Mycorrhiza (2016) 26:831–846
DOI 10.1007/s00572-016-0717-0

ORIGINAL ARTICLE

Experimental evidence of ericoid mycorrhizal potential within Serendipitaceae (Sebacinales)

Martin Vohník[1,2] · Matěj Pánek[3,4] · Judith Fehrer[5] · Marc-André Selosse[6,7]

Received: 14 April 2016 / Accepted: 14 June 2016 / Published online: 21 June 2016
© Springer-Verlag Berlin Heidelberg 2016

Abstract The Sebacinales are a monophyletic group of ubiquitous hymenomycetous mycobionts which form ericoid and orchid mycorrhizae, ecto- and ectendomycorrhizae, and nonspecific root endophytic associations with a wide spectrum of plants. However, due to the complete lack of fungal isolates derived from Ericaceae roots, the Sebacinales ericoid mycorrhizal (ErM) potential has not yet been tested experimentally. Here, we report for the first time isolation of a serendipitoid (formerly Sebacinales Group B) mycobiont from Ericaceae which survived in pure culture for several years. This allowed us to test its ability to form ericoid mycorrhizae with an Ericaceae host in vitro, to describe its development and colonization pattern in host roots over time, and to compare its performance with typical ErM fungi and other serendipitoids derived from non-Ericaceae hosts. Out of ten serendipitoid isolates tested, eight intracellularly colonized *Vaccinium* hair roots, but only the Ericaceae-derived isolate repeatedly formed typical ericoid mycorrhiza morphologically identical to ericoid mycorrhiza commonly found in naturally colonized Ericaceae, but yet different from ericoid mycorrhiza formed in vitro by the prominent ascomycetous ErM fungus *Rhizoscyphus ericae*. One Orchidaceae-derived isolate repeatedly formed abundant hyaline intracellular microsclerotia morphologically identical to those occasionally found in naturally colonized Ericaceae, and an isolate of *Serendipita* (= *Piriformospora*) *indica* produced abundant intracellular chlamydospores typical of this species. Our results confirm for the first time experimentally that some Sebacinales can form ericoid mycorrhiza, point to their broad endophytic potential in Ericaceae hosts, and suggest possible ericoid mycorrhizal specificity in Serendipitaceae.

Keywords Ericaceae · Ericoid mycorrhiza · Sebacinales · Serendipitaceae · Endophytes · In vitro re-synthesis

✉ Martin Vohník
vohnik@ibot.cas.cz

[1] Department of Mycorrhizal Symbioses, Institute of Botany ASCR, Průhonice 25243, Czech Republic

[2] Department of Experimental Plant Biology, Faculty of Science, Charles University in Prague, Prague 12844, Czech Republic

[3] Department of Forest Ecology, Faculty of Forestry and Wood Technology, Mendel University of Agriculture and Forestry in Brno, Zemědělská 3, Brno 61300, Czech Republic

[4] Crop Research Institute, Drnovská 507, Praha 16106, Czech Republic

[5] DNA Laboratory, Institute of Botany ASCR, Průhonice 25243, Czech Republic

[6] Institut de Systématique, Évolution, Biodiversité, Muséum National d'Histoire Naturelle, Sorbonne Universités, 57 rue Cuvier, CP50, Paris 75005, France

[7] Department of Plant Taxonomy and Nature Conservation, University of Gdansk, Wita Stwosza 59, Gdansk 80308, Poland

Introduction

The Sebacinales are a ubiquitous monophyletic group of basal Hymenomycetes involved in a wide range of plant root symbiotic associations (Oberwinkler et al. 2013; Weiss et al. 2016). These include arbutoid (Richard et al. 2005; Kühdorf et al. 2014), cavendishioid (Setaro et al. 2006), ericoid (see below), jungermannoid (Kottke et al. 2003; Newsham and Bridge 2010), orchid (e.g., Warcup and Talbot 1967; Dearnaley et al. 2009; Wright et al. 2010), and pyroloid (e.g., Tedersoo et al. 2007; Vincenot et al. 2008; Hashimoto et al. 2012) mycorrhizae, as well as ectomycorrhizae (e.g.,

Selosse et al. 2002; Urban et al. 2003; Wei and Agerer 2011). Sebacinales are phylogenetically divided into two families, namely, Sebacinaceae (formerly Group or Clade A) and Serendipitaceae (Group/Clade B), which also differ in ecology (including mycorrhizal potential) and cultivability (Weiss et al. 2004; Oberwinkler et al. 2013; Oberwinkler et al. 2014; Weiss et al. 2016).

Serendipitaceae (hereafter, serendipitoids) are frequently detected in Ericaceae roots all over the world (Berch et al. 2002; Allen et al. 2003; Selosse et al. 2007; Ishida and Nordin 2010; Wurzburger et al. 2011; Setaro et al. 2013; Bruzone et al. 2015). Combined microscopic and molecular evidence suggests that they form ericoid mycorrhiza (Selosse et al. 2007), an endomycorrhizal type specific to Ericaceae characterized by formation of hyphal coils within the rhizodermal cells of host thin roots, the so-called hair roots (Smith and Read 2008). However, transmission electron microscopy revealed that serendipitoid hyphae often co-occur with ascomycetous hyphae in ericoid mycorrhizal (ErM) rhizodermal cells (Bonfante-Fasolo 1980; Selosse et al. 2007). This, together with the fact that Ascomycetes, but not Basidiomycetes, were originally isolated in vitro as typical ErM fungi (Pearson and Read 1973; Read 1983; Read 1996), cast some doubts on their true ErM potential (Smith and Read 2008). Moreover, all Sebacinales, and especially serendipitoids, have retained an ancestral potential to grow in roots as endophytes (Selosse et al. 2009; Weiss et al. 2011). Endophytes grow within living plant tissues, cause an unapparent infection, and especially do not form mycorrhizae, or cause any obvious disease symptoms (Wilson 1995; Rodriguez et al. 2009). Serendipitoids are endophytic in numerous plant hosts (e.g., Selosse et al. 2009; Garnica et al. 2013; Riess et al. 2014), and *Serendipita* (= *Piriformospora*) *indica* (Verma et al. 1998) is a much-studied model serendipitoid endophyte (Varma et al. 1999) with plant growth-promoting potential (e.g., Zuccaro et al. 2011; Qiang et al. 2012; Varma et al. 2013). It has no host specificity (Oelmüller et al. 2009), as is typical of many fungal endophytes. Thus, molecular detection of serendipitoid DNA in ErM roots might be connected with endophytic colonization rather than with a true ErM lifestyle (cf. Grunewaldt-Stöcker and von Alten 2016).

The ErM potential of the Sebacinales has not yet been experimentally verified, mainly due to the complete lack of viable isolates derived from Ericaceae (Weiss et al. 2016). This lack is surprising given that all Sebacinales mycobionts detected from Ericaceae hair roots by culture-independent methods so far belong to the Serendipitaceae, which contains many already isolated and cultivated lineages, although obtained mostly from Orchidaceae mycorrhizal hosts (Dearnaley et al. 2013) and, to a lower extent, as plant endophytes (Weiss et al. 2016). We are only aware of one report on isolation of a serendipitoid mycobiont from Ericaceae hair roots, but this isolate (Sebacinales sp. JPK 89; GenBank JQ926170) soon lost its viability, preventing its use in re-synthesis experiments (Vohník et al. 2012a). Sebacinales sp. JPK 89 was isolated from a sheathed ericoid mycorrhiza which was formed by a non-Sebacinales basidiomycete, further emphasizing the above-mentioned potential endophytic lifestyle of serendipitoids within ericoid mycorrhizae actually formed by other mycobionts.

The final, rigorous demonstration by re-synthesis that serendipitoids are mycorrhizal mycobionts of Ericaceae hair roots is pending (Smith and Read 2008) and is hampered by the fact that available serendipitoid strains were not isolated from Ericaceae roots but mostly from orchids. Indeed, field comparisons of sympatric Orchidaceae and Ericaceae show that they tend to associate with different serendipitoid guilds (Kottke et al. 2008; Setaro et al. 2013). Thus, the many serendipitoid isolates available from orchids may not be relevant for re-synthesis of ericoid mycorrhizae.

We obtained an Ericaceae-derived serendipitoid isolate during an attempt to isolate new strains of the basidiomycete recently described to form sheathed ericoid mycorrhiza (Vohník et al. 2012a). Subsequently, we established a small collection of serendipitoids already isolated from other sources (including the endophyte *Serendipita indica*) in order to test their symbiotic potential in an ericaceous host plant, the European blueberry (*Vaccinium myrtillus*). Here, we describe (1) a simple procedure we routinely use for the isolation of Ericaceae root mycobionts, which led to the isolation of the Ericaceae-derived serendipitoid mycobiont, and (2) the colonization patterns and performances of serendipitoid isolates from the above-mentioned collection in the Ericaceae host, with comparison to the typical ascomycetous ErM fungi *Meliniomyces variabilis* and *Rhizoscyphus ericae*.

Materials and methods

Isolation and identification of a serendipitoid mycobiont from *Vaccinium* hair roots

Root sampling was performed in a regenerating Norway spruce (*Picea abies* (L.) Karst.) forest located in the foothills of Forbordfjellet, close to Stjørdal in mid-Norway (63°31.125′ N, 010°53.287′E; approx. 400 m above sea level) in May 2011. The forest soil is a typical podzol, with a layer of humus overlying mineral soil of sand and gravel. Ground ericaceous vegetation is dominated by *V. myrtillus* L. with co-occurring *Vaccinium vitis-idaea* L., *Calluna vulgaris* (L.) Hull., and *Empetrum nigrum* L. A mixed hair root sample from *Vaccinium* shrubs overgrowing a partially decomposed spruce stump was transported to the laboratory and carefully washed free of adhering substrate. Hair roots were subsequently cut into approx. 2.5-mm pieces, surface-sterilized with 10 %

SAVO (household bleach, 4.5 % available chlorine) for 30 s, rinsed three times in sterile water, and placed on modified Melin Norkrans Medium (MMN; Molina and Palmer 1982) amended with 4 mg/L benomyl (Sigma-Aldrich, Germany), which suppresses the growth of most ascomycetes. There were five plastic Petri dishes, each with ten root pieces, i.e., 50 root pieces in total. The dishes were sealed with an air-permeable tape and incubated in the dark at room temperature for 6 weeks. Emerging mycelia were transferred to new dishes with MMN, kept at room temperature in the dark, and periodically checked for growth and possible contamination.

DNA was extracted from all obtained isolates using an Extract-N-Amp Plant Kit (Sigma-Aldrich) following the manufacturer's instructions. The ITS1-5.8S-ITS2 nuclear ribosomal DNA (rDNA) region was amplified using the ITS1F+ ITS4 primer pair, with polymerase chain reaction (PCR) parameters and gel electrophoresis as in Vohník et al. (2012a). PCR products were purified and sequenced by Macrogen Europe Laboratory (Macrogen Inc., the Netherlands/South Korea) using the ITS1F forward primer. Obtained sequences were screened in Finch TV v1.4.0 (geospiza.com/finchtv) and manually edited when needed. Subsequently, they were subjected to BLAST searches in the GenBank database (Altschul et al. 1997). The only serendipitoid isolate obtained (labeled as Sebacinales sp. JPK 132) was further used in the following re-synthesis experiments.

Isolates used for re-synthesis experiments

Twelve different fungal isolates were used in this study. Of these, ten belonged to the Serendipitaceae (*Serendipita* = *Piriformospora indica* isolate PIND, Sebacinales sp. isolates #26, #29, #39, #42, #165, #316, #443, JPK 132, and PB3a) and two represented typical ascomycetous ErM fungi (*M. variabilis* isolate MVA-4 and *R. ericae* isolate RER-1). Seven serendipitoid isolates originated from roots of different species of Australian orchids, one originated from rhizosphere soil of *Phyllanthus* sp. (Phyllanthaceae, Malpighiales) from Australia, one from Ericaceae hair roots (Sebacinales sp. JPK 132; see above), and *S. indica* PIND was derived from the original *S. indica* isolate obtained from the rhizosphere of *Prosopis juliflora* and *Zizyphus nummularia* in northwest India. The two reference ascomycetous isolates, *R. ericae* RER-1 and *M. variabilis* MVA-4, were originally isolated from *C. vulgaris* hair roots and a *P. abies* root tip, respectively (for details and references, see Table 1). All isolates were sub-cultured on malt-yeast-peptone (MYP) medium (malt extract 7 g, peptone 1 g, yeast extract 0.5 g, and agar 10 g per 1 l of sterile deionized water) and subsequently transferred to MMN, i.e., the same medium as used for re-synthesis experiments. In total, three independent in vitro re-synthesis experiments were performed so as to compare/verify results in different experimental microcosms (cf. Vohník et al. 2012a) and to test as many available isolates as possible.

Phylogenetic placement of the serendipitoid isolates used in this study

The D1/D2 regions of the nuclear-encoded large subunit (nucLSU) of 28S rDNA, i.e., the standard regions for Sebacinales phylogenetic studies (Weiss et al. 2004), were

Table 1 Fungal isolates used in this study

Isolate	Source (host/country)	Reference (isolated by)	GenBank no.
Meliniomyces variabilis isolate MVA-4	*Picea abies* (Pinaceae) root tip/CZE	Vohník et al. (2013)	EF093169
Serendipita (= *Piriformospora*) *indica* isolate PIND	rhizosphere soil/IND	Verma et al. (1998)	KT762618
Rhizoscyphus ericae isolate RER-1	*Calluna vulgaris* (Ericaceae) hair root/ENG	Pearson and Read (1973)	AJ319078
Sebacinales sp. isolate #26	*Caladenia latifolia* (Orchidaceae) root/AUS	#026AC2 (B. Newman, K. Dixon)	KT762613
Sebacinales sp. isolate #29	*Eriochilus scaber* (Orchidaceae) root/AUS	MAFF305829 [J.H. Warcup (0714)]	KT762620
Sebacinales sp. isolate #39	*Phyllanthus calycinus* (Phyllanthaceae) rhizosphere soil/AUS	MAFF305839 [J.H. Warcup (0914)]	KT762611
Sebacinales sp. isolate #42	*Microtis unifolia* (Orchidaceae) root/AUS	MAFF305842 [J.H. Warcup (0977)]	KT762612
Sebacinales sp. isolate #165	*Caladenia longicauda longicauda* (Orchidaceae) root/AUS	#165 CD9 (B. Newman, K. Dixon)	KT762615
Sebacinales sp. isolate #316	*Caladenia huegelii* (Orchidaceae) root/AUS	#316 FB4 (B. Newman, K. Dixon)	KT762617
Sebacinales sp. isolate #443	*Caladenia huegelii* (Orchidaceae) root/AUS	#443 HB11 (B. Newman, K. Dixon)	KT762619
Sebacinales sp. isolate JPK 132	*Vaccinium* sp. (Ericaceae) hair root/NOR	this study (J. J. Sadowsky)	KT762614
Sebacinales sp. isolate PB3a	*Caladenia attingens gracillima* (Orchidaceae) root/AUS	this study (N. Swarts)	KT762616

AUS Australia, *CZE* Czech Republic, *ENG* England, *IND* India, *NOR* Norway

used to place the serendipitoid isolates into a phylogenetic context. To obtain their sequences, DNA was extracted from all serendipitoid isolates as described above. The nucLSU rDNA was amplified using the LR0R + LR5 primer pair (Vilgalys and Hester 1990). PCR products were purified and sequenced by Macrogen Europe Laboratory using the LR0R forward primer. The sequences obtained were edited using Chromas 1.45 (Technelysium Pty Ltd., Australia) and aligned in Bioedit V7.0.9.0 (Hall 1999) to a dataset of Sebacinales sequences kindly provided by M. Weiss (Tübingen, Germany) and originally used in Weiss et al. (2011). The sequences were deposited in GenBank (accession numbers KT762611–KT762620).

Preliminary neighbor joining analyses were performed in PAUP* v. 4.0b10 (Swofford 2002) in order to reduce the large dataset (comprising 277 taxa including our samples) to a smaller, representative set of sequences emphasizing the position of the investigated isolates. To achieve this, the number of Sebacinaceae sequences was greatly reduced, retaining only representatives of various genera, with taxonomic adaptations following Oberwinkler et al. (2014) and Weiss et al. (2016). Further reductions were made in the Serendipitaceae (e.g., deleting sequences dissimilar to any of our samples, excluding very similar or identical sequences). Generally, fungal isolates were preferred to uncultured material, and a broad range of host mycorrhizal types and geographic regions was covered. The dataset was complemented by the newly described species *Serendipita hermamans* (Riess et al. 2014) and *Serendipita* (= *Piriformospora*) *williamsii* (Basiewicz et al. 2012). Samples of the complex "*Sebacina vermifera*" were renamed "*Serendipita vermifera*" following Weiss et al. (2016). Subsequently, BLAST searches with each of the serendipitoid isolates were performed against GenBank to ensure that all similar serendipitoid sequences were included. Since some of our isolates (Sebacinales sp. PB3a, #316 and #443, and Sebacinales sp. #29 and #39, respectively) were identical in sequence, only one representative sequence was included for tree construction. Members of the orders Auriculariales, Trechisporales, and Geastrales were used as outgroups. The final dataset comprising 91 sequences (including four outgroup taxa) was subjected to phylogenetic analyses based on maximum likelihood (ML), Bayesian inference, and maximum parsimony (MP) criteria.

ML analysis was performed with raxmlGUI v. 1.3 (Silvestro and Michalak 2012) based on RAxML (Stamatakis 2006) version 7.4.2 (released by Alexandros Stamatakis on 23rd November 2012). Ten ML runs with 1000 thorough bootstrap replicates were done using a GTR substitution matrix and a gamma model of rate heterogeneity. Bayesian analysis was done using MrBayes (Ronquist and Huelsenbeck 2003) v. 3.2.2. x64 in a GTR+I+G model of the presumed molecular evolution. Two parallel MCMC runs with four chains each were run for 5 million generations, sampling every 1000th tree. All statistical parameters indicated that convergence was reached. The first 25 % of trees per run were discarded as burn-in and a consensus tree based on the remaining 10,002 trees was generated. MP analysis was done in PAUP* v. 4.0b10 (Swofford 2002) as a heuristic search with 100 random addition sequence replicates and TBR branch swapping, saving no more than 100 trees with length greater than or equal to 1 per replicate. Bootstrapping was done using the same settings and 1000 replicates, but without branch swapping.

Re-synthesis Experiment I: testing mycorrhizal ability of the Ericaceae-derived Sebacinales sp. JPK 132

This experiment was set up (1) to verify the ErM status of the Ericaceae-derived Sebacinales sp. JPK 132 isolate and (2) to observe the development of host root colonization over time as compared to a representative of the serendipitoid isolates derived from non-Ericaceae hosts (Sebacinales sp. PB3a) and the typical ErM ascomycete *R. ericae*.

Seeds of wildly growing European blueberry of local origin (Lusatian Mountains, Northern Bohemia, Czech Republic) were surface-sterilized in 10 % SAVO, rinsed in sterile water, and sown on MMN without malt extract. Eighty-three-day-old seedlings were transferred to square Petri dishes (12 × 12 cm) with MMN without malt extract and glucose (nine seedlings per dish), sealed with an air-permeable tape, and left to root in a growth chamber under a 21 °C–16-h light/15 °C–8-h dark cycle and irradiation of 200 μmol m^{-2} s^{-1}. After 10 days, nine plugs (diameter approx. 5 mm) dissected from mycelial cultures of the selected isolates grown on full MMN were placed in each dish close to the seedlings' roots (one plug per seedling). There were three dishes with a total of 27 seedlings for each of the three inoculated fungi, and control seedlings (27 in three dishes) were inoculated with MMN plugs without fungal mycelium. The inoculated dishes were resealed, randomly placed in the growth chamber, and incubated as above.

The seedlings were evaluated at three consecutive harvests: one seedling was randomly picked per dish (i.e., three per inoculation treatment) 22 days after inoculation, another three (nine per treatment) 56 days after inoculation, and, finally, the five remaining seedlings (15 per treatment) were harvested 112 days after inoculation. At each harvest, seedlings were carefully washed free of the remaining agar medium and their roots were separated from shoots. The roots were subsequently cleared in 10 % KOH at 121 °C for 15 min, rinsed in water, acidified for 20 s in 3 % HCl, rinsed in water, and placed on glass slides in 0.05 % Trypan blue solution in lactoglycerol (lactic acid/glycerol/deionized water in a mixing ratio of 1:1:3). The percentage of intracellular hyphal colonization was assessed using an Olympus BX60 microscope equipped with differential interference contrast at ×400 magnification in 100 random fields of view. Photographs of root fungal

colonization were taken with an Olympus DP70 camera at magnifications of ×200, ×400, and ×1000. Subsequently, they were assembled and modified for clarity as needed in Paint.NET and Adobe Photoshop.

At the final harvest, a small piece of the growth medium with fungal mycelium was picked from the rhizosphere of the remaining seedlings, transferred to a 6-cm Petri dish filled with MMN, incubated in the dark at room temperature, and periodically screened for hyphal growth during the next 2 months. This was done to ensure that the inoculated fungi remained alive during the whole experiment as some Sebacinales isolates often die when transferred from MYP (a medium not suitable for plant growth) to MMN.

Re-synthesis Experiment II: comparative mycorrhizal abilities of several serendipitoids

In this experiment, we mainly wanted to compare the root symbiotic potential of the Ericaceae-derived Sebacinales sp. JPK 132 with other non-Ericaceae serendipitoid isolates using a peat-based substrate that better reflects natural conditions. Nine fungal isolates were pre-cultivated for 24 days in plastic Petri dishes with MMN as above: the reference ErM isolates *M. variabilis* MVA-4 and *R. ericae* RER-1, the *S. indica* isolate PIND, and the serendipitoid isolates Sebacinales sp. #26, #29, #42, #165, JPK 132, and PB3a. Sterile 50-ml polypropylene Neptune centrifuge tubes (Pretech Instruments, Sollentuna, Sweden) were filled with 5 ml of MMN without malt extract and glucose, inoculated with plugs dissected from the pre-cultivated fungal cultures, and incubated in the dark at room temperature. Control tubes were inoculated with plugs dissected from Petri dishes without fungal mycelium. After 1 month of incubation, 1.5 g (fresh weight, FW) of gamma-sterilized peat + vermiculite (1:9, *v*/*v*) substrate was added to each tube; the tubes were each watered with 3 ml of sterile deionized water and incubated in the dark at room temperature for 10 days to let the fungal mycelium develop into the newly added substrate. Eventually, developed seedlings of European blueberry (five to six leaves, prepared as above) were inserted into the tubes, these were sealed with an air-permeable tape, randomly placed in the growth chamber, and incubated as above for 3 months. There were nine tubes for each inoculated fungus and non-inoculated control. After 3 months, the seedlings were destructively harvested and their roots were treated as above, including graphic documentation.

Re-synthesis Experiment III: comparative effects on host plants of several serendipitoids

This experiment was set up to compare the root symbiotic potential of the Ericaceae-derived Sebacinales sp. JPK 132 with the rest of the serendipitoid isolates available in our culture collection, and to screen their effect on the host plant growth, in larger, better ventilated cultivation vessels. We used seven isolates pre-cultivated on MMN in more realistic soil-like conditions: *R. ericae* RER-1, *S. indica* PIND, and the serendipitoid isolates Sebacinales sp. #39, #316, #443, JPK 132, and PB3a. Fifty milliliters of MMN without malt extract and with 1 g of glucose per liter were poured into sterile plant culture containers Magenta GA7-3 (275 ml; Sigma-Aldrich), solidified at room temperature, and then inoculated with one of the six fungal strains using five mycelial plugs (diameter approx. 5 mm) of the respective strain per container and three containers per fungal strain. Control containers were inoculated with plugs without mycelium. All containers were incubated in the dark at room temperature for 2 weeks, which allowed the mycelium of the inoculated fungi to cover the surface of the medium. Subsequently, 13 g (FW) of gamma-sterilized peat + vermiculite substrate (approx. one fifth of the container depth) was transferred onto the surface of the MMN medium, watered with 15 ml of sterilized tap water, and three 1-month-old European blueberry seedlings (prepared as above) were aseptically planted in each container. The containers were randomly placed and incubated in the growth chamber under the same regime as described above. The seedlings were harvested after 2, 3, and 4 months by picking one randomly selected seedling from each container at each harvest. Their roots were gently washed under running tap water, dried with paper towels, weighed to obtain fresh root weight (FRW), and transferred to 10 % KOH solution overnight. The following day, the roots were acidified as above, stained 3 days in Trypan blue solution in lactoglycerol, and de-stained for 3 days in lactoglycerol (all at room temperature). Their percentage colonization was estimated by random screening of 500 rhizodermal cells per root system (50 cells per ten randomly selected hair roots) using the equipment described above. Shoots were dried in a laminar flow hood at room temperature overnight and weighed to obtain dry shoot weight (DSW). Kruskal–Wallis test followed by multiple comparisons of mean ranks was conducted in Statistica 12.6 (StatSoft Inc., Tulsa, USA).

Results

Isolation of a serendipitoid mycobiont from *Vaccinium* hair roots

In total, we obtained 28 isolates from Forbordfjellet's *Vaccinium*. BLAST searches in GenBank revealed that most of them belonged to non-serendipitoid Basidiomycetes belonging to the Agaricales (*Gymnopilus* spp. and *Mycena* spp.) and Polyporales (*Hypochnicium geogenium*; details not

shown). Only one ITS sequence had 98–99 % similarity to deposited sequences belonging to Sebacinales/Serendipitaceae (e.g., AM181401 and HQ211970), and the respective isolate (Sebacinales sp. JPK 132) was subjected to further investigations.

Phylogenetic analyses

The serendipitoid isolates used in this study covered most of the phylogenetic diversity of the family (Fig. 1). Sequences of the "*Serendipita vermifera*" complex (formerly "*Sebacina vermifera*"), a non-monophyletic species in need of taxonomic revision (Weiss et al. 2004), were scattered across Serendipitaceae. Most of the isolates screened in this study (#26, #29, #39, #42, #316, #443, and PB3a) had sequences identical or nearly identical to previously known *S. vermifera* strains (Fig. 1). One Australian Orchidaceae-derived isolate (#165) was most similar to an uncultured symbiont from an Australian orchid (AY643801, 98 % identity) and to available "*S. vermifera*" sequences from Australian orchids (e.g., AY505550 and AY505551, 96–97 % identity). The sequence of *S. indica* PIND was, as expected, identical to that of the original isolate (see "Materials and methods"). Apart from cultivated *Serendipita* fungi, the basal part of the Serendipitaceae contained sequences from fungi mycorrhizal with orchids or endophytic with different hosts. The terminal crown group of Serendipitaceae showed a more versatile assemblage of host mycorrhizal types (cavendishioid, ericoid, jungermannoid, and orchid mycorrhizal) and endophytic associations. The new Sebacinales sp. JPK 132 isolate clustered in this crown, among taxa that were exclusively derived from ErM hosts (especially the host genus *Vaccinium*) from all over the world. In this part of the Serendipitaceae phylogenetic tree, no other in vitro isolated fungus was present.

Re-synthesis Experiment I: testing mycorrhizal ability of the Ericaceae-derived Sebacinales sp. JPK 132

This experiment shows that the *Vaccinium*-derived isolate Sebacinales sp. JPK 132 forms ericoid mycorrhiza with typical intracellular hyphal coils and documents its development in host roots over time. Intracellular hyphal colonization was always limited to the host rhizodermis and started by the formation of loose loops (Fig. 2a), which became denser over time (Fig. 2b), eventually filling the whole lumen of the colonized cells (Fig. 2c). In later stages, hyphae started to lose their contours and the intracellular dense coils appeared to undergo stages of digestion (Fig. 2d; cf. McLennan 1935). Intracellular hyphal colonization often started right behind the apical part of the colonized hair root (Fig. 2e) and was often markedly high around zones where lateral hair roots emerged from main roots (Fig. 2f). In contrast, the intracellular colonization pattern of the Orchidaceae-derived serendipitoid isolate typically comprised loose loops formed by constricted hyphae which became denser over time (Fig. 3a), eventually forming hyaline microsclerotia (Fig. 3b–d), which are occasionally found in naturally colonized Ericaceae hair roots. Very rarely, Sebacinales sp. PB3a formed dense intracellular coils which might be interpreted as ericoid mycorrhizae (Fig. 3e). However, such colonization was limited to a few rhizodermal cells within all the root systems. Roots of control plants were free of any visible colonization. Beyond sparse hyphae, no fungal sheath was ever seen around the roots.

Although Sebacinales sp. JPK 132 formed typical ericoid mycorrhizae as in naturally colonized roots, its colonization pattern differed from that of the ErM fungus *R. ericae*, whose hyphae often filled whole rhizodermal cells, but the contours of the resulting dense coils were not distinct and it was often difficult to distinguish single hyphae (Fig. 4a, b). On the other hand, the latter ErM morphology is also frequently seen in naturally colonized roots.

Twenty-two days after inoculation, the average root intracellular colonization was 45.3 ± 24.0 % (mean ± SD) for plants inoculated with *R. ericae* RER-1, 33.3 ± 1.52 % for Sebacinales sp. PB3a, and 7.7 ± 8.6 % for Sebacinales sp. JPK 132. After 56 days, it was 46.9 ± 26.6 % for RER-1, 26.9 ± 20.1 % for PB3a, and 11.3 ± 11.1 % for JPK 132. At the final harvest, 112 days after inoculation, it was 34.9 ± 16.7 % for RER-1, 19.13 ± 11.7 % for PB3a, and 26.8 ± 19.4 % for JPK 132. Apparently, although the colonization levels of the serendipitoid isolate JPK 132 were the lowest in the first half of the experimental time span, they eventually reached values comparable to those of the typical ErM fungus *R. ericae* (Table 2).

All 15 plugs dissected from the rhizosphere of the seedlings inoculated with Sebacinales sp. PB3a and *R. ericae* RER-1 and ten plugs dissected from the rhizosphere of those inoculated with Sebacinales sp. JPK 132 produced viable fungal mycelium morphologically identical to the respective inoculated mycobiont, confirming that the inoculum had remained viable.

Re-synthesis Experiment II: comparative mycorrhizal abilities of several serendipitoids

Sebacinales sp. JPK 132, Sebacinales sp. PB3a, and *R. ericae* RER-1 formed intracellular hyphal colonization identical to Experiment 1 (Figs. 2, 3, and 4a, b). *M. variabilis* MVA-4 very rarely (a few rhizodermal cells for the whole experimental set) formed intracellular coils of hyaline hyphae (Fig. 4c),

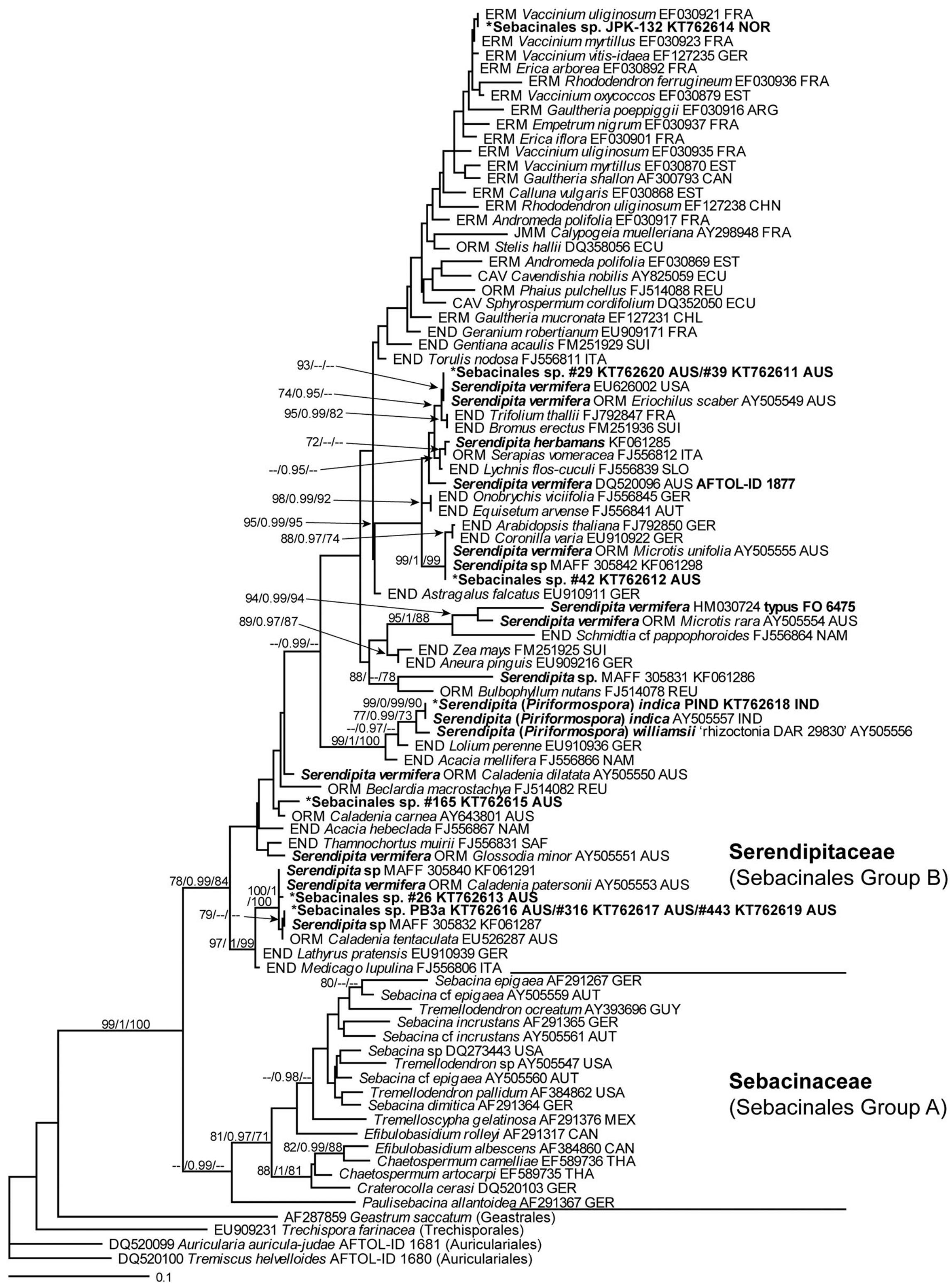

Fig. 1 Phylogenetic placement of serendipitoid isolates investigated in this study. Phylogenetic analyses were based on sequences of the D1/D2 regions of the nuclear LSU repeat of 28S ribosomal DNA. The best maximum likelihood tree is shown with bootstrap support values ≥70 %, Bayesian posterior probabilities ≥0.95, and bootstrap support values of maximum parsimony analysis ≥70 % near the respective branches. For easier orientation, sequences derived from the isolates investigated in this study are indicated with an *asterisk* and in **bold**, and sequences of *Serendipita* (including former *Piriformospora*) isolates determined at species level are in **bold** and *italics*. Further sequence labels indicate putative symbiotic status for sequences derived from isolates (*CAV* cavendishioid mycorrhiza, *END* endophytic, *ERM* ericoid mycorrhiza, *JMM* jungermannoid mycorrhiza, *ORM* orchid mycorrhiza), host plant (where available), GenBank accession number, and country of origin (following ISO 3166-1 alpha-3)

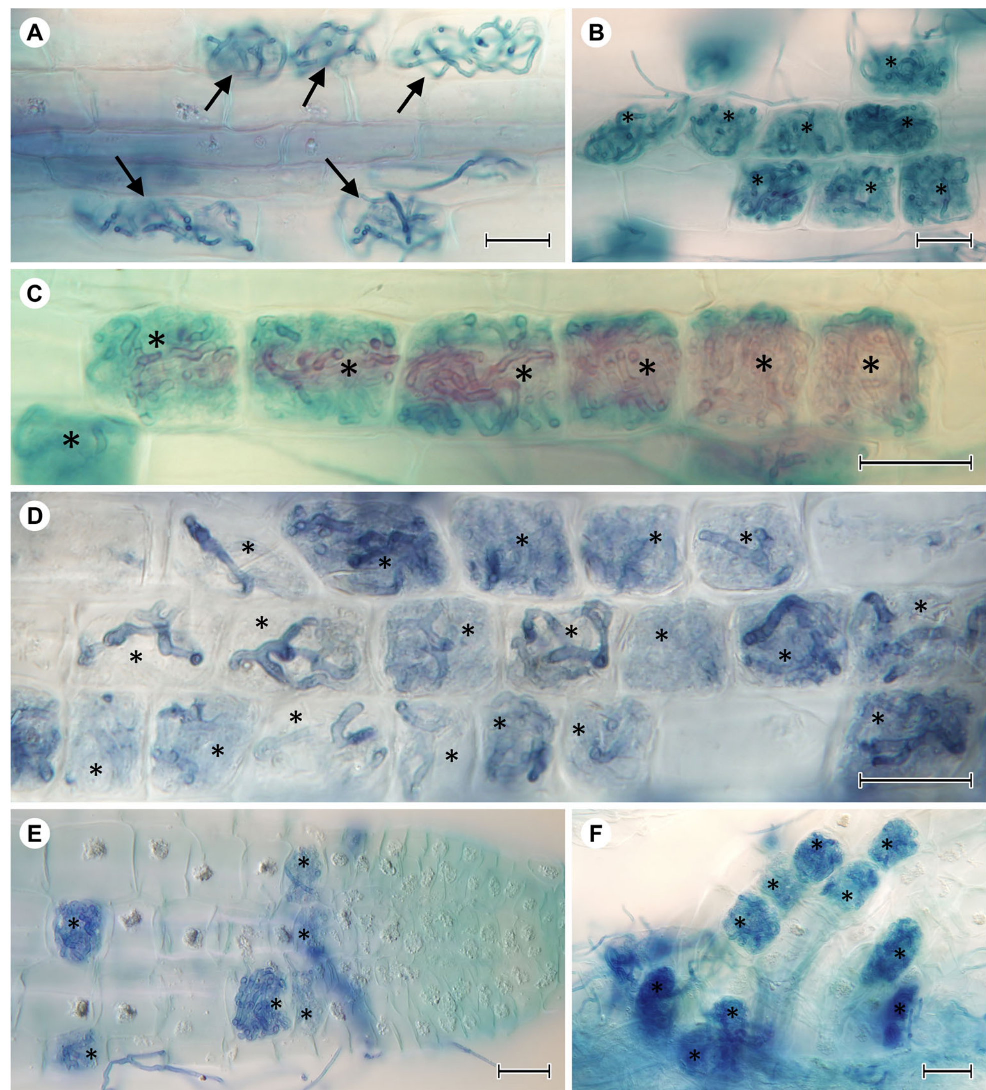

Fig. 2 Ericoid mycorrhizal colonization in *Vaccinium myrtillus* hair roots inoculated with Sebacinales sp. JPK 132. **a** Early stages of colonization characterized by the formation of loose intracellular hyphal loops (*arrows*) in the host rhizodermis. **b**, **c** Later stages of intracellular colonization characterized by the development of dense hyphal coils (*asterisks*) typical of ericoid mycorrhiza. **d** The final phase of intracellular colonization when contours of some hyphal coils become less visible (*asterisks*). Hyphal colonization often started in the rhizodermal cells developing immediately behind the root cap (**e**) or at the basis of lateral roots (**f**). All roots stained with Trypan blue and observed with differential interference contrast. *Bars* = 20 μm; all pictures from three consecutive harvests in Experiment I

which differed from Sebacinales sp. JPK 132 and *R. ericae* RER-1, but resembled the infrequent intracellular colonization of Sebacinales sp. PB3a (Fig. 3e).

While in the seedlings inoculated with Sebacinales sp. #42 there was no apparent root colonization, Sebacinales sp. #26, #29, and #165 rarely formed intracellular loose loops similar to Sebacinales sp. PB3a, i.e., with irregularly lobed hyphae (Fig. 5a, b). However, the frequency of these structures was much lower (individual rhizodermal cells per screened root system) than in Sebacinales sp. PB3a and formation of typical hyaline microsclerotia was absent. *S. indica* PIND typically formed intracellular round bodies, which we interpret as intracellular chlamydospores (Figs. 6a–e; cf. Verma et al. 1998; Varma et al. 1999), one to four per single colonized cell, often in the cells right below the rhizodermis. Chlamydospores formed up to the apex of the colonized hair roots (Fig. 6a) and were discontinuously present throughout the whole colonized roots (Fig. 6b–e). *S. indica* PIND also produced intracellular coiled hyphae resembling the intracellular colonization of Sebacinales sp. PB3a and *M. variabilis* MVA-4 (Fig. 6b–e) and "coiled and branched intracellular hyphae" (Fig. 6b–e) as reported by Varma et al. (1999). Control roots were free of any apparent fungal or bacterial colonization.

Re-synthesis Experiment III: comparative effects on host plants of several serendipitoids

All previously tested isolates (*S. indica* PIND, *R. ericae* RER-1, and Sebacinales sp. JPK 132 and PB3a) displayed similar or identical colonization patterns also in Experiment III, i.e., intracellular chlamydospores, ericoid mycorrhizal coils with barely visible contours, ericoid mycorrhizal coils with clearly visible hyphae, and hyaline microsclerotia, respectively. Sebacinales sp. #39 infrequently produced loose to dense intracellular coils formed by narrow hyphae (Fig. 5c), which were similar to those of *M. variabilis* MVA-4 and Sebacinales sp. PB3a. In contrast to Sebacinales sp. #316,

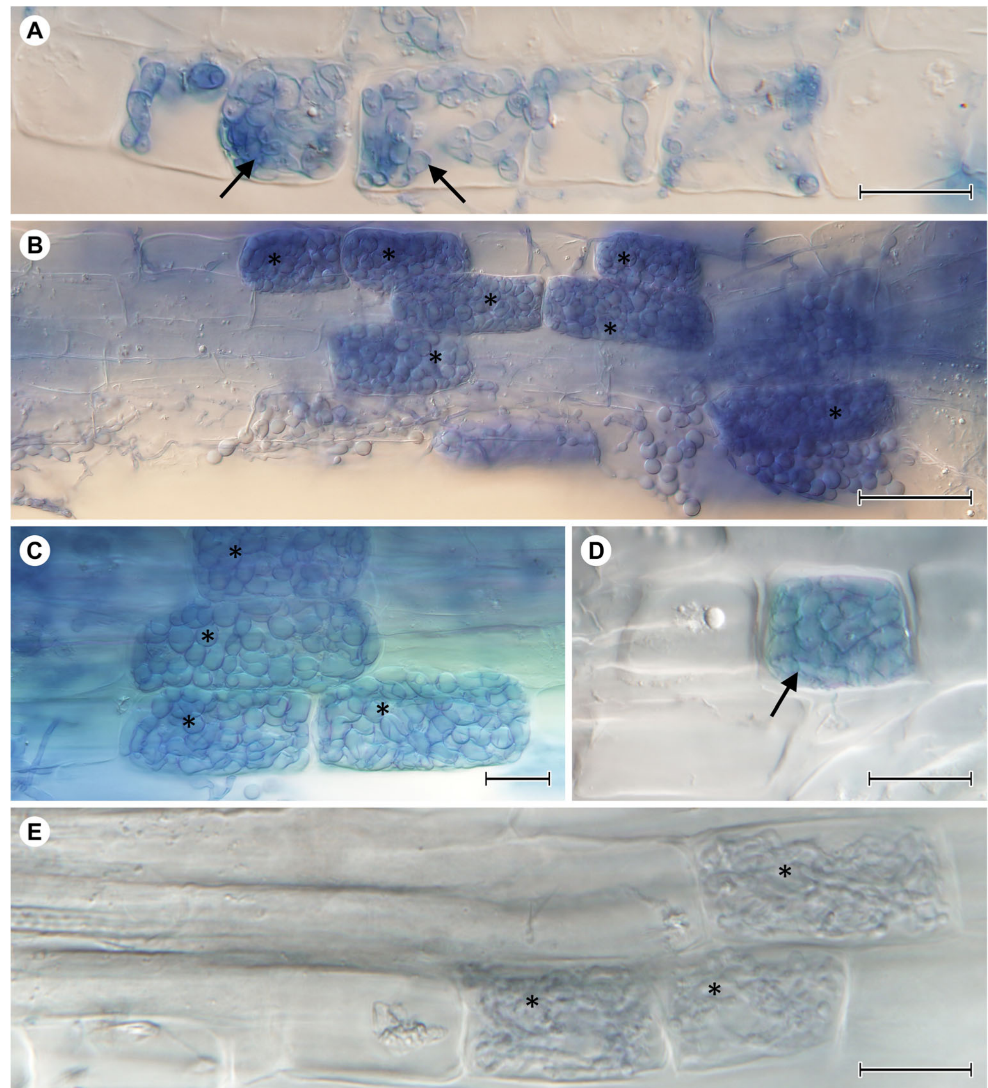

Fig. 3 Colonization pattern of Sebacinales sp. PB3a in *Vaccinium myrtillus* hair roots. **a** Thick septate intracellular hyphae forming loose loops to microsclerotia in the host rhizodermis (*arrows*). *Bar* = 20 μm, Experiment II. **b** Host rhizodermal cells filled with microsclerotia (*asterisks*). Bar = 50 μm, Experiment I. **c**, **d** Host rhizodermal cells filled with microsclerotia in detail (*asterisks* in (**c**) and *arrow* in (**d**)). *Bars* = 20 μm, Experiments I and III, respectively. **e** The Sebacinales sp. isolate PB3a rarely formed dense intracellular hyphal coils morphologically similar to ericoid mycorrhizae (*asterisks*). *Bar* = 20 μm, Experiment III. All roots stained with Trypan blue and observed with DIC

which did not form any visible intracellular colonization, Sebacinales sp. #443 produced thick constricted intracellular hyphae, which often developed into hyaline microsclerotia (Fig. 5d), and occasionally produced loose intracellular loops of narrow hyphae (Fig. 5e). All results concerning the interactions between the inoculated fungi and the host roots at the morphological/cellular level are summarized in Table 3.

The differences in shoot and root growth among the inoculated variants were statistically non-significant (Table 2), probably due to the high variability inherent to genetically non-homogeneous seedlings and the low number of replicates. Similarly, while the Kruskal–Wallis test showed significant differences in the colonization rates after the first and third harvests, multiple comparisons of mean ranks were unable to detect differences between the inoculation variants. Nevertheless, the serendipitoid isolate JPK 132 eventually reached colonization comparable to *R. ericae* RER-1 (40.1 ± 19.9 and 51.7 ± 7.0, respectively), and these levels were by far the highest among all inoculated plants (Table 2). In contrast, the plants inoculated with these two fungi produced the lowest dry shoot biomass (comparable to non-inoculated control but approx. twofold lower than the rest; Table 2), suggesting an (albeit statistically non-significant) negative correlation between root colonization and host shoot growth, i.e., a fungal carbon drain typical of mycorrhizal mycobionts (cf. Colpaert et al. 1996).

Discussion

To our knowledge, there are only two reports of a successful isolation of a serendipitoid mycobiont from Ericaceae hair roots, even though Sebacinales are considered as common ErM fungi worldwide (Selosse et al. 2007). Both reports are from the same area in mid-Norway and use the same isolation method as here on *Vaccinium* roots. The isolate from the first report (Vohník et al. 2012a) soon lost its viability, in contrast

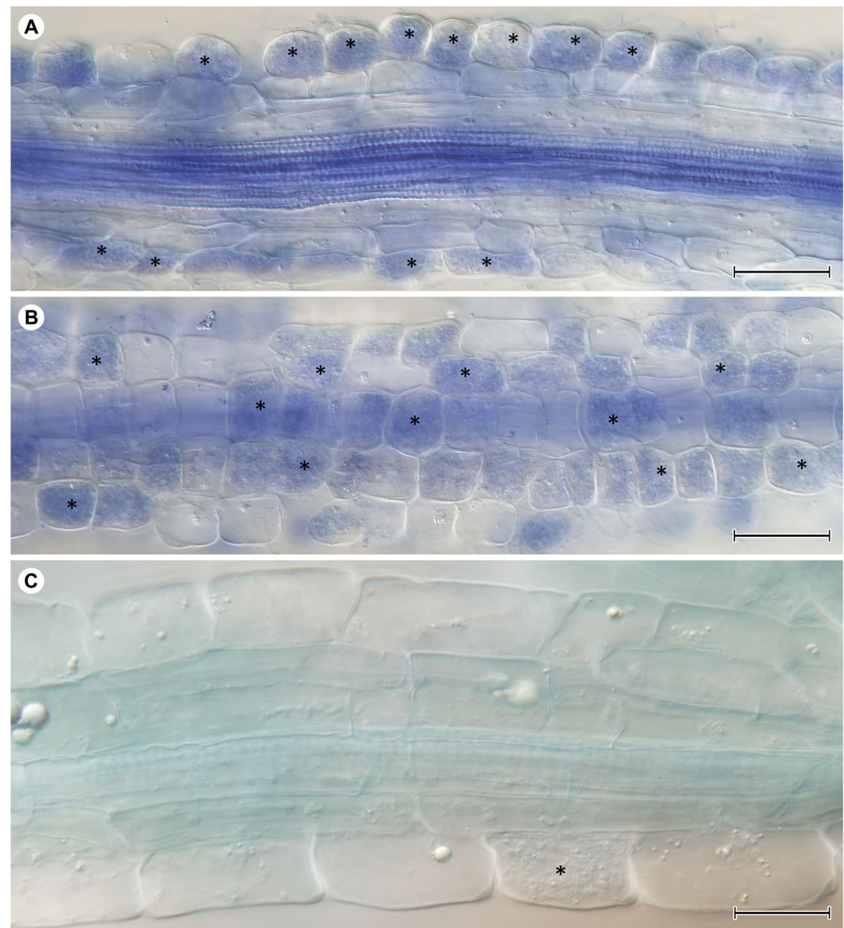

Fig. 4 Colonization patterns of *Rhizoscyphus ericae* RER-1 and *Meliniomyces variabilis* MVA-4 in *Vaccinium myrtillus* hair roots. **a**, **b** Dense intracellular hyphal coils typical of ericoid mycorrhizal colonization abundantly formed by *R. ericae* isolate RER-1 in the rhizodermis of *V. myrtillus* (representatives marked by *asterisks*). *Bars* = 50 μm, Experiment I. **c** Single dense hyaline hyphal coil formed by *M. variabilis* isolate MVA-4 in a rhizodermal cell of *V. myrtillus*. This colonization pattern was infrequent in the host roots, but can be attributed to ericoid mycorrhiza. *Bar* = 20 μm, Experiment II. All roots stained with Trypan blue and observed with DIC

Table 2 *Vaccinium myrtillus* growth response to inoculation and root fungal colonization (Experiment III)

Isolate	FRW (mg)			DSW (mg)			Colonization (%)		
	1 (NS) $H = 1.29$, $p = 0.990$	2 (NS) $H = 6.17$, $p = 0.520$	3 (NS) $H = 5.85$, $p = 0.557$	1 (NS) $H = 10.51$, $p = 0.160$	2 (NS) $H = 6.78$, $p = 0.451$	3 (NS) $H = 13.03$, $p = 0.071$	1 (S) $\mathbf{H = 11.76}$, $\mathbf{p = 0.011}$	2 (NS) $H = 11.06$, $p = 0.055$	3 (S) $\mathbf{H = 18.79}$, $\mathbf{p = 0.008}$
S. indica PIND	7.7 ± 7.2	19.2 ± 17.7	156.1 ± 137.2	4.8 ± 2.5	11.5 ± 4.1	92.6 ± 38.2	2.5 ± 3.7	3.0 ± 2.7	5.7 ± 1.4
***R. ericae* RER-1**	**5.4 ± 4.6**	**35.7 ± 26.4**	**97.2 ± 82.0**	**7.1 ± 4.7**	**12.5 ± 5.6**	**41.6 ± 5.5**	**36.1 ± 15.2**	**31.5 ± 27.5**	**51.7 ± 7.0**
Sebacinales sp. #30	5.9 ± 2.3	16.3 ± 11.1	214.9 ± 122.5	3.4 ± 0.4	12.4 ± 10.1	62.1 ± 10.1	0	0	7.8 ± 4.0
Sebacinales sp. #316	5.7 ± 3.3	8.1 ± 1.1	150.5 ± 129.1	3.0 ± 0.7	14.6 ± 1.7	111.2 ± 37.1	0	0	0.2 ± 0.3
Sebacinales sp. #443	4.4 ± 2.7	22.4 ± 16.5	117.9 ± 93.9	2.0 ± 1.1	21.2 ± 12.1	99.9 ± 20.9	2.1 ± 2.6	2.1 ± 3.7	3.1 ± 2.7
Sebacinales sp. JPK 132	**3.6 ± 2.8**	**103.1 ± 126.5**	**122.8 ± 116.0**	**4.2 ± 2.6**	**24.0 ± 14.2**	**55.7 ± 39.2**	**0**	**23.9 ± 12.1**	**40.1 ± 19.9**
Sebacinales sp. PB3a	5.1 ± 4.2	34.5 ± 34.1	269.4 ± 89.8	4.3 ± 1.2	21.8 ± 10.1	96.5 ± 34.6	0	4.7 ± 5.5	6.5 ± 8.6
Control	4.5 ± 1.8	14.1 ± 17.1	74.8 ± 104.6	1.9 ± 0.8	9.6 ± 4.8	66.9 ± 18.9	0	0	0

Columns within each variable (FRW, DSW, Colonization) contain measurements from the first (*1*), second (*2*) and third (*3*) harvests, respectively ($n = 3$ replicates each). The two isolates forming abundant unambiguous ericoid mycorrhizal structures, i.e., *R. ericae* RER-1 and Sebacinales sp. JPK 132, are in bold

FRW fresh root weight, *DSW* dry shoot weight, *Colonization* % intracellular fungal colonization of the host hair roots rhizodermis

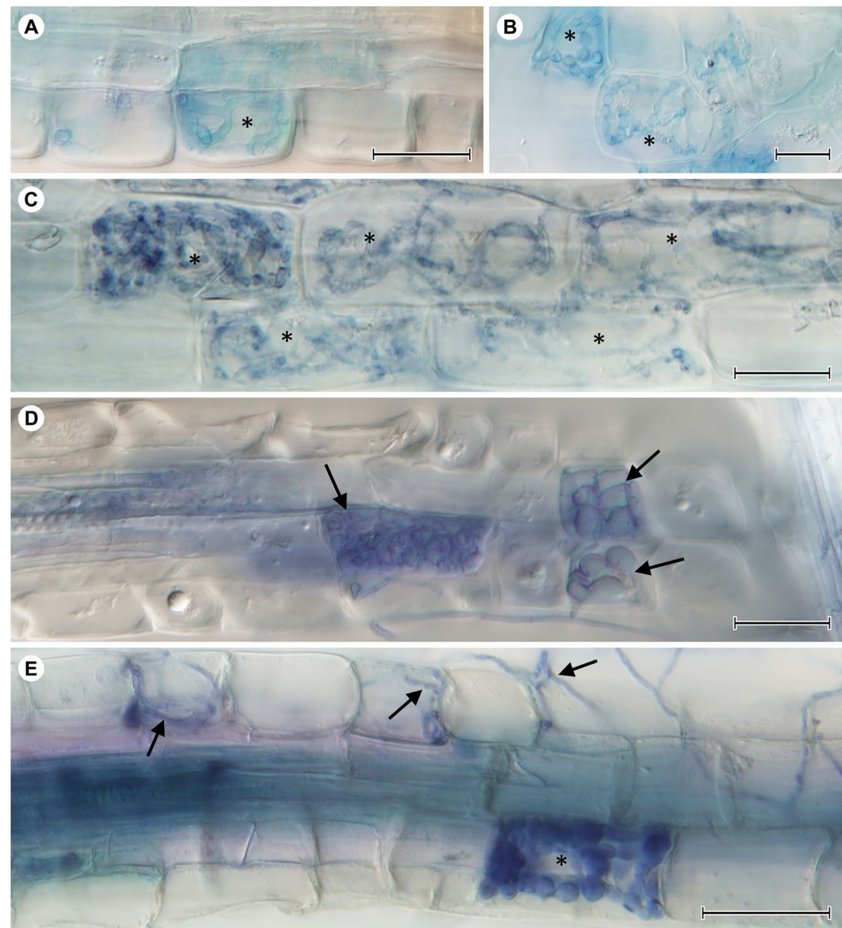

Fig. 5 Colonization pattern of Sebacinales sp. #26, #29, #39, and #443 in *Vaccinium myrtillus* hair roots. **a** Loose intracellular hyphal loops formed by Sebacinaceae sp. #26 (*asterisk*), Experiment II. **b** Loose intracellular hyphal loops formed by Sebacinaceae sp. #29 (*asterisks*), Experiment II. **c** Loose intracellular hyphal loops and coils formed by Sebacinaceae sp. #39 (*asterisks*), Experiment III. These structures would be interpreted as ericoid mycorrhizae when observed in naturally colonized Ericaceae roots. **d** Intracellular microsclerotia formed by Sebacinaceae sp. #443 (*arrows*), Experiment III. **e** Primordium of an intracellular microsclerotium (*asterisk)* and some sparse intracellular hyphae (*arrows*) formed by Sebacinaceae sp. #443, Experiment III. All roots stained with Trypan blue and observed with DIC. *Bars* = 20 μm

to the present study (the JPK 132 isolate has now survived in pure culture for approx. 5 years). The same methodology was also used in our previous study in NW Patagonia on *Gaultheria* roots, but despite detection of serendipitoid DNA using specific primers, no serendipitoid isolate was obtained (Bruzone et al. 2015). In general, Ericaceae-associated Basidiomycetes grow slowly and commonly represent a minority of the cultivable mycobiont communities from Ericaceae hair roots, even when Ascomycetes-suppressing media are used for isolation. For example, using a benomyl-amended medium, Bruzone et al. (2015) found that fast-growing Ascomycetes still represented approx. 77 % of isolates from *Gaultheria* roots. In contrast, in the studies where serendipitoid isolates were obtained, Ascomycetes represented only approx. 38 % (Vohník et al. 2012a) or were totally absent (this study). It thus seems that successful isolation of a serendipitoid mycobiont is not a matter of a special procedure but of competition between different guilds of cultivable root mycobionts. Therefore, the source hair roots have to contain only low amounts of fast-growing, competing (ascomycetous) mycobionts, especially the dark septate endophytes (DSE) related to *Phialocephala fortinii*/*Acephala applanata*. These mycobionts are nearly omnipresent in Ericaceae roots (e.g., Walker et al. 2011; Vohník and Albrechtová 2011; Gorzelak et al. 2012), do not form ericoid mycorrhiza (Lukešová et al. 2015), but can mask typically slow-growing ErM fungi, including the Serendipitaceae (cf. Bruzone et al. 2015). In the current study, such an environment was represented by a dead spruce stump overgrown by Ericaceae dwarf shrubs which apparently favored root symbioses with Basidiomycetes and suppressed Ascomycetes.

The nucLSU rDNA sequence of the *Vaccinium*-derived isolate tested here for its ability to form ericoid mycorrhiza clustered among sequences that were exclusively derived from ErM hosts (Fig. 1), including samples from all over the world (cf. Selosse et al. 2007). Interestingly, the most similar sequences (99.8 % identity) were also derived from *Vaccinium*. We are not able to judge whether this similarity entails conspecificity because the intraspecific variation range of nucLSU remains unknown for Sebacinales. Nevertheless, this observation, together with the fact that only the serendipitoid isolate derived from *Vaccinium* roots formed ericoid mycorrhiza, points to possible ErM specificity within clades of the Serendipitaceae, perhaps similar to the ErM specificity of *R. ericae* within the *R. ericae* aggregate (cf. Vohník et al. 2013). On the one hand, experimental corroboration as well as investigation of the mechanisms underlying the evolution of this specificity are limited by the absence of

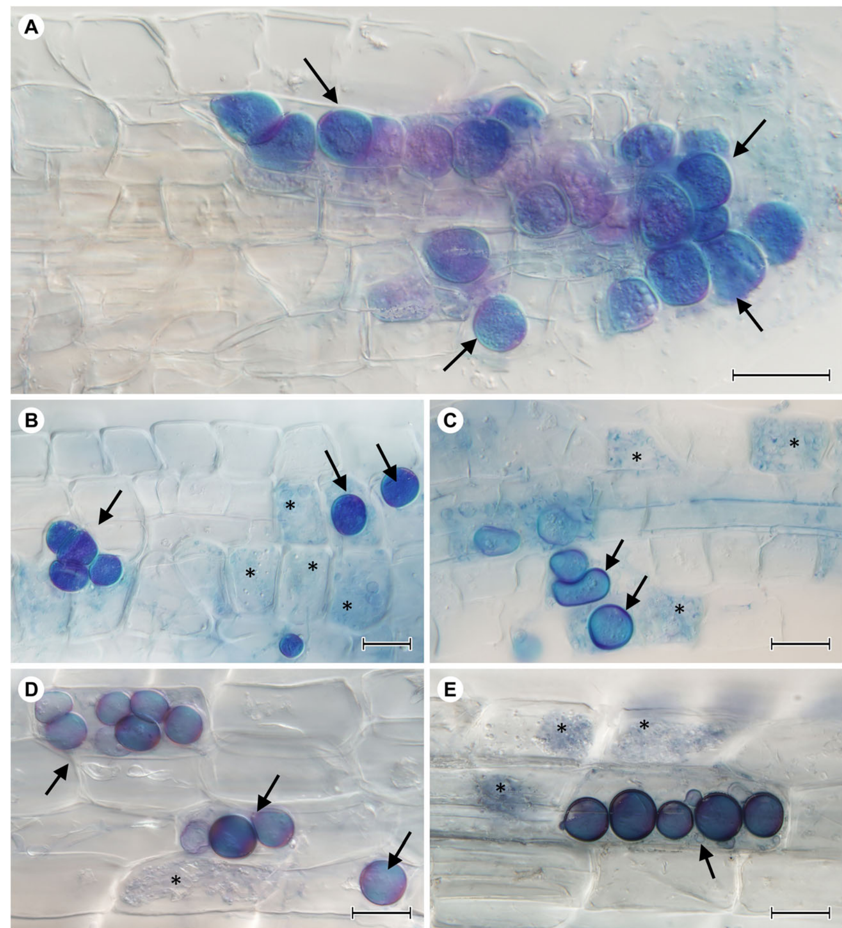

Fig. 6 Colonization pattern of *Serendipita* (= *Piriformospora*) *indica* PIND in *Vaccinium myrtillus* hair roots. **a** Terminal part of a *V. myrtillus* hair root heavily colonized by *S. indica* PIND intracellular chlamydospores, both in the rhizodermis and the root cortex (*arrows*), Experiment II. **b**–**e** Intracellular chlamydospores (*arrows*) were frequently accompanied by fine intracellular hyphae forming loops and coils (*asterisks*), sometimes morphologically resembling ericoid mycorrhizae, Experiments II and III. All roots stained with Trypan blue and observed with DIC. *Bars* = 20 μm

Table 3 Results of microscopic observations with conclusions about the symbiotic status of the investigated isolates

Isolate	Experiment I	Experiment II	Experiment III	Symbiotic status[a]
M. variabilis MVA-4	nu	Infrequent IC hyaline coils resembling ErM	nu	ErM or endophytic
S. indica PIND	nu	IC spores, infrequent IC hyphal loops	IC spores, infrequent IC hyphal loops	Endophytic
***R. ericae* RER-1**	**Typical fine IC ErM coils**	**Typical fine IC ErM coils**	**Typical fine IC ErM coils**	**Typical ErM**
Sebacinales sp. #26	nu	Infrequent IC hyphal loops	nu	Endophytic
Sebacinales sp. #29	nu	Infrequent IC coarse hyphae, often constricted	nu	Endophytic
Sebacinales sp. #39	nu	nu	Infrequent fine IC coils resembling ErM	Endophytic
Sebacinales sp. #42	nu	No colonization	nu	No interaction
Sebacinales sp. #165	nu	Scarce hyaline IC hyphal loops	nu	Endophytic
Sebacinales sp. #316	nu	nu	No colonization	No interaction
Sebacinales sp. #443	nu	nu	IM, loose often constricted IC hyphal loops	endophytic
Sebacinales sp. JPK 132	**Typical coarse IC ErM coils**	**Typical coarse IC ErM coils**	**Typical coarse IC ErM coils**	**Typical ErM**
Sebacinales sp. PB3a	IM, infrequent IC coiled hyphae	Infrequent IC coarse coiled hyphae	IM, coarse and fine coils resembling ErM	Endophytic

The two isolates forming abundant unambiguous ErM structures, *R. ericae* RER-1 and Sebacinales sp. JPK 132, are in bold

ErM ericoid mycorrhizal, *IC* intracellular, *IM* intracellular microsclerotia, *nu* not used

[a] Proposed symbiotic status of the investigated isolates in Ericaceae hair roots based on our microscopic observations and host growth response

other serendipitoid isolates derived from Ericaceae and our generally poor knowledge of the global diversity and distribution of Ericaceae root mycobionts (cf. van der Heijden et al. 2015). On the other hand, several studies suggest that Ericaceae roots do host serendipitoid fungi distinct from those of neighboring plants, e.g., in sympatric orchids and ericaceous plants in Ecuador (Kottke et al. 2008; Setaro et al. 2013). This is surprising, considering the generally broad spectrum of Ericaceae root mycobionts and the apparent ability of Ericaceae to associate with many nonspecific root endophytes co-occurring in the roots of the surrounding vegetation (Bougoure et al. 2007; Tedersoo et al. 2009; Vohník et al. 2013). Indeed, most of the Orchidaceae-derived isolates tested in this study were able to intracellularly colonize apparently healthy roots of the model Ericaceae host.

In terms of morphology, ericoid mycorrhiza is characterized by the formation of dense intracellular hyphal coils in the host rhizodermis (Smith and Read 2008), and this ability is used as a trait sufficient to classify a tested fungus as an ErM mycobiont (cf. Dalpé 1986, 1989). However, many apparently non-ErM fungi (e.g., endophytic, saprotrophic, and ectomycorrhizal) can form these structures in Ericaceae roots in vitro, usually at (very) low colonization levels (e.g., Vohník et al. 2007a, 2012a; Zhang et al. 2009). In this study, several serendipitoid isolates derived from non-Ericaceae hosts infrequently formed dense intracellular hyphal coils, which might be interpreted as ericoid mycorrhiza (cf. Figs. 3e, 5c, and 6d). We suggest, more conservatively, that the fact that an isolate/strain of a certain fungal species infrequently forms intracellular hyphal coils in the Ericaceae rhizodermis in vitro is not, on its own, sufficient to prove the ErM status of that fungal species (cf. Grunewaldt-Stöcker and von Alten 2016). Instead, a true ErM fungal species has first to be isolated from surface-sterilized (i.e., contaminant-free) Ericaceae hair roots in pure culture and, subsequently, it has to form intracellular hyphal coils similar to those observed under natural conditions in a monoxenic culture, with an apparently healthy Ericaceae host, at reasonable levels of colonization, i.e., similar to those of already verified ErM isolates (that is, in a way fulfilling Koch's postulates; Koch 1878). We make this conservative suggestion in an attempt to reduce the number of cases where claims of new ericoid mycorrhizal fungi are debatable, especially when those belong to typical saprobes (Zhang et al. 2009) and typical ectomycorrhizal fungi (Bougoure et al. 2007; Villarreal-Ruiz et al. 2012; cf. Vohník et al. 2012a), or even DSE (Usuki and Narisawa 2005; cf. Lukešová et al. 2015). In this study, among the serendipitoid fungi tested, only the Ericaceae-derived Sebacinales sp. JPK 132 isolate met all these criteria.

Although the Sebacinales sp. JPK 132 colonization pattern differed from the *R. ericae* RER-1 intracellular hyphal colonization, it corresponded to typical ericoid mycorrhiza often seen in naturally colonized Ericaceae hair roots (e.g., Figs. 14–20 in Massicotte et al. 2005). Moreover, it allowed blueberries to reach a biomass similar to that seen in some other treatments, even higher (albeit non-significantly) than in plants colonized by the ErM isolate *R. ericae* RER-1. To discuss the impact on biomass in more detail is beyond the scope of this study because host biomass depends on the cost of the fungal partner (which may be related to colonization level) and growth conditions (especially nutrient concentrations; van der Heijden et al. 2015) and engaging in mycorrhizal symbiosis does not need to result in altered/ increased biomass of the host (e.g., Smith et al. 2003). Yet, it is important that no evidence for parasitism was observed in terms of growth. Although we did not apply any method to test whether the colonized rhizodermal cells were living (cf. Bonfante-Fasolo 1980; Grunewaldt-Stöcker and von Alten 2016), they were turgescent and without any obvious microscopic symptoms of plant-pathogen defense. Moreover, the colonization rates of plants inoculated with the JPK 132 serendipitoid isolate corresponded to those inoculated with *R. ericae* RER-1 at the end of all three experiments.

Intriguingly, although the *M. variabilis* MVA-4 isolate originating from a non-Ericaceae host used here as a positive ErM control in Experiment II also formed intracellular hyphal coils, the incidence of these structures in the host rhizodermis was very low. This is in contrast with our recent study (Vohník et al. 2013) where it formed vigorous ericoid mycorrhizae and even enhanced the growth of *V. myrtillus*, but in agreement with two previous studies where two other non-Ericaceae *M. variabilis* isolates behaved in the same manner (Vohník et al. 2007a, b). Apparently an isolate originating from an Ericaceae host must be used in future experiments for comparison, in agreement with our suggestions made above.

Several serendipitoid isolates tested in this study formed hyaline intracellular microsclerotia, and *S. indica* PIND-1 formed intracellular rounded structures interpreted as chlamydospores, similar to those developed by this species 1–2 weeks after inoculation on barley (Waller et al. 2005). Additionally, the majority of the tested non-Ericaceae serendipitoid isolates apparently had the ability to colonize the *Vaccinium* rhizodermis intracellularly, often forming irregularly lobbed hyphal loops in living cells, although at low colonization levels. Similar structures are found infrequently in naturally colonized Ericaceae hair roots (M. Vohník, unpublished observations), and this finding, coupled with the formation of these structures under monoxenic controlled conditions, suggests a potential of the Serendipitaceae for a root endophytic lifestyle in Ericaceae. However, since these mycobionts originated from orchids, a wider selection of serendipitoid isolates derived from Ericaceae roots and

behaving in the same manner is needed to corroborate this hypothesis. Yet our observations tally with the general view that Sebacinales are common root endophytes (Selosse et al. 2009; Weiss et al. 2011) and are congruent with the hypothesis that the Ericaceae evolved ErM associations with representatives of fungal taxa that were endophytic in their ancestors. According to the so-called waiting room hypothesis, some mycorrhizal lineages evolved from former root endophytes because root endophytism acts as a symbiotic "waiting room" predisposing the fungus to evolve a tighter mutualism with some hosts (Selosse et al. 2009; van der Heijden et al. 2015). Several of these isolates increased the production of shoot biomass in the inoculated plants, most probably not by enhancing symbiotic nutrient transfer but rather by increased CO_2 concentration in the cultivation vessels (cf. Lukešová et al. 2015), non-symbiotic nutrient release (cf. Vohník et al. 2012b), or a combination of both.

Conclusions

This study confirms for the first time experimentally that some members of the Serendipitaceae (formerly Sebacinales Group B) form ericoid mycorrhizae. Further investigations of the Sebacinales lifestyle in Ericaceae hair roots, as well as corroboration of the hypotheses concerning the possible ericoid mycorrhizal specificity and broad endophytic potential within the Seren dipitaceae, are conditioned by isolation of more serendipitoid mycobionts from ericaceous hosts, if possible from different phylogenetic backgrounds within the Seren dipitaceae. Yet we show that this could be achieved by a relatively easy method commonly used in ericoid mycorrhiza research. The Sebacinales sp. JPK 132 isolate derived from *Vaccinium* hair roots still survives in pure culture under artificial conditions, opening a space for testing its physiological traits and comparing its performance with the prominent ErM fungus *R. ericae*.

Acknowledgments This study is part of the long-term research project of the Institute of Botany ASCR (RVO 67985939) and the Department of Experimental Plant Biology, Faculty of Science, Charles University in Prague (MŠMT LO1417), and Marc-André Selosse's research on Sebacinales in collaboration with the Institute of Botany ASCR supported by CNRS (PICS INEE 'Tracking carbon exchanges in mycorrhizal networks'). The authors thank Rolf Nestby for help during field sampling in Norway; Jesse J. Sadowsky for help with isolation of the Sebacinales sp. JPK 132 isolate; Yi Ding, Kingsley Dixon, Libor Mrnka, Belinda Newman, Nigel Swarts, and Alga Zuccaro for kindly sharing serendipitoid isolates; two anonymous referees for helpful suggestions; David Marsh for English corrections; and Jiří Machač for assembling the figures.

References

Allen TR, Millar T, Berch SM, Berbee ML (2003) Culturing and direct DNA extraction find different fungi from the same ericoid mycorrhizal roots. New Phytol 160:255–272

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402

Basiewicz M, Weiss M, Kogel K-H, Langen G, Zorn H, Zuccaro A (2012) Molecular and phenotypic characterization of a range of *Sebacina vermifera* strains associated with orchids, and the description of *Piriformospora williamsii* sp. nov. Fungal Biol 116:204–213

Berch SM, Allen TR, Berbee ML (2002) Molecular detection, community structure and phylogeny of ericoid mycorrhizal fungi. Plant Soil 244:55–66

Bonfante-Fasolo P (1980) Occurrence of a basidiomycete in living cells of mycorrhizal hair roots of *Calluna vulgaris*. Trans Br Mycol Soc 75:320–325

Bougoure DS, Parkin PI, Cairney JWG, Alexander IJ, Anderson IC (2007) Diversity of fungi in hair roots of Ericaceae varies along a vegetation gradient. Mol Ecol 16:4624–4636

Bruzone MC, Fontenla SB, Vohník M (2015) Is the prominent ericoid mycorrhizal fungus *Rhizoscyphus ericae* absent in the Southern Hemisphere's Ericaceae? A case study on the diversity of root mycobionts in *Gaultheria* spp. from northwest Patagonia, Argentina. Mycorrhiza 25:25–40

Colpaert JV, van Laere A, van Assche JA (1996) Carbon and nitrogen allocation in ectomycorrhizal and non-mycorrhizal *Pinus sylvestris* L. seedlings. Tree Physiol 16:787–793

Dalpé Y (1986) Axenic synthesis of ericoid mycorrhiza in *Vaccinium angustifolium* Ait. by *Oidiodendron* species. New Phytol 103:391–396

Dalpé Y (1989) Ericoid mycorrhizal fungi in the Myxotrichaceae and Gymnoascaceae. New Phytol 113:523–527

Dearnaley JDW, Murray AJ, Mathieson MT (2009) Molecular identification of a mycorrhizal Sebacinaceae from the endangered *Caladenia atroclavia* (black clubbed spider orchid). Australas Mycol 28:45–50

Dearnaley JDW, Martos F, Selosse M-A (2013) Orchid mycorrhizas: molecular ecology, physiology, evolution and conservation aspects. In: Hock B (ed) Fungal associations, 2nd edn. The mycota IX. Springer, Berlin, pp 207–230

Garnica S, Riess K, Bauer R, Oberwinkler F, Weiss M (2013) Phylogenetic diversity and structure of sebacinoid fungi associated with plant communities along an altitudinal gradient. FEMS Microbiol Ecol 83:265–278

Gorzelak MA, Hambleton S, Massicotte HB (2012) Community structure of ericoid mycorrhizas and root-associated fungi of *Vaccinium membranaceum* across an elevation gradient in the Canadian Rocky Mountains. Fungal Ecol 5:36–45

Grunewaldt-Stöcker G, von Alten H (2016) Is the root-colonizing endophyte *Acremonium strictum* an ericoid mycorrhizal fungus? Mycorrhiza 26:429–440

Hall TA (1999) BioEdit, a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Hashimoto Y, Fukukawa S, Kunishi A, Suga H, Richard F, Sauve M, Selosse M-A (2012) Mycoheterotrophic germination of *Pyrola asarifolia* dust seeds reveals convergences with germination in orchids. New Phytol 195:620–630

Ishida TA, Nordin A (2010) No evidence that nitrogen enrichment affect fungal communities of *Vaccinium* roots in two contrasting boreal forest types. Soil Biol Biochem 42:234–243

Koch R (1878) Untersuchungen Über die Aetiologie der Wundinfectionskrankheiten. FCW Vogel, Leipzig

Kottke I, Beiter A, Weiss M, Haug I, Oberwinkler F, Nebel M (2003) Heterobasidiomycetes form symbiotic associations with hepatics: Jungermanniales have sebacinoid mycobionts while *Aneura pinguis* (Metzgeriales) is associated with a *Tulasnella* species. Mycol Res 107:957–968

Kottke I, Haug I, Setaro S, Suárez J, Weiss M, Preussing M, Nebel M, Oberwinkler F (2008) Guilds of mycorrhizal fungi and their relation to trees, ericads, orchids and liverworts in a neotropical mountain rain forest. Basic Appl Ecol 9:13–23

Kühdorf K, Münzenberger B, Begerow D, Karasch-Wittmann C, Gómez-Laurito J, Hüttl RF (2014) *Sebacina* sp. is a mycorrhizal partner of *Comarostaphylis arbutoides* (Ericaceae). Mycol Prog 13:733–744

Lukešová T, Kohout P, Větrovský T, Vohník M (2015) The potential of Dark Septate Endophytes to form root symbioses with ectomycorrhizal and ericoid mycorrhizal middle European forest plants. PLoS One 10:e0124752

Massicotte HB, Melville LH, Peterson RL (2005) Structural characteristics of root-fungal interactions for five ericaceous species in eastern Canada. Can J Bot 83:1057–1064

McLennan EI (1935) Non-symbiotic development of seedlings of *Epacris impressa* Labill. New Phytol 34:55–63

Molina R, Palmer JG (1982) Isolation, maintenance and pure culture manipulation of ectomycorrhizal fungi. In: Shenk NC (ed) Methods and principles of mycorrhizal research. American Phytopathological Society, Minnesota, pp 115–129

Newsham KN, Bridge PD (2010) Sebacinales are associates of the leafy liverwort *Lophozia excisa* in the southern maritime Antarctic. Mycorrhiza 20:307–313

Oberwinkler F, Riess K, Bauer R, Selosse M-A, Weiss M, Garnica S, Zuccaro A (2013) Enigmatic Sebacinales. Mycol Prog 12:1–27

Oberwinkler F, Riess K, Bauer R, Garnica S (2014) Morphology and molecules: the Sebacinales, a case study. Mycol Prog 13:445–470

Oelmüller R, Sherameti I, Tripathi S, Varma A (2009) *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. Symbiosis 49:1–17

Pearson V, Read DJ (1973) Biology of mycorrhiza in Ericaceae 1. Isolation of endophyte and synthesis of mycorrhizas in aseptic culture. New Phytol 72:371–379

Qiang X, Weiss M, Kogel K-H, Schäfer P (2012) *Piriformospora indica*—a mutualistic basidiomycete with an exceptionally large plant host range. Mol Plant Pathol 5:508–518

Read DJ (1983) The biology of mycorrhiza in the Ericales. Can J Bot 61: 985–1004

Read DJ (1996) The structure and function of the ericoid mycorrhizal root. Ann Bot 77:365–374

Richard F, Millot S, Gardes M, Selosse M-A (2005) Diversity and specificity of ectomycorrhizal fungi retrieved from an old-growth Mediterranean forest dominated by *Quercus ilex*. New Phytol 166: 1011–1023

Riess K, Oberwinkler F, Bauer R, Garnica S (2014) Communities of endophytic Sebacinales associated with roots of herbaceous plants in agricultural and grassland ecosystems are dominated by *Serendipita herbamans* sp. nov. PLoS One 9:e94676

Rodriguez RJ, White JF Jr, Arnold AE, Redman RS (2009) Fungal endophytes: diversity and functional roles. New Phytol 182:314–330

Ronquist F, Huelsenbeck J (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574

Selosse M-A, Bauer R, Moyersoen B (2002) Basal hymenomycetes belonging to the Sebacinaceae are ectomycorrhizal on temperate deciduous trees. New Phytol 155:183–195

Selosse M-A, Setaro S, Glatard F, Richard F, Urcelay C, Weiss M (2007) Sebacinales are common mycorrhizal associates of Ericaceae. New Phytol 174:864–887

Selosse M-A, Dubois M-P, Alvarez N (2009) Do Sebacinales commonly associate with plant roots as endophytes? Mycol Res 113:1062–1069

Setaro S, Weiss M, Oberwinkler F, Kottke I (2006) Sebacinales form ectendomycorrhizas with *Cavendishia nobilis*, a member of the Andean clade of Ericaceae, in the mountain rain forest of southern Ecuador. New Phytol 169:355–365

Setaro S, Suárez JP, Herrera P, Cruz D, Kottke I (2013) Distinct but closely related Sebacinales form mycorrhizae with coexisting Ericaceae and Orchidaceae in a Neotropical mountain area. In: Varma A, Kost G, Oelmüller R (eds) *Piriformospora indica*: Sebacinales and their biotechnological applications. Soil biology, vol. 33. Springer, Berlin, pp 81–105

Silvestro D, Michalak I (2012) raxmlGUI: a graphical front-end for RAxML. Org Divers Evol 12:335–337

Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd edn. Academic, London

Smith SE, Smith FA, Jakobsen I (2003) Mycorrhizal fungi can dominate phosphate supply to plants irrespective of growth responses. Plant Physiol 133:16–20

Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22:2688–2690

Swofford DL (2002) PAUP*: Phylogenetic analysis using parsimony (and other methods), version 4.0 Beta. Sinauer, Sunderland

Tedersoo L, Pellet P, Kõljalg U, Selosse M-A (2007) Parallel evolutionary paths to mycoheterotrophy in understorey Ericaceae and Orchidaceae: ecological evidence for mixotrophy in Pyroleae. Oecologia 151:206–217

Tedersoo L, Pärtel K, Jairus T, Gates G, Põldmaa K, Tamm H (2009) Ascomycetes associated with ectomycorrhizas: molecular diversity and ecology with particular reference to the Helotiales. Environ Microbiol 11:3166–3178

Urban A, Weiss M, Bauer R (2003) Ectomycorrhizae involving sebacinoid mycobionts. Mycol Res 107:3–14

Usuki F, Narisawa K (2005) Formation of structures resembling ericoid mycorrhizas by the root endophytic fungus Heteroconium chaetospira within roots ofRhododendron obsutum var. kaempferi. Mycorrhiza 15:61–64

van der Heijden MGA, Martin FM, Selosse MA, Sanders IR (2015) Mycorrhizal ecology and evolution: the past, the present, and the future. New Phytol 205:1406–1423

Varma A, Verma S, Sudha SN, Bütehorn B, Franken P (1999) *Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. Appl Environ Microbiol 65:2741–2744

Varma A, Kost G, Oelmüller R (eds) (2013) *Piriformospora indica*: Sebacinales and their biotechnological applications. Soil biology, vol. 33. Springer, Berlin

Verma S, Varma A, Rexer K-H, Hassel A, Kost G, Sarbhoy A, Bisen P, Bütehorn B, Franken P (1998) *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. Mycologia 90:896–903

Vilgalys R, Hester M (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J Bacteriol 172:4238–4246

Villarreal-Ruiz L, Neri-Luna C, Anderson IC, Alexander IJ (2012) In vitro interactions between ectomycorrhizal fungi and ericaceous plants. Symbiosis 56:67–75

Vincenot L, Tedersoo L, Richard F, Horcine H, Kõljalg U, Selosse M-A (2008) Fungal associates of *Pyrola rotundifolia*, a mixotrophic Ericaceae, from two Estonian boreal forests. Mycorrhiza 19:15–25

Vohník M, Albrechtová J (2011) The co-occurrence and morphological continuum between ericoid mycorrhiza and dark septate endophytes in roots of six European *Rhododendron* species. Folia Geobot 46: 373–386

Vohník M, Fendrych M, Albrechtová J, Vosátka M (2007a) Intracellular colonization of *Rhododendron* and *Vaccinium* roots by *Cenococcum*

geophilum, *Geomyces pannorum* and *Meliniomyces variabilis*. Folia Microbiol 52:407–414

Vohník M, Fendrych M, Kolařík M, Gryndler M, Hršelová H, Albrechtová J, Vosátka M (2007b) The ascomycete *Meliniomyces variabilis* isolated from a sporocarp of *Hydnotrya tulasnei* (Pezizales) intracellularly colonizes roots of ecto- and ericoid mycorrhizal host plants. Czech Mycol 59:215–226

Vohník M, Sadowsky JJ, Kohout P, Lhotáková Z, Nestby R, Kolařík M (2012a) Novel root-fungus symbiosis in Ericaceae: sheathed ericoid mycorrhiza formed by a hitherto undescribed basidiomycete with affinities to Trechisporales. PLoS One 7:e39524

Vohník M, Sadowsky JJ, Lukešová T, Albrechtová J, Vosátka M (2012b) Inoculation with wood decomposing basidiomycete, but not with root symbiotic ascomycetes, positively affects growth of highbush blueberry (Ericaceae) grown in a pine litter substrate. Plant Soil 355: 341–352

Vohník M, Mrnka L, Lukešová T, Bruzone MC, Kouhout P, Fehrer J (2013) The cultivable endophytic community of Norway spruce ectomycorrhizas from microhabitats lacking ericaceous hosts is dominated by ericoid mycorrhizal *Meliniomyces variabilis*. Fungal Ecol 6:281–292

Walker JF, Aldrich-Wolfe L, Riffel A, Barbare H, Simpson NB, Trowbridge J, Jumpponen A (2011) Diverse Helotiales associated with the roots of three species of Arctic Ericaceae provide no evidence for host specificity. New Phytol 191:515–527

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Hückelhoven R, Neumann C, von Wettstein D, Franken P, Kogel KH (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci U S A 102:13386–13391

Warcup J, Talbot PHB (1967) Perfect states of Rhizoctonias associated with orchids. New Phytol 66:631–641

Wei J, Agerer R (2011) Two sebacinoid ectomycorrhizae on Chinese pine. Mycorrhiza 21:105–115

Weiss M, Selosse M-A, Rexer K-H, Urban A, Oberwinkler F (2004) Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. Mycol Res 108:1003–1010

Weiss M, Sýkorová Z, Garnica S, Riess K, Martos F, Krause C, Oberwinkler F, Bauer R, Redecker D (2011) Sebacinales everywhere: previously overlooked ubiquitous fungal endophytes. PLoS One 6:e16793

Weiss M, Waller F, Zuccaro A, Selosse M-A (2016) Sebacinales—one thousand and one interactions with land plants. New Phytol 211:20–40

Wilson D (1995) Endophyte: the evolution of a term, and clarification of its use and definition. Oikos 73:274–276

Wright MM, Cross R, Cousens RD, May TW, McLean CB (2010) Taxonomic and functional characterization of fungi from the *Sebacina vermifera* complex from common and rare orchids in the genus *Caladenia*. Mycorrhiza 20:375–390

Wurzburger N, Higgins BP, Hendrick RL (2011) Ericoid mycorrhizal root fungi and their multicopper oxidases from a temperate forest shrub. Ecol Evol 2:65–79

Zhang C, Yin LJ, Dai SL (2009) Diversity of root-associated fungal endophytes in *Rhododendron fortunei* in subtropical forests of China. Mycorrhiza 19:417–423

Zuccaro A, Lahrmann U, Güldener U, Langen G, Pfiffi S, Biedenkopf D, Wong P, Samans B, Grimm C, Basiewicz M, Murat C, Martin F, Kogel K-H (2011) Endophytic life strategies decoded by genome and transcriptome analyses of the mutualistic root symbiont *Piriformospora indica*. PLoS Pathog 7:e1002290