

**Diversity and identification of mycorrhizal fungi of the Ericaceae
Comarostaphylis arbutoides from Costa Rica**

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1

Introduction

1.1 The Ericaceae and their mycorrhizal symbioses

The Ericaceae family has a nearly worldwide distribution and comprises ten subfamilies, 126 genera, and over 4,000 species. It is the largest family in the order Ericales (Stevens 2001) and is the only family in the order that harbors multiple types of mycorrhizas (Obase et al. 2013): arbutoid, arbuscular, cavendishoid, ericoid, and monotropoid. Most of the ericad subfamilies (Cassiopoideae, Ericoideae, Styphelioideae, and Vaccinioideae) form ericoid mycorrhiza, whereby some genera e.g. *Styphelia* (Styphelioideae), *Vaccinium* (Vaccinioideae), and *Rhododendron* (Ericoideae) have been reported to form arbuscular mycorrhiza occasionally as well (Koske et al. 1990; Chaurasia et al. 2005). However, in the Enkianthoideae arbuscular mycorrhizal associations are common (Obase et al. 2013). Other genera of the Vaccinioideae (e.g. *Cavendishia*, *Disterigma*) are known to form cavendishoid mycorrhiza (Setaro et al. 2006a, b), whereas arbutoid mycorrhiza occur in the Arbutoideae as well as in the tribe Pyrolae, and monotropoid associations in the Monotropeoideae subfamily (Peterson et al. 2004).

Besides the involved host plant, the five mycorrhizal types can be distinguished by their different developed anatomical structures, as well as the diverse fungal partners that are participating (Peterson et al. 2004). The ericoid mycorrhiza is restricted to the hair root system of the plant host. Here, the fungal hyphae penetrate only the epidermal cells and form a branched hyphal complex in each colonized cell. Fungi of the *Rhizoscyphus ericae* (formerly *Hymenoscyphus ericae*) aggregate (Vrålstad et al. 2002; Smith and Read 2008), *Oidiodendron maius* (Usuki et al. 2003) as well as the Sebaciniales (Weiss et al. 2004; Selosse

et al. 2007) are known to be partners of the ericoid mycorrhiza. Arbuscular mycorrhiza is, however, exclusively formed by members of the Glomeromycota (Chaurasia et al. 2005). In this association the hypha of a germinated spore penetrates the epidermal cell of the plant root and forms typical intracellular arbuscules and/or vesicles.

The arbutoid, monotropoid, and cavendishoid mycorrhiza have much in common anatomically. In contrast to the first-mentioned mycorrhizal types, they possess a hyphal mantle, as well as a Hartig net and, herein, resemble ectomycorrhiza. However, all three types form intracellular structures what clearly distinguish them from ectomycorrhiza. The arbutoid and cavendishoid mycorrhiza show intracellular hyphae confined to the epidermal cells, while monotropoid mycorrhiza forms intrusions into the epidermal cell walls, so called fungal pegs, which in contrast, never actually penetrate the plant cell wall. Likewise, arbutoid, monotropoid, and cavendishoid mycorrhiza differ in their fungal partners. According to Setaro et al. (2006a, b), Leotiales as well as Sebaciales of the group B are proven so far to be involved in cavendishoid mycorrhiza, whereas monotropoid and arbutoid mycorrhizas are formed by typical ectomycorrhizal fungi of the ascomycetes and basidiomycetes, as well as Sebaciales exclusively from group A (Bidartondo 2005; Richard et al. 2005; Selosse et al. 2007; Oberwinkler et al. 2013).

1.2 Mycorrhizal associations of the Arbutoideae

The Arbutoideae subfamily is a distinct and natural group within the Ericaceae (Hileman et al. 2001; Kron et al. 2002) based on flower and fruit morphology, as well as anatomy and phytochemistry (Cox 1948; Stevens 1971). Their distribution is restricted to the northern hemisphere (Tab. 1.1) and comprises dry-adapted sclerophyllous taxa. The Arbutoideae includes the genera *Arbutus*, *Arctostaphylos*, *Arctous*, *Comarostaphylis*, *Ornithostaphylis*, and *Xylococcus*.

Members of the genera *Arbutus* and *Arctostaphylos* are well known to form arbutoid mycorrhiza. Associations with a wide range of typical ectomycorrhizal fungi like *Amanita*, *Amphinema*, *Boletus*, *Cantharellus*, *Cenococcum*, *Cortinarius*, *Elaphomyces*, *Hebeloma*, *Hydnellum*, *Hysterangium*, *Inocybe*, *Laccaria*, *Lactarius*, *Leccinum*, *Melanogaster*, *Paxillus*, *Piloderma*, *Pisolithus*, *Rhizopogon*, *Russula*, *Scleroderma*, *Sebacina*, *Suillus*, *Tomentella*, *Tricholoma*, *Tuber*, and *Xerocomus* have been reported and/or described by numerous authors (Molina and Trappe 1982a, b; Ascai and Largent 1983; Horton et al. 1999, Massicotte et al.

Tab. 1.1 Genera of the Arbutoideae subfamily and their general distribution. Modified after Hileman et al. (2001)

Genus	Distribution
<i>Arbutus</i>	West coast of North America through Mexico and Central America; western Europe through the Mediterranean region, northern Africa and parts of the Middle East
<i>Arctostaphylos</i>	Circumboreal Region of North America and Eurasia; California floristic province (from southwestern Oregon, California, western Nevada to northern Baja California)
<i>Arctous</i>	Circumarctic distribution
<i>Comarostaphylis</i>	Southern California to northern Baja California and from the Mexican highlands to western Panama
<i>Ornithostaphylos</i>	Southern California to northern Baja California
<i>Xylococcus</i>	Southern California to northern Baja California

1993; Molina et al. 1997; Mühlmann and Göbl 2006; Krpata et al. 2007; Kennedy et al. 2012; Lancellotti et al. 2014; Gomes et al. 2015).

Comarostaphylis is an evergreen shrub to a small tree and closely related to *Arbutus* and *Arctostaphylos* (Hileman et al. 2001; Osmundson et al. 2007). The genus was also suggested to form arbutoid mycorrhiza with diverse ascomycetes and basidiomycetes (Bidartondo and Bruns 2001; Halling and Mueller 2003) but evidence was still missing. Later, Osmundson et al. (2007) provide the first and so far only description of the mycorrhizal association of *Comarostaphylis arbutoides*-*Leccinum monticola* from the Cerro de la Muerte in Costa Rica.

1.3 Ectomycorrhizal community in Costa Rica

Costa Rica is a hot spot of global plant diversity (Kappelle et al. 1992), but harbors very few native ectomycorrhizal host plants, such as *Quercus* species, *Alnus acuminata*, and *Comarostaphylis arbutoides* (Mueller et al. 2006). The montane forests of the Cordillera de Talamanca in Costa Rica are dominated by *Quercus* trees in the canopy, originating from the temperate zone, whereas their understory and subcanopy is principally composed of tropical originating species (Kappelle et al. 1992; Halling and Mueller 2002). Ectomycorrhizal fungi migrated from North America to the south together with their host tree (oaks) and therefore, are distributed into Costa Rica up to southern Colombia. In neotropical montane forests endemic species can be expected as well as local endemics with restricted distributions (Mueller et al. 2006).

In Costa Rica *Comarostaphylis arbutoides* grows in the montane cloud forest to the páramo of the Talamanca montane range and can form small woods. At the Cerro de la Muerte *Comarostaphylis arbutoides* co-occurs as understory with dominant *Quercus* (oak) tree species (Fig. 1.1). However, still little data are available concerning the fungal communities of *Comarostaphylis arbutoides* in sub-alpine tropical forests of Central America and elsewhere.

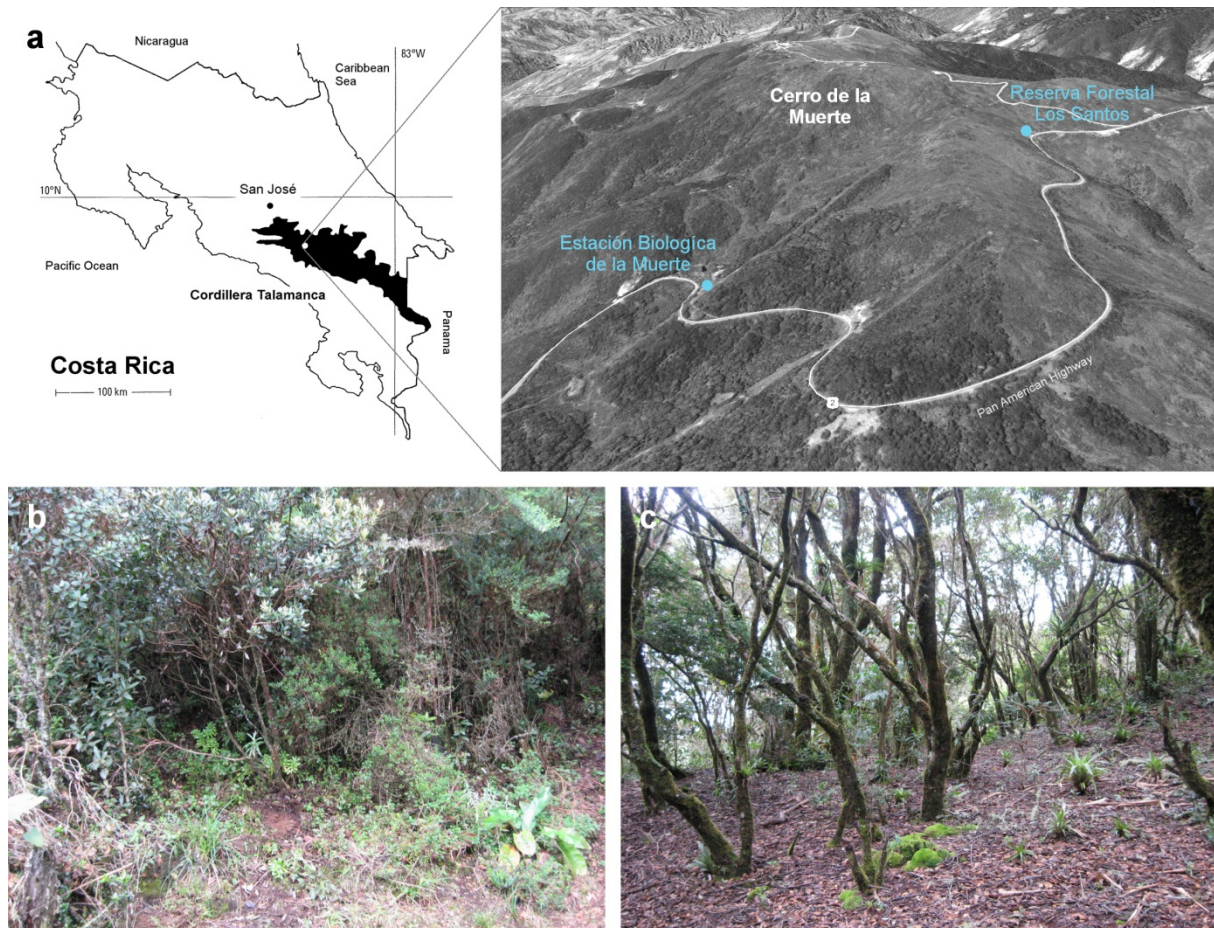


Fig. 1.1 a Study area at the Cerro de la Muerte in the Talamanca montane range of Costa Rica. Sampling sites are indicated as *blue dots*. b Estación Biológica de la Muerte (site I). Single tree of *Comarostaphylis arbutoides* (in front) in a *Quercus*-dominated forest, mixed with understory vegetation. c Reserva Forestal Los Santos (site II). Small *Comarostaphylis arbutoides*-dominated forest with single *Quercus* trees at the edge

1.4 Aim of thesis

Since the late 1980s, macrofungi of the Cordillera de Talamanca have been studied (Mueller and Halling 1995; Halling and Mueller 2005) to better understand neotropical fungi and to contribute to forest conservation, reforestation and land management activities (Halling and Mueller 2005; Mueller et al. 2006). Since then, many ectomycorrhizal fungal species were identified of which several are presumed to be endemic in the tropical montane *Quercus*-

dominated forest. Most of these fungi have been reported to be mycorrhizal with *Quercus* but, according to Halling and Mueller (1999, 2005) some were also assumed to be associated with *Comarostaphylis arbutoides*, e.g. *Cortinarius comarostaphylis*, *Cortinarius glaucopus*, *Cortinarius* sp. (subgenus *Phlegmacium*), *Hysterangium* sp., *Laccaria amethystina*, *Phaeocollybia longistipitata*, *Tricholoma columbetta*, *Tricholoma equestre*, and *Tricholoma palustre*. However, these assumptions have never been proven genetically based on mycorrhizal systems and host tree identification.

This study aims to contribute in revealing the occurrence of further ectomycorrhizal fungi at the Cerro de la Muerte in Costa Rica, especially regarding the mycorrhizal association with the Ericaceae *Comarostaphylis arbutoides*. Therefore, mycorrhizal systems from two sampling sites have been investigated genetically to identify the associated fungi and to confirm the involved host plant. Selected genera will be presented in more detail since they contribute to a better understanding of biodiversity in this region and may reveal hitherto unknown and undescribed species based on their mycorrhizal systems.

1.5 Identified and selected taxa from the Cerro de la Muerte

In the years 2010 and 2011 a total of 399 mycorrhizal root tips were analyzed genetically. Out of these, 223 sequence types could be assigned to genus level, comprising 20 to 24 different genera. Further 71 could only be identified to family, order or division level, and another 21 sequence types remain as unidentified ectomycorrhizal fungi (Fig. 1.2). For the present study we choose three genera (*Sebacina*, *Leotia*, and *Cortinarius*) which were described in more detail phylogenetically, as well as morphologically and anatomically. The reason for their selection is given in the following sections.

By the year 2001, the basidiomycete family Sebacinaceae was assigned to the wood-decaying order Auriculariales, but molecular phylogenetic studies revealed that it belongs to the new order Sebacinales (Weiß and Oberwinkler 2001; Weiß et al. 2004). In the following years it became more evident that members of the genus *Sebacina* have a diverse ecology since they are known to form various mycorrhizal types and also occur as endophytes. A wide range of diverse plant families are involved as host associates. Although *Sebacina* has a worldwide distribution, the genus has never been reported from Costa Rica before. In this study we present a hitherto probably unknown *Sebacina* species as mycorrhizal partner of the Ericaceae *Comarostaphylis arbutoides*.

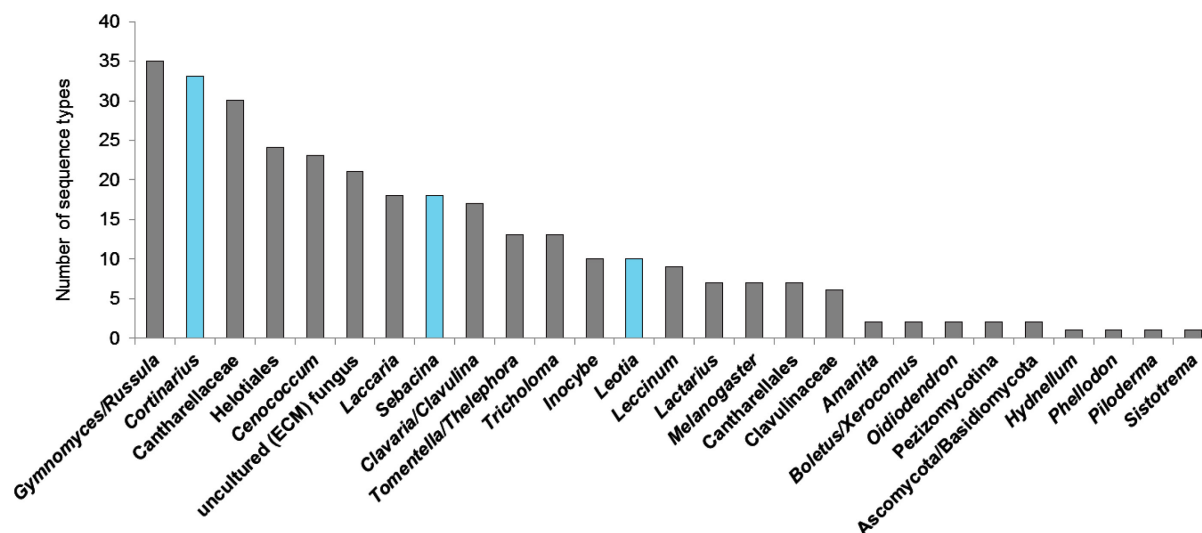


Fig. 1.2 Taxa resulted from 315 single root tips from the two sampling sites around the Cerro de la Muerte in Costa Rica collected in 2010 and 2011. Results obtained by BLASTn search in NCBI and UNITE database based on ITS sequences. Additional 84 samples failed during analysis. Mycorrhizal systems of the genera *Cortinarius*, *Leotia*, and *Sebacina* (blue) were examined in more detail phylogenetically, as well as morphologically and anatomically

Members of the genus *Leotia* were generally rated as saprotrophic so far. However, mycorrhizal associations of the species *Leotia lubrica* were reported sporadically with ectomycorrhizal plants, but findings based exclusively on molecular data and have not been proven morphologically. Even though fruit bodies of *Leotia lubrica* are routinely encountered in oak forests of the Talamanca montane range as well as in the páramo under *Comarostaphylis arbutoides* trees (Halling and Mueller 1999), this ascomycete was not yet recognized to be associated with any mycorrhizal host plant in Costa Rica. In our study we provide, the first evidence that *Leotia lubrica* is indeed a mycorrhizal species and show *Comarostaphylis arbutoides* as host plant.

The genus *Cortinarius* comprises over 2000 species which are all ectomycorrhizal, typically associated with broadleaf or coniferous trees. Although the genus occurs worldwide little is known about tropical species. Halling and Mueller (1999, 2005) and Ammirati et al. (2007) already reported or described several Costa Rican *Cortinarii* from the Talamanca montane range, but assumed *Comarostaphylis arbutoides* only occasionally as host tree. Here, we could confirm *Comarostaphylis arbutoides* as associate of diverse cortinarioid species and present four different mycorrhizal associations of the genus *Cortinarius* with the Ericaceae.

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2

Sebacina* sp. is a mycorrhizal partner of *Comarostaphylis arbutoides* (Ericaceae)¹*Abstract**

Sebacina (Sebacinales) forms ectomycorrhiza, arbutoid, ericoid, jungermannoid, cavendishoid 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. *Comarostaphylis 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 *Comarostaphylis 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.-*Comarostaphylis arbutoides* was described morphologically and anatomically.

Keywords

Arbutoid mycorrhiza, anatomy, morphology, ITS, LSU, *Quercus costaricensis*, Costa Rica

¹ Based on: Kühndorf K, Münzenberger B, Begerow D, Karasch-Wittmann C, Gómez-Laurito J, Hüttl RF (2014) *Mycol Prog* 13:733–744 (with kind permission of Springer)

2.1 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), jungermannoid (JMM), cavendishoid (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 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 para-epidermal 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 Monotropeoideae; 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), *Comarostaphylis arbutoides* grows together with *Quercus costaricensis*. As it is known from fruitbody collections (Halling and Mueller 2005), these oak trees probably share their ectomycorrhizal fungi with *Comarostaphylis arbutoides*, well known from other EEM forming plants (Smith and Read 2008). Thus, *Comarostaphylis 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 *Comarostaphylis arbutoides* (Halling and Mueller 2003; den Bakker et al. 2004). *Comarostaphylis 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 *Comarostaphylis arbutoides* are still missing.

We collected EEMs from *Comarostaphylis arbutoides* at the Cerro de la Muerte (Cordillera de Talamanca), Costa Rica, and characterized the morphotype of *Comarostaphylis 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 *Comarostaphylis arbutoides*, sebacinoid ECMs of *Quercus costaricensis* were also found, and were therefore included for comparison purposes.

2.2 Materials and methods

2.2.1 Sampling sites and sampling

Two forest sites with *Comarostaphylis 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 *Quercus costaricensis* mixed with solitary individuals of *Comarostaphylis arbutoides*. At sampling site II, *Comarostaphylis arbutoides* itself is the dominating species, mixed with a few species of *Quercus 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 turgescient mycorrhizas, sampling took place during the rain seasons in October 2010 and 2011. Fine root systems of *Comarostaphylis 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.

2.2.2 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'-TCCTGAGGGAACTTCG-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 *Comarostaphylis arbutoides* and seven with *Quercus*. All eight sebacinoid sequences of *Comarostaphylis arbutoides* were deposited in NCBI GenBank under the accession numbers KF419105-KF419112 (LSU), KF419113-KF419120 (ITS), as well as *Comarostaphylis arbutoides* (KF419121).

2.2.3 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 *Comarostaphylis 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.

2.2.4 Microscopy

The morphological and anatomical description of the sebacinoid mycorrhizas of *Comarostaphylis 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 *Quercus costaricensis* could be used for further anatomical comparison. 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, Wolfartshausen, 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.

2.3 Results

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

Morphological characters (Fig. 2.1a) *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.

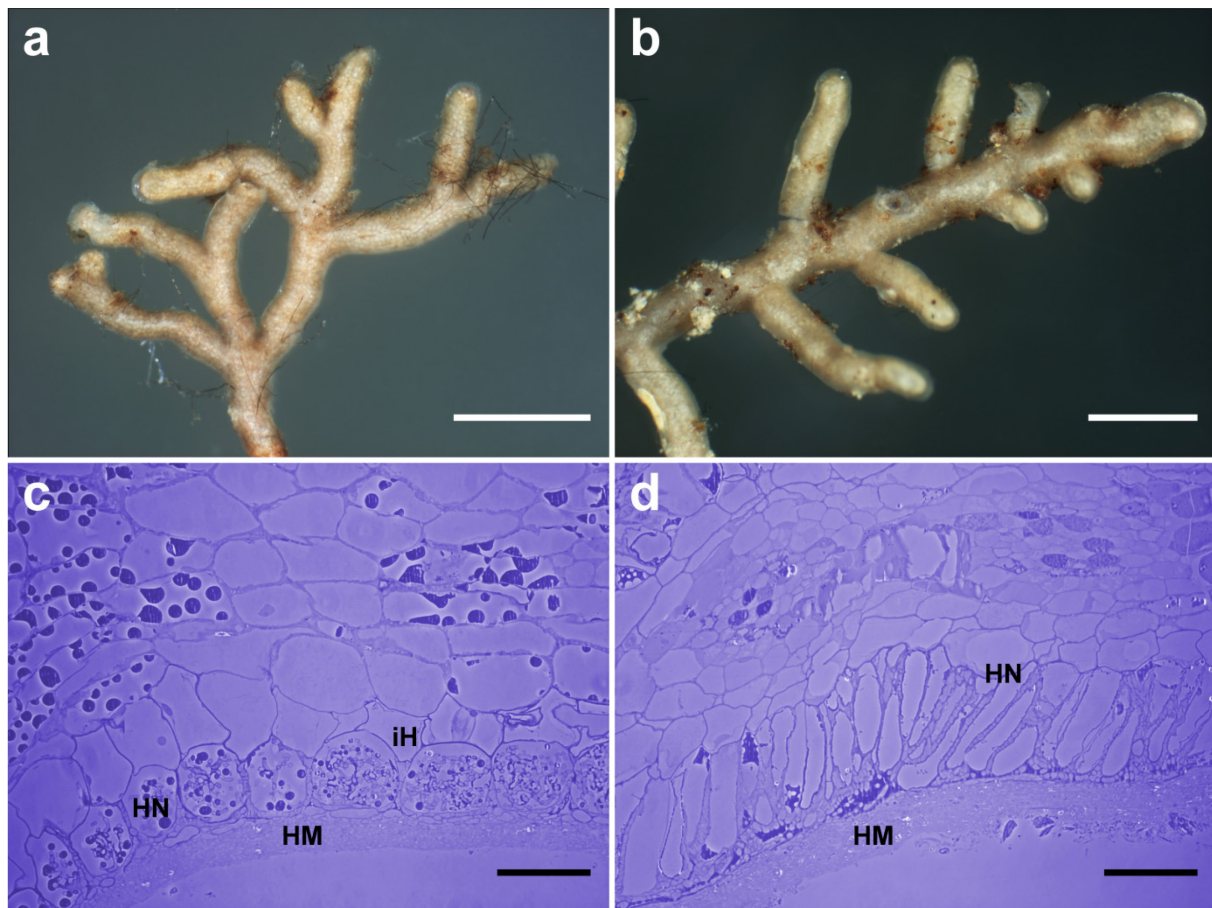


Fig. 2.1 Habit and semi-thin section of the mycorrhiza *Sebacina* sp.-*Comarostaphylis arbutoides* compared to *Sebacina* sp.-*Quercus costaricensis*. **a** Habit of *Sebacina* sp.-*Comarostaphylis arbutoides*. Mycorrhiza arbutoid ramified, mantle smooth and transparent with foreign brown hyphae; bar 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 μ m. **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 μ m

Anatomical characters of the mantle in plan views (Fig. 2.2a–f) Mantle with three distinct layers, hyphae in all layers colorless and clampless. *Outer mantle layers* (Fig. 2.2a, 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 20–44 μ m long, 0.5–2.7 μ m diam., cell walls 0.3–0.6 μ m thick; surface smooth; laticifers lacking. *Middle mantle layers* (Fig. 2.2b, 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 μ m long, 1.2–1.6 μ m diam., cell walls 0.2–0.5 μ m thick. *Inner mantle layers* (Fig. 2.2c, f) transitional type between plectenchymatous and pseudoparenchymatous, no gelatinous matrix; hyphae densely arranged and frequently ramified, mostly epidermoid, no pattern discernible, hyphae

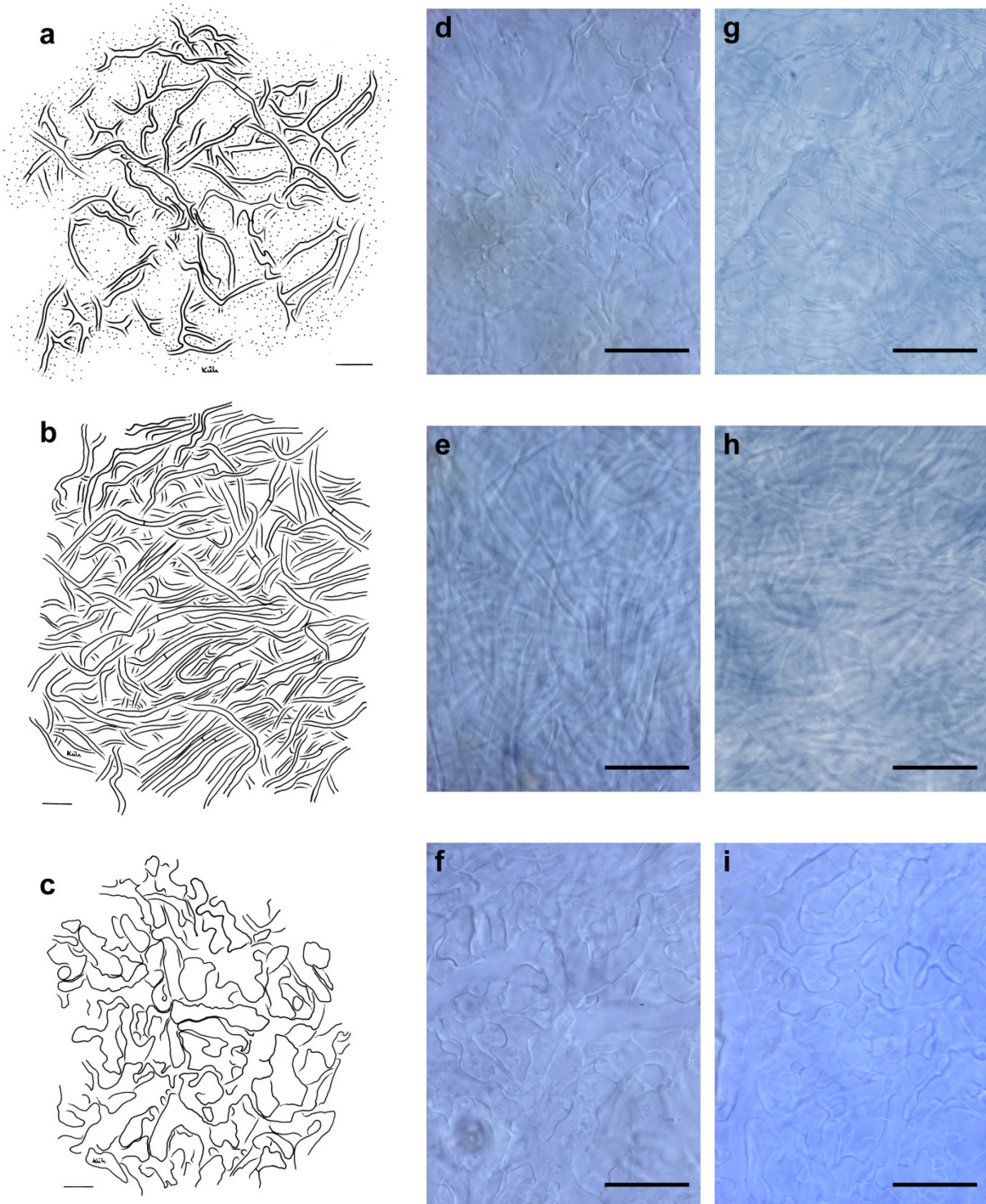


Fig. 2.2 Arbutoid mycorrhiza of *Sebacina* sp.-*Comarostaphylis arbutoides* compared to the ectomycorrhiza *Sebacina* sp.-*Quercus costaricensis*. **a-f** *Sebacina* sp.-*Comarostaphylis arbutoides*; **a-c** Plan view of different mantle layers; bars 10 µm; **a** outer mantle layer with irregularly inflated and repeatedly branched hyphae in a gelatinous matrix. **b** Middle mantle layer with densely parallel or irregularly branched 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

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. 2.1c) 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 para-epidermal 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).

2.3.2 Ultrastructure

Five sebacinoid EEM of *Comarostaphylis arbutoides* were investigated by transmission electron microscopy (TEM). TEM analyses of these mycorrhizas revealed a dolipore with an imperforate cap typical for Sebaciniales (Fig. 2.3).



Fig. 2.3 Ultrastructure of *Sebacina* sp.-*Comarostaphylis arbutoides* showing a dolipore with imperforate cap (white arrow), 50,000 magnification

2.3.3 Phylogenetic analyses

Sequencing of the LSU region of the eight EEMs of *Comarostaphylis 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. 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 Tab. 2.1. The sebacinoid ECMs of *Quercus costaricensis* completely correspond with the obtained sequences for LSU and ITS regions of the *Comarostaphylis arbutoides* mycobionts. Therefore, both host trees are associated with the same sebacinoid sequence type.

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. 2.4). 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 *Sebacina dimitica*, *Sebacina epigaea*, *Sebacina helvelloides*, *Sebacina incrustans*, *Sebacina vermifera* and *Sebacina 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 *Sebacina vermifera* can be found in this clade as identified *Sebacina* species. All eight investigated EEMs (KF419105-KF419112) of *Comarostaphylis arbutoides* are grouped together (PP 1/BS 100) and nest within the *Sebacina* complex of Clade A (Fig. 2.4). 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 *Sebacina incrustans* (AJ966753), which is grouped together with two *Sebacina* ECMs (HQ154314; JQ420940) in the Bayesian analysis (PP

Tab. 2.1 Identification of LSU rDNA and ITS sequences with the NCBI and UNITE databases obtained from the mycobiont of *Comarostaphylis arbutoides*

	NCBI			UNITE				
	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)	1452	0.0/100	95	<i>Sebacina dimitica</i> (UDB016422)	1469	0.0/100	95
					<i>Sebacina</i> sp. (UDB16385)	1469	0.0/100	95
					<i>Sebacina in crustans</i> (UDB014122)	1469	0.0/100	95
ITS: KF419117; KF419118; KF419120	Uncultured <i>Sebacina</i> sp. (JQ347196)	1092	0.0/100	95	<i>Sebacina dimitica</i> (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 Accesses 9 December 2013

KF419120) of *Comarostaphylis 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 *Comarostaphylis arbutoides* (PP 0.96/BS 74).

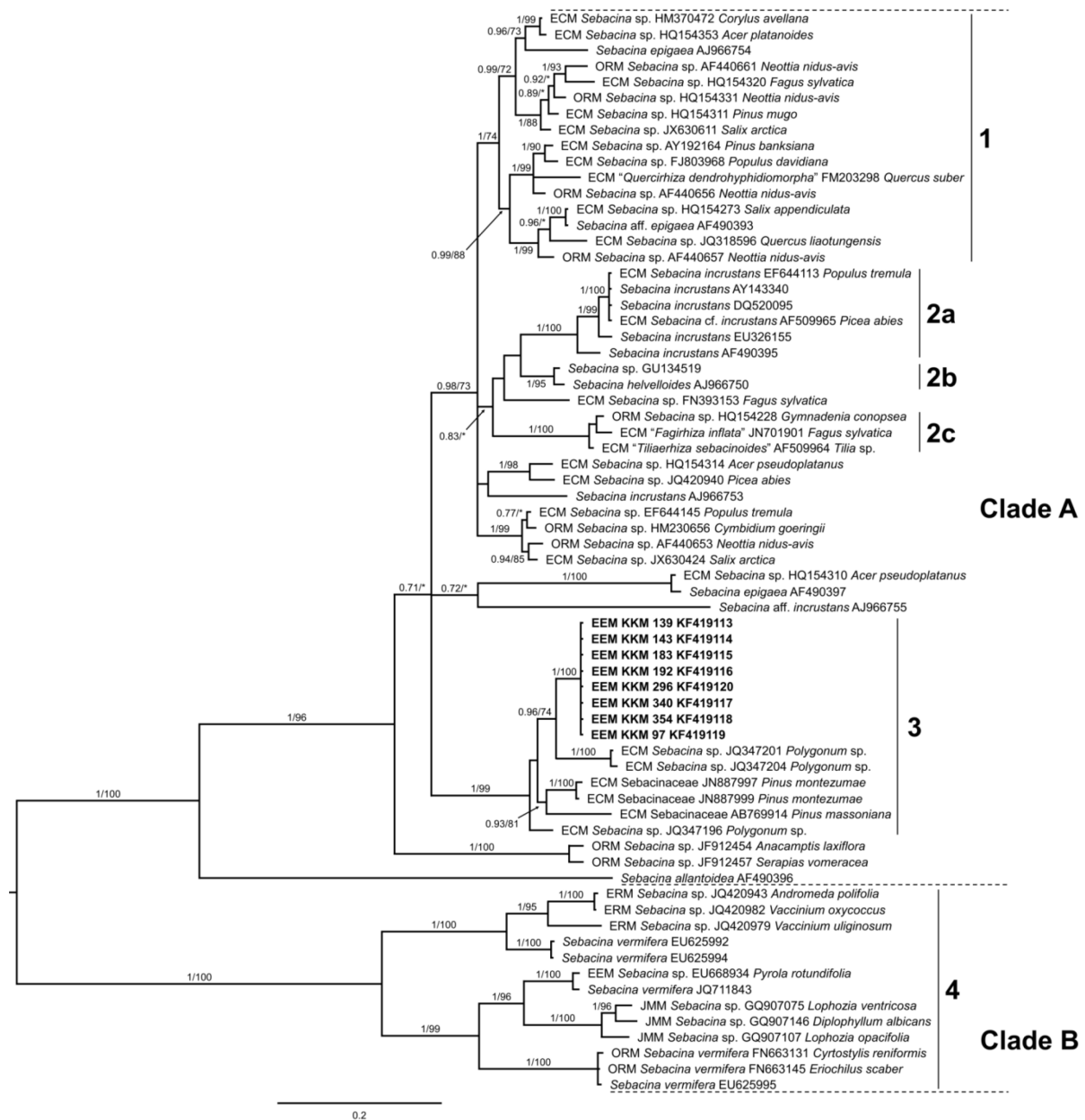


Fig. 2.5 Phylogenetic relationship of eight sebacinoid mycobionts of *Comarostaphylis arbutoides* within members of *Sebacina*. Complexes with identified *Sebacina* species are indicated by clusters (1–4). Phylogram was obtained from Bayesian analysis based on ITS sequences. Branch support values were calculated as posterior probability from 2,000,000 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 *Comarostaphylis arbutoides* are marked in bold. Mycorrhizal types: ectomycorrhiza (ECM), ectendomycorrhiza (EEM), ericoid mycorrhiza (ERM), jungermannioid mycorrhiza (JMM), orchid mycorrhiza (ORM)

2.4 Discussion

2.4.1 Mycorrhiza of *Comarostaphylis arbutoides* and *Quercus costaricensis* formed with *Sebacina* sp.

Genetically investigations showed that sebacinoid mycorrhizas of *Comarostaphylis arbutoides* and *Quercus costaricensis* are formed by the same hitherto unknown *Sebacina* species (see below). The mycorrhizal systems of this *Sebacina* sp. formed with *Comarostaphylis arbutoides* (Ericaceae) and *Quercus costaricensis* (Fagaceae), respectively, do not differ significantly in their features. The sebacinoid ECM of *Quercus costaricensis* is morphologically identical to the EEM of *Comarostaphylis 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 *Comarostaphylis arbutoides* shows an arbutoid ramification, whereas that of *Quercus costaricensis* is monopodial-pyramidal ramified (Fig. 2.1a, b). Also, the semi-thin sections reflect the typical characteristic difference of the arbutoid mycorrhiza and ectomycorrhiza by the presence (*Comarostaphylis arbutoides*) or lack (*Quercus costaricensis*) of intracellular hyphae in the outer cortical cells (Fig. 2.1c, d). In contrast, both sebacinoid mycorrhizal types again show the same hyphal pattern in all three mantle layers (Fig. 2.2). 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).

2.4.2 Morpho-anatomical comparison of sebacinoid EEM of *Comarostaphylis arbutoides* with other sebacinoids

The investigated EEM of *Comarostaphylis arbutoides* belongs, according to phylogenetic studies (Figs. 2.4 and 2.5), 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*", "*Pinirhiza nondextrinoidea*" (Wei and Agerer 2011), and "*Fagirhiza inflata*" (Leberecht et al.

2012). Furthermore, a species of *Sebacina*, *Sebacina* 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 (*Sebacina incrustans*) yellowish (“*Tiliaerhiza sebacinoides*”) or orange ochre (“*Fagirhiza inflata*”) to grayish orange-brown (“*Pinirhiza multifurcata*”), brownish (“*Quercirhiza dendrohyphidiomorpha*”) and cinnamon brownish (“*Pinirhiza nondextrinoidea*”). On neither of these ECMs are the cortical cells visible, only Wei and Agerer (2011) describe an opaque to semitransparent mantle in “*Pinirhiza multifurcata*”.

All mycorrhizas, except “*Pinirhiza 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 “*Pinirhiza 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 *Comarostaphylis 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 “*Pinirhiza multifurcata*” can also be considered as cystidia. Further samples with only emanating hyphae are “*Pinirhiza nondextrinoidea*” and “*Tiliaerhiza sebacinoides*”. *Sebacina* cf. *incrustans* and “*Quercirhiza 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 “*Fagirhiza inflata*” and together with “*Pinirhiza multifurcata*” and “*Pinirhiza 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 *Comarostaphylis 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 *Sebacina* cf. *incrustans* and “*Tiliaerhiza sebacinoides*”, which only feature hyaline cell walls and a superficial hyphal net of thick-walled, lobed, and frequently branched hyphae. All samples differ from “*Quercirhiza 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 *Comarostaphylis arbutoides*. In “*Fagirhiza 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 “*Tiliaerhiza 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 *Comarostaphylis arbutoides* shares the plectenchymatous-pseudoparenchymatous inner mantle layer of “*Quercirhiza dendrohyphidiomorpha*” and “*Fagirhiza 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 (“*Quercirhiza dendrohyphidiomorpha*”: 2.3–3.5 μm diam.; “*Fagirhiza inflata*”: 2–4.5 μm) and clearly thicker cell walls, with a thickness of 0.4–2 μm (“*Quercirhiza dendrohyphidiomorpha*”: 0.3–0.5 μm ; “*Fagirhiza inflata*”: 0.3 μm).

The EEM of *Comarostaphylis arbutoides* showed a dextrinoid reaction, a common feature with the other sebacinoid ECMs, with the exception of “*Pinirhiza nondextrinoidea*”, where no reaction with Melzer’s reagent was observed. However, this feature was not checked in *Sebacina* cf. *incrustans* and “*Tiliaerhiza sebacinoides*”. Urban et al. (2003) describe a doliporus with imperforate parentheses in “*Tiliaerhiza sebacinoides*”, which is typical for Sebaciales (Oberwinkler et al. 2013). We also found this ultrastructure in the sebacinoid EEM of *Comarostaphylis arbutoides*.

Hitherto, “*Tiliaerhiza sebacinoides*” and “*Pinirhiza 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 *Comarostaphylis arbutoides* are quite dense and do not have emanating elements. This makes the measurements reliable, but perhaps not comparable to “*Tiliaerhiza sebacinoides*” and “*Pinirhiza nondextrinoidea*”, since both show emanating hyphae.

2.4.3 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 Sebaciniales. We also found this typical structure in the sebacinoid mycobionts of *Comarostaphylis arbutoides* (Fig. 2.3), 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).

2.4.4 Phylogenetic position

The two well-supported clades of Sebacinaceae generated by the analyses of LSU rDNA (Fig. 2.4) 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 *Comarostaphylis arbutoides* belong to *Sebacina*, because they cluster in the *Sebacina* complex (PP 1/BS 82) within the Sebacinaceae family (Fig. 2.4). Together with an EEM of *Arbutus unedo*, they belong to Clade A, whereas

Clade B contains all sebacinoid ERM 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 *Comarostaphylis 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. 2.5), over half of the *Sebacina* samples are only identified as *Sebacina* sp. Nevertheless, some well-supported and circumscribed clusters (cluster 1–4, Fig. 2.5) are identifiable. Clade B, for instance, comprises three different mycorrhizal types (EEM; ERM; CVM) that are all obviously formed by *Sebacina vermifera*, which is regarded as a cryptic species (Weiß et al. 2004; Weiß et al. 2011). Surprisingly, an EEM (EU668934) of *Pyrola rotundifolia* (Monotropeoideae, 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 sebacinoid fungal partners of the Ericaceae subfamily Monotropeoideae also include Clade B Sebacinales. In Clade A, several samples recorded as *Sebacina epigaea* form a huge, well-supported (PP 1/BS 74) group (cluster 1, Fig. 2.5). The former morphologically and anatomically discussed “*Quercirhiza dendrohyphidiomorpha*” (FM203298; Azul et al. 2006) can also be found in this lineage, and may be identified as *Sebacina epigaeae*. Another large cluster (cluster 2, Fig. 2.5) achieved a 0.83 posterior probability and a bootstrap support of 33 %. It is formed by one big subcluster of *Sebacina incrustans* (2a; PP 1/BS 100), and two further smaller subclusters of *Sebacina helvelloides* (2b; PP 1/BS 95) and three, not yet identified, species (2c; PP 1/BS 100). In the last-mentioned group, “*Fagirhiza inflata*” (JN701901; Leberecht et al. 2012) and “*Tiliaerhiza 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 *Sebacina* cf. *incrustans* (AF509965; Urban et al. 2003), however, fits perfectly in the subcluster of *Sebacina incrustans* (2a) within the *Sebacina incrustans/helvelloides* complex of cluster 2 (Fig. 2.5). Thus, our data is in accordance with Urban et al. (2003).

The sebacinoid ECMs “*Pinirhiza multifurcata*” (GU269908) and “*Pinirhiza 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. 2.4. However, “*Pinirhiza nondextrinoidea*” shows some affinity to *Sebacina* 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 “*Fagirhiza inflata*”, “*Tiliaerhiza sebacinoides*”, “*Pinirhiza multifurcata*”, and “*Pinirhiza nondextrinoidea*” the eight EEMs (KF419113-KF419120) of *Comarostaphylis arbutoides* cannot be assigned to any known species. As shown in Fig. 2.5, 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 *Comarostaphylis 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 *Polygonum 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 *Bistorta vivipara* (Freeman and Hinds 2005). If *Bistorta 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; Blaaid et al. 2012).

It has to be mentioned that species of the *Sebacina* shown in Figs. 2.4 and 2.5, 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 *Comarostaphylis 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 *Sebacina epigaea* and *Sebacina 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 *Comarostaphylis arbutoides*. Additionally, we found sebacinoid ECMs of *Quercus costaricensis* that are identical in morphology, anatomy and genetics with the arbutoid *Sebacina* samples investigated in this study. Thus, we can confirm that *Comarostaphylis arbutoides* is a refuge plant for ECM fungi after clearance of the economically important oaks.

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3

***Leotia cf. lubrica* forms arbutoid mycorrhiza with *Comarostaphylis arbutoides* (Ericaceae)²**

Abstract

Arbutoid mycorrhizal plants are commonly found as understory vegetation in forests worldwide where ectomycorrhiza-forming trees occur. *Comarostaphylis arbutoides* (Ericaceae) is a tropical woody plant and common in tropical Central America. This plant forms arbutoid mycorrhiza, whereas only associations with *Leccinum monticola* as well as *Sebacina* sp. are described so far. We collected arbutoid mycorrhizas of *Comarostaphylis arbutoides* from the Cerro de la Muerte (Cordillera de Talamanca), Costa Rica, where this plant species grows together with *Quercus costaricensis*. We provide here the first evidence of mycorrhizal status for the ascomycete *Leotia cf. lubrica* (Helotiales) that was so far under discussion as saprophyte or mycorrhizal. This fungus formed arbutoid mycorrhiza with *Comarostaphylis arbutoides*. The morphotype was described morphologically and anatomically. *Leotia cf. lubrica* was identified using molecular methods, such as sequencing the internal-transcribed spacer (ITS) and the large subunit (LSU) ribosomal DNA regions, as well as phylogenetic analyses. Specific plant primers were used to confirm *Comarostaphylis arbutoides* as the host plant of the leotioid mycorrhiza.

Keywords

Arbutoid mycorrhiza, anatomy, morphology, phylogeny, Costa Rica, Leotiomyces

² Based on: Kühndorf K, Münzenberger B, Begerow D, Gómez-Laurito J, Hüttl RF (2015) *Mycorrhiza* 25:109–120

3.1 Introduction

The tropical woody plant *Comarostaphylis arbutoides* Lindl. (Ericaceae) is common in tropical Central America (c. 2,500–3,430 m height above sea level (a.s.l.)), where it can form extensive thickets. Based on fruit and flower morphology, anatomy, and phytochemistry, this plant is a member of the subfamily Arbutoideae (Hileman et al. 2001; Fig. 3.1a). The Arbutoideae also include the circumboreal *Arctostaphylos uva-ursi* and species of *Arbutus* that are all known to form arbutoid mycorrhiza (Molina and Trappe 1982a; Münzenberger et al. 1992; Osmundson et al. 2007). The mycorrhizal fungus induces the branching of the lateral roots to form mostly, with some exceptions (Molina and Trappe 1982a), a pinnate-cruciate branching pattern that is typical for this mycorrhizal type (Massicotte et al. 1993). The arbutoid mycorrhiza is characterized by a hyphal mantle, a para-epidermal Hartig net and intracellular hyphae penetrating the living epidermal cells of the host (Münzenberger et al. 1992; Selosse et al. 2007). Suberization of the outer cortical layer leads to the formation of an exodermis and prevents penetration of the fungus into deeper root cell layers (Münzenberger et al. 1992; Massicotte et al. 1993).

Comarostaphylis arbutoides is a refuge plant for ectomycorrhizal fungi as it shares these fungi with ectomycorrhizal tropical trees such as *Quercus costaricensis* (Halling and Mueller 2003; Kühdorf et al. 2014). After forest clearcutting of the economically important forest trees, arbutoid mycorrhizal plants host the ectomycorrhizal fungi that contribute to forest recovery later on (Dahlberg 1990; Visser 1995; Molina et al. 1997; Horton et al. 1999; Hagerman et al. 2001). Ectomycorrhizal fungi of Arbutoideae show identical morphology (apart from, e.g., host-dependent branching pattern and habit dimensions) and hyphal mantle anatomy as the ectomycorrhiza (ECM) of other hosts such as *Pinus*, *Picea*, *Pseudotsuga*, and *Quercus* (Zak 1976a, b; Molina and Trappe 1982b; Mühlmann and Göbl 2006; Kühdorf et al. 2014). The ericaceous hosts *Arbutus menziesii* and *Arctostaphylos uva-ursi* lack mycorrhizal specificity, which means numerous ectomycorrhizal fungi are able to colonize these ericads (Molina and Trappe 1982a; Acsai and Largent 1983a, b; Massicotte et al. 1994; Richard et al. 2005; Kennedy et al. 2012).

Osmundson et al. (2007) were the first to describe the arbutoid mycorrhiza of *Comarostaphylis arbutoides* and *Leccinum monticola* anatomically and molecularly. To date, only one more morphotype has been described in literature (Kühdorf et al. 2014). However, Halling and Mueller (2005) mention that species of other mycorrhizal genera such as



Fig. 3.1 **a** Leaves and flowers of the Ericaceae *Comarostaphylis arbutoides*. Photo by Roy E. Halling. **b** Two fruit bodies and one initial (arrowhead) of *Leotia lubrica* found at Estación Biológica de la Muerte (site I), Costa Rica. Ascomata 10–40 mm. Photo by Katja Kühndorf. **c** Habit of the mycorrhiza *Leotia* cf. *lubrica*-*Comarostaphylis arbutoides*. Mycorrhiza arbutoid ramified, mantle smooth to moderately hairy, and transparent with foreign brown hyphae; bar 0.5 mm

Tricholoma, *Cortinarius*, *Russula*, and *Laccaria* are likely associates of *Comarostaphylis arbutoides*.

The ecology of the helotialean fungi is diverse. Members of the Helotiales have been described as plant pathogens, ECM and ericoid mycorrhiza formers, terrestrial and aquatic saprobes, and wood rot fungi (Wang et al. 2006a). The genus *Leotia* (Helotiales) is globally distributed (Wang et al. 2006a, b), whereas the ecological role of the ascomycete is not clear. Species of *Leotia* and *Microglossum* are found usually on humus-rich soil, sometimes on decaying wood, but rarely on leaf litter (Arora 1986). *Leotia* was already recorded as mycorrhiza-forming fungus by Molina et al. (1992). This status was later speculated to be saprotrophic by Rinaldi et al. (2008) based on insufficient evidence. Both Zeller et al. (2007) and Seitzman et al. (2011) later found the ^{13}C signature of the species *Leotia lubrica* comparable with those of ectomycorrhizal fungi and therefore, conclude that this fungus should be mycorrhizal. Furthermore, *Leotia lubrica* was reported to form ECM with diverse plant species such as *Quercus rotundifolia*, *Polygonum* sp., and *Nothofagus menziesii* (Branco and Ree 2010; Gao and Yang 2010; Orlovich et al. 2013). However, these records have been proven by DNA sequencing only and were not further described.

The genus *Leotia* contains three species of inoperculate discomycetes with stipitate-capitate ascomata. They are constructed, at least in part, of tissues built-up of hyphae imbedded in a gelatinous matrix (Zhong and Pfister 2004). They are distinguished by the color of their fresh ascomata: species with a yellow stipe and yellow hymenia are assigned to *Leotia lubrica*, those with an entirely green ascomata to *Leotia atrovirens*. Species of *Leotia viscosa* have a yellow stalk and green hymenia (Durand 1908; Mains 1956; Grund and Harrison 1967).

Here, we describe arbutoid mycorrhizal systems morphologically and anatomically according to Agerer (1991), formed by *Leotia* cf. *lubrica* and *Comarostaphylis arbutoides* from the Cerro de la Muerte (Cordillera de Talamanca), Costa Rica. *Leotia* cf. *lubrica* was identified using molecular methods such as internal-transcribed spacer (ITS) and large subunit (LSU) sequencing as well as phylogenetic analysis. Plant primers were used to sequence the ITS region of *Comarostaphylis arbutoides* from the same arbutoid mycorrhiza as used for fungal analysis.

3.2 Materials and methods

3.2.1 Site description and sampling

Sampling was conducted at two forest sites 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. Both 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). Site I is dominated by *Quercus costaricensis* mixed with solitary individuals of *Comarostaphylis arbutoides*. At sampling site II, *Comarostaphylis arbutoides* itself is the dominating species, mixed with a few individuals of *Quercus costaricensis*. The understorey species consist of members of the families Araliaceae (*Schefflera* and *Oreopanax*), Cunoniaceae (*Weinmannia*), Ericaceae (*Cavendishia*, *Disterigma*, and *Vaccinium*), Poaceae (*Chusquea*), Primulaceae (*Myrsine*), and Winteraceae (*Drimys*).

Fine root systems of *Comarostaphylis arbutoides* were collected during the rainy seasons in October 2010 and 2011. For this, a soil corer (diameter, 3 cm; length, 40 cm) was used 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 and apparently healthy morphotypes were sorted out using a stereomicroscope. Systems with the same morphological features (e.g., color, hydrophobicity presence, emanating elements, and rhizomorphs) were assigned to one morphotype. For further analyses, the morphotypes were preserved in 2 % glutaraldehyde with 0.1 M sodium cacodylate buffer (Münzenberger et al. 2009) for light microscopy or dried on silica gel for DNA extraction, respectively. Identification of each morphotype is based on their respective sequence type. The genus *Leotia* was proven genetically in six soil cores.

3.2.2 DNA extraction, PCR, and sequencing

One unramified root tip per morphotype was used for DNA extraction using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. For phylogenetic analysis at family and species level, the ribosomal nuclear LSU and the ITS region from the ribosomal DNA (rDNA) were amplified. For this purpose, the primer combinations LR0R/LR5 (Moncalvo et al. 2000) and ITS1F/ITS4 (Gardes and Bruns 1993; White et al. 1990) were used. To identify the plant from mycorrhizal roots without co-amplifying fungal DNA, the angiosperm-specific ITS primer pair ITS-5A/ITS-241r was amplified (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).

3.2.3 Identification and phylogenetic analysis

All fungal sequences obtained for ITS and LSU rDNA were analyzed and edited using Chromas Lite v2.01 software (<http://technelysium.com.au>). Sequence comparisons were performed in the NCBI database (<http://www.ncbi.nlm.nih.gov/>) using Megablast, and the database UNITE (Kõljalg et al. 2005; <http://unite.ut.ee/>) using blastn. To calculate the phylogenetic tree of the ITS region, the 100 most similar sequences for each reference sequence in NCBI database were downloaded and complemented with an additional search in the nucleotide database and sequences of other publications as well. Alignment was 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 analyses 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 analyses were 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.

3.2.4 Microscopy

The morphological and anatomical description of the mycorrhizas was carried out according to Agerer (1987–2012, 1991), and using the online key of DEEMY (Agerer and Rambold 2004–2014). The iodine reaction specially adapted to ascomycetes was done after Baral (1987, 2009). Anatomical studies are based on 15 mycorrhizal systems. Drawings were performed by using an interference contrast microscope (BX50F-3, Olympus Corporation, Tokyo, Japan) connected with a drawing tube. All drawings were made at a thousand-fold magnification.

For semi-thin sections, the mycorrhizas were fixed with 2 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at room temperature until further processing. Thereafter, six washes in 0.1 M sodium cacodylate buffer were performed. Samples were postfixed in 1 % osmium tetroxide in the same buffer for 1 h under light exclusion 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 (Münzenberger et al. 2009). The mycorrhizal tips were embedded in Spurr's plastic (Spurr 1969) and sectioned with a diamond knife on an Ultracut Reichert Ultramicrotome (W. Reichert-LABTEC, Wolfratshausen, Germany). The sections (0.5 µm thick) were stained with crystal violet and investigated by use of a light microscope (Zeiss Axioskop 50, Oberkochen, Germany).

3.2.5 Fruit bodies of *Leotia lubrica*

During 2 years of sampling, fruit bodies of *Leotia lubrica* were found only once and were coincidentally documented. For that reason, no further genetic investigations were carried out to clearly identify the fungus. As the three species of the genus *Leotia* are easy to distinguish morphologically, the ascomata (Fig. 3.1b) were preliminary identified as *Leotia lubrica*, based on the yellow color of both the stipe and the hymenium.

3.3 Results

3.3.1 Morpho-anatomical description of the mycorrhiza *Leotia* cf. *lubrica* + *Comarostaphylis arbutoides*

Morphological characters (Fig. 3.1c) *Mycorrhizal systems* arbutoid, with 0–1 (4) orders of ramification, solitary or in small numbers, up to 2.8 mm long, main axis at 0.3 mm diameter; mantle surface hydrophilic and smooth to moderately hairy, of contact exploration type or short-distance exploration type. *Unramified ends* straight to slightly bent, cylindrical, not inflated, 0.1–0.7 (1.4) μm long, 0.2–0.3 (0.1) μm diameter, mantle consistently transparent and yellowish to light orange, older parts dark orange to ochre. *Surface of unramified ends* smooth to occasionally hairy, cortical cells (correspond to epidermal cells) visible. *Cystidia* not distinct under stereomicroscope magnification. *Rhizomorphs* not found. *Sclerotia* not observed.

Anatomical characters of the mantle in plan views (Figs. 3.2 and 3.3a–d) Mantle plectenchymatous throughout, all hyphae clampless and smooth; laticifers are lacking. *Outer mantle layers* (Figs. 3.2a and 3.3a) hyphae arranged net-like; hyphae frequently branched, often with merged hyphal tips, matrix lacking (mantle type E; Agerer 1991); hyphae septate, even and straight, not constricted at septa; hyphae with closed anastomoses, anastomosal bridge long, mostly thinner than hyphae; hyphae with numerous oily droplets, droplets light yellow to light orange; cytoplasm colorless; hyphae 10–90 μm long, 0.7–3.1 μm diameter; cell walls 0.2–0.4 μm thick; septa as thick as cell walls and often difficult to discern due to frequent droplets. *Middle mantle layers* (Figs. 3.2d, e and 3.3b) contain a nongelatinous matrix, hyphae irregularly interwoven, repeatedly branched and septate, colorless, no discernible pattern; frequently merged hyphal tips, hyphae at distal end simple; hyphae 15–50 (90) μm long, 0.9–2.4 μm diameter; cell walls 0.2 μm thick; septa as thick as cell walls; anastomoses frequently and very variable in shape; anastomoses open or closed by a simple septum; anastomosal bridge long, short, or almost lacking; bridge thinner or thicker than hyphae or as thick as hyphae; cell walls of anastomoses as thick as remaining wall; anastomoses close to hyphal tips not found. *Inner mantle layers* (Figs. 3.2f, h and 3.3c) ring-like arrangement of hyphal bundles; hyphae even or irregularly inflated, at distal end simple or slightly inflated; hyphae rarely septate, colorless; hyphae (7) 20–130 μm long, 1.5–3.9 μm diameter; cell walls 0.2–0.3 μm thick, septa as thick as cell walls; anastomoses frequently, anastomoses open, with short bridge or bridge almost lacking, bridge thinner or thicker than hyphae, or as thick as hyphae; cell walls of anastomoses as thick as remaining walls;

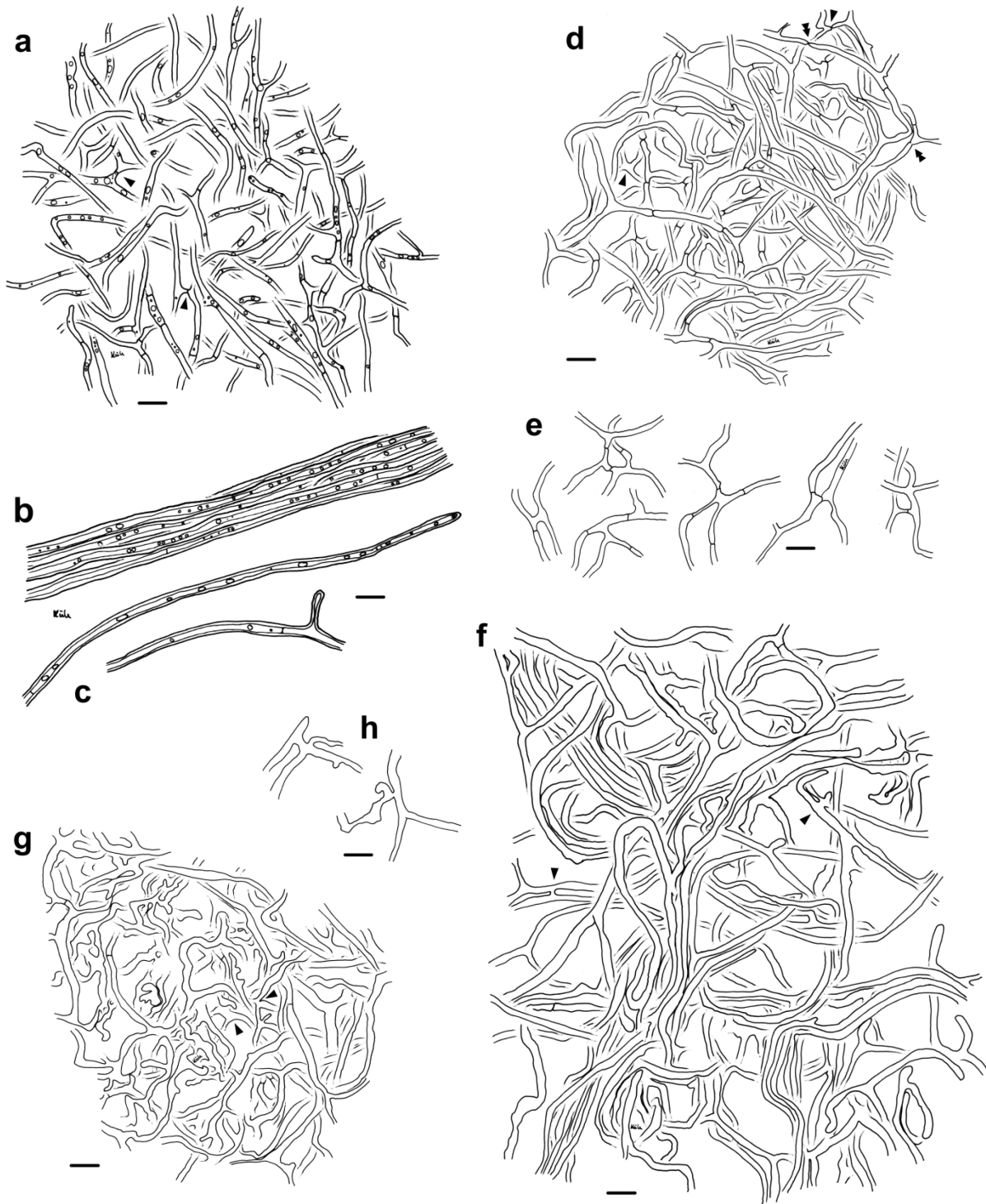


Fig. 3.2 Arbutoid mycorrhiza of *Leotia cf. lubrica*-*Comarostaphylis arbutoides*. **a-h** Plan view of different mantle layers, emanating hyphae and anastomoses; bars 10 μ m: **a** outer mantle layer with ramified hyphae, irregularly arranged; hyphae with numerous oily droplets; anastomoses with closed long bridge (*arrowheads*); **b** bundle of emanating hyphae, hyphae with oily droplets; **c** simple and branched emanating hyphae; **d** middle mantle layer with repeatedly branched hyphae, densely arranged; anastomoses with short or long bridge, bridge closed or open (*single arrowheads*); merged hyphal tips, some with remnants of a partly solved septum (*double arrowheads*); **e** different anastomoses of the middle mantle layer; **f** inner mantle layer with ring-like arrangement of hyphal bundles, some hyphae irregularly inflated; anastomoses (*arrowheads*); **g** inner mantle layer close to the very tip with many irregularly inflated hyphae, many hyphae with hyphal tips; **h** anastomoses of both the inner mantle layer and the inner mantle layer close to very tip, anastomoses close to the hyphal tip

anastomoses close to hyphal tips also present. *Very tip* (Figs. 3.2g, h and 3.3d) inner mantle layers similar to remaining part but without hyphal bundles; hyphae often irregularly inflated, many hyphae with hyphal tips; outer and middle mantle layers organized as older parts of the mantle.

Anatomical characters of emanating elements (Fig. 3.2b, c) *Rhizomorphs* lacking. *Emanating hyphae* rare to frequently, not specifically distributed; hyphae even, not striking, sometimes one-side branched, sometimes with open anastomoses; hyphal tips sometimes merged with hyphae; some hyphae in bundles; hyphae at distal end simple; hyphae straight and even, up to 500 μm long, maybe longer since often observed without hyphal tip, 2.3–4.2 μm diameter; cell walls 0.5–1.2 μm thick, not constricted at septa, septa 0.2–0.3 (0.6) μm thick, septa often difficult to discern due to frequent droplets; cell wall light yellow to light orange, cytoplasm light dirty blue, oily droplets same color as cell wall; lacking are clamps, elbow-like protrusions, and intrahyphal hyphae. *Cystidia* not found.

Anatomical characters of longitudinal section (Fig. 3.3e, f) Mantle plectenchymatous, 5–17 μm thick. Mantle of very tip plectenchymatous, 8–20 μm thick. Epidermal layer with intracellular hyphae, epidermal cells radially oval to elliptic; Hartig net around epidermal cells para-epidermal in one row; hyphal cells roundish to cylindrical. Tannin cells lacking.

Color reactions with different reagents (mantle preparations and emanating hyphae) Acetic acid: no reaction; congo red: no reaction; cotton blue: hyphae of outer mantle layer with blue cytoplasm and blue (sometimes reddish) oily droplets, emanating hyphae with violet (sometimes reddish) cell walls, blue cytoplasm, and red to red brown oily droplets; ethanol 70 %: no reaction; Fe(II)SO_4 : no reaction; guaiac: no reaction; KOH 10 %: no reaction; lactic acid: no reaction; Lugol's solution: no reaction; Melzer's reagent: no reaction; NH_4OH : no reaction; sulpho-vanillin: no reaction; H_2SO_4 concentration: no reaction; toluidine blue: hyphae of outer mantle layer with patchy pale blue to blue cytoplasm and red oily droplets, emanating hyphae with violet cell walls, patchy pale blue to blue cytoplasm, and red oily droplets.

Reference specimen Costa Rica, province of San José, canton of Pérez Zeledón, at mountain Cerro de la Muerte, Reserva Forestal Los Santos (3,300 m a.s.l.; precipitation c. 2,812 mm/year; inceptisol (USDA)), in a secondary cloud forest with *Qercus costaricensis*, soil core exc., myc. isol. Katja Kühdorf; KKM 337 and KKM 348, 18 October 2011; mycorrhiza deposited by B. Münzenberger (ZALF Müncheberg, Germany). *Further material*

studied same location, soil core exc., myc. isol. Katja Kühdorf; KKM 334 and KKM 347, 18 October 2011; mycorrhiza deposited by B. Münzenberger (ZALF Müncheberg, Germany).

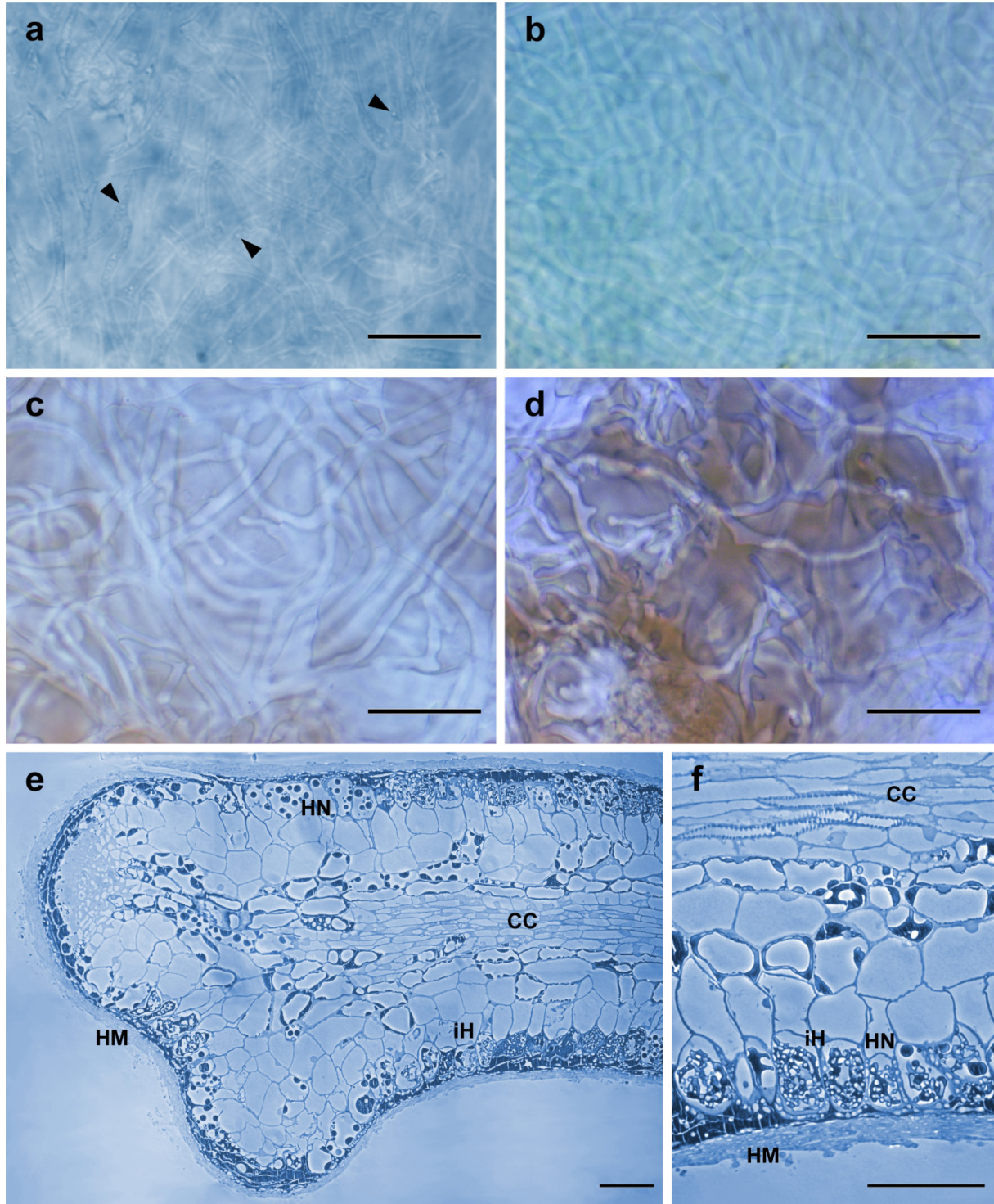


Fig. 3.3 a–d Interference contrast of the three mantle layers of leotioid mycorrhiza; bars 20 μm; a outer mantle layer, hyphae containing numerous oily droplets (arrowheads); b middle mantle layer; c inner mantle layer; d inner mantle layer close to very tip with brownish matrix (remnants of root cap cells). e–f Semi-thin sections of the arbutoid mycorrhiza *Leotia* cf. *lubrica*-*Comarostaphylis arbutoides*; bars 50 μm; e hyphal mantle (HM), Hartig net (HN), intracellular hyphae (iH), and central cylinder (CC); f anatomical features in detail

3.3.2 Phylogenetic analyses

A total of 399 root tips were analyzed genetically, of which ten were identified as *Leotia* cf. *lubrica*. All leotiid sequences were deposited in NCBI GenBank under the accession numbers KF836622-KF836631 (LSU) and KF836612-KF836621 (ITS), respectively. In all samples, *Comarostaphylis arbutoides* (KF419121) was proven as host tree.

The sequenced LSU rDNA region of the ten arbutoid mycorrhizas of *Comarostaphylis arbutoides* resulted in sequences with a length of 890–913 bp (KF836622–KF836631), in which the overlapping area was different in five positions within these samples. Sequence comparison with BLASTn in NCBI database resulted in matches mainly belonging to the order Helotiales, where the Leotiaceae *Leotia lubrica* (AY789359) showed the highest similarity values (Tab. 3.1). In UNITE, best values were reached with the Helotiaceae *Unguiculariopsis thallophila* (UDB016232), which also belongs to the Helotiales. In the sequenced 563–616 bp long ITS region (KF836612-KF836621) of the arbutoid mycorrhizas of *Comarostaphylis arbutoides*, 30 positions were different in the overlapping area. Sequences obtained from NCBI and UNITE comparison with ITS sequences belong to members of the genus *Leotia* (Tab. 3.1), whereby UNITE provides lower similarity values.

The Bayesian and RAxML phylogenies, generated by ITS sequences are concordant. Both trees show the same grouping structure, supported by high posterior probabilities (PP) in the Bayesian analysis and by typically lower bootstraps (BS) in the RAxML analysis (Fig. 3.4). The phylogenetic analysis in Fig. 3.4 reveals that the three species of the genus *Leotia* are paraphyletic and split in several groups. Members of the species *Leotia atrovirens* can be found in two groups (III and IV), whereby both are highly supported (PP 1/BS 100, each). Samples of *Leotia lubrica* are also divided and can be found exclusively in group II (PP 1/BS 99) on the one hand, and as a large complex together with *L. viscosa* samples in group I (PP 1/BS 78) on the other. In this *Leotia lubrica/viscosa* complex, again several subgroups (a–f) are formed, containing exclusively *Leotia lubrica* (b, d, e, f; PP 1/BS 90-100) and *Leotia viscosa* (c; PP 1/BS 98) species, respectively, as well as a mixture of *Leotia lubrica* and *Leotia viscosa* samples (a).

All investigated leotiid arbutoid mycorrhizas of *Comarostaphylis arbutoides* can be found in group I, whereby eight group together (PP 1/BS 98) and nest within samples of *Leotia lubrica* (subgroup e; PP 1/BS 90). Therefore, these samples were identified as *Leotia* cf. *lubrica* species. The other two mycorrhizas do not group to a specific *Leotia* species. KKM 317 (KF836615) can be found among various *Leotia lubrica* and *Leotia viscosa* samples within

Tab. 3.1 Comparison of ITS and LSU sequences with NCBI and UNITE database obtained from ten mycobionts of *Comarostaphylis arbutoides*

Samples with accession numbers (ITS, LSU)	NCBI (ITS)				UNITE (ITS)				NCBI (LSU)				UNITE (LSU)			
	Closest match ^a	Highest maximum score	E value/ query coverage (%)	Identity (%)	Closest match ^a	Highest bit-score	E value/ query coverage (%)	Identity (%)	Closest match ^a	Highest maximum score	E value/ query coverage (%)	Identity (%)	Closest match ^a	Highest bit-score	E value/ query coverage (%)	Identity (%)
KKM 145 (KF836612; KF836622)	<i>Leotia lubrica</i> (EU819412)	1,072	0.0/99	99	<i>Leotia</i> sp. (UDB013464)	504	e-143/56	94	<i>Leotia lubrica</i> (AY789359)	1,650	0.0/100	99	<i>Unguculariopsis thallophila</i> (UDB016232)	985	0.0/*	*
KKM 147 (KF836613; KF836623)	<i>Leotia viscosa</i> (AY144536)	942	0.0/100	97	<i>Leotia</i> sp. (UDB013464)	478	e-135/60	94	<i>Leotia lubrica</i> (AY789359)	1,644	0.0/100	99	<i>Unguculariopsis thallophila</i> (UDB016232)	979	0.0/*	*
KKM 152 (KF836614; KF836624)	<i>Leotia lubrica</i> (AY144544)	1,064	0.0/99	99	<i>Leotia</i> sp. (UDB013464)	496	e-140/56	94	<i>Leotia lubrica</i> (AY789359)	1,644	0.0/100	99	<i>Unguculariopsis thallophila</i> (UDB016232)	977	0.0/*	*
KKM 317 (KF836615; KF836625)	<i>Leotia lubrica</i> (AY144551)	1,083	0.0/99	99	<i>Leotia</i> sp. (UDB013464)	533	e-152/55	95	<i>Leotia lubrica</i> (AY789359)	1,631	0.0/99	99	<i>Unguculariopsis thallophila</i> (UDB016232)	971	0.0/*	*
KKM 334 (KF836616; KF836626)	<i>Leotia lubrica</i> (EU819412)	1,092	0.0/99	99	<i>Leotia</i> sp. (UDB013464)	496	e-140/55	94	<i>Leotia lubrica</i> (AY789359)	1,639	0.0/99	99	<i>Unguculariopsis thallophila</i> (UDB016232)	971	0.0/*	*
KKM 337 (KF836617; KF836627)	<i>Leotia lubrica</i> (EU819412)	1,092	0.0/99	99	<i>Leotia</i> sp. (UDB013464)	496	e-140/55	94	<i>Leotia lubrica</i> (AY789359)	1,629	0.0/100	99	<i>Unguculariopsis thallophila</i> (UDB016232)	961	0.0/*	*
KKM 347 (KF836618; KF836628)	<i>Leotia lubrica</i> (EU819412)	1,022	0.0/99	99	<i>Leotia</i> sp. (UDB013464)	496	e-140/58	94	<i>Leotia lubrica</i> (AY789359)	1,604	0.0/100	99	<i>Unguculariopsis thallophila</i> (UDB016232)	938	0.0/*	*
KKM 348 (KF836619; KF836629)	<i>Leotia lubrica</i> (EU819412)	1,092	0.0/99	99	<i>Leotia</i> sp. (UDB013464)	496	e-140/55	94	<i>Leotia lubrica</i> (AY789359)	1,602	0.0/100	99	<i>Unguculariopsis thallophila</i> (UDB016232)	938	0.0/*	*
KKM 349 (KF836620; KF836630)	<i>Leotia lubrica</i> (EU819412)	1,024	0.0/99	99	<i>Leotia</i> sp. (UDB013464)	496	e-140/58	94	<i>Leotia lubrica</i> (AY789359)	1,602	0.0/100	99	<i>Unguculariopsis thallophila</i> (UDB016232)	938	0.0/*	*
KKM 427 (KF836621; KF836631)	<i>Leotia lubrica</i> (EU819412)	1,092	0.0/99	99	<i>Leotia</i> sp. (UDB013464)	496	e-140/55	94	<i>Leotia lubrica</i> (AY789359)	1,637	0.0/100	99	<i>Unguculariopsis thallophila</i> (UDB016232)	969	0.0/*	*

Closest match was chosen according to the highest maximum score or bit-score

* Information locked by the reference author

^a Accessed 28 January 2014

subgroup a, which is not well supported, whereas the sample KKM 147 (KF836613) does not cluster in one of the above mentioned subgroups.

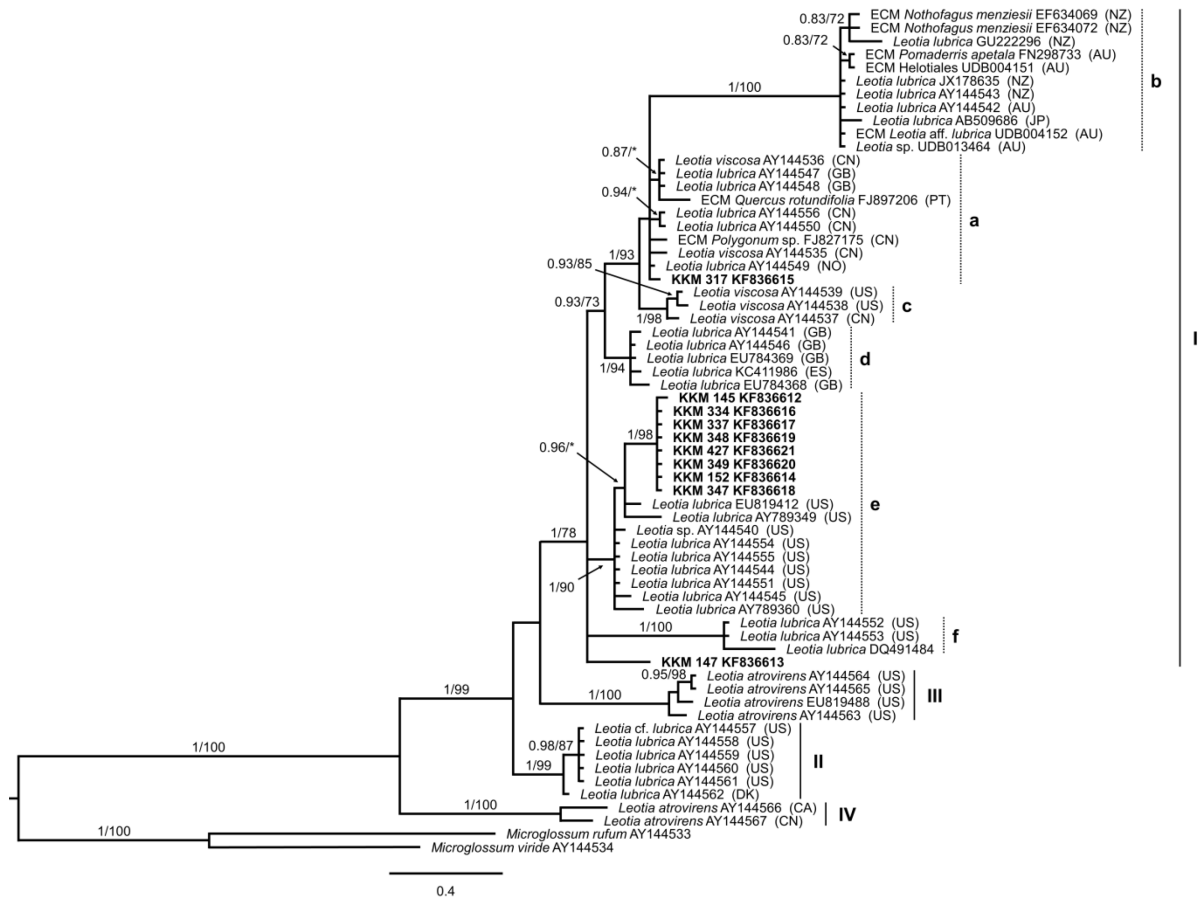


Fig. 3.4 Phylogenetic relationship of ten leotioid mycobionts of *Comarostaphylis arbutoides* within the genus *Leotia*. Phylogram was obtained from Bayesian analysis based on ITS sequences. Branch support values were calculated as posterior probability from 2,000,000 generations of Bayesian analysis (*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 *Microglossum rufum* and *Microglossum viride*. Sequences were obtained from NCBI and UNITE database complemented by the name of corresponding host plant, if available. Investigated arbutoid mycobionts of *Comarostaphylis arbutoides* from Costa Rica are marked in bold. Mycorrhizal type: ectomycorrhiza (ECM). Country codes, if applicable: Australia (AU), Canada (CA), China (CN), Denmark (DK), Spain (ES), UK (GB), Japan (JP), Norway (NO), New Zealand (NZ), Portugal (PT), and USA (US). Indicated groups (I-IV) and subgroups (a-f) after Zhong and Pfister (2004)

3.4 Discussion

The classification of Ascomycota was traditionally based on the morphology of their fruit bodies, whereas, molecular studies showed that such morphologically defined groups can be phylogenetically misleading (Wang et al. 2006a, b). Currently, the five orders Cyttariales, Erysiphales, Helotiales, Rhytismatales, and Thelebolales are placed in the ascomycetous class Leotiomyces (Hibbett et al. 2007). The genus *Leotia* Pers. belongs to the Leotiaceae (Helotiales) whose final composition of genera is not fully resolved. Currently, Lumbsch and Huhndorf (2009) place the genera *Geocoryne*, *Gelatinipulvinella*, *Leotia*, *Microglossum*, *Neobulgaria*, and *Pezoloma* into this family. Sufficient molecular information of currently included genera is rarely or not available at all in NCBI database. Therefore, further reinterpretations within the Leotiaceae family are expected. Nevertheless, the obtained LSU rDNA sequences of the ten arbutoid mycorrhizas of *Comarostaphylis arbutoides* (KF836622–KF836631) identify them as members of the genus *Leotia* (Tab. 3.1).

Thus far, a detailed phylogenetic investigation within the genus *Leotia* has been carried out only by Zhong and Pfister (2004). They combined morphological information with phylogenetic analyses and found four groups within the genus *Leotia*. Group I comprises all *Leotia viscosa* and some *Leotia lubrica* samples, which are characterized by a yellow stipe in fresh and dry condition, respectively. Another group (II) exclusively formed by *Leotia lubrica* samples, however, showed a green stipe when dry. The species *Leotia atrovirens* is divided into two groups (III and IV) and differ from each other by the presence or absence of gel in their stipes.

Genetically, eight of ten arbutoid mycorrhizas of *Comarostaphylis arbutoides* collected in Costa Rica were identified as *Leotia* cf. *lubrica*. The other two leotioid mycorrhizas samples KKM 147 (KF836613) and KKM 317 (KF836615) still remain unidentified, whereby the first one represents a further genotype within the *Leotia lubrica/viscosa* complex, because it does not cluster in a specific subgroup within group I. However, those two leotioid mycorrhizas are also assumed to be *Leotia lubrica* species, since they were found as mycorrhizal partner of *Comarostaphylis arbutoides*. This suggests a high genetic variability of *Leotia lubrica* species assigned to group I.

The stipes of the *Leotia lubrica* fruit bodies found at site I, were not further investigated regarding a possible color change in dried condition or genetically. As pointed out by Zhong and Pfister (2004), it is difficult to distinguish between *Leotia lubrica* species of group I and

II, as both have a yellow stipe in fresh conditions. Thus, it is not possible to clearly assign the found fruit bodies to one of both groups. However, thus far mycorrhizal associations are only known from group I *Leotia lubrica* samples, not from *Leotia lubrica* species belonging to group II. Among *Comarostaphylis arbutoides*, the species *Quercus rotundifolia*, *Polygonum* sp., and *Nothofagus menziesii* are reported as host plants of leotioid ECMs (Branco and Ree 2010; Gao and Yang 2010; Orlovich et al. 2013). Orlovich et al. (2013) additionally indicate that *Leotia* perhaps interact in some way with other ectomycorrhizal fungi, since the fungus was found with either *Russula*, *Clavulina*, or *Laccaria* at the same root tip. Tedersoo et al. (2009) identified *Leotia lubrica* from ectomycorrhizal roots formed by Basidiomycota and the Rhamnaceae *Pomaderris apetala* (FN298733) and suggest a secondary colonization of ECMs by this fungal species (Tedersoo et al. 2010). However, such secondary mycorrhizal association for the described leotioid arbutoid mycorrhizas of *Comarostaphylis arbutoides* is not assumed. Color of leotioid mycorrhiza is similar to the fruit bodies of *Leotia lubrica* and all investigated samples show the same morpho- and anatomotype. Additionally, semi-thin sections reveal a mantle, Hartig net, as well as intracellular hyphae, typical for mycorrhizas of the Arbutoideae (Fig. 3.3e, f). However, only samples of *Leotia cf. lubrica* clustering in subgroup e were investigated, so no conclusion can be made if there are morphological or anatomical differences regarding the leotioid mycorrhizas KKM 147 (KF836613) and KKM 317 (KF836615).

The *Leotia cf. lubrica* arbutoid mycorrhiza is morphologically characterized by a hydrophilic, consistently transparent and yellowish colored mantle. According to Agerer and Rambold (2004–2014), these features are in common with the ECM of the basidiomycete *Entoloma nitidum* (Montecchio et al. 2006). Given their smooth to moderately hairy surface, the leotioid arbutoid mycorrhiza is assigned to the contact exploration type or short distance exploration type (Agerer 2001). By contrast, the mantle of the *Entoloma nitidum* ECM features abundant rhizomorphs and is therefore assigned to the medium distance exploration type (Montecchio et al. 2006). Anatomically, all mantle layers lack a matrix and offer hyphae with clamps, which make it also different from the leotioid mycorrhiza.

Anatomically, the *Leotia cf. lubrica* arbutoid mycorrhiza is characterized by a continuous plectenchymatous mantle, wherein the middle mantle layer is embedded in a nongelatinous matrix. Anastomoses can be found in all layers, showing various types. Emanating hyphae also show anastomoses, which confirm them as such structures and not as cystidia (Agerer 1999). The yellowish droplets are another characteristic feature, only found in emanating hyphae and in hyphae of the outer mantle layer. The ECM of the hypogeous ascomycete

Gautieria inapire (Palfner and Horak 2001) also show a plectenchymatous mantle in all layers with clampless hyphae and oily droplets, which do not stain in sulpho-vanillin (Agerer and Rambold 2004–2014). However, it differs from *Leotia cf. lubrica* arbutoid mycorrhiza in having cystidia, emanating hyphae, and rhizomorphs. A matrix is also present, but shows, by contrast, a gelatinous condition and can be found only in the inner mantle layer.

Color reactions of the leotioid arbutoid mycorrhiza are observed with cotton blue and toluidine blue, which are restricted to the hyphae of the outer mantle layer, emanating hyphae, and the droplets within. Both chemicals cause a metachromatic reaction due to the color change of droplets to red. Additionally, cell walls of the emanating hyphae turn violet in cotton blue and are thus cyanophil. In contrast, other applied chemicals cause no reaction, for instance, dissolution of the droplets.

Evidence of amyloidity or dextrinoidity in fungal structures is an important characteristic feature in taxonomy (Baral 1987; Agerer and Rambold 2004–2014). These blue (amyloid) or red to red-brown (dextrinoid) iodine-based reactions are induced by Melzer's reagent as well as Lugol's solution. According to Baral (1987) there exists a special case of amyloidity, called hemiamyloidity. Here, Lugol's solution provokes a red reaction, whereas Melzer's reagent yields no reaction at all due to chloral hydrate contained within. However, a pretreatment with KOH causes a blue reaction in both, Lugol's and Melzer's. This hemiamyloid color reaction is so far only known in ascomycetes, including many Helotiales (Baral 2009). Among mycologists, Melzer's reagent is preferred over Lugol's solution. This makes it more difficult to observe clearly a hemiamyloid reaction as Melzer's reagent solely used may falsely indicate inamyloidity (Baral 2009). For that reason, a possible hemiamyloid reaction of ascomycete mycorrhizas could be overlooked. Regardless which treatment was applied, the arbutoid mycorrhiza of *Leotia cf. lubrica* reacts inamyloid. Zhong and Pfister (2004) confirm this reaction also for the cap of the ascoma for all three species of the genus *Leotia*.

The present study is the first morpho-anatomical proof that the species *Leotia cf. lubrica* is actually mycorrhizal. Besides *Comarostaphylis arbutoides* (Ericaceae) as mycorrhizal partner, an association likewise with *Quercus costaricensis* has not been found as it was shown for the basidiomycete *Sebacina* sp. (Kühdorf et al. 2014). However, an ectomycorrhizal association with *Quercus costaricensis* is likewise assumed, since Branco and Ree (2010) reported a leotioid ECM with *Quercus rotundifolia*. Nevertheless, it is interesting that mycorrhizas of *Leotia lubrica* were overlooked so far, although the fungus occurs worldwide. One possibility

could be a low competitive ability, e.g., due to slow colonization of root tips (Kennedy 2010). In addition, a dual lifestyle (mycorrhizal as well as saprotrophic) may be also possible, as is known for the ectomycorrhizal basidiomycete *Laccaria bicolor* (Vincent et al. 2012). However, these assumptions need further investigation. Further sampling could reveal if group II *Leotia lubrica* species also have the potential to be mycorrhizal or if this ability is limited to members of group I.

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4

Arbutoid mycorrhizas of the genus *Cortinarius* from Costa Rica³

Abstract

Arbutoid mycorrhizas of *Comarostaphylis arbutoides* (Arbutoidea, Ericaceae) from neotropical montane forests are rarely described. To date, only mycorrhizal associations with the fungal species *Leccinum monticola*, *Leotia lubrica* and *Sebacina* sp. are known from literature. The genus *Cortinarius* is one of the most species-rich ectomycorrhizal taxa with over 2000 assumed species. In this study, two sites in the Cordillera de Talamanca of Costa Rica were sampled, where *Comarostaphylis arbutoides* is endemic and grows together with *Quercus costaricensis*. Using a combined method of rDNA sequence analysis and morphotyping, 33 sampled mycorrhizal systems of *Cortinarius* were assigned to the subgenera *Dermocybe*, *Phlegmacium* and *Telamonia*. Specific plant primers were used to identify the host plant. Here, we present the phylogenetic data of all found Cortinariii and describe four of the arbutoid mycorrhizal systems morphologically and anatomically.

Keywords

Anatomy, Central America, morphology, secondary cloud forest

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4.1 Introduction

Comarostaphylis arbutoides is a tropical woody plant of Central America, occurring in dry oak-pine and cloud forests, as well as in the páramo at an elevation of c. 2500–3430 m a.s.l. Together with *Arbutus* and *Arctostaphylos*, it belongs to the ericaceous subfamily Arbutoidea, which are known to form arbutoid mycorrhizas with ectomycorrhizal fungi (Molina and Trappe 1982). Although, Bidartondo and Bruns (2001) infer that *Comarostaphylis arbutoides* forms arbutoid mycorrhizas with diverse species of basidiomycetes and ascomycetes, only mycorrhizal associations with *Leccinum monticola*, *Sebacina* sp. and *Leotia* cf. *lubrica* have been described so far (Osmundson et al. 2007; Kühdorf et al. 2014, 2015). However, typical as well as presumable ectomycorrhizal forming species of the genera *Cortinarius*, *Hysterangium*, *Laccaria*, *Tricholoma* and *Phaeocollybia*, have also been mentioned from the páramo by Halling and Mueller (1999). Therefore, further mycorrhizal associations with other fungal species for *Comarostaphylis arbutoides* can be assumed.

The genus *Cortinarius* is assumed to be the species-richest genus of Agaricales, containing over 2000 species (Garnica et al. 2005) with a worldwide distribution (Peintner et al. 2004). The taxonomy of *Cortinarius* is largely based on macromorphological characters, spore morphology as well as on chemical characters (Brandrud 1996). The subdivision of *Cortinarius* into subgeneric units causes many problems, induced by high morphological variation within species, as well as the different weighting of morphological characters by different taxonomists (Peintner et al. 2004). However, molecular investigation of the genus *Cortinarius* is just at the beginning (Liimatainen 2013; Zotti et al. 2014). As proposed by Peintner et al. (2004), studies should, first of all, focus on natural units (e.g. sections), bringing DNA sequence data as well as morphological and ecological data in accordance, as already done by several authors (e.g. Garnica et al. 2009, 2011; Suárez-Santiago et al. 2009; Niskanen et al. 2013a, b; Dima et al. 2014; Stensrud et al. 2014; Liimatainen et al. 2015).

Cortinarius is an important ectomycorrhizal fungal genus associated with trees, shrubs and a number of herbaceous plants of many different plant families (Liimatainen 2013), whereby also host specificity occurs (e.g. Brandrud 1996; Garnica et al. 2003; Frøslev et al. 2007; Niskanen et al. 2011; Liimatainen 2013). Based on fruit body collections, Halling and Mueller (1999; 2005), Mueller et al. (2006) and Ammirati et al. (2007) have reported and/or described 18 different Costa Rican *Cortinari*. These species were collected in the Talamanca mountain range of Costa Rica, where *Comarostaphylis* and *Quercus* trees occur.

In our samples collected in the Cerro de la Muerte (Cordillera de Talamanca, Costa Rica) several different *Cortinarius* species formed mycorrhizas with *Comarostaphylis arbutoides* and *Quercus* sp. The genus *Cortinarius* was identified using molecular methods such as large subunit (LSU) and internal transcribed spacer (ITS) sequencing as well as phylogenetic analysis. Plant primers were used to sequence the ITS region of the host plant from the same mycorrhizal system as used for fungal analysis. According to Agerer (1991), we present a morphological and anatomical description of four cortinarioid mycorrhizal systems associated with *Comarostaphylis arbutoides*.

4.2 Materials and methods

4.2.1 Site location and sampling

Sampling was conducted in a secondary cloud forest around the Mountain Cerro de la Muerte (3491 m a.s.l.) in the Cordillera de Talamanca of Costa Rica, 54 km southeast of the capital city of San José. Site I (Estación Biológica de la Muerte; 3100 m a.s.l.; 9° 33' N, 83° 45' W) is dominated by *Quercus costaricensis* mixed with solitary individuals of *Comarostaphylis arbutoides*. At site II (Reserva Forestal Los Santos; 3300 m a.s.l.; 9° 34' N, 83° 45' W), *Comarostaphylis arbutoides* itself is the dominating species, mixed with a few isolated *Quercus costaricensis*. Members of the Araliaceae (*Schefflera* and *Oreopanax*); Cunoniaceae (*Weinmannia*); Ericaceae (*Cavendishia*, *Disterigma* and *Vaccinium*); Poaceae (*Chusquea*); Primulaceae (*Myrsine*) and Winteraceae (*Drimys*) form the understory vegetation.

Fine root systems of *Comarostaphylis arbutoides* were collected during the rainy seasons in October 2010 and 2011. For this, a soil corer (diameter 3 cm; length 40 cm) was used at distances of 50 and 100 cm from the trunk. At the University of Costa Rica, turgid and apparently healthy morphotypes were sorted out using a stereomicroscope. Systems with the same morphological features (e.g. colour, hydrophobicity presence, emanating elements and rhizomorphs) were assigned to one morphotype. For further analyses, the morphotypes were preserved in 2 % glutaraldehyde with a 0.1 M sodium cacodylate buffer (Münzenberger et al. 2009) for light microscopy or dried on silica gel for DNA extraction, respectively. Identification of each morphotype is based on their respective sequence type. Within these 2 years a total of 60 soil cores were taken and analysed. The genus *Cortinarius* was proven genetically in 23 soil cores.

4.2.2 Molecular analyses

Genomic DNA was isolated from one unramified root tip per morphotype, using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's recommendations. To identify the mycorrhizal fungi at both family and species level, PCR amplification and sequencing of the internal transcribed spacer (ITS) region and the ribosomal nuclear large subunit (LSU) were performed. Here, the primer combinations ITS1F/ITS4 (Gardes and Bruns 1993; White et al. 1990) as well as LR0R/LR5 (Moncalvo et al. 2000) were used. In order to identify the plant from mycorrhizal root tips without coamplifying fungal DNA the angiosperm-specific ITS primer pair ITS-5A/ITS-241r was amplified (Osmundson et al. 2007). Sequencing service was facilitated by GATC Biotech AG (Konstanz, Germany). A total of 399 root tips were analysed genetically, of which 33 were identified as members of the genus *Cortinarius*. All sequences were deposited in NCBI GenBank under the accession numbers KM456990-KM457022 (ITS), KM457023-KM457055 (LSU), KF419121 (*Comarostaphylis arbutoides*) and KM978077 (*Quercus* sp.).

Sequences were analysed and edited using Chromas Lite v2.01 software (<http://technelysium.com.au>). Identity of obtained sequence data was confirmed 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 analysis at species level, the datasets of ITS sequences provided by Peintner et al. (2004), Garnica et al. (2005) and Ammirati et al. (2007) were used. The dataset was complemented by best match results obtained by NCBI and UNITE blast search for each sequence. Alignment was performed with MAFFT v7 (Kato et al. 2002) using the FFT-NS-2 alignment algorithm. To estimate phylogenetic relationships, maximum likelihood and Bayesian approaches were applied. Maximum likelihood analyses were performed using RAxML (v7.7.1; Stamatakis 2006) in a parallelized version supplied by RAxML BlackBox (Stamatakis et al. 2008) with trees inferred from 100 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, sampling every 1000. Analyses were performed until average standard deviation of split frequencies was <0.01 and stationarity was checked using Tracer v1.6.1 (Rambaut et al. 2014). The first 50,000 trees were discarded before calculating the posterior

probabilities. The potential scale reduction factor (PSRF) values for all inferences were ~ 1.0 , indicating a good posterior probability distribution sample.

4.2.3 Microscopy

The morphological and anatomical description of the mycorrhizas was carried out according to Agerer (1987–2012; 1991), and the online key of DEEMY (Agerer and Rambold 2004–2015). Anatomical studies are based on multiple arbutoid mycorrhizal systems. Drawings were performed with an interference contrast microscope (BX50F-3, Olympus Corporation, Tokyo, Japan) connected with a drawing tube. All drawings were done in thousandfold magnification.

For semi-thin sections, the mycorrhizas were fixed with 2 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at room temperature until further processing. Thereafter, six washes in 0.1 M sodium cacodylate buffer were performed. Samples were postfixed in 1 % osmium tetroxide in the same buffer for 1 h under light exclusion at room temperature. After six washes in double-distilled water, samples were dehydrated by immersion for 15 min in 25, 50, 70 and 95 % acetone and three times for 1 h in 100 % acetone, respectively. The mycorrhizal tips were embedded in Spurr's plastic (Spurr 1969) and sectioned with a diamond knife on an Ultracut Reichert Ultramicrotome (W. Reichert-LABTEC, Wolfratshausen, Germany). The sections (0.5 μm thick) were stained with crystal violet and investigated using a light microscope (Zeiss Axioskop 50, Oberkochen, Germany).

4.3 Results

4.3.1 Phylogenetic analysis

A total of 399 root tips were analysed genetically, of which 33 were assigned to the genus *Cortinarius* after sequence comparison with BLASTn in the NCBI database and UNITE. In NCBI, best matches were mainly received with samples originally from North America, whereas comparison in UNITE almost exclusively resulted in European species (Tab. 4.1). In NCBI, 11 samples (KKM 109, KKM 117, KKM 149, KKM 167, KKM 198, KKM 204, KKM 298, KKM 335, KKM 407, KKM 429, KKM 437) achieved their highest match with no further identified *Cortinarius* sp. or Cortinariaceae samples, whereby this was the case only

Tab. 4.1 Comparison of ITS sequences with NCBI and UNITE database obtained from 33 cortinarioid mycobionts associated with *Comarostaphylis arbutoides* and *Quercus* sp.

Samples	NCBI			UNITE		
	Best match	Accession numbers	Identity score in % (max. score)	Best match	Accession numbers	Identity in % (bit-score)
KKM 81 ^I	<i>Cortinarius</i> sp.	KM402900	98 (1,129-1,147)	<i>C. tillamookensis</i>	UDB015917	98 (1,113-1,131)
KKM 156 ^{II}	<i>C. aurantiobasis</i>	DQ481866				
KKM 255 ^{III}		DQ481899				
KKM 262 ^{III}		FJ157139				
KKM 336 ^{IV}		GQ159787				
KKM 344 ^{IV}		HM068560				
KKM 359 ^{IV}						
KKM 381 ^{IV}						
KKM 388 ^{IV}						
KKM 392 ^{IV}						
KKM 431 ^{IV}						
KKM 439 ^{IV}						
KKM 109 ^{II}	<i>Cortinarius</i> sp.	HQ285378	99 (1,147-1,179)	<i>C. fulvescens</i>	UDB018657	95-96 (1,034-1,047)
KKM 204 ^{II}						
KKM 335 ^{IV}						
KKM 437 ^{IV}						
KKM 117 ^{II}	<i>Cortinarius</i> sp.	EF619685	99 (1,038-1,040)	<i>C. ochrophyllus</i>	UDB000675	96 (921)
KKM 167 ^{II}	Cortinariaceae	DQ377381				
KKM 132 ^{II}	<i>C. exlugubris</i>	NR119791	89 (819)	<i>C. terpsichores</i>	UDB015909	88 (838)
KKM 144 ^{II}	<i>C. comarostaphylis</i>	EF420151	100 (1,216)	<i>C. leucophanes</i>	UDB019884	99 (1,177)
		EF420153			UDB020289	
		NR131800				
	<i>C. oregonensis</i>	GQ159798				
KKM 149 ^{II}	<i>Cortinarius</i> sp.	JQ711769	99 (1,195)	<i>C. croceus</i>	UDB017892	99 (1,162-1,177)
KKM 429 ^{IV}					UDB021432 ^a	
KKM 177 ^{II}	<i>C. tillamookensis</i>	KP087981	98 (1,074)	<i>C. croceus</i>	UDB001554	98 (1,061)
KKM 198 ^{II}	<i>Cortinarius</i> sp.	GU998260	95 (887)	<i>C. casimiri</i>	UDB018229	95 (848)
		GU998441				
		GU998522				
KKM 298 ^{III}	<i>Cortinarius</i> sp.	FJ196918	95 (883)	<i>C. anisatus</i>	UDB001317	93 (825)
KKM 330 ^{III}	<i>C. camphoratus</i>	HQ604694	94 (850)	<i>C. raphanoides</i>	UDB018234	94 (874)
KKM 333 ^{III}					UDB018300	
KKM 358 ^{IV}	<i>C. aff. pauperculus</i>	GQ159858	98 (1,125)	<i>C. fulvescens</i>	UDB018657	95 (1,050)
KKM 373 ^{IV}	<i>C. obtusus</i>	HQ604668	95 (1,027)	<i>C. acutus</i>	UDB017978	93 (964)
KKM 376 ^{IV}						
KKM 407 ^{IV}	<i>Cortinarius</i> sp.	EF619685	99 (813)	<i>C. cf. bayeri</i>	UDB018640	99 (764)
	Cortinariaceae	DQ377381				
KKM 419 ^{IV}	<i>Cortinarius</i> sp.	DQ481700	96 (941)	<i>C. alboviolaceus</i>	UDB018227	96 (913)
		FJ152517				
		KP403013				
	<i>C. cf. biformis</i>	HQ604700				
	<i>C. biformis</i>	FJ157106				
		FJ717531				
		FJ717532				
KKM 432 ^{IV}	<i>C. cedriolens</i>	HQ604729	99 (1,029)	<i>Cortinarius</i> sp.	UDB016059	98 (987)

Best match was chosen according to the highest maximum score or bit-score. All showed matches obtained an E-value of 0.0

Sites and year of sampling: site I in 2010 (^I) and 2011 (^{II}); site II in 2010 (^{III}) and 2011 (^{IV})

^aSequence locked by author

Accessed 9 July 2015

for KKM 432 in UNITE. The highest identity match in NCBI (100 %) is obtained by KKM 144 with *Cortinarius comarostaphylis* from Costa Rica; in UNITE *Cortinarius leucophanes* from Finland is the closest match (99 %) for this sample. KKM 132 achieved the lowest identity match in both databases (89 as well as 88 %) with *Cortinarius exlugubris* from New Zealand and *Cortinarius terpsichores* from Sweden, respectively.

The Bayesian and RAxML phylogenies, generated by ITS sequences are concordant. Both trees show the same grouping structure, supported by mainly higher posterior probabilities (PP) in the Bayesian analysis and lower bootstraps (BS) in the RAxML analysis (Fig. 4.1). Within the genus *Cortinarius*, the 33 samples can be assigned to three different subgenera: *Dermocybe*, *Phlegmacium* and *Telamonia*.

Around half of the analysed cortinarioid sequences belong to the *Dermocybe* subgenus (PP 1/BS 91), whereas 13 samples are assigned to subgroup “Dermocybe I” (PP 1/BS 100), and a further two samples (KKM 149, KKM 429) to subgroup “Dermocybe II” (PP 0.99/BS 73) (Fig. 4.1). The samples KKM 132 and KKM 144 are assigned to subgenus *Phlegmacium*, but can be found in two different clades. There, KKM 144 is grouped in a very well-supported cluster (PP 1/BS 100), whereas the cluster with KKM 132 received a good PP (0.93), but a weak BS support (53). The remaining 16 cortinarioid samples are assigned to the subgenus *Telamonia* and are distributed amongst three very well-supported clusters (PP 1/BS 96 to 100) as shown in Fig. 4.1.

Except for the telamonioid samples KKM 198, KKM 373 and KKM 376, where *Quercus* sp. was proven as host plant (Fig. 4.1), all mycorrhizal systems of the cortinarioid samples are formed with *Comarostaphylis arbutoides*.

4.3.2 Morpho-anatomical descriptions of arbutoid mycorrhizas formed by various *Cortinarius* sp. species with the Ericaceae *Comarostaphylis arbutoides*

Of the 33 sequence types, four morphotypes were described in detail as, here, sufficient mycorrhizal materials were available. Assignment to the respective subgenus is based on phylogenetic analysis (Fig. 4.1).

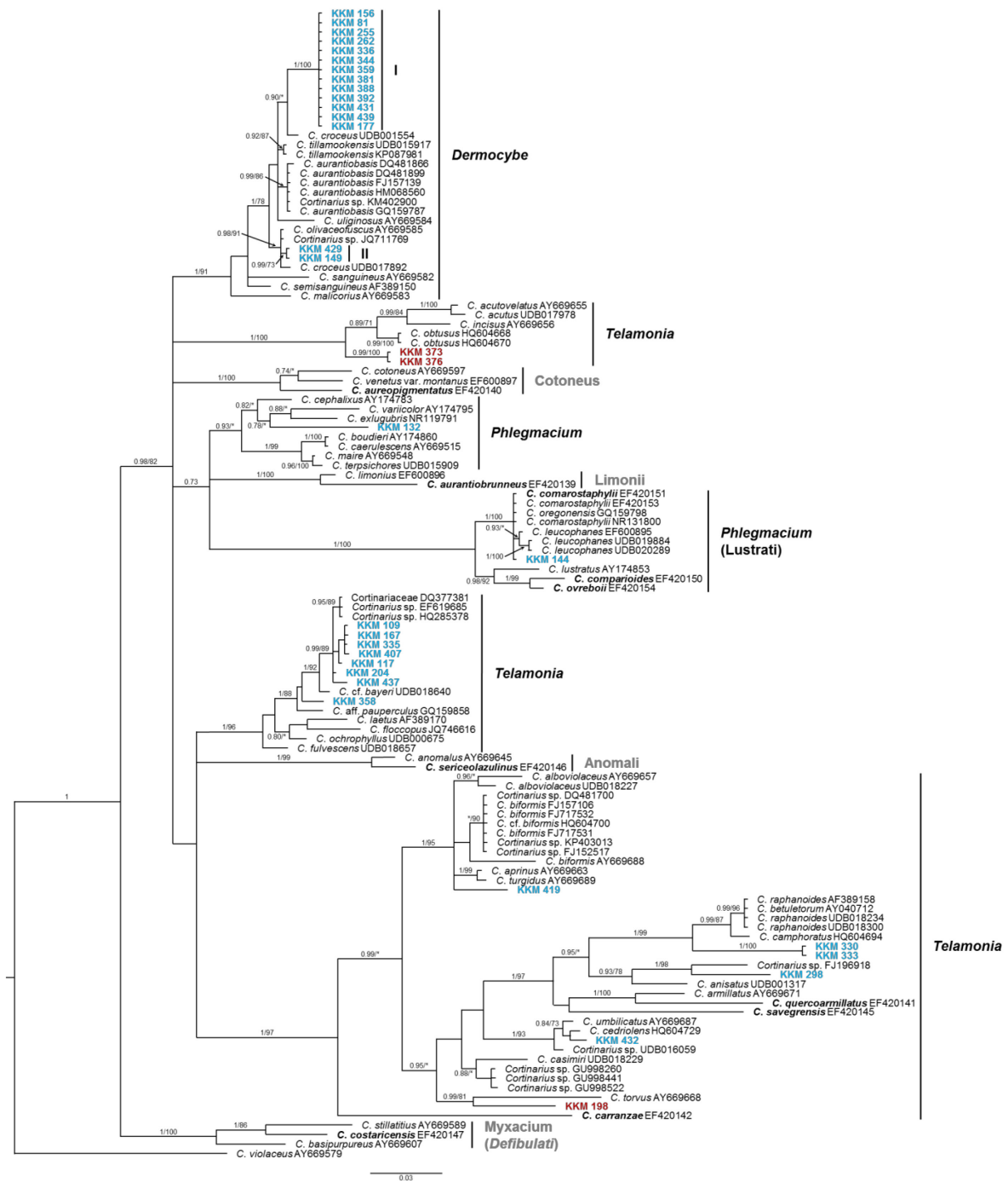


Fig. 4.1 Phylogenetic relationship of 33 cortinarioid mycobionts associated with *Comarostaphylis arbutoides* (blue) or *Quercus* sp. (red) within selected representatives of the genus *Cortinarius*. Phylogram was obtained from Bayesian analysis based on ITS sequences. Branch support values were calculated 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 *Cortinarius violaceus*. Costa Rican *Cortinarius* species from a previous study are marked in *bold* (Ammirati et al. 2007). Assignment to taxonomic units according to Peintner et al. (2004), Garnica et al. (2005), and Ammirati et al. (2007)

Identification key for the cortinarioid mycorrhizas:

1 Mycorrhizal system usually not silvery, but densely stringy; older parts of mantle not transparent; rhizomorphs abundant, with hairy or even fan-like margins: **KKM 132** (*Phlegmacium*)

1* Mycorrhizal system silvery, rapidly displaced by water when touched, then mantle transparent; epidermal cells generally visible through mantle along the whole mycorrhizal system; rhizomorphs frequent, with smooth margins: **2**

2 Mycorrhizal system densely silvery; all mantle layers with open anastomoses; no colour reaction with KOH and NH₄OH: **KKM 298** (*Telamonia*)

2* Mycorrhizal system slightly silvery; only inner mantle layer with open anastomoses; colour reaction with KOH and NH₄OH: **3**

3 Mycorrhizal system ochre to yellowish brown, very tip yellowish; rhizomorphs ochre to yellowish brown; anastomoses of emanating elements likewise closed by a clamp or open: **KKM 255; KKM 359; KKM 388** (*Dermocybe*)

3* Mycorrhizal system brown, very tip brownish to greyish; rhizomorphs ochre to reddish brown; anastomoses of emanating elements closed by a clamp or rarely open: **KKM 149** (*Dermocybe*)

4.3.2.1 KKM 132 (*Phlegmacium*) + *Comarostaphylis arbutoides*

Morphological characters (Fig. 4.2a) *Mycorrhizal systems* irregularly pinnate to dichotomous, with 0–1 orders of ramification, systems abundant and dense, up to 6.8 mm long, strongly hydrophobic, of medium distance fringe exploration type. *Main axes* 0.3–0.5 mm diameter. *Unramified ends* sinuous to tortuous, not inflated, cylindric, up to 1.1 (2.1) mm long and 0.2–0.3 mm diameter; mantle ochre to brownish, very tip ochre to yellowish, older parts dark brown and mycorrhizas not carbonising. *Surface of unramified ends* densely stringy, not smooth, very tip transparent and epidermal cells visible through mantle, older parts of mantle not transparent and occasionally, very tip partly silvery due to enclosed air. *Rhizomorphs* abundant, up to 0.12 mm diameter, roundish to flat in cross-section, emanating from all parts of the mycorrhiza, connection oblique, distal rhizomorphs connected over a

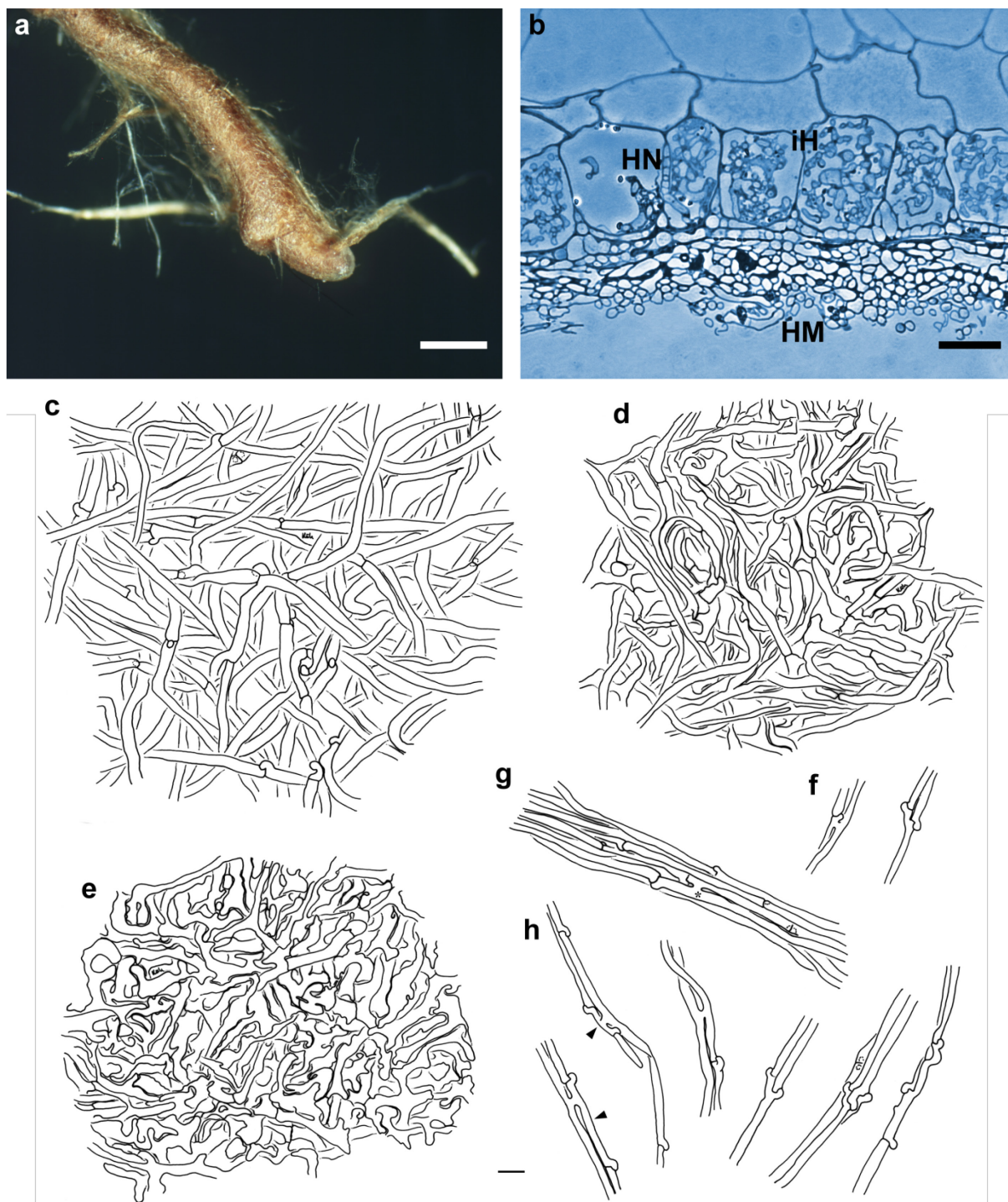


Fig. 4.2 Arbutoid mycorrhiza of *Cortinarius* sp.-*Comarostaphylis arbutoides* (sample KKM 132, subgenus *Phlegmacium*). **a** Habit of the phlegmacioid mycorrhiza with ochre to brownish coloured mantle, densely stringy mantle surface and ochre to brownish rhizomorphs; *bar* 0.5 mm. **b** Semi-thin section of phlegmacioid mycorrhiza with hyphal mantle (HM), Hartig net (HN) and intracellular hyphae (iH); *bar* 20 μ m. **c–h** Plan view of different mantle layers and emanating elements of the phlegmacioid mycorrhiza; *bar* 10 μ m; **c** outer mantle layer with densely and irregularly arranged hyphae, hyphae with clamps; **d** middle mantle layer with densely arranged hyphae, some hyphae in bundles, forming ring-like structures; **e** inner mantle layer with densely arranged hyphae, hyphae irregular in shape; **f** emanating hyphae with open anastomosis and backwards-oriented ramification; **g** rhizomorph with open anastomosis (*asterisk*); **h** hyphae of rhizomorphs with backwards-oriented clamps (*arrowheads*), open anastomoses and acute as well as backwards-oriented ramifications

long distance with mantle surface, growing into soil or organic layers, ochre to brownish, repeatedly ramified into smaller filaments, with hairy or even fan-like margins; rhizomorphs appear very wiry to brittle and are frequently found in short broken fragments around the mycorrhizal systems. *Cystidia* lacking. *Sclerotia* not observed.

Anatomical characters of the mantle in plan views (Fig. 4.2c–e) Mantle lacks cells densely filled with oily droplets or brownish content, blue granules, needle-like contents, matrix, crystals and exudated pigments, as well as cystidia. *Outer mantle layers* densely plectenchymatous, hyphae irregularly to somewhat star-like arranged, rarely ramified, some hyphae in bundles, without any special pattern (type B, Agerer 1991) and yellow; hyphae (17) 25–39 (57) μm long, 2.7–4.4 μm in diameter, cells walls 0.3 (0.4) μm thick; hyphae with clamps and constricted at septa, septa as thick as cell walls. *Middle mantle layers* densely plectenchymatous, hyphae in bundles, forming ring-like structures, hyphae 7–39 (50) μm long, 1.6–4.6 (6.4) μm in diameter, cell walls (0.2) 0.3–0.4 μm thick, smooth and yellow; hyphae rarely septate, clampless and constricted at septa, septa as thick as cell walls. *Inner mantle layers* densely plectenchymatous, hyphae irregular in shape, hyphae (4) 8–27 (63) μm long, 1.8–6.8 (8.3) μm in diameter, cell walls 0.3–0.4 μm thick, smooth and yellow; septa not observed. *Very tip* like other parts of the mantle.

Anatomical characters of emanating elements (Fig. 4.2f–h) Lacking are gelatinized hyphae, matrix, rhizomorphal nodia, simple septa, intrahyphal hyphae, crystals, brownish substances and secreted pigments; elbow-like protrusions not observed. *Rhizomorphs* undifferentiated, hyphae loosely interwoven and of uniform diameter (type A/B, Agerer and Rambold 2004–2015); hyphae smooth, cells (10) 65–113 μm long, 2.4–3.8 μm diameter and cell walls 0.3–0.4 μm ; ramification backwards-oriented or acute, one or two hyphal diameter below the septum and ramifications one side branch at septum; septa with clamps, constricted at septa, backwards-oriented clamps observed only twice and septa as thick as hyphal walls; anastomoses frequent and open with a short bridge, bridge thinner or as thick as hyphae, cell walls of anastomoses as thick as remaining walls. *Emanating hyphae* straight to wavy, smooth, surface occasionally with few soil particles, cells 30–120 μm long, 2.0–3.4 (4.3) μm diameter, cell walls 0.3 μm , and distal ends of hyphae simple; ramification backwards-oriented or acute, with one side branch at septum, one or two hyphal diameter below the septum; septa with clamps, constricted at septa, backwards-oriented clamps not observed and septa as thick as hyphal walls; anastomoses rare and open with a short bridge, bridge as thick as hyphae, cell walls of anastomoses as thick as remaining walls. *Cystidia* not found.

Anatomical characters of longitudinal section (Fig. 4.2b) Mantle plectenchymatous, 13–27 µm thick. Mantle of very tip plectenchymatous, 16–20 µm thick. Epidermal layer with intracellular hyphae and epidermal cells radially oval to elliptic; Hartig net around epidermal cells para-epidermal in one row and hyphal cells roundish to cylindrical. Tannin cells are lacking.

Colour reactions with different reagents (mantle preparations and emanating elements)
Cotton blue: hyphae blue or greenish; toluidine blue: hyphae blue to violet. No reaction was observed with: acetic acid, ethanol 70 %, Fe(II)SO₄, guaiac, KOH 10 %, lactic acid, Lugol's solution, Melzer's reagent, NH₄OH conc., sulpho-vanillin, H₂SO₄.

Reference specimen Costa Rica, province of San José, canton of Pérez Zeledón, at mountain Cerro de la Muerte, Reserva Forestal Los Santos (3300 m a.s.l.; precipitation c. 2812 mm/year; inceptisol (USDA)), in a secondary cloud forest with *Quercus costaricensis*, soil core exc., myc. isol. Katja Kühdorf; KKM 132, 12 October 2010; mycorrhiza deposited by B. Münzenberger (ZALF Müncheberg, Germany).

4.3.2.2 KKM 298 (*Telamonia*) + *Comarostaphylis arbutoides*

Morphological characters (Fig. 4.3a) *Mycorrhizal systems* irregularly pinnate to dichotomous, with 0–4 orders of ramification, systems nest-like, forming hyphal mats, up to 8.4 mm long, strongly hydrophobic, of medium distance fringe exploration type. *Main axes* 0.3–0.4 mm diameter. *Unramified ends* bent or sinuous, not inflated, cylindric, up to 1.8 (3) mm long, 0.2–0.3 (0.4) mm diameter; mantle and very tip white and yellowish to ochre, older parts light orange, not carbonising. *Surface of unramified ends* densely stringy or forming rings, not smooth, fan-like cottony between side branches and main axis, densely silvery by enclosed air, rapidly displaced by water when touched, then mantle transparent and epidermal cells visible through mantle. *Rhizomorphs* frequent, up to 0.25 mm diameter; flat in cross-section, emanating from all parts of the mycorrhiza, connection oblique, distal rhizomorphs connected over a long distance with mantle surface, growing into soil or organic layers, white, repeatedly ramified into smaller filaments, with smooth margins. *Cystidia* lacking. *Sclerotia* not observed.

Anatomical characters of the mantle in plan views (Fig. 4.3c–e) Mantle lacks cells densely filled with oily droplets or brownish content, blue granules, needle-like contents, crystals and

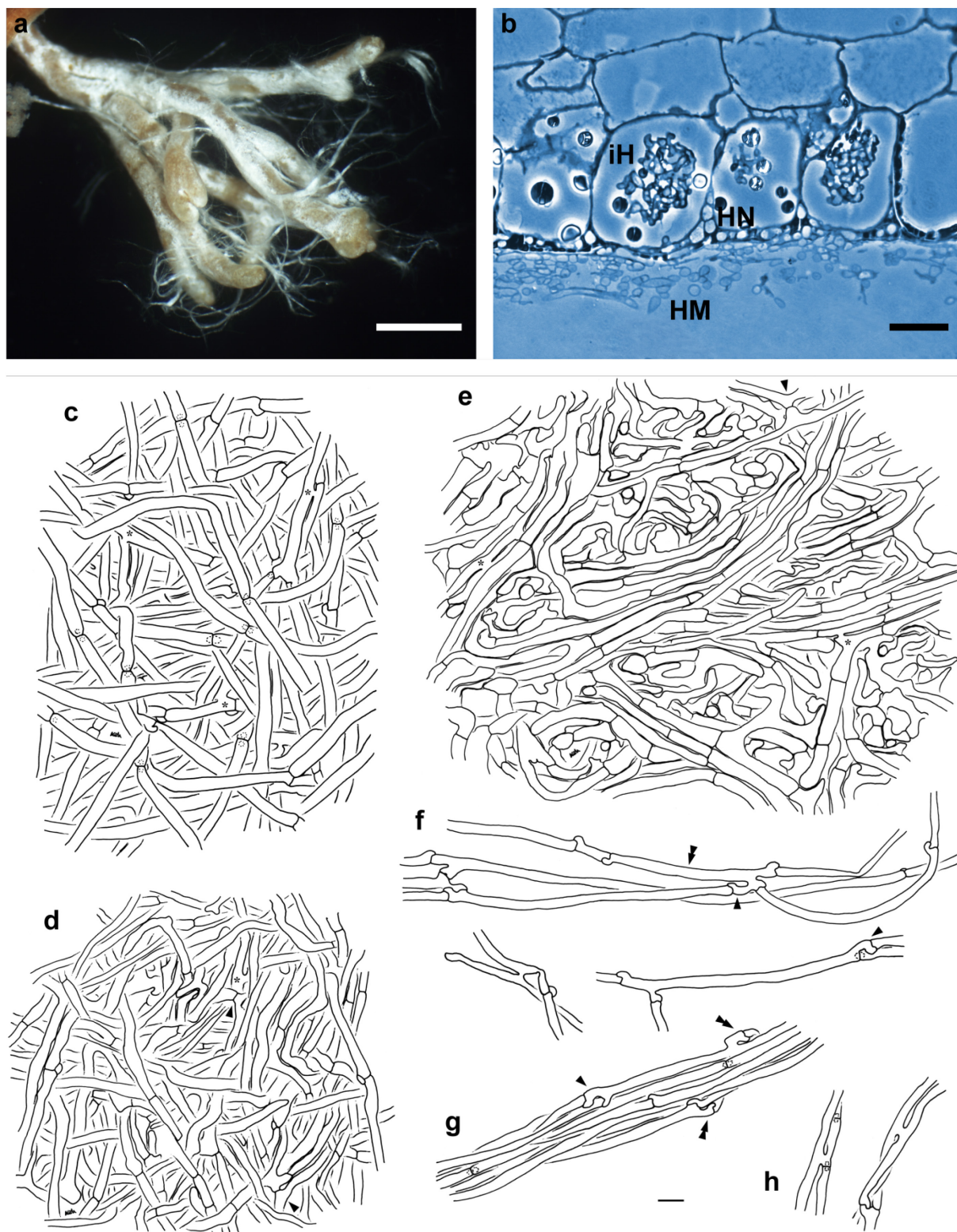


Fig. 4.3 Description see next page

Fig. 4.3 Arbutoid mycorrhiza of *Cortinarius* sp.-*Comarostaphylis arbutoides* (sample KKM 298, subgenus *Telamonia*). **a** Habit of the telamonioid mycorrhiza with transparent, yellowish mantle, partly densely stringy mantle surface, and white rhizomorphs; *bar* 1 mm. **b** Semi-thin section of telamonioid mycorrhiza with hyphal mantle (HM), Hartig net (HN) and intracellular hyphae (iH); *bar* 20 μ m. **c–h** Plan view of different mantle layers and emanating elements of the telamonioid mycorrhiza; *bar* 10 μ m; **c** outer mantle layer with densely and irregularly arranged hyphae, hyphae with clamps and open anastomoses (*asterisks*); **d** middle mantle layer with densely arranged hyphae, some hyphae in bundles, hyphae with open (*asterisk*) or closed anastomoses (*arrowheads*); **e** inner mantle layer with densely arranged hyphae, hyphae in bundles, forming ring-like structures, hyphae with open (*asterisks*) or closed (*arrowhead*) anastomoses; **f** emanating hyphae with open anastomoses, backwards-oriented ramifications (*single arrowheads*) and backwards-oriented clamps (*double arrowhead*); **g** rhizomorph with acute (*single arrowhead*) as well as backwards-oriented (*double arrowheads*) ramifications; **h** hyphae of rhizomorphs with open anastomoses

exudated pigments, matrix, as well as cystidia. *Outer mantle layers* densely plectenchymatous, hyphae irregularly to somewhat star-like arranged, occasionally ramified, without any special pattern, often with bundles of parallel hyphae (type B, Agerer 1991) and colourless, with few soil particles; hyphae smooth and cylindric, hyphae (32) 85–140 μ m long, (2.7) 3.3–5.8 (6.6) μ m diameter, cell walls 0.2–0.3 μ m thick; hyphae with clamps, constricted at septa, septa as thick as cell walls and anastomoses open, with a short bridge, bridge thinner or as thick as hyphae. *Middle mantle layers* densely plectenchymatous, hyphae irregularly interwoven, some hyphae in bundles, hyphae (14) 20–32 (50) μ m long, (1.9) 2.3–4.3 (5.2) μ m in diameter, cell walls 0.3 μ m thick, irregularly inflated, smooth and colourless; hyphae with simple septa, occasionally with clamps, constricted at septa and septa as thick as cell walls and anastomoses open or closed, with short bridge, bridge thinner or as thick as hyphae. *Inner mantle layers* densely plectenchymatous, hyphae in bundles, forming ring-like structures, hyphae uneven in diameter, some hyphae epidermoid, sometimes ampullate at one side of septum and hyphae up to (9) 20–115 (200) μ m long, 2.4–5.3 μ m in diameter, cell walls 0.3 μ m thick and colourless; hyphae with simple septa, rarely with clamp connection, constricted at septa, septa as thick as cell walls and anastomoses open, with short bridge, bridge thinner or as thick as hyphae, anastomoses closed, with long bridge, bridge bigger than hyphae. *Very tip* like other parts of the mantle.

Anatomical characters of emanating elements (Fig. 4.3f–h) Lacking are gelatinized hyphae, matrix, rhizomorphal nodia, simple septa, intrahyphal hyphae, crystals, brownish substances and secreted pigments; elbow-like protrusions not observed. *Rhizomorphs* undifferentiated, hyphae loosely interwoven and of uniform diameter (type A/B, Agerer and Rambold 2004–2015); hyphae smooth, cells 60–100 μ m long, 2.8–5.2 μ m diameter and cell walls 0.3 μ m; ramification backwards-oriented or acute, one or two hyphal diameter below the septum or in considerable distance from the septum ramification and ramifications one side branch at septum; septa with clamps, backwards-oriented clamps not observed and septa as thick as

hyphal walls; anastomoses are frequent, open with a short bridge, bridge slightly thicker than hyphae and cell walls of anastomoses as thick as remaining walls; surface of peripheral hyphae with few soil particles. *Emanating hyphae* straight to wavy, smooth, surface occasionally with few soil particles, cells (25) 65–115 µm long, 2.4–4.8 µm diameter, cell walls 0.3 µm and distal ends of hyphae simple; ramification acute or approximately 90 °, with one side branch at septum and one or two hyphal diameter below the septum; septa with clamps, constricted at septa, backwards-oriented clamp observed only once, septa as thick as hyphal walls; anastomoses frequent, open with a short bridge, bridge thinner or as thick as hyphae and cell walls of anastomoses as thick as remaining walls. *Cystidia* not found.

Anatomical characters of longitudinal section (Fig. 4.3b) Mantle plectenchymatous, 10–22 µm thick. Mantle of very tip plectenchymatous, 10–17 µm thick. Epidermal layer with intracellular hyphae and epidermal cells radially oval to elliptic; Hartig net around epidermal cells para-epidermal in one row and hyphal cells roundish to cylindrical. Tannin cells are lacking.

Colour reactions with different reagents (mantle preparations and emanating elements)
Cotton blue: hyphae blue; toluidine blue: hyphae violet and cell content pink. No reaction was observed with: acetic acid, ethanol 70 %, Fe(II)SO₄, guaiac, KOH 10 %, lactic acid, Lugol's solution, Melzer's reagent, NH₄OH conc., sulpho-vanillin, H₂SO₄.

Reference specimen Costa Rica, province of San José, canton of Pérez Zeledón, at mountain Cerro de la Muerte, Estación Biológica de la Muerte (3100 m a.s.l.; precipitation c. 2812 mm/year; lithosol (FAO)), in a secondary cloud forest with *Quercus costaricensis*, soil core exc., myc. isol. Katja Kühdorf; KKM 298, 4 October 2011; mycorrhiza deposited by B. Münzenberger (ZALF Müncheberg, Germany).

4.3.2.3 KKM 255, KKM 359 and KKM 388 (*Dermocybe*) + *Comarostaphylis arbutoides*

Morphological characters (Fig. 4.4a) *Mycorrhizal systems* irregularly pinnate to dichotomous, with 0–2 orders of ramification, systems solitary or in small numbers to abundant and dense, up to 9.9 mm long, slightly hydrophobic, of medium distance fringe exploration type. *Main axes* 0.2–0.6 mm diameter. *Unramified ends* bent or sinuous, not inflated, cylindric, up to 1.5 (2.7) mm long, 0.2–0.3 mm diameter; mantle ochre to yellowish brown, very tip yellowish, older parts dark orange and mycorrhizas not carbonising. *Surface*

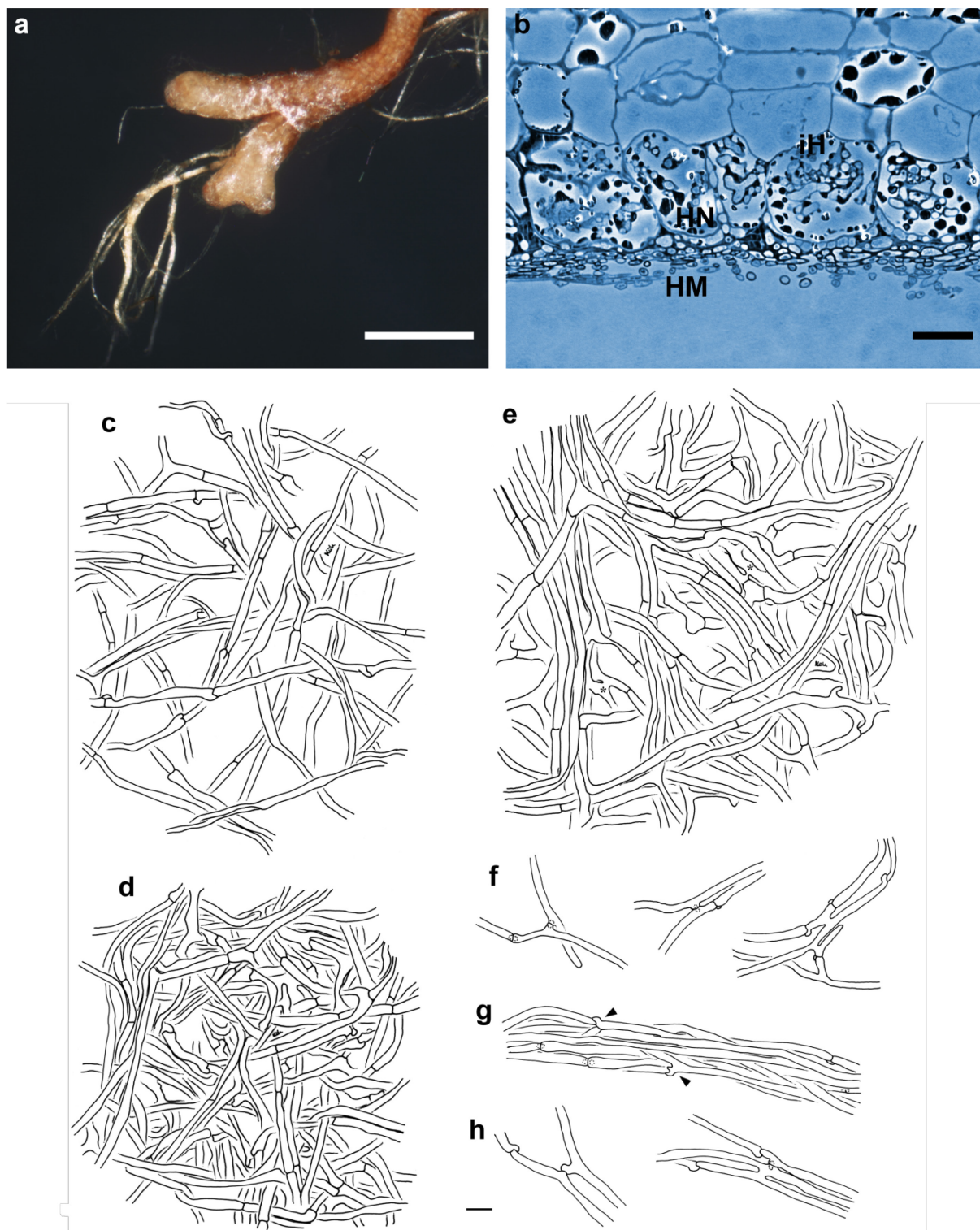


Fig. 4.4 Arbutoid mycorrhiza of *Cortinarius* sp.-*Comarostaphylis arbutoides* (samples KKM 255, KKM 359 and KKM 388, subgenus *Dermocybe*). **a** Habit of the dermocyboid mycorrhiza with transparent, ochre to yellowish brown mantle, loosely stringy mantle surface and ochre to yellowish brown rhizomorphs; *bar* 0.5 mm. **b** Semi-thin section of dermocyboid mycorrhiza with hyphal mantle (HM), Hartig net (HN) and intracellular hyphae (iH); *bar* 20 μ m. **c-h** Plan view of different mantle layers and emanating elements of the dermocyboid mycorrhiza; *bar* 10 μ m; **c** outer mantle layer with loosely and irregularly arranged hyphae, some hyphae in bundles and hyphae with clamps; **d** middle mantle layer with densely arranged hyphae, some hyphae in bundles; **e** inner mantle layer with densely arranged hyphae, hyphae in bundles, forming ring-like structures, hyphae with open anastomoses (*asterisks*); **f** emanating hyphae with contact clamps and open anastomoses; **g** rhizomorph with acute ramifications (*arrowheads*); **h** hyphae of rhizomorphs with contact clamps and open anastomosis

of unramified ends loosely stringy to loosely cottony, not smooth, between side branches and main axis sometimes fan-like cottony, slightly silvery by enclosed air, rapidly displaced by water when touched, then mantle generally transparent; epidermal cells visible through mantle. *Rhizomorphs* frequent, up to 0.11 mm diameter; flat in cross-section, emanating from all parts of the mycorrhiza, connection oblique, distal rhizomorphs connected over a long distance with mantle surface, growing into soil or organic layers, ochre to yellowish brown, repeatedly ramified into smaller filaments, with smooth margins. *Cystidia* lacking. *Sclerotia* not observed.

Anatomical characters of the mantle in plan views (Fig. 4.4c–e) Mantle lacks cells densely filled with oily droplets or brownish content, blue granules, needle-like contents, matrix, crystals and exudated pigments, as well as cystidia. *Outer mantle layers* loosely plectenchymatous, hyphae irregularly arranged, some hyphae in bundles, rarely ramified, without any special pattern (type B, Agerer 1991), colourless and with few soil particles; hyphae 25–90 μm long, 1.6–4.2 μm in diameter and cell walls 0.3 μm thick and hyphae with clamps, constricted at septa, septa as thick as cell walls. *Middle mantle layers* densely plectenchymatous, hyphae irregularly interwoven, some hyphae in bundles, occasionally ampullate at one side of septum, hyphae 15–50 μm long, 2.2–3.8 μm in diameter, cell walls 0.3 μm thick, smooth and colourless; hyphae with simple septa, rarely with clamps and constricted at septa, septa as thick as cell walls. *Inner mantle layers* densely plectenchymatous, hyphae in bundles, forming ring-like structures, occasionally ampullate at one side of septum, hyphae 17–200 μm long, 1.9–4.6 (5.3) μm in diameter, cell walls 0.3 (0.4) μm thick, smooth and colourless; hyphae with simple septa, constricted at septa and septa as thick as cell walls; anastomoses open, with short bridge, bridge thinner or as thick as hyphae. *Very tip* like other parts of the mantle.

Anatomical characters of emanating elements (Fig. 4.4f–h) Lacking are gelatinized hyphae, matrix, rhizomorphal nodia, simple septa, intrahyphal hyphae, crystals, brownish substances and secreted pigments; elbow-like protrusions are not observed. *Rhizomorphs* undifferentiated, hyphae loosely interwoven and of uniform diameter (type A/B, Agerer and Rambold 2004–2015); hyphae smooth, cells 30–125 μm long, 2.8–4.3 μm diameter and cell walls 0.3 μm ; ramification acute, one or two hyphal diameter below the septum and ramifications one side branch at septum; septa with clamps, constricted at septa, septa as thick as hyphal walls; anastomoses frequent, open or closed by a clamp (contact clamp), with a short bridge or bridge almost lacking, bridge as thick as hyphae, cell walls of anastomoses as

thick as remaining walls. *Emanating hyphae* straight to wavy, smooth, surface occasionally with few soil particles, cells 40–110 μm long, 2.1–3.8 μm diameter, cell walls 0.3 μm and distal ends of hyphae simple; ramification acute or approximately 90 °, with one side branch at septum and one or two hyphal diameter below the septum; septa with clamps, constricted at septa, septa as thick as hyphal walls; anastomoses are frequent, open or closed by a clamp (contact clamp), with a short bridge or bridge almost lacking, bridge as thick as hyphae, cell walls of anastomoses as thick as remaining walls. *Cystidia* not found.

Anatomical characters of longitudinal section (Fig. 4.4b) Mantle plectenchymatous, 5–19 μm thick. Mantle of very tip plectenchymatous, 4–14 μm thick. Epidermal layer with intracellular hyphae, epidermal cells radially oval to elliptic; Hartig net around epidermal cells para-epidermal in one row and hyphal cells roundish to cylindrical. Tannin cells are lacking.

Colour reactions with different reagents (mantle preparations and emanating elements)
Cotton blue: hyphae blue to patchy greenish; KOH 10 %: rhizomorphs bright pink; NH₄OH conc.: rhizomorphs bright pink after a few minutes; toluidine blue: hyphae violet, cell content pink. No reaction was observed with: acetic acid, ethanol 70 %, Fe(II)SO₄, guaiac, lactic acid, Lugol's solution, Melzer's reagent, sulpho-vanillin, H₂SO₄.

Reference specimen Costa Rica, province of San José, canton of Pérez Zeledón, at mountain Cerro de la Muerte, Estación Biológica de la Muerte (3100 m a.s.l.; precipitation c. 2812 mm/year; lithosol (FAO)), in a secondary cloud forest with *Quercus costaricensis*, soil core exc., myc. isol. Katja Kühdorf; KKM 255, 4 October 2011 and mycorrhiza deposited by B. Münzenberger (ZALF Müncheberg, Germany). Further material studied Costa Rica, province of San José, canton of Pérez Zeledón, at mountain Cerro de la Muerte, Reserva Forestal Los Santos (3300 m a.s.l.; precipitation c. 2812 mm/year; inceptisol (USDA)), in a secondary cloud forest with *Quercus costaricensis*, soil core exc., myc. isol. Katja Kühdorf; KKM 359 and KKM 388, 18 October 2011; mycorrhiza deposited by B. Münzenberger (ZALF Müncheberg, Germany).

4.3.2.4 KKM 149 (*Dermocybe*) + *Comarostaphylis arbutoides*

Morphological characters (Fig. 4.5a) *Mycorrhizal systems* irregularly pinnate to dichotomous, with 0–2 orders of ramification, systems abundant and dense, up to 11.3 mm long, slightly hydrophobic and of medium distance fringe exploration type. *Main axes* 0.3–0.5

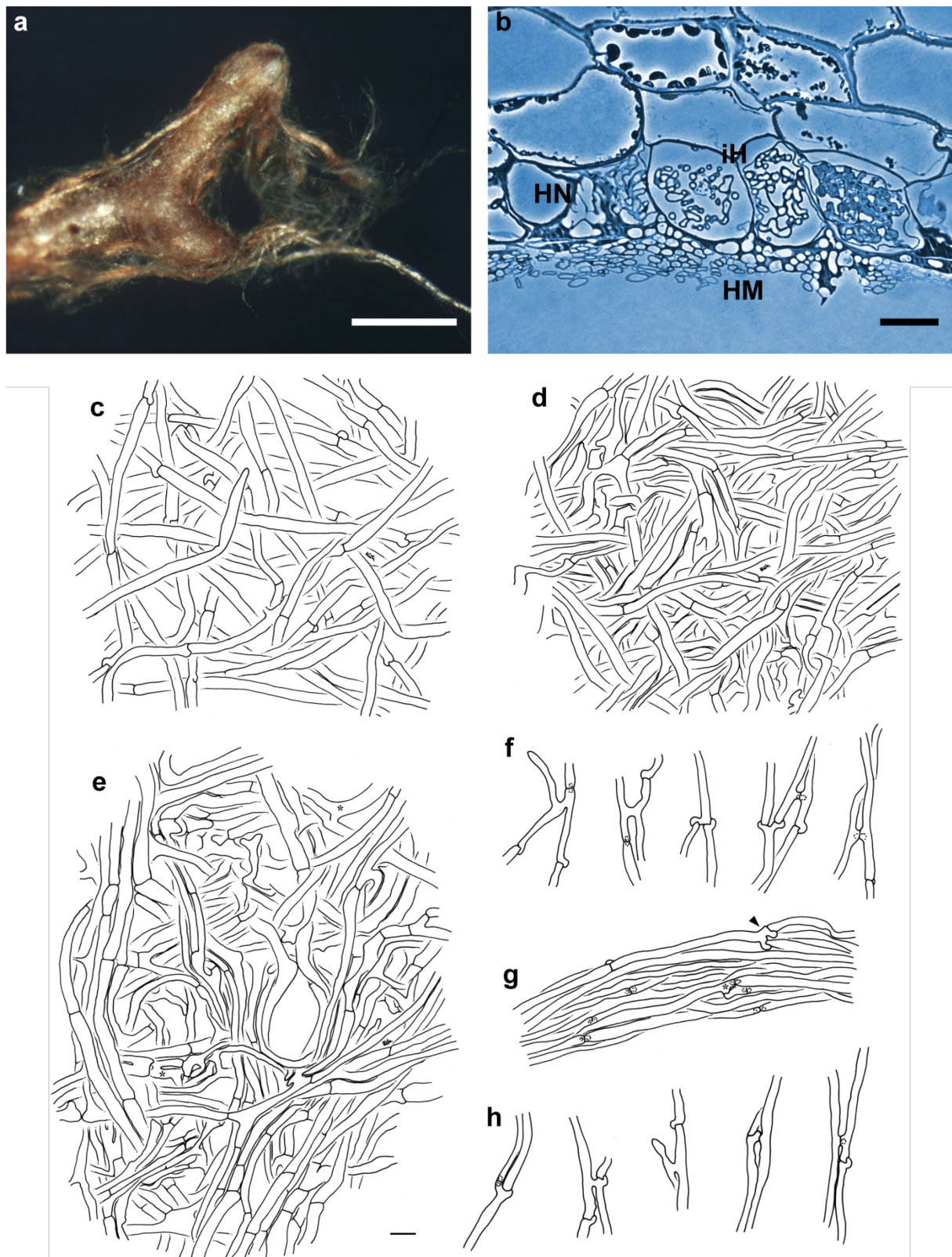


Fig. 4.5 Description see next page

Fig. 4.5 Arbutoid mycorrhiza of *Cortinarius* sp.-*Comarostaphylis arbutoides* (sample KKM 149, subgenus *Dermocybe*). **a** Habit of the dermocyboid mycorrhiza with transparent, brown-coloured mantle, loosely stringy mantle surface and ochre to reddish brown rhizomorphs; *bar* 0.5 mm. **b** Semi-thin section of dermocyboid mycorrhiza with hyphal mantle (HM), Hartig net (HN) and intracellular hyphae (IH); *bar* 20 μ m. **c–h** Plan view of different mantle layers and emanating elements of the dermocyboid mycorrhiza; *bar* 10 μ m; **c** outer mantle layer with loosely and irregularly arranged hyphae, hyphae with clamps; **d** middle mantle layer with densely arranged hyphae, some hyphae in bundles; **e** inner mantle layer with densely arranged hyphae, hyphae in bundles, forming ring-like structures, hyphae with open anastomoses (*asterisks*); **f** emanating hyphae with open anastomoses (*single arrowheads*), backwards-oriented ramification and with contact clamps; **g** rhizomorph with contact clamp (*asterisk*) and acute ramification (*arrowhead*); **h** hyphae of rhizomorphs with acute ramification, open anastomoses and contact clamps

mm diameter. *Unramified ends* sinuous to tortuous, not inflated, cylindrical, up to 1.6 (2.5) mm long, 0.3 mm diameter; mantle brown, very tip brownish to greyish, older parts dark brown and mycorrhizas not carbonising. *Surface of unramified ends* loosely stringy to loosely cottony, not smooth, between side branches and main axis sometimes fan-like cottony, slightly silvery by enclosed air, rapidly displaced by water when touched, then mantle generally transparent and epidermal cells visible through mantle. *Rhizomorphs* frequent, up to 0.13 mm diameter, flat in cross-section, emanating from all parts of the mycorrhiza, connection oblique, distal rhizomorphs connected over a long distance with mantle surface, growing into soil or organic layers, ochre to reddish brown, repeatedly ramified into smaller filaments, with smooth margins. *Cystidia* lacking. *Sclerotia* not observed.

Anatomical characters of the mantle in plan views (Fig. 4.5c–e) Mantle lacks cells densely filled with oily droplets or brownish content, blue granules, needle-like contents, matrix, crystals and exudated pigments, as well as cystidia. *Outer mantle layers* loosely plectenchymatous, hyphae irregularly arranged, rarely ramified, without any special pattern (type B, Agerer 1991), colour yellowish to colourless and with few soil particles; hyphae 20–59 (100) μ m long, 3.5–4.7 μ m in diameter, cell walls 0.3 (0.4) μ m thick; hyphae with clamps, constricted at septa and septa as thick as cell walls. *Middle mantle layers* densely plectenchymatous, some hyphae in bundles or irregularly interwoven, sometimes ampullate at one side of septum, hyphae 20–110 μ m long, 2.8–5.8 μ m in diameter, cell walls 0.3 μ m thick, smooth and colourless; hyphae with simple septa, rarely with clamps, constricted at septa and septa as thick as cell walls. *Inner mantle layers* densely plectenchymatous, hyphae in bundles, forming ring-like structures, sometimes ampullate at one side of septum, hyphae uneven in diameter, hyphae 30–200 μ m long, 1.3–5.1 μ m in diameter, cell walls 0.3–0.4 μ m thick, smooth and colourless; hyphae with simple septa, constricted at septa and septa as thick as cell walls; anastomoses open with short bridge, bridge thinner or as thick as hyphae. *Very tip* like other parts of the mantle.

Anatomical characters of emanating elements (Fig. 4.5f–h) Lacking are gelatinized hyphae, matrix, rhizomorphal nodia, simple septa, intrahyphal hyphae, crystals, brownish substances and secreted pigments; elbow-like protrusions not observed. *Rhizomorphs* undifferentiated, hyphae loosely interwoven and of uniform diameter (type A/B, Agerer and Rambold 2004–2015); hyphae smooth, cells 20–90 µm long, 2.4–3.8 µm diameter, cell walls 0.3 µm; ramification acute, one or two hyphal diameter below the septum and ramifications one side branch at septum; septa with clamps, constricted at septa and septa as thick as hyphal walls; anastomoses are frequent, closed by a clamp or rarely open, with a short bridge or bridge almost lacking, bridge as thick as hyphae and cell walls of anastomoses as thick as remaining walls. *Emanating hyphae* straight to wavy, smooth, surface occasionally with few soil particles, cells 20–95 µm long, 2.1–3.8 (4.7) µm diameter, cell walls 0.3 µm, and distal ends of hyphae simple; ramification acute or approximately 90 °, with one side branch at septum, one or two hyphal diameter below the septum, backwards-oriented ramification observed only once; septa with clamps, constricted at septa and septa as thick as hyphal walls; anastomoses frequent, closed by a clamp (contact clamp) or rarely open, with a short bridge or bridge almost lacking, bridge as thick as hyphae and cell walls of anastomoses as thick as remaining walls. *Cystidia* not found.

Anatomical characters of longitudinal section (Fig. 4.5b) Mantle plectenchymatous, 4–16 µm thick. Mantle of very tip plectenchymatous, 4–11 µm thick. Epidermal layer with intracellular hyphae and epidermal cells radially oval to elliptic; Hartig net around epidermal cells para-epidermal in one row and hyphal cells roundish to cylindrical. Tannin cells are lacking.

Colour reactions with different reagents (mantle preparations and emanating elements)
Cotton blue: hyphae blue to patchy greenish; KOH 10 %: rhizomorphs bright pink; NH₄OH conc.: rhizomorphs bright pink after a few minutes; toluidine blue: hyphae violet and cell content pink. No reaction was observed with: acetic acid, ethanol 70 %, Fe(II)SO₄, guaiac, lactic acid, Lugol's solution, Melzer's reagent, sulpho-vanillin, H₂SO₄.

Reference specimen Costa Rica, province of San José, canton of Pérez Zeledón, at mountain Cerro de la Muerte, Reserva Forestal Los Santos (3300 m a.s.l.; precipitation c. 2812 mm/year; inceptisol (USDA)), in a secondary cloud forest with *Quercus costaricensis*, soil core exc., myc. isol. Katja Kühdorf; KKM 149, 12 October 2010; mycorrhiza deposited by B. Münzenberger (ZALF Müncheberg, Germany).

4.4 Discussion

Despite the efforts in investigating the biodiversity of Costa Rican *Cortinarii*, only Ammirati et al. (2007) supplied genetic data for their reported species. This makes it difficult to address mycorrhizal findings to already known species. With one exception, none of the cortinarioid mycorrhizas, found in Costa Rica, can be determined to species level.

However, due to sequence identity of 100 %, the mycorrhizal sample KKM 144 can most probably be assigned to *Cortinarius comarostaphyliae*. This species occurs in the Costa Rican páramo (Halling and Mueller 1999) and, according to Ammirati et al. (2007), *Cortinarius comarostaphyliae* seems to be restricted to the Ericaceae *Comarostaphylis arbutoides*. This plant was also confirmed genetically as host plant for the mycorrhiza KKM 144. Due to strict host specificity of many *Cortinarius* species (e.g. Brandrud 1996; Liimatainen 2013), identification with the European species *Cortinarius leucophanes* (Fig. 4.1) is excluded, since this species is associated with conifers (Ammirati et al. 2007).

Based on phylogenetic analyses, the sample KKM 132 is placed in the subgenus *Phlegmacium*, but fitted laboriously into this subgenus during processing. Also, the databases NCBI and UNITE reached comparatively low identity values, which confirms the insufficient sequence data quantity of Costa Rican *Cortinarii*. However, beside *Cortinarius glaucopus*, Halling and Mueller (1999) mention another phlegmacioid *Cortinarii* from the Costa Rican páramo. Since no sequence data is available, it remains unclear, whether or not this unidentified *Phlegmacium* species correspond to our mycorrhizal sample KKM 132.

4.4.1 Mycorrhiza assigned to subgenus *Phlegmacium*

The mycorrhiza KKM 132 strongly differs in morphology from phlegmacioid ectomycorrhizas (ECMs) hitherto described in DEEMY (*Cortinarius alnobetulae* (Wiedmer and Senn-Irlet 1999a), *Cortinarius calochrous* ssp. *coniferarum* (Kernaghan 2001), *Cortinarius kuehneri* (Wiedmer and Senn-Irlet 1999b), *Cortinarius odorifer* (Uhl 1988; Egli 1992), as well as *Cortinarius varicolor* (Agerer 1969, 1988)). All these ECMs offer a silvery surface, whereas our *Phlegmacium* sample from Costa Rica lacks this feature. However, amongst this feature and its dense stringy surface, the ochre to yellowish colour, as well as the lack of sclerotia, KKM 132 resembles the Cortinariaceae “*Nothofagirhiza reticulosa*” from Chile (Palfner 2001; Agerer and Rambold 2004–2015).

Anatomically, KKM 132 resembles “*Nothofagirhiza reticulosa*” in showing similar plectenchymatous mantle structures. However, in contrast to KKM 132, “*Nothofagirhiza reticulosa*” possess anastomoses in the outer mantle layer, a rough hyphal surface in the outer mantle layer as well as in the emanating elements, and simple septa as well as intrahyphal hyphae in the emanating hyphae.

The phlegmacioid mycorrhiza of KKM 132 shows no reaction with KOH, an attribute in common with *Cortinarius alnobetulae* and “*Nothofagirhiza reticulosa*”. However, both samples differ from KKM 132 in lacking reaction with cotton blue and toluidine blue, and a positive reaction with sulpho-vanillin, respectively. The likewise absent reaction with NH₄OH of KKM 132 was not investigated in the other mycorrhizal samples of *Phlegmacium*.

4.4.2 Mycorrhiza assigned to subgenus *Telamonia*

In DEEMY and literature, the following ECMs of the subgenus *Telamonia* are described as follows: *Cortinarius armillatus* (Cuvelier 1990; Cuvelier and Agerer 1991), *Cortinarius atropusillus* (Wiedmer and Senn-Irlet 1999c), *Cortinarius badiovestitus* (Wiedmer and Senn-Irlet 1999d), *Cortinarius bibulus* (Wiedmer and Senn-Irlet 2001a), *Cortinarius bulliardii* (Raidl et al. 2006), *Cortinarius cinnabarinus* (Ceruti et al. 1988; Brand 1991, 1992), *Cortinarius helvelloides* (Wiedmer and Senn-Irlet 2001b), *Cortinarius hinnuleus* (Kovács et al. 2002), *Cortinarius malachius* (Uhl 1988), *Cortinarius obtusus* (Agerer 1987a; Gronbach 1988; Gronbach and Agerer 1988) and *Cortinarius saturninus* (Seress et al. 2012). Here, *Cortinarius obtusus* resembles our telamonioid mycorrhiza KKM 298 with the silvery surface, white rhizomorphs with smooth margins, and the medium distance fringe exploration type.

Anatomically, the KKM 298 differs clearly from the 11 *Cortinarius* subgenus *Telamonia* ECMs. *Cortinarius badiovestitus*, *Cortinarius bibulus*, *Cortinarius hinnuleus* and *Cortinarius obtusus* have contact clamps in their emanating elements, whereas KKM 298 possesses no contact clamps at all. The anastomoses of *Cortinarius cinnabarinus* are not only open with a short bridge but are also closed by a clamp with a long bridge. Moreover, together with *Cortinarius bulliardii* and *Cortinarius malachius* and *Cortinarius cinnabarinus* show differentiated rhizomorphs of type D or C, whereas these of KKM 298 are assigned to type A/B. The mycorrhiza of *Cortinarius saturninus* is the only one which offers a matrix in the mantle layers as well as in the rhizomorphs. Together with *Cortinarius armillatus*, both

systems feature not only clamps but also secondary septa in the emanating elements which were, in contrast, not observed in KKM 298. None of the *Cortinarius* species possess open anastomoses in all three mantle layers, whereby, in the case of *Cortinarius cinnabarinus*, *Cortinarius hinnuleus* and *Cortinarius saturninus* no middle mantle layer was described. Secondly, a distinctly ring-like arrangement of the inner mantle layer, as was also shown for KKM 298, was not described in any of the 11 telamonioid *Cortinarius* ECMs.

For chemical reactions, only KOH was tested in all described *Cortinarius* subgenus *Telamonina* ECMs. Here, no reaction was observed for KKM 298, as well as for most of the other telamonioid species. Exceptions are *Cortinarius bulliardii*, *Cortinarius cinnabarinus* and *Cortinarius malachus* which show violet pink, red-violet colour reactions or dissolving incrustations. A likewise negative reaction with NH₄OH, as shown for KKM 298, cannot be conformed since the other telamonioid systems were not tested. For *Cortinarius armillatus*, no information on chemical reactions is given at all.

4.4.3 Mycorrhizas assigned to subgenus *Dermocybe*

Currently, nine ECMs of the subgenus *Dermocybe* are described in DEEMY: *Dermocybe cinnamomea* (Agerer 1987b; Agerer and Gronbach 1987; Gronbach 1988; Berg 1989; Weiss 1988), *Dermocybe cinnamomeolutea* (Waller and Agerer 1993), *Dermocybe crocea* (Uhl and Agerer 1987, 1988; Uhl 1988), *Dermocybe holoxantha* (Uhl 1988; Agerer 1995), *Dermocybe huronensis* (Kuss et al. 2004), *Dermocybe palustris* (Uhl and Agerer 1987; Agerer 1995), *Dermocybe phoenicea* (Cuvelier 1990), *Dermocybe sanguinea* (Agerer 1987b, c), as well as *Dermocybe semisanguinea* (Uhl 1988; Agerer and Uhl 1989; Agerer 1995). Due to the ochre to yellowish brown-coloured mantle and rhizomorphs, the mycorrhizal systems of KKM 255, KKM 359 and KKM 388 ("Dermocybe I") are similar to *Dermocybe cinnamomeolutea*. In contrast, the system of KKM 149 ("Dermocybe II") shows a brown to dark brown mantle with a reddish tint, as is also common with *Dermocybe phoenicea*. However, both described mycorrhizas show a distinctly transparent mantle clearly distinguishing them from the other *Dermocybe* species described in DEEMY so far.

Anatomically, the mycorrhizas of "Dermocybe I" and "Dermocybe II" do not differ much. Both types show a plectenchymatous mantle throughout undifferentiated rhizomorphs, infrequently with smooth margins (types A or B) and both form short anastomoses with contact clamps. These features are typical for *Dermocybe* species (Agerer 1995). According

to DEEMY, our samples herein resemble *Dermocybe cinnamomeolutea*, *Dermocybe holoxantha* and *Dermocybe huronensis*. Nevertheless, these three species also show clear differences. Hyphae of emanating elements (*Dermocybe huronensis*), as well as of the outer mantle layer (*Dermocybe cinnamomeolutea*) possess a rough surface. *Dermocybe cinnamomeolutea* and *Dermocybe holoxantha* have a matrix in the middle and inner mantle layers and, furthermore, none of these three species show a ring-like inner mantle layer as occurs in both, "Dermocybe I" and "Dermocybe II". Anastomoses open or closed by a clamp, are very common in the emanating elements, whereas in "Dermocybe II", open anastomoses are less frequently observed than in "Dermocybe I". Within the described *Dermocybe* ECM only, *Dermocybe phoenicea* also feature these two types of anastomoses.

The rhizomorphs of "Dermocybe I" and "Dermocybe II" show a bright pink colour reaction with KOH, as well as with NH₄OH. A colour reaction with NH₄OH was only tested in *Dermocybe cinnamomea*, where no reaction was observed. A reaction with KOH was investigated in seven of the nine *Dermocybe* species, where again, *Dermocybe cinnamomea*, as well as *Dermocybe palustris* showed a reaction, but which only affected the hyphal content or the mantle, respectively.

According to Ammirati et al. (2007), Costa Rica has a limited number of confirmed host plants for ectomycorrhizal fungi, such as *Quercus* sp., *Comarostaphylis arbutoides* and *Alnus acuminata*. Even though oak species have an extensive distribution and are the most common ectomycorrhizal host plant for *Cortinarius*, they only occur in small numbers. Additionally, some *Cortinarius* species have very special habitat preferences (Liimatainen 2013) and, amongst topographical factors, often only exist in small, geographically isolated populations (Frøslev et al. 2005). Beside insufficient recorded biodiversity, this explains why the *Cortinarius* findings in this study primarily differ from Ammirati et al. (2007), and why species of, e.g. subgenus *Dermocybe* have not been reported yet for Costa Rica. Moreover, in contrast to Ammirati et al. (2007), who described Costa Rican *Cortinari* sporocarps exclusively found in June, we found no fruit bodies at all. This suggests that sampling in October, within the rainy season, is suitable for mycorrhizal investigations but not for *Cortinarius* fruit bodies in this area.

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5

General discussion

5.1 Mycorrhiza versus fruit body: limits in gathering the diversity of ectomycorrhizal fungi

To proclaim new macrofungal species, fruit body collections are still indispensable. However, seasonal and yearly fruit body production of ectomycorrhizal fungi is impossible to predict (Mueller et al. 2006), since it depends on abiotic factors and condition of the associated host tree (Niskanen 2008; Lim 2010; Egli 2011; Martínez-Peña 2012). Moreover, some fungi produce fruit bodies that are hard to recognize or even probably lack fruit bodies at all (Tedersoo and Nara 2010). Thus, even longtime field studies do not always reveal the whole diversity spectrum of ectomycorrhizal fungi of a local area. Therefore, studies on mycorrhizas could be very suitable to get a more comprehensive insight of the ectomycorrhizal fungal component as their development is more independent from seasonal conditions and thus, can be found in soil throughout the year (Tedersoo and Nara 2010; Garay-Serrano et al. 2012).

In theory, the simplest way to identify ectomycorrhizas is to trace their hyphae or rhizomorphs to their respective formed fruit bodies, which in turn can be determined (Agerer 1991, 1993). In addition to fruit body limitation as already mentioned above, Agerer (1991) points out that this procedure is not applicable for every genus (e.g. *Hygrophorus*, *Inocybe*, *Lactarius*, and *Russula*) and foreign ectomycorrhizas can be intermixed as well. Moreover, determination of already known species can be very difficult even for experts (Halling and Mueller 2005), and due to the limitation of knowledgeable mycologists, time as well as socio-political, economic and political aspects (e.g. war events) the investigation and description of new species cannot always be afforded (Hawksworth 1997, 2012; Comandini et al. 2012). Therefore, the determination of mycorrhizal fungi to their appropriate species has to be

performed by sequence comparison within databases. However, this approach is limited due to amount and quality of data. Many compared sequences resulted in insufficient determined species or remain as unknown since many query sequences do not originate from already known species. Additionally, molecular data of species from Asia, Central America, Afrika, and the southern hemisphere are still underrepresented in these databases (Garnica et al. 2005), as shown for Costa Rican species of the cosmopolitan genus *Cortinarius*. Consequently, even though genetics offer a putative simple and fast way to determine species, an adequate species assignment of mycorrhizal systems is only possible when these data are based on fruit body collections that were previously determined.

Recently, next generation high-throughput sequencing is used to reveal fungal species richness in tropical habitats directly from soil samples (McGuire et al. 2012, 2013; Teasdale et al. 2013; Geml et al. 2014; Barberán et al. 2015). With regard to ectomycorrhizal studies, this method cannot provide any ensured evidence that the detected fungi forms indeed mycorrhizas with a certain host tree but may indicate range of dispersion of these fungi (Teasdale et al. 2013).

5.2 Ectomycorrhizal diversity at the Cerro de la Muerte

This study reveals that numerous fungal genera occur as ectomycorrhiza at the Cerro de la Muerte. Here, species were detected belonging to, inter alia, *Amanita*, *Cenococcum*, *Cortinarius*, *Hydnellum*, *Inocybe*, *Laccaria*, *Lactarius*, *Leccinum*, *Melanogaster*, *Phellodon*, *Piloderma*, *Sebacina*, *Sistotrema*, and *Tricholoma*. Samples of the *Gymnomyces/Russula* complex can most probably be assigned to the genus *Russula*, since *Gymnomyces* is a synonym of this genus (Calonge and Martín 2000; Miller et al. 2001). Other mycorrhizal systems found at the Cerro de la Muerte, concerning samples of the *Boletus/Xerocomus* as well as *Tomentella/Thelephora* complex, could not be clearly assigned genetically to a specific genus since they are too closely related to each other, respectively. In contrast it is surprising that mycorrhizas referred to the *Clavaria/Clavulina* complex cannot be distinguished even though both belong to different families and even orders. One reason could be that the ITS region is too homologous and therefore, not suitable to separate both genera genetically. Likewise, an incorrect taxonomic determination of reference sequences within the databases cannot be excluded. However, members of *Clavulina* (Clavulinaceae, Cantharellales) are well known to form ectomycorrhiza, whereas the ecological status of *Clavaria* (Clavariaceae, Agaricales) changed over the years from mycorrhizal (Englander and

Hull 1980), to saprotrophic (Rinaldi et al. 2008), and is currently referred to “not known” (Tedersoo et al. 2010). Therefore, investigation of the mycorrhizal systems assigned to this complex may help to clarify this discrepancy.

Likewise, the ascomycete *Leotia lubrica* was long considered to be saprotrophic. The species was reported from the Cerro de la Muerte, together with other confirmed or assumed saprotrophic genera e.g. *Gymnopus*, *Hygrocybe*, *Mycena*, and *Poronia*. However, very few ascomycetes are known or described to be ectomycorrhizal (Agerer and Rambold 2004–2016; Tedersoo et al. 2010). In this study *Leotia lubrica* was confirmed to be actually ectomycorrhizal. Perhaps such a transfer can be performed for other species, whose status is still unclear or not known yet, e.g. members of *Clavaria* or *Hygrocybe* (Halling and Mueller 2005; Seitzman et al. 2011; Tello et al. 2014). However, it must be noted that some genera are known to comprise both ectomycorrhizal and saprotrophic species (Tedersoo et al. 2010).

The species *Sistotrema confluens*, well-known from North America and Europe, was discovered for the first time in Central America, in the Costa Rican páramo of the Talamancas by Halling and Mueller (1999). This species is strongly suspected to be mycorrhizal (Nilsson et al. 2006; Bubner et al. 2014). We found mycorrhizal systems assigned to this genus only once at the Cerro de la Muerte. Further investigations are required whether this sample can be also assigned to *Sistotrema confluens* or not.

Numerous species of the genera *Cortinarius* (21), *Russula* (16), *Amanita* (12), *Boletus* (12), *Lactarius* (8), *Leccinum* (8), *Tricholoma* (6), *Laccaria* (3), *Inocybe* (2), *Hydnellum* (1), and *Melanogaster* (1), were already described from the Talamanca, some species even exclusively from the Cerro de la Muerte (Halling and Mueller 1999, 2005; Ammirati et al. 2007). To what extent our findings comprise already known or even new species within these genera, as we have shown for the genus *Cortinarius* is, however, uncertain and has to be clarified.

As already mentioned above, several species of the genus *Cortinarius* were reported from Costa Rica, but only one of our approximately 15 cortinarioid species could be assigned to species level. However, only few of these Costa Rican species were investigated genetically, which makes it difficult to compare our mycorrhizal findings to already known species. In our study we were able to describe four different *Cortinarius* species based on morphology and anatomy of their mycorrhizal systems, respectively. These included inter alia two species of the subgenus *Dermocybe*. However, only one unidentified Costa Rican *Cortinarius* sp. was assigned to this subgenus so far, found at the western slope of Volcán Poás in the Cordillera Central (Halling and Mueller 1999). Therefore, we assume that at least one new species for

the subgenus *Dermocybe* was found at the Cerro de la Muerte. How far our other mycorrhizal findings imply new or endemic species of the genus *Cortinarius* remains unresolved due to the lack of genetic data.

The genera *Cenococcum*, *Clavaria* and/or *Clavulina*, *Phellodon*, *Piloderma*, *Sebacina*, *Tomentella* and/or *Thelephora*, as well as *Xerocomus* have never been reported from Costa Rica before and are therefore, documented genetically for the first time for this country. Fruit bodies of the genus *Sebacina* mostly remain unnoticed unless specifically searched for. Based on mycorrhizal systems, we were able to show that *Sebacina* is represented by one, possibly new species at the Cerro de la Muerte. The hitherto non-report of *Cenococcum* is not surprising. Although this ascomycete is ubiquitous and cosmopolitan, fruit bodies are not known and, therefore, this genus is regarded as asexual (Jany et al. 2002).

On the other hand, samples of *Hysterangium* or *Phaeocollybia*, already known as mycorrhizal fungi from the Cerro de la Muerte and additionally, supposed to be associated with *Comarostaphylis arbutoides*, have not been proven as mycorrhizal systems. Here, insufficient data availability within NCBI and UNITE cannot be excluded and could be a reason, beside non-collection during sampling, for their non-record when compared with databases. Equally affected are numerous samples determined only to family or higher level, or matched no taxonomic designation at all. This concerns almost one third of our samples (29.21 %) and confirms the underrepresentation of (identified) neotropical species in these databases (Hawksworth 1997, 2012; Mueller et al. 2006). Therefore, a repeated comparison with databases is highly recommended since they are permanently updated and may result in a more adequate identification.

The two found samples identified as *Oidiodendron*, a typical ericoid mycorrhizal fungal genus, are considered as artifacts. Even though, some genera within the Ericaceae family are known to form different types of mycorrhiza, with different fungal taxa, the ericoid association is anatomically known from hair root systems only. However, we investigated ectomycorrhizal fine root systems and suspect that, e.g. spores or hyphae of *Oidiodendron* adhered to the collected mycorrhizal systems, which were sequenced instead.

5.3 *Comarostaphylis arbutoides* as host tree

Besides identification of the mycorrhizal fungus, the associated host plant can also be determined or confirmed genetically. Our study reveals that the Ericaceae *Comarostaphylis*

arbutoides is, beside the species *Leccinum monticola*, indeed mycorrhizal with other genera. Up to now *Leotia* cf. *lubrica*, an unknown *Sebacina* sp., and diverse species of the genus *Cortinarius* have been proven to be associates. It is assumed that *Comarostaphylis* is likewise associated with members of further genera since many collected mycorrhizal systems possess a coralloid ramification, typical for mycorrhizas associated with members of the Arbutoideae, but this has to be verified genetically. However, based on current morpho/anatomical descriptions and hitherto genetically detected genera in this study, the mycorrhizal genus community of *Comarostaphylis arbutoides* seems to be similar with those of the related Arbutoideae *Arbutus* and *Arctostaphylos*.

As shown for *Sebacina* sp., *Comarostaphylis arbutoides* shares its mycorrhizal fungal partners with *Quercus* tree species. Therefore, *Comarostaphylis arbutoides* could be very important with regard to reforestation, as it is able to serve as refuge for ectomycorrhizal fungi. However, how far this applies for species of other fungal genera has still to be verified.

5.4 Description of ectomycorrhizas

The classical fungal taxonomy is based on fruit body investigations, concentrating on hymenium, pileus, stipe, spores, as well as odor, taste, and the reaction with specific chemicals. According to Agerer (1991), ectomycorrhizal structures consist of fungal tissues, the arrangement and organization of which can be used to describe fungal species in the same way as any other taxonomically suitable feature. Actually, mycorrhizas offer a wide range of features that can be used for determination. Here, Agerer (1991) quotes the most informative features with the structure and anatomy of the outer mantle layer as well as emanating elements (rhizomorphs, hyphae, and cystidia), followed by morphological characteristics (color and surface of the ectomycorrhizal sheath), and the reaction with chemicals, like Melzer's reagent, KOH, and sulpho-vanillin.

Even though Agerer (1987-2012) and Agerer and Rambold (2004-2016) provide a very detailed instruction guide for the description of ectomycorrhizal systems, the comparison and hence, their identification is sometimes challenging. For example, some descriptions are very old, not detailed enough or incomplete and therefore, meet the present description standard only to some degree. Moreover, not all investigations are available in English, but only in non-scientific languages.

Other problems concern the implementation of description. Some feature (e.g. color impressions in general) underlies highly subjective estimates. Drawing sections are sometimes either too small and therefore, do not reflect the whole description, or even contain further details that are not explicitly mentioned in the text. Consequently, it might also be useful to mention not only observed features but also non-observed ones. For instance, many descriptions of *Cortinarius* species often do not explicitly contain additional information about the presence/absence of anastomosal structures and clamps in the middle and inner mantle layers, as well as backward-oriented ramifications and clamps in emanating elements, but which were found in the Costa Rican species. Whether and how far this information is needed for species determination remains to be seen but they are easy to record and, if necessary, to deny. This makes interpretation and comparison with other descriptions challenging or even impossible and thus, prevents clear evidence for an assignment to already described species.

On the basis of our investigation an improvement for describing ectomycorrhizal systems can also be proposed here. The use of Lugol's solution is not a standard yet in investigating color reactions, but should be recommended at least for mycorrhizal systems formed by ascomycetes, as it was discussed for the mycorrhiza formed by *Leotia lubrica*.

However, the determination of species based on their ectomycorrhizal systems are still fragmentarily, since the amount of described genera based on their ectomycorrhizal systems is not yet adequate, especially characterizations of ectomycorrhizas of native fungi in the neotropics are still limited (Garay-Serrano et al. 2012).

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Summary

In this study the mycorrhizal community of the Ericaceae *Comarostaphylis arbutoides* was investigated to reveal further ectomycorrhizal fungal species that are associated with this tropical plant. Mycorrhizal systems of selected genera were investigated in more detail regarding their phylogeny, morphology, and anatomy as well as the genetic confirmation of the associated host tree.

The genetic investigation of mycorrhizal systems collected at the Cerro de la Muerte (Costa Rica) detected a diverse fungal community. Most common were species of the genera *Russula*, *Cortinarius*, and *Cenococcum*, as well as not further determined members of the Cantharellaceae and Helotiales. The genera *Cenococcum*, *Clavaria/Clavulina*, *Phellodon*, *Piloderma*, *Sebacina*, *Tomentella/Thelephora*, as well as *Xerocomus* could be proven genetically for the first time from Costa Rica based on mycorrhizal systems.

Comarostaphylis arbutoides was proven to be mycorrhizal with *Leotia* cf. *lubrica*, *Sebacina* sp., and diverse species of the genus *Cortinarius*. Species of other genera are also assumed, but their confirmation is still to be made.

On the basis of genetics and the investigation of its mycorrhizal systems, the collected Costa Rican *Sebacina* sp. may constitute a hitherto not described species. However, to confirm this assumption fruit body investigations are necessary. *Sebacina* sp. was proven in this study to be also mycorrhizal with *Quercus* sp.

The ascomycete *Leotia lubrica* was proven to be mycorrhizal for the first time. However, mycorrhizal findings of this species are still rare in literature and further studies are necessary to underpin its new status. As *Leotia lubrica* occurs worldwide, other host plants, beside the proven *Comarostaphylis arbutoides*, are assumed for this species as well.

The mycorrhizal samples of the genus *Cortinarius* could comprise approximately 15 species. Their final determination is still outstanding, but it is assumed that also hitherto not known species were detected in this study. A host preference is indicated as species of *Cortinarius* are found to be associated either with *Comarostaphylis arbutoides* or *Quercus* sp., but needs to be confirmed by including mycorrhizal samples of *Quercus* trees.

One third of the sequenced mycorrhizal systems still remain unidentified. Therefore, repeated comparison with sequence databases is recommended. The phylogenetic and morphological/anatomical investigation of already identified as well as unidentified mycorrhizal systems is advised to deliver additional descriptions of Costa Rican ectomycorrhizal fungi and therefore contribute to the knowledge of tropical fungal species.

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Anhang

I. Zusammenfassung

In der vorliegenden Studie wurde die Mykorrhizagemeinschaft der Ericacee *Comarostaphylis arbutoides* untersucht, um weitere Mykorrhizapilzarten die mit dieser Pflanze assoziiert sind, nachzuweisen. Hierfür wurden die Mykorrhizen ausgewählter Pilzgattungen hinsichtlich ihrer Phylogenie, Morphologie, sowie Anatomie näher untersucht und die involvierte Wirtspflanze der Mykorrhiza genetisch verifiziert.

Die genetische Untersuchung der Mykorrhizen, welche am Cerro de la Muerte (Costa Rica) gesammelt wurden, offenbarten eine mannigfaltige Pilzgemeinschaft. Die am häufigsten nachgewiesenen Arten konnten den Gattungen *Russula*, *Cortinarius* und *Cenococcum* zugeordnet werden, sowie nicht weiter bestimmbar Mitgliedern der Cantharellaceae und Helotiales. Die Gattungen *Cenococcum*, *Clavaria/Clavulina*, *Phellodon*, *Piloderma*, *Sebacina*, *Tomentella/Thelephora*, sowie *Xerocomus* konnten, basierend auf ihren Mykorrhizen, erstmalig genetisch für Costa Rica nachgewiesen werden.

Comarostaphylis arbutoides konnte als pflanzlicher Mykorrhizapartner für *Leotia* cf. *lubrica*, *Sebacina* sp. und verschiedene Arten der Gattung *Cortinarius* verifiziert werden. Es wird vermutet, dass Arten weiterer Pilzgattungen mit *Comarostaphylis arbutoides* assoziiert sind, was jedoch erst noch bestätigt werden muss.

Die in Costa Rica gesammelte *Sebacina* sp. könnte, basierend auf der Untersuchung ihrer Mykorrhiza und Genetik, zu einer bisher noch nicht beschriebenen Art zählen. Um dies jedoch zu bestätigen sind Untersuchungen an Fruchtkörpern dieser Art notwendig. *Sebacina* sp. konnte ebenso als Mykorrhizapartner von *Quercus* sp. nachgewiesen werden.

Der Askomyzet *Leotia lubrica* konnte erstmalig als Mykorrhizapilz nachgewiesen werden. Nachweise dieser Art sind in der Literatur noch selten, was die Notwendigkeit weiterer Untersuchungen zur Festigung des neuen Status als Mykorrhizabildner unterstreicht. Da *Leotia lubrica* weltweit vorkommt, können neben *Comarostaphylis arbutoides* auch weitere Wirtspflanzen angenommen werden, die mit diesem Pilz assoziiert sind.

Die Mykorrhizen der Gattung *Cortinarius* umfassen circa 15 Arten, deren endgültige Bestimmung noch aussteht. Es wird jedoch angenommen, dass in dieser Studie auch bisher noch nicht bekannte *Cortinarius* Arten erfasst worden sind. Da die Arten der Gattung *Cortinarius* entweder mit *Comarostaphylis arbutoides* oder mit *Quercus* sp. assoziiert waren, kann hier eine Wirtsspezifität für die Arten dieser Pilzgattung angenommen werden. Um dies zu bestätigen müssten zukünftige Studien allerdings ebenso Mykorrhizen von *Quercus* Arten mit einbeziehen.

Ein Drittel der sequenzierten Mykorrhizen konnte nicht identifiziert werden. Daher wird ein wiederholter Abgleich mit Datenbanken empfohlen. Die phylogenetische und morphologisch/anatomische Untersuchung bereits genetisch identifizierter sowie auch nichtidentifizierter Mykorrhizen wird angeraten, da solche Beschreibungen von costa-ricanischen Ektomykorrhizapilzen zur Erweiterung des Wissens zu tropischen Pilzarten beitragen.

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Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation selbstständig verfasst und alle in Anspruch genommenen Hilfsmittel und Quellen angegeben habe.

Ich erkläre hiermit, dass die Veröffentlichung der Dissertation bestehende Schutzrechte nicht verletzt.



Katja Kühdorf, Müncheberg, den 31. Mai 2016