomycota and Basidiomycota existed in the Neoproterozoic-Palaeozoic, which in geological time-scale more or less roughly coincides with the origin of Ascomycota and Basidiomycota (Samarakoon et al. 2016). Most likely, Entorrhizomycota is more closely related to Basidiomycota than to Ascomycota, and both phyla share, amongst others, the ultrastructural trait of dolipores in the septa of hyphae (Bauer et al. 2015). Considering the hypothesised origin of the Entorrhizomycota and the origin of land plants (Kenrick and Crane 1997), the ancestors of this fungal phylum were probably marine species or appeared in the early land plant ecosystems.

Origin of the Entorrhizales and their host plant families

The extant members of Entorrhizomycota are associated with two phylogenetically rather distant groups of land plants, the Caryophyllales (families Aizoaceae and Limeaceae) and the Poales (families Cyperaceae and Juncaceae). The age estimations conducted in this study imply that the divergence and split of the two extant main lineages of the Entorrhizomycota, namely Talbotiomycetales and Entorrhizales, occurred long after the divergence of the phylum and corresponded only approximately with the divergence of their respective host plants. Thus, the split of Talbotiomycetales and Entorrhizales (Fig. 3) happened somewhat later than the divergence of the families Aizoaceae and Limeaceae (which include the host plants of *Talbotiomyces*); the latter divergence was dated to the Cretaceous, according to most recent estimations [Aizoaceae—74.88 Mya (72.5–77.87 Mya), Limeaceae—85.04 Mya (80.62–90.26 Mya), see Magallón et al. 2015]. The split of the two major lineages within the Entorrhizales (corresponding to the genera *Entorrhiza* and *Juncorrhiza*) took place somewhat later than the split between the host families Cyperaceae and Juncaceae, which was estimated to have happened during the Late Cretaceous and Paleogene according to recent datings [Cyperaceae—82.3 Mya (73.7–87.6 Mya), Juncaceae—71.8 Mya (51–87 Mya), according to Bouchenak-Khelladi et al. 2014 or Cyperaceae—55.16 Mya (38.19–74.58 Mya), Juncaceae—55.16 Mya (38.19–74.58 Mya), according to Magallón et al. 2015].

Although the Entorrhizales has deep evolutionary roots, only recent evolutionary divergence of its current members is visible. Based on the numbers of taxa in the three dikaryan phyla, rapid speciation resulted in the highly speciose phyla Ascomycota and Basidiomycota in contrast with a low number of known species in the Entorrhizomycota. It cannot be excluded that Entorrhizomycota were more diverse in the past and, as highly specialised biotrophs, they disappeared, together with their hosts, during one of the mass extinction events, for example in the Cretaceous-Paleogene extinction event. It cannot also be excluded that a high number of hidden species remain to be discovered.

Co-phylogeny between the Entorrhizales and host plants

The present study showed co-speciation and lineage sorting events between Entorrhizales infecting Cyperaceae and Juncaceae. This genetic split is congruent with phenotypic characters, and the new genus *Juncorrhiza* was described to reflect the monophyletic group on Juncaceae, distinguished from *Entorrhiza* (hosts in Cyperaceae) by host preference and spore ornamentation. Age estimates between the Entorrhizales (this study) and their host families (Bouchenak-Khelladi et al. 2014 or Magallón et al. 2015, see above) are not congruent, and interpretations of fungus–host relationships as a result of co-evolution seem problematic (de Vienne et al. 2013). In fact, most recent co-phylogenetic analyses of different groups of biotrophic pathogens (Peterson et al. 2010; Choi and Thines 2015; Escudero 2015; McTaggart et al. 2016; Li et al. 2017) suggest host-shift speciation, rather than co-speciation, as the driving force of their diversification. It is noteworthy that the major radiation of the Entorrhizales in the Oligocene-Miocene (as estimated in this study) is partly correlated with the time of the evolutionary switch of species of Cyperaceae from dry to wet environments, which took place from the early Eocene to the late Oligocene (~23–55 Mya) (Bouchenak-Khelladi et al. 2014). The co-occurrence of host plants in the same wet habitats may have promoted host switches of wet-adapted Entorrhizales between closely related host species.

Hidden diversity in the Entorrhizales

Although this study is based on a relatively small sampling (26 fungal collections), it covers eight of the 14 hitherto known members of the Entorrhizales. Additionally, three new species are described based on the discovery of either taxa on previously unknown hosts, such as *Entorrhiza fuirenae* on *Fuirena ciliaris*, or cryptic species within a species complex, such as *Juncorrhiza casparyana*. The latter fact suggests that Entorrhizales are probably host species specific like many biotrophic pathogens (e.g. Lutz et al. 2008; Choi et al. 2011;

Beenken et al. 2012; Piątek et al. 2013a, b, 2016; Rouxel et al. 2013; Mardones et al. 2017; Ziegler et al. 2018; Kruse et al. 2018). Therefore, further splitting of polyphagous species, especially *Juncorrhiza casparyana* reported from 17 host plants (Vánky 2012), may increase the number of species in this order. The addition of *Fuirena* to the range of host genera reported previously for the Entorrhizales (*Carex*, *Eleocharis*, *Isolepis*, *Juncus* and *Pycreus*; see Vánky 2012) suggests that the host range for members of this order may be much wider than is currently known. All these aspects open new avenues to reveal hidden diversity in the Entorrhizales. Finally, the fungal ITS + LSU sequences generated in this study, which are linked to reliably identified specimens (including six type specimens), together with the knowledge of the host plant species and geographical origin (Table 1), will serve as valuable reference data for future studies on this enigmatic fungal lineage.

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