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ORIGINAL ARTICLE

Sebacina sp. is a mycorrhizal partner of *Comarostaphylis arbutoides* (Ericaceae)

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Abstract *Sebacina* (Sebacinales) forms ectomycorrhiza, arbutoid, ericoid, jungermannioid, cavendishioid and orchid mycorrhiza with diverse plant species. The woody plant *Comarostaphylis arbutoides* (Ericaceae) forms arbutoid mycorrhiza with *Leccinum monticola*. However, further morphotypes have hitherto not been described. *C. arbutoides* grows in tropical Central America at an elevation of 2,500–3,430 m a.s.l., where it is found as understory vegetation in forests or forms extensive thickets. It shares ectomycorrhizal fungi with *Quercus* species, thereby being a refuge for these fungi after forest clearance of the oaks. We collected arbutoid mycorrhizas of *C. arbutoides* from the Cerro de la Muerte (Cordillera de Talamanca) in Costa Rica, where it grows together with *Quercus costaricensis*. *Sebacina* sp. was identified after sequencing the internal transcribed spacer (ITS) and large subunit (LSU) rDNA regions, and their phylogenetic analyses. The morphotype *Sebacina* sp.-*C. arbutoides* was described morphologically and anatomically.

Keywords Arbutoid mycorrhiza · Anatomy · Morphology · ITS · LSU · *Quercus costaricensis* · Costa Rica

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Introduction

Members of the Sebacinaceae are species with exidioid basidia and hyphae without clamp connections (Weiß and Oberwinkler 2001), and were included within the Auriculariales by Bandoni (1984) as a group of wood-decaying fungi. However, Weiß et al. (2004) created the new order Sebacinales and divided the Sebacinaceae into two ecologically different groups (Clade A and Clade B). Clade A contains the sebacinoid ectomycorrhizas, arbutoid mycorrhizas and endomycorrhizas with heterotrophic orchids. Clade B contains the endomycorrhizas with autotrophic orchids and members of the Ericaceae and liverworts (Selosse et al. 2007). Both clades also contain members that are also known to be endophytic (Selosse et al. 2009; Weiß et al. 2011). *Sebacina* contains resupinate species with occasional inconspicuous or macroscopically invisible basidiomes (Oberwinkler 1964) that are often overlooked (Weiß et al. 2004) and are only known from members of clade A.

Today, we know that *Sebacina* forms ectomycorrhiza (ECM), ectendomycorrhiza (EEM), ericoid (ERM), jungermannioid (JMM), cavendishioid (CVM) and orchid mycorrhiza (ORM) with diverse plant species (Selosse et al. 2002a, b; Kottke et al. 2003; Urban et al. 2003; Richard et al. 2005; Setaro et al. 2006; Selosse et al. 2007; Wright et al. 2010). Species of Sebacinaceae form ECMs with temperate

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deciduous trees (Selosse et al. 2002a). The hyphal mantle of a sebacinoid ECM was 6–10 cells thick, and the hyphae missed clamp connections. Ultrastructure of the septal pore revealed a dolipore with imperforate caps (Selosse et al. 2002a).

Wei and Agerer (2011) described two sebacinoid ECMs on Chinese pine morphologically and anatomically. The outer hyphal mantle was plectenchymatic and hyphae were embedded in a gelatinous matrix, they were clampless and with tick-walled emanating hyphae. Morpho-anatomical features of ECMs are still rare in Sebacinales (Wei and Agerer 2011). Additionally, little is known on interactions between tropical plants and Sebacinales (Selosse et al. 2009).

Comarostaphylis arbutoides Lindl. is a tropical woody plant of Central America at an elevation of c. 2,500–3,430 m a.s.l. It belongs to the subfamily Arbutoideae (Ericaceae), and is related to the circumboreal *Arctostaphylos uva-ursi* and to species of *Arbutus*. All these species have in common that they form EEM (Molina and Trappe 1982a; Münzenberger et al. 1992; Osmundson et al. 2007). This mycorrhizal type is characterized by a hyphal mantle, a paraepidermal Hartig-net and intracellular hyphae in living cells of the epidermis (Münzenberger et al. 1992; Selosse et al. 2007). The fungal partner induces the branching of the lateral roots, thus forming typical mycorrhizal clusters (Molina and Trappe 1982a; Massicotte et al. 1993). Those EEMs of the basal Ericaceae (Arbutoideae and Monotropoideae; Selosse et al. 2007) are formed by basidiomycetes and ascomycetes that also form ECMs with commercial tree species (Richard et al. 2005; Bidartondo 2005). Therefore, morphology and hyphal mantle anatomy of EEMs of *Arbutus* and *Arctostaphylos*, as well as ECMs of conifers, are identical (Zak 1976a, b; Molina and Trappe 1982b).

At the study sites at the Cerro de la Muerte (Costa Rica), *C. arbutoides* grows together with *Quercus costaricensis*. As it is known from fruitbody collections (Halling and Mueller 2004), these oak trees probably share their ectomycorrhizal fungi with *C. arbutoides*, well known from other EEM forming plants (Smith and Read 2008). Thus, *C. arbutoides* is a refuge plant for ectomycorrhizal fungi after forest clearance of the economically important oak trees (Hagerman et al. 2001). *Leccinum monticola* can be collected in the timberline of oak forests in Costa Rica and is strictly associated with *C. arbutoides* (Halling and Mueller 2003; den Bakker et al. 2004). *C. arbutoides-Leccinum monticola* was the first arbutoid morphotype described by Osmundson et al. (2007) morphologically/anatomically, as well as molecularly. Further characterization and identification of morphotypes of *C. arbutoides* are still missing.

We collected EEMs from *C. arbutoides* at the Cerro de la Muerte (Cordillera de Talamanca), Costa Rica, and characterized the morphotype of *C. arbutoides-Sebacina* sp. morphologically and anatomically, according to Agerer (1991). For identification of the fungus *Sebacina* sp., molecular methods, such as internal transcribed spacer (ITS) and large subunit (LSU) rDNA sequencing, and phylogenetic analyses, were used. In addition to the EEMs of *C. arbutoides*, sebacinoid ECMs of *Q. costaricensis* were also found, and were therefore included for comparison purposes.

Materials and methods

Sampling sites and sampling

Two forest sites with *C. arbutoides* were chosen around the Mountain Cerro de la Muerte (3,491 m a.s.l.) in the Cordillera de Talamanca of Costa Rica, 54 km southeast of the capital San José. The sites are secondary cloud forests and about 1.4 km apart from each other: Estación Biológica de la Muerte (site I; 3,100 m a.s.l.; 9°33′N, 83°45′W) and Reserva Forestal Los Santos (site II; 3,300 m a.s.l.; 9°34′N, 83°45′W). The vegetation community at site I is dominated by *Q. costaricensis* mixed with solitary individuals of *C. arbutoides*. At sampling site II, *C. arbutoides* itself is the dominating species, mixed with a few species of *Q. costaricensis*. Members of the genera *Weinmannia*, *Schefflera*, *Drimys*, *Myrsine*, *Cavendishia*, *Disterigma*, *Vaccinium*, *Oreopanax*, and *Chusquea* are other common plants, more often represented at site I than at site II.

To receive turgescent mycorrhizas, sampling took place during the rain seasons in October 2010 and 2011. Fine root systems of *C. arbutoides* were collected with a soil corer (diameter 3 cm; length 40 cm) at distances of 50 and 100 cm from the trunk. Within these 2 years, a total of 60 soil cores were taken and analyzed. At the University of Costa Rica, turgid, apparently healthy, arbutoid morphotypes were sorted out using a stereomicroscope. For transport and further analyses, the morphotypes were preserved in 2 % buffered glutaraldehyde (light microscopy and ultrastructure) and dried on silica gel (DNA extraction), respectively.

DNA extraction, PCR, and sequencing

One unramified root tip was used for DNA extraction using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. PCR was performed with the AccuPrime™ *Taq* DNA Polymerase System (Life Technologies GmbH, Carlsbad, California, USA). To identify the fungal partner at family level (Urban et al. 2003), the ribosomal nuclear large subunit (LSU) was amplified using the PCR primer pair LR0R: 5′-ACCCGCTGAACTTAAGC-3′ and LR5: 5′-TCCTGAGGGAAACTTCG-3′ (Moncalvo et al. 2000). For phylogenetic analysis at species level, the ITS region is regarded as useful (Schoch et al. 2012). Therefore, the primer pair ITS1F: 5′-CTTGGTCATTTAGAGGAAGTAA-3′ (Gardes and Bruns 1993) and ITS4: 5′-TCCTCCGCTTATTGATATGC-3′ (White et al. 1990) was

used. The angiosperm-specific ITS primer pair ITS-5A: 5′-CCTTATCATTTAGAGGAAGGAG-3′ and ITS-241r: 5′-CAGTGCCTCGTGGTGCGACA-3′ was amplified to identify the plant from mycorrhizal roots without co-amplifying fungal DNA (Osmundson et al. 2007). Direct sequencing of PCR products was performed using the PCR primers as sequencing primers. Sequencing service was facilitated by GATC Biotech AG (Konstanz, Germany).

A total of 399 root tips were analyzed genetically, of which 15 were identified as *Sebacina* sp. Eight of these sebacinoid samples were associated with *C. arbutoides* and seven with *Quercus*. All eight sebacinoid sequences of *C. arbutoides* were deposited in NCBI GenBank under the accession numbers KF419105-KF419112 (LSU), KF419113-KF419120 (ITS), as well as *C. arbutoides* (KF419121).

Phylogenetic analyses

All fungal sequences obtained for ITS and LSU rDNA were analyzed and edited using Chromas Lite v2.01 software (http://technelysium.com.au), and confirmed as sebacinoid sequences by BLASTn search against the NCBI database (http://www.ncbi.nlm.nih.gov/) and the database UNITE (Kõljalg et al. 2005; http://unite.ut.ee/). For phylogenetic analyses of the sebacinoid mycobionts of *C. arbutoides*, the 100 most similar sequences for each genome region in the NCBI database were downloaded and complemented with an additional search in the nucleotide database and sequences of other papers as well. Alignments were performed with the program MAFFT v7 (Katoh et al. 2002) using the FFT-NS-2 alignment algorithm. To estimate phylogenetic relationships, we used maximum likelihood and Bayesian approaches. Maximum likelihood analysis was performed using RAxML (v7.3.2; Stamatakis 2006; Stamatakis et al. 2008) in a parallelized version supplied by Bioportal (http://bioportal.uio.no/) with eight parallel processors and trees inferred from 10,000 rapid bootstrap analyses as starting trees in a heuristic search for the tree with the highest likelihood. GTRCAT was used in the heuristic search and the final evaluation of the best tree found was based on the GTR + Gamma model. The Bayesian analysis was performed using MrBayes v3.2.1 (Ronquist et al. 2012) on an iMac (2.9 GHz Quad-Core Intel Core i5). The GTR + Gamma model was in effect and four chains in two parallel runs were performed for 2,000,000 generations. The first 50,000 trees were discarded before calculating the posterior probabilities.

Microscopy

The morphological and anatomical description of the sebacinoid mycorrhizas of *C. arbutoides* was carried out according to Agerer (1987–2012, 1991) and with the online key of DEEMY (Agerer and Rambold 2004–2013). Anatomical studies are based on multiple arbutoid mycorrhizal systems. Two samples of sebacinoid ECMs of *Q. costaricensis* could be used for further anatomical comparision. Drawings were performed with an interference contrast microscope (BX50F-3, Olympus Corporation, Tokyo, Japan) connected with a drawing tube. All drawings were made with one-thousand-fold magnification.

For semi-thin sections, the sebacinoid mycorrhizas were fixed with 2 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at room temperature. Following six transfers in 0.1 M sodium cacodylate buffer, samples were postfixed in 1 % osmium tetroxide in the same buffer for 1 h in the dark at room temperature. After six washes in double-distilled water, samples were dehydrated by immersion for 15 min each in 25 %, 50 %, 70 %, and 95 % acetone and three times for 1 h in 100 % acetone. Samples were embedded in Spurr's plastic (Spurr 1969) and sectioned with a glass or diamond knife on an Ultracut Reichert Ultramicrotome (W. Reichert-LABTEC, Wolfratshausen, Germany). Sections (0.5 μm thick) were stained with crystal violet for light microscopy. For ultrastructure (transmission electron microscopy; TEM), serial sections (80 nm thick) were mounted on formvar-coated, single-slot copper grids, stained with uranyl acetate for 1 h and lead citrate at room temperature for 5 min, and washed with double-distilled water. The ultrastructure of sebacinoid mycorrhizas was studied with a ZEISS 109 transmission electron microscope (Zeiss, Oberkochen, Germany) at 80 kV.

Results

Morpho-anatomical description of *Sebacina* sp.-*Comarostaphylis arbutoides*

Morphological characters (Fig. 1a) Mycorrhizal systems arbutoid, with 0–3 orders of ramification, solitary or in small numbers, up to 3 mm long, main axis 0.3–0.4 mm diam.; mantle surface smooth and hydrophilic, of contact exploration type. *Unramified ends* straight to bent, cylindric, not inflated, 0.2–1.3 mm long, 0.2–0.3 mm diam., mantle consistently transparent and colorless to light yellowish. *Surface of unramified ends* smooth, cortical cells visible. *Emanating hyphae* lacking. *Cystidia* not distinct under stereoscope magnification. *Rhizomorphs* not found. *Sclerotia* not observed.

Anatomical characters of the mantle in plan views (Fig. 2a–f) Mantle with three distinct layers, hyphae in all layers colorless and clampless. *Outer mantle layers* (Fig. 2a, d) plectenchymatous, irregularly inflated and repeatedly branched hyphae with a gelatinous matrix, branched hyphal segments inflated (mantle type E/C, Agerer 1991), shape of individual underlying hyphae poorly visible due to the gelatinous appearance of the mantle; hyphae of the net 20–

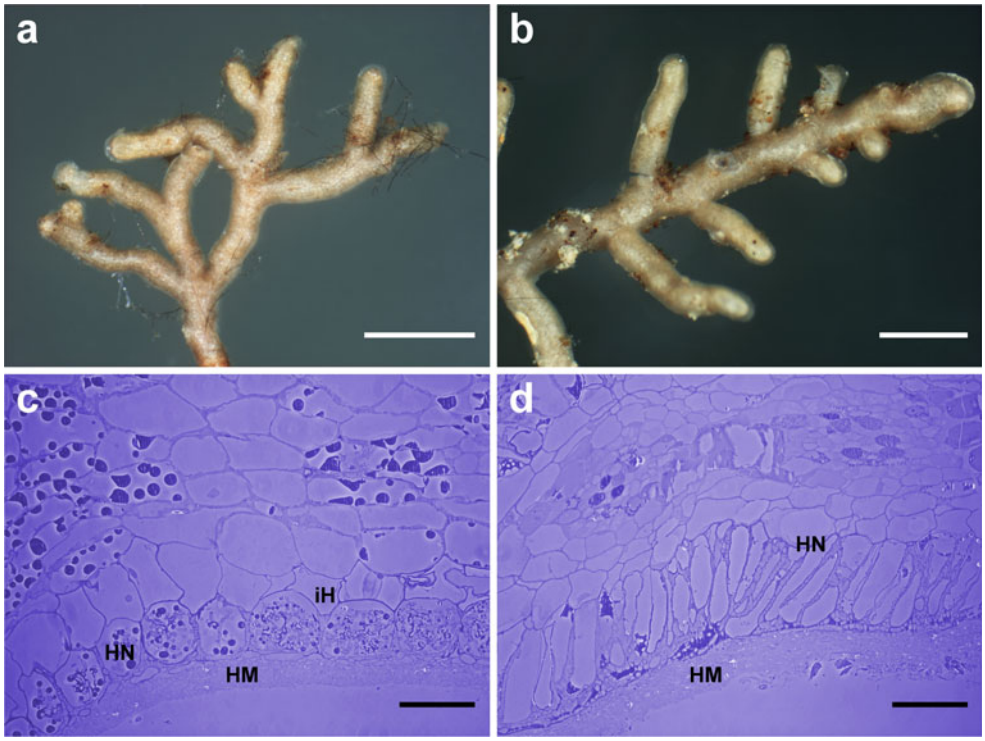

Fig. 1 Habit and semi-thin section of the mycorrhiza *Sebacina* sp.-*Comarostaphylis arbutoides* compared to *Sebacina sp.-Quercus costaricensis*. **a** Habit of *Sebacina* sp.-*Comarostaphylis arbutoides*. Mycorrhiza arbutoid ramified, mantle smooth and transparent with foreign brown hyphae, bar 1 mm. **b** Habit of *Sebacina sp.-Quercus costaricensis*. Mycorrhiza monopodial-pyramidal ramified, mantle smooth and yellowish transparent with soil particles, bar 1 mm. **c** Semi-thin section of *Sebacina* sp.-*Comarostaphylis arbutoides* with hyphal mantle (HM), Hartig net (HN) and isodiametric outer cortical cells with intracellular hyphae (iH), bar 20 μm. **d** Semi-thin section of *Sebacina* sp.-*Quercus costaricensis* with hyphal mantle (HM), Hartig net (HN) and longitudinal outer cortical cells without hyphae, bar 20 μm

44 μm long, 0.5–2.7 μm diam., cell walls 0.3–0.6 μm thick; surface smooth; laticifers lacking. *Middle mantle layers* (Fig. 2b, e) plectenchymatous, with gelatinous matrix, hyphae densely arranged, parallel or irregularly interwoven, infrequently branched, no discernible pattern (mantle type B/C, Agerer 1991); hyphae 10–80 μm long, 1.2–1.6 μm diam., cell walls 0.2–0.5 μm thick. *Inner mantle layers* (Fig. 2c, f) transitional type between plectenchymatous and pseudoparenchymatous, no gelatinous matrix; hyphae densely arranged and frequently ramified, mostly epidermoid, no pattern discernible, hyphae 10–31 μm long, 2.5–9.1 μm diam., cell walls 0.4–1.2 μm thick. *Very tip* similar to remaining parts of the mantle.

Anatomical characters of longitudinal section (Fig. 1c) Mantle plectenchymatous, 14–40 μm thick. Mantle of very tip plectenchymatous, 15–30 (40) μm thick. Cortical cells in 3–4 rows, rectangular to radially oval; Hartig net around cortical cells paraepidermal in one row; hyphal cells around cortical cells roundish to cylindrical; outer cortical cells with intracellular hyphae. Tannin cells lacking.

Color reactions with different reagents (preparations of mantle) Melzer's reagent: hyphae of outer mantle layers dextrinoid; $FeSO_4$: no reaction; lactic acid: no reaction; KOH: no reaction.

Reference specimen Costa Rica, province of San José, canton of Pérez Zeledón, at mountain Cerro de la Muerte (3,100–3,300 m a.s.l.; precipitation c. 2,812 mm/year; inceptisol (USDA)), in a secondary cloud forest with *Quercus costaricensis*, soil core exc., myc. isol. Katja Kühdorf; KKM 139, 12 October 2010; mycorrhiza deposited by B. Münzenberger (ZALF Müncheberg, Germany). *Further material studied* same location, soil core exc., myc. isol. Katja Kühdorf; KKM 183, KKM 192, 12 October 2010; KKM 296, 4 October 2011; KKM 340, 18 October 2011; mycorrhiza deposited by B. Münzenberger (ZALF Müncheberg, Germany).

Ultrastructure

Five sebacinoid EEM of *C. arbutoides* were investigated by transmission electron microscopy (TEM). TEM analyses of these mycorrhizas revealed a dolipore with an imperforate cap typical for Sebacinales (Fig. 3).

Phylogenetic analyses

Sequencing of the LSU region of the eight EEMs of *C. arbutoides* resulted in sequences with a length of 918–932 bp (KF419105-KF419112), whereas sequences of the ITS region were 611–650 bp (KF419113-KF419120) long.

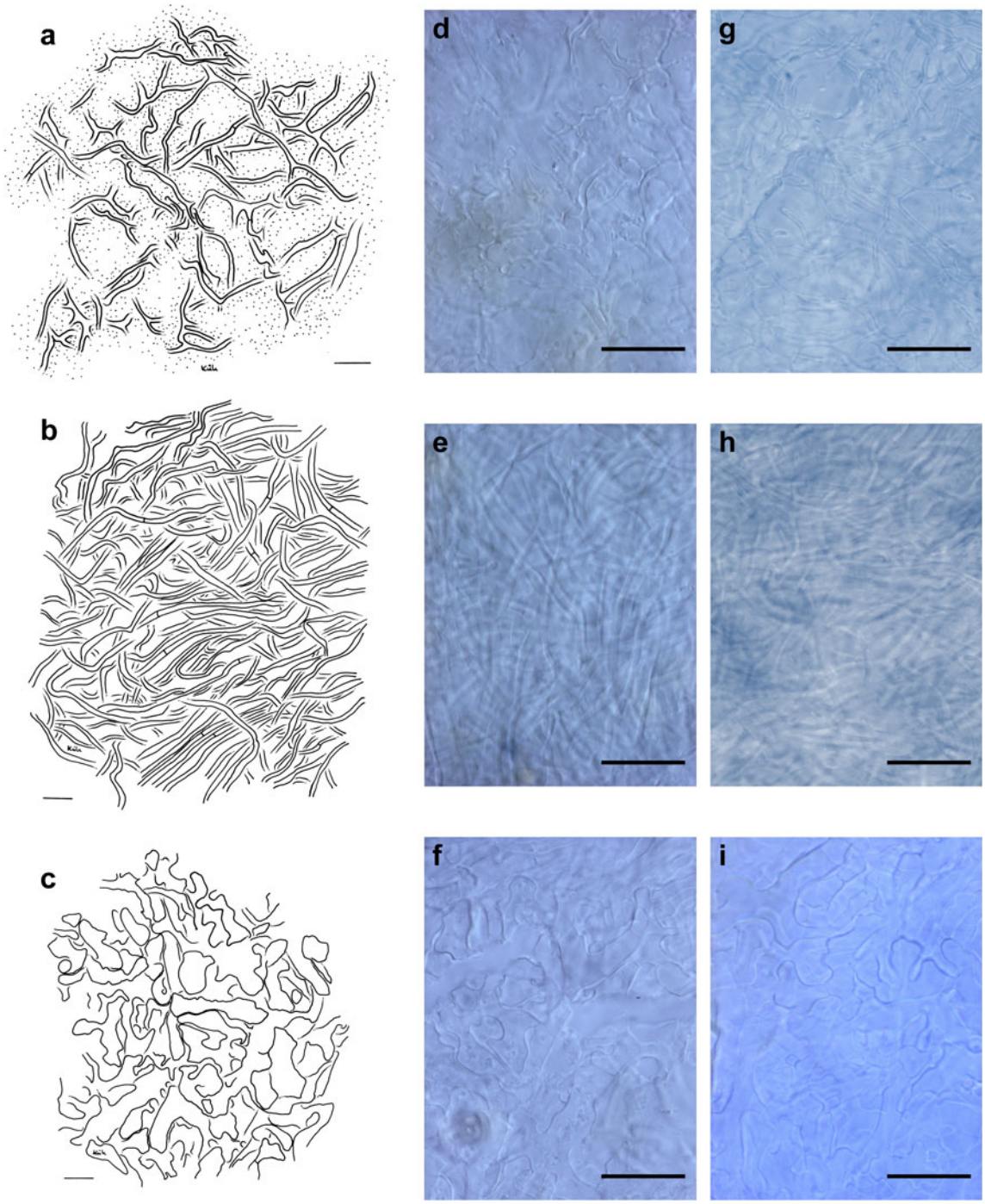

Fig. 2 Arbutoid mycorrhiza of *Sebacina* sp.-*Comarostaphylis arbutoides* compared to the ectomycorrhiza *Sebacina* sp.-*Quercus costaricensis*. **a-f** *Sebacina* sp.-*Comarostaphylis arbutoides*; **a–c** Plan view of different mantle layers, bars 10 μm; **a** outer mantle layer with irregularly inflated and repeatedly branched hyphae in a gelatinous matrix. **b** middle mantle layer with densely parallel or irregularly hyphae, infrequently branched. **c** inner mantle layer with epidermoid shaped cells. **d–f** Interference contrast of the three mantle layers, bars 20 μm; **d** outer mantle layer, **e** middle mantle layer, **f** inner mantle layer. **g–i** Interference contrast of the three mantle layers of *Sebacina* sp.-*Quercus costaricensis*, bars 20 μm; **g** outer mantle layer, **h** middle mantle layer, **i** inner mantle layer

All investigated samples of each genome region belong to the same sequence haplo-type, as sequences of LSU rDNA and ITS, respectively, are identical. From sequence comparison with BLASTn in NCBI and the UNITE database, all sequences of LSU and ITS showed the best matches with members of Sebacinaceae as well as *Sebacina*, as shown in Table 1. The sebacinoid ECMs of *Q. costaricensis* completely correspond with the obtained sequences for LSU and ITS regions of the *C. arbutoides* mycobionts. Therefore, both host trees are associated with the same sebacinoid sequence type.

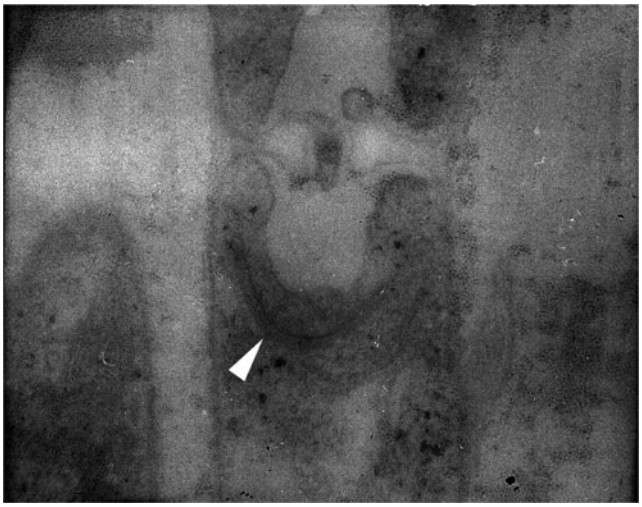

Fig. 3 Ultrastructure of *Sebacina* sp.-*Comarostaphylis arbutoides* showing a dolipore with imperforate cap (*white arrow*), 50,000 magnification

The topologies of the Bayesian and the RAxML phylogenies as generated by the analysis of LSU rDNA sequences are concordant. Groups receiving high bootstraps (BS) in the Bayesian analysis and were also supported as posterior probability (PP) in RAxML analysis, whereby here the values are typically lower (Fig. 4). The tree indicates that the genus *Sebacina* is polyphyletic, and the whole family can be split into the two usual clades. Clade A (PP 0.99/BS 81) comprises the *Sebacina* complex, which includes sebacinoid ECMs and ORMs (only heterotrophic orchids), as well as one EEM of *Arbutus unedo* (EF030911). Several identified species such as *S. dimitica*, *S. epigaea*, *S. helvelloides*, *S. incrustans*, *S. vermifera* and *S. allantoidea* are also included. Additionally, *Craterocolla cerasi* and *Efibulobasidium rolleyi* are sister groups to a large *Sebacina* group, and also clusters in Clade A (PP 0.99/BS 78 and PP 0.76/BS 59, respectively). Clade B (PP 1/BS 97) contains all sebacinoid ORMs of autotrophic orchids, as well as ERMs, CVMs, and JMMs. Only *S. vermifera* can be found in this clade as identified *Sebacina* species. All eight investigated EEMs (KF419105-KF419112) of *C. arbutoides* are grouped together (PP 1/BS 100) and nest within the *Sebacina* complex of Clade A (Fig. 4). The next relative is a Sebacinaceae ECM of *Tilia cordata* from Estonia (AJ534932) with a posterior probability of 0.98 (BS 89).

The Bayesian and RAxML phylogenies, generated by ITS sequences are largely concordant. In both analyses, all samples show the same grouping structure supported with high BS in the Bayesian tree. The only exception is *S. incrustans* (AJ966753), which is grouped together with two *Sebacina* ECMs (HQ154314; JQ420940) in the Bayesian analysis (PP 0.50), but stands alone in the RAxML phylogeny. *Sebacina* is again clearly divided into Clade A and Clade B (PP 1/BS 100, each) in the phylogenies of ITS sequences, as shown in Fig. 5. Clade B considers all sebacinoid ERMs, JMMs, the *S. vermifera* species, and the ORMs of autotrophic orchids as well. An EEM of *Pyrola rotundifolia* can also be found in this association. The Clade A again comprises ECMs and ORMs (only heterotrophic orchids) of sebacinoid samples, as well as the species *S. epigaea*, *S. incrustans*, *S. helvelloides*, and *S. allantoidea.* The eight investigated EEMs (KF419113-KF419120) of *C. arbutoides* are grouped in a well-supported (PP 1/BS 100) monophyletic lineage, together with sebacinoid ECMs of *Pinus* and *Polygonum* species. Within this cluster, two *Sebacina* sp. ECMs of *Polygonum* sp. (JQ347201; JQ347204) are very close to the EEMs of *C. arbutoides* (PP 0.96/BS 74).

Table 1 Identification of LSU rDNA and ITS sequences with the NCBI and UNITE databases obtained from the mycobiont of *Comarostaphylis arbutoides*

	NCBI				UNITE			
Reference sequences	Closest match[a]	Highest maximum score	E value/query coverage (%)	Identity (%)	Closest match[a]	Highest bit-score	E value/query coverage (%)	Identity (%)
LSU: KF419105, KF419107, KF419111	Uncultured Sebacinaceae (FJ207513)	1,452	0.0/100	95	*Sebacina dimitica* (UDB016422)	1,469	0.0/100	95
					Sebacina sp. (UDB16385)	1,469	0.0/100	95
					Sebacina incrustans (UDB014122)	1,469	0.0/100	95
ITS: KF419117, KF419118, KF419120	Uncultured *Sebacina* sp. (JQ347196)	1,092	0.0/100	95	*Sebacina dimitica* (UDB016422)	537	e-153/71	91

Closest match was chosen according to the highest maximum score or bit-score. Only reference sequences revealing the highest maximum score or bit-score are shown

[a] Accessed 9 December 2013

Fig. 4 Phylogenetic relationship of eight sebacinoid mycobionts of *Comarostaphylis arbutoides* within selected members of Sebacinaceae. Phylogram was obtained from Bayesian analysis based on LSU rDNA sequences. Branch support values were calculated as posterior probability from 2,000,000 generations of Bayesian analysis (first number), and as bootstrap support from RAxML analysis (second number). Values below 70 % are indicated with *asterisks* or omitted. The phylogram was rooted with *Geastrum saccatum* and *Endoperplexa enodulosa*. Sebacinoid sequences were obtained from NCBI and UNITE database complemented by the name of the corresponding host plant, if available. Investigated arbutoid mycobionts of *C. arbutoides* are marked in *bold*. Mycorrhizal types: cavendishioid mycorrhiza (CVM), ectomycorrhiza (ECM), ectendomycorrhiza (EEM), ericoid mycorrhiza (ERM), jungermannioid mycorrhiza (JMM), orchid mycorrhiza (ORM)

Discussion

Mycorrhiza of *C. arbutoides* and *Q. costaricensis* formed with *Sebacina* sp.

Genetically investigations showed that sebacinoid mycorrhizas of *C. arbutoides* and *Q. costaricensis* are formed by the same hitherto unknown *Sebacina* species (see below). The mycorrhizal systems of this *Sebacina* sp. formed with *C. arbutoides* (Ericaceae) and *Q. costaricensis* (Fagaceae), respectively, do not differ significantly in their features. The sebacinoid ECM of *Q. costaricensis* is morphologically identical to the EEM of *C. arbutoides*, and both show, for example, the typical smooth and hydrophilic surface, the lack of any emanating elements, as well as the colorless to light yellowish transparency of the mantle. Only different mycorrhizal ramification types are observed, which are typical for the involved host plants (Agerer 1987–2012; Osmundson et al. 2007). Therefore, the system of *C. arbutoides* shows an arbutoid ramification, whereas that of *Q. costaricensis* is monopodial-

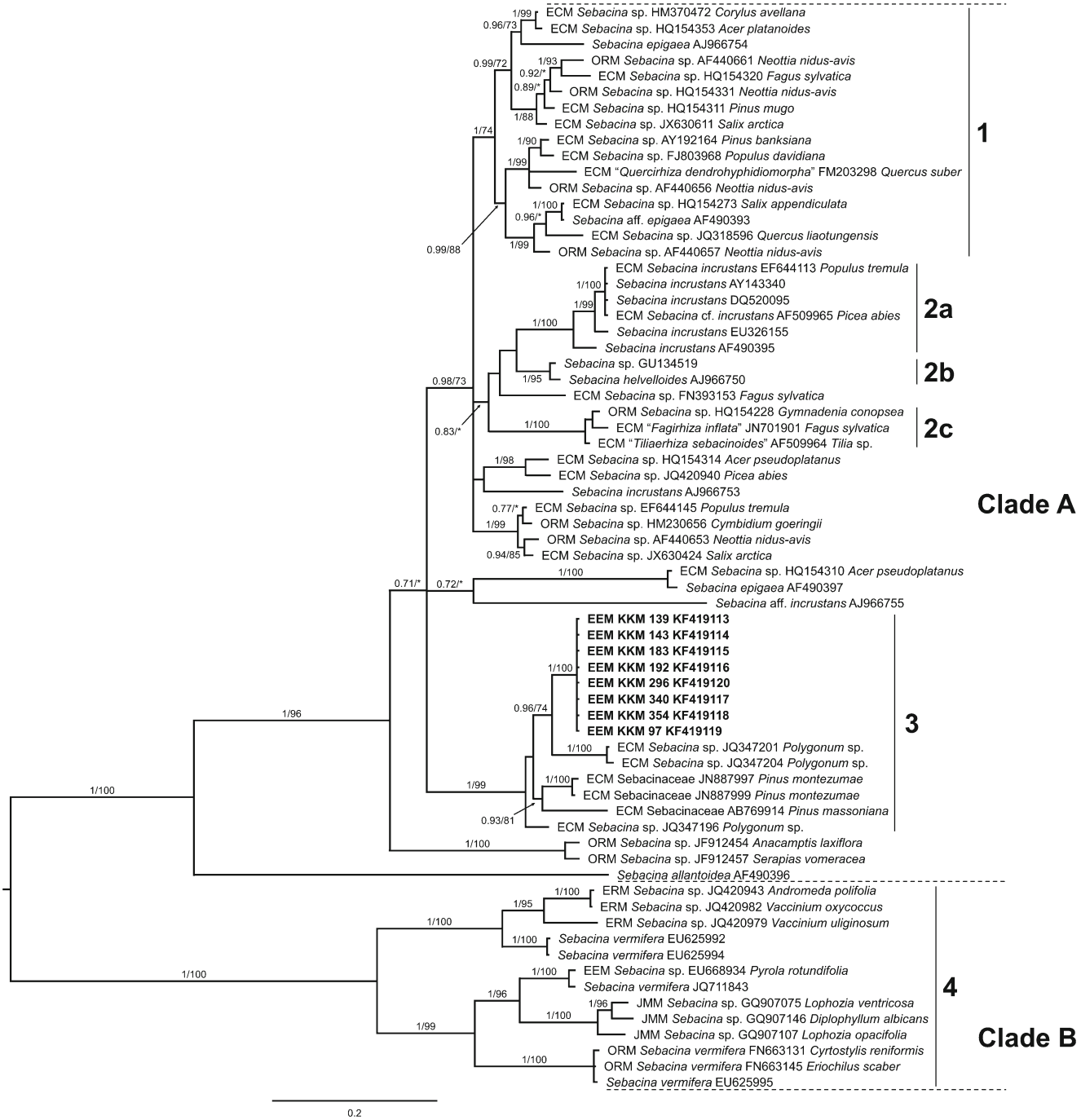

Fig. 5 Phylogenetic relationship of eight sebacinoid mycobionts of *Comarostaphylis arbutoides* within members of *Sebacina.* Complexes with identified *Sebacina* species are indicated by clusters (1–4). Phylogram was obtained from Bayesian analysis based on ITS sequences. Branch support values were calculated as posterior probability from 2,000,000 generations of Bayesian analysis (first number), and as bootstrap support from RAxML analysis (second number). Values below 70 % are indicated with *asterisks* or omitted. The phylogram was rooted with *Sebacina* members of Clade B. Sequences were obtained from NCBI and UNITE database complemented by the name of the corresponding host plant, if available. Investigated arbutoid mycobionts of *C. arbutoides* are marked in *bold.* Mycorrhizal types: ectomycorrhiza (ECM), ectendomycorrhiza (EEM), ericoid mycorrhiza (ERM), jungermannioid mycorrhiza (JMM), orchid mycorrhiza (ORM)

pyramidal ramified (Fig. 1a, b). Also, the semi-thin sections reflect the typical characteristic difference of the arbutoid mycorrhiza and ectomycorrhiza by the presence (*C. arbutoides*) or lack (*Q. costaricensis*) of intracellular hyphae in the outer cortical cells (Fig. 1c, d). In contrast, both sebacinoid mycorrhizal types again show the same hyphal pattern in all three mantle layers (Fig. 2). The same morphology and hyphal mantle anatomy of different mycorrhizal types formed by the same fungal species is in accordance with Zak (1976a, b), and Molina and Trappe (1982b).

Morpho-anatomical comparison of sebacinoid EEM of *C. arbutoides* with other sebacinoids

The investigated EEM of *C. arbutoides* belongs, according to phylogenetic studies (Figs. 4 and 5), to *Sebacina* (see below). Several features confirm the affiliation to this family, such as the dextrinoid reaction with Melzer's reagent and the presence of a gelatinous matrix (Agerer and Rambold 2004–2013), as well as the lack of clamps and cystidia (Weiß et al. 2004). In the literature, five identified sebacinoid ECM fungi are described in detail: "*Tiliaerhiza sebacinoides*" (Urban et al. 2003; Agerer and Rambold 2004–2013), "*Quercirhiza dendrohyphidiomorpha*" (Azul et al. 2006), "*Pinirhiza multifurcata*", "*P. nondextrinoidea*" (Wei and Agerer 2011), and "*Fagirhiza inflata*" (Leberecht et al. 2012). Furthermore, a species of *Sebacina*, *S.* cf. *incrustans*, is portrayed by Urban et al. (2003) and Agerer and Rambold (2004–2013), respectively.

Numerous morphological and anatomical characters clearly differentiate the investigated sebacinoid mycorrhiza from described sebacinoid ECM in the literature. The mantle of the sebacinoid EEM is consistently colorless to light yellowish and transparent so that cortical cells are visible. The six sebacinoid ECMs show a wide array of colors, and range from pale cream, faintly yellowish (*S. incrustans*) yellowish ("*T. sebacinoides*") or orange ochre ("*F. inflata*") to grayish orange-brown ("*P. multifurcata*"), brownish ("*Q. dendrohyphidiomorpha*") and cinnamon brownish ("*P. nondextrinoidea*"). On neither of these ECMs are the cortical cells visible, only Wei and Agerer (2011) describe an opaque to semitransparent mantle in "*P. multifurcata*".

All mycorrhizas, except "*P. nondextrinoidea*", have in common that they lack rhizomorphs. The habits of the other ECMs are mostly described as loosely up to densely cottony, as well as loosely hairy, caused by emanating hyphae. Therefore, all these ECMs belong to the short distance exploration type (Agerer 2001). In addition to emanating hyphae, only "*P. nondextrinoidea*" also features rhizomorphs, but those are described as anatomically similar to the exposed emanating hyphae. Hence, Wei and Agerer (2011) assign this ECM to the short distance exploration type, or to the smooth subtype of medium distance exploration type. In contrast, the sebacinoid EEM of *C. arbutoides* is smooth and belongs to the contact exploration type. Common to all mycorrhizas is their hydrophilic surface.

All six mycorrhizas previously found in literature are described with characteristic emanating elements, whereas the clear distinction between emanating hyphae and cystidia is ambiguous because of the great variety of shapes of that last-mentioned (Agerer 1987–2012; Agerer 1991). As noted by Wei and Agerer (2011), the emanating hyphae described in "*P. multifurcata*" can also be considered as cystidia. Further samples with only emanating hyphae are "*P. nondextrinoidea*" and "*T. sebacinoides*". *S.* cf. *incrustans* and "*Q. dendrohyphidiomorpha*" expose both types of emanating elements, but do not deliver any supporting drawings of the described emanating hyphae. Thus, it is difficult to make a satisfactory distinction between both elements. Leberecht et al. (2012) found only cystidia in "*F. inflata*" and together with "*P. multifurcata*" and "*P. nondextrinoidea*", they are well documented in drawings and could serve as an orientation guide. However, Oberwinkler et al. (2013) describe cystidia as unexpected, because they are lacking in all known basidiocarps documented, for instance, by Wells and Oberwinkler (1982) and Riess et al. (2013). The EEM of *C. arbutoides* does not have any emanating elements and exhibits a smooth mantle surface instead. Therefore, it exposes a further morphotype of sebacinoid mycorrhizas.

Anatomically, many similarities can be found with other sebacinoid ECMs. All mycorrhizas show a plectenchymatic outer mantle layer with multiple branched and irregularly inflated hyphae. In addition, the hyphae lay in a gelatinous matrix, with the exception of *S.* cf. *incrustans* and "*T. sebacinoides*", which only feature hyaline cell walls and a superficial hyphal net of thick-walled, lobed, and frequently branched hyphae. All samples differ from "*Q. dendrohyphidiomorpha*" in having a plectenchymatous, rather than a pseudoparenchymatous middle mantle layer. The inner mantle layer of this sample is a transitional type between plectenchymatous and pseudoparenchymatous; again, a feature in common with the EEM of *C. arbutoides*. In "*F. inflata*" both middle and inner layers show a plectenchymatous-pseudoparenchymatous pattern, too.

Hyphae of samples with a plectenchymatous middle mantle layer are described as multi-ramified, whereas the hyphae of the EEM are infrequently branched and show a parallel or irregularly interwoven pattern. Thus, the EEM resembles "*T. sebacinoides*", which has also parts with streaks of parallel hyphae, but with a larger hyphal diameter (2–3 μm versus 1.2–2.6 μm) and thinner cell walls (0.2 μm versus 0.2–0.5 μm).

The sebacinoid EEM of C. arbutoides shares the plectenchymatous- pseudoparenchymatous inner mantle layer of "*Q. dendrohyphidiomorpha*" and "*F. inflata*", whereas the hyphae of the EEM are shaped more epidermoid. Thus, the arbutoid samples show a considerably larger hyphal diameter of up to 9.1 μm ("*Q. dendrohyphidiomorpha*": 2.3–3.5 μm diam.; "*F. inflata*": 2–4.5 μm) and clearly thicker cell walls, with a thickness of 0.4–2 μm ("*Q. dendrohyphidiomorpha*": 0.3–0.5 μm; "*F. inflata*": 0.3 μm).

The EEM of *C. arbutoides* showed a dextrinoid reaction, a common feature with the other sebacinoid ECMs, with the exception of "*Q. dendrohyphidiomorpha*", where no reaction with Melzer's reagent was observed. However, this feature was not checked in *S.* cf. *incrustans* and "*T. sebacinoides*". Urban et al. (2003) describe a doliporus with imperforate

parenthesome in "*T. sebacinoides*", which is typical for Sebacinales (Oberwinkler et al. 2013). We also found this ultrastructure in the sebacinoid EEM of *C. arbutoides*.

Hitherto, "*T. sebacinoides*" and "*P. nondextrinoidea*" show the most abundant mantle thickness of 15–20 (25) μm and 9–22 μm, respectively. The sebacinoid EEM samples reveal a considerably thicker mantle of up to 40 μm. However, the inner and outer delimitations of the mantle are difficult to discern, as pointed out by Agerer and Rambold (2004–2013). The reasons for this are adjacent root cells at or within the mantle, a generally very loosely woven mantle, or emanating hyphae. Such measurements are seen as not very essential, especially for very loosely woven mantles. But, mantles of the sebacinoid mycorrhizas of *C. arbutoides* are quite dense and do not have emanating elements. This makes the measurements reliable, but perhaps not comparable to "*T. sebacinoides*" and "*P. nondextrinoidea*", since both show emanating hyphae.

Ultrastructure of EEM with Arbutoideae

The ultrastructure of an arbutoid mycorrhiza of *Laccaria amethystea-Arbutus unedo* was described in detail by Münzenberger et al. (1992). They showed that living fungal hyphae penetrate the living outer cortical cells of the host plant. Those intracellular hyphae are surrounded by the plasmalemma of the host cell, forming an interface that is used for exchange processes between plant and fungi (Münzenberger et al. 1992). Selosse et al. (2007) was able to show this interaction for *A. unedo* with a sebacinoid mycobiont. Here, the associated fungus has dolipores and imperforate caps, as it is typical for Sebacinales. We also found this typical structure in the sebacinoid mycobionts of *C. arbutoides* (Fig. 3), but were not able to show adequate intracellular interactions. However, we assume the same structures, since we observed intracellular colonization of fungal hyphae into living cortical root cells, according to the descriptions of Münzenberger et al. (1992).

Phylogenetic position

The two well-supported clades of Sebacinaceae generated by the analyses of LSU rDNA (Fig. 4) concur with the results of Weiß et al. (2004), Setaro et al. (2006), Selosse et al. (2007), and Garnica et al. (2012). In accordance with Weiß and Oberwinkler (2001) *Efibulobasidium* and *Craterocolla* are placed at the basal position within Clade A. All eight investigated EEMs (KF419105-KF419112) of *C. arbutoides* belong to *Sebacina*, because they cluster in the *Sebacina* complex (PP 1/BS 82) within the Sebacinaceae family (Fig. 4). Together with an EEM of *Arbutus unedo*, they belong to Clade A, whereas Clade B contains all sebacinoid ERMs and CVMs of the other Ericaceae. This corresponds well with the phylogenetic analysis of Selosse et al. (2007), who investigated sebacinoid mycorrhizas of different Ericaceae lineages from all over the world. The EEMs of *C. arbutoides* are grouped together with a single sample provided as Sebacinaceae ECM (AJ534932) of *Tilia cordata* (PP 0.98/BS 89). This allows no closer identification of the sebacinoid EEMs at this level.

In the analysis of ITS sequences (Fig. 5), over half of the *Sebacina* samples are only identified as *Sebacina* sp. Nevertheless, some well-supported and circumscribed clusters (cluster 1–4, Fig. 5) are identifiable. Clade B, for instance, comprises three different mycorrhizal types (EEM; ERM; CVM) that are all obviously formed by *S. vermifera*, which is regarded as a cryptic species (Weiß et al. 2004; Weiß et al. 2011). Surprisingly, an EEM (EU668934) of *Pyrola rotundifolia* (Monotropoideae, Ericaceae) can be found here, which conflicts with the assumption that all sebacinoid EEM fungi associated with Ericaceae belong to Clade A. As pointed out by Oberwinkler et al. (2013), it could be possible that sebacinalean fungal partners of the Ericaceae subfamily Monotropoideae also include Clade B Sebacinales. In Clade A, several samples recorded as *S. epigaea* form a huge, well-supported (PP 1/BS 74) group (cluster 1, Fig. 5). The former morphologically and anatomically discussed "*Q. dendrohyphidiomorpha*" (FM203298; Azul et al. 2006) can also be found in this lineage, and may be identified as *S. epigaeae*. Another large cluster (cluster 2, Fig. 5) achieved a 0.83 posterior probability and a bootstrap support of 33 %. It is formed by one big subcluster of *S. incrustans* (2a; PP 1/BS 100), and two further smaller subclusters of *S. helvelloides* (2b; PP 1/BS 95) and three, not yet identified, species (2c; PP 1/BS 100). In the last-mentioned group, "*F. inflata*" (JN701901; Leberecht et al. 2012) and "*T. sebacinoides*" (AF509964; Urban et al. 2003) are included, together with a *Sebacina* sp. ORM (HQ154228) of *Gymnadenia conopsea*. This constellation does not allow one to assign them to one of the already identified species or to a third one. Therefore, the sebacinoid ECMs of Urban et al. (2003) and Leberecht et al. (2012) still remain to be addressed. The morpho-anatomically discussed *S.* cf. *incrustans* (AF509965; Urban et al. 2003), however, fits perfectly in the subcluster of *S. incrustans* (2a) within the *S. incrustans*/*helvelloides* complex of cluster 2 (Fig. 5). Thus, our data is in accordance with Urban et al. (2003).

The sebacinoid ECMs "*P. multifurcata*" (GU269908) and "*P. nondextrinoidea*" (GU269909) presented by Wei and Agerer (2011) have not yet been investigated with regard to the ITS region. Only LSU rDNA sequences are provided, and therefore allow no clear affiliation to any specific species, as shown in Fig. 4. However, "*P. nondextrinoidea*" shows some affinity to *S.* cf. *epigaea* (AY505560) with a posterior probability of 0.76 (BS 31). Finally, no identification is possible for either of the two "*Pinirhiza*" ECMs.

In addition to "*F. inflata*", "*T. sebacinoides*", "*P. multifurcata*", and "*P. nondextrinoidea*" the eight EEMs (KF419113-KF419120) of *C. arbutoides* cannot be assigned to any known species. As shown in Fig. 5, they can be found in cluster 3, together with several Sebacinaceae/*Sebacina* sp. ECMs from Mexico or China in a clearly differentiated/resolved and well-supported cluster (PP 1/BS 99). Here, two *Sebacina* sp. ECMs (JQ347201; JQ347204) of *Polygonum* sp. from southwest China are grouped next to the EEMs of *C. arbutoides*, and the monophyly is supported with a 0.96 posterior probability (BS 74). However, further information about morphology or anatomy is not given for the two *Polygonum* ECMs. Within the genus *Polygonum*, only the herbaceous species *P. viviparum* (alpine bistort) is known to form along arbuscular mycorrhiza, also ECM (Massicotte et al. 1998; Kagawa et al. 2006; Ronikier and Mleczko 2006). The plant is common in arctic and alpine environments of Europe, Asia, and North America. Currently, the species is included in the genus *Bistorta* (Polygonaceae) and renamed as *B. vivipara* (Freeman and Hinds 2005). If *B. vivipara* is identical with the two *Polygonum* sp. species in our phylogenetic analysis, then numerous fungal genera are reported to form ECM with this plant: *Cortinarius*, *Inocybe*, *Cenococcum*, *Russula*, *Tomentella*, *Laccaria*, and *Sebacina* as well (Mühlmann et al. 2008; Brevik et al. 2010; Blaalid et al. 2012).

It has to be mentioned that species of the *Sebacina* shown in Figs. 4 and 5, well known to be mycorrhizal, were also demonstrated to be endophytic by several authors (Selosse et al. 2009; Weiß et al. 2011; Garnica et al. 2012). Thus, it is possible that plants, forming their respective corresponding mycorrhizal types, can also be hosts for endophytic *Sebacina* species (Dearnaley et al. 2012). Oberwinkler et al. (2013) give an overview for diverse plant families with a proven sebacinoid non-mycorrhizal interaction.

The present study is the first report that a *Sebacina* species forms EEM with the tropical Ericaceae *C. arbutoides* (Costa Rica). Furthermore, another new morphotype and anatomotype for a *Sebacina* mycorrhiza is described in detail, and helps to contribute to this field of research. An identification to species level was not possible, because ITS sequences are not yet provided for every known *Sebacina* species. Since Riess et al. (2013) reveal a high intraspecific genetic variation within *S. epigaea* and *S. incrustans*, it is not yet clear if there are also morpho-anatomical variations within one *Sebacina* species regarding their formed mycorrhizas. These variations have not yet been observed or confirmed regarding the sebacinoid EEMs of *C. arbutoides*. Additionally, we found sebacinoid ECMs of *Q. costaricensis* that are identical in morphology, anatomy and genetics with the arbutoid *Sebacina* samples investigated in this study. Thus, we can confirm that *C. arbutoides* is a refuge plant for ECM fungi after clearance of the economically important oaks.

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