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ORIGINAL PAPER

Two sebacinoid ectomycorrhizae on Chinese pine

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Abstract Sebacinoid fungi show a broad mycorrhizal capacity; therefore, they play a very important role in natural systems. Worldwide, fungi of Sebacinales are present under different environmental conditions and associate with diverse plant hosts, however, are hitherto poorly studied in China. Two sebacinoid ectomycorrhizae (ECM), *Pinirhiza multifurcata* and *Pinirhiza nondextrinoidea*, are described in detail morphologically and anatomically in the present study. They share a plectenchymatous outer mantle with multiply ramified hyphae in a gelatinous matrix, clampless, thin, thick-walled emanating hyphae with mostly Y-shaped ramifications and triangular inflations at the point of ramification. *P. multifurcata* and *P. nondextrinoidea* can be distinguished by thick cells in mantle layers, the ramification of emanating hyphae, the presence or absence of rhizomorphs, as well as the differing color reaction in Melzer's reagent. The putative molecular phylogenetic relationships of *P. multifurcata* and *P. nondextrinoidea* were inferred by analyses of the partial large subunit nuclear rDNA (nLSU); however, an affiliation to fungal species was not possible. This is the first report of sebacinoid ECM on Chinese pine.

Keywords Anatomy · Sebacinoid ectomycorrhiza · Morphology · Molecular phylogenetic analyses

Introduction

The order Sebacinales, assigned to the Agaricomycetes (Blackwell et al. 2006), ecologically is characterized by the capacity of its members to form a diversity of mycorrhizae (Table 1). Fungi of Sebacinales are present under different environmental conditions and associate with diverse plant hosts, however, are hitherto poorly studied in China.

Most of our knowledge on Sebacinales and their diverse host species come from molecular ecology studies from direct amplification of fungal ribosomal DNA of environmental samples (Selosse et al. 2007). Sebacinoid fungi form similar structure of hyphal coils (pelotons) in cortical cells in orchid mycorrhiza (OM), ericoid mycorrhiza (ERM), and ectendomycorrhiza (EEM) (e.g., Selosse et al. 2004, 2007; Setaro et al. 2006; Taylor et al. 2003). Hyphal coils show at present limited information to make further taxonomical analyses, although additional evidence of features like clampless hyphae with imperforate parenthesomes has been applied (e.g., Selosse et al. 2007; Setaro et al. 2006). However, morpho-anatomical features of ectomycorrhiza (ECM), which are recognized as being important for function and can also be used to hypothesize fungal relationships at different taxonomic levels (Agerer 2006) provide us a hard-won chance to study in detail the sebacinoid group whose study of the fruitbody is limited till now (Weiß and Oberwinkler 2001; Weiß et al. 2004).

That an ectomycorrhizal status might be a common feature in the Sebacinaceae has already been assumed by Weiß and Oberwinkler (2001), and has been repeatedly proven (Table 1). Nevertheless, morpho-anatomical features of ECM are still little known in Sebacinales. Sebacinoid ECM have been described in detail only by Urban et al. (2003) providing characterizations of *Seba-*

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Table 1 Sebacinoid mycorrhiza

MC	Host	Type of ecosystem	Geographic localization	Edaphic condition	Reference
ECM	*Betula* sp.	Wooded meadow	Estonia	mollisihumi- rendzic leptosol	Tedersoo et al. 2006
	Corylus avellana L., *Carpinus betulus* L.	Deciduous forest	Lorraine, France	rendzine	Selosse et al. 2002a
	Eucalyptus marginata Donn ex Sm.	*Eucalyptus* forest	Western Australia	–	Avis et al. 2003
	Picea abies L.*	Deciduous forest	Vienna, Austria	calcareous soil	Urban et al. 2003
	Pinus sylvestris L.	Mixed forest	Eastern Austria	serpentine soil	Urban et al. 2008
	Pinus thunbergii Parl.	Coastal pine forests	Korea	maritime sand	Obase et al. 2009
	Quercus douglasii Hook & Arn.	Oak woodlands	Yuba, USA	metavolcanic rocks, pH 5.7–6.2	Smith et al. 2007
	Quercus ilex L.	Mediterranean forest	Corsica, France	alocrisols, pH 5.7–6.4	Richard et al. 2005
	Quercus macrocarpa Michaux	Oak savanna	Cedar Creek, USA	metalliferous soils, pH 6.55	Glen et al. 2002
	Quercus suber L. *	Oak forest	Portugal	–	Azul et al. 2006
	Tilia sp. *	Deciduous forest	Vienna, Austria	calcareous soil	Urban et al. 2003
OM	*Cephalanthera damasonium* (Mill.) Druce	Deciduous forest	France	calcareous soil	Julou et al. 2005
	Epipactis microphylla (Ehrh.) Schinz &Thell.	Deciduous forest	France	calcareous soil	Selosse et al. 2004
	Hexalectris spicata (Walter) Barnhart	Dessert	USA	–	Taylor et al. 2003
	Neottia nidus-avis (L.) Rich.	Mixed forest	France	–	Selosse et al. 2002b
	Neottia nidus-avis	Pine forest	Bavaria, Germany	Leptosols, pH 7	Bidartondo et al. 2004
ERM	*Agauria* sp., *Andromeda* spp. *Calluna vulgaris* (L.) Hull *Chiogenes hispidula* (Linn.) Torr. *Empetrum nigrum* L., *Erica* spp. *Gaultheria* spp. *Kalmia* sp. *Rhododendron* spp. *Vaccinium* spp.	–	Austria, Estonia, France, Spain, La Réunion Island Canada, Argentina	–	Selosse et al. 2007
JM	*Calypogeia muelleriana* (Schiffn.) K. Müll.	–	Germany, France	soil covered by needle litter	Kottke et al. 2003
	Lophozia incisa (Schrad.) Dum.	–	Swedish Lapland	sandy soil	
	Lophozia sudetica (Nees) Grolle	–	Pyrenean	wet soil	
EEM	*Arbutus unedo* L.	Forest	France	–	Selosse et al. 2007
	Cavendishia nobilis var. *capitata* (Bentham) Luteyn	Mountain rain forest	Andes in southern Ecuador	schists and sandstones, pH 3–5.5	Setaro et al. 2006
	Orthilia secunda (L.) House	Forest, lake	France, Canada	–	Selosse et al. 2007
	Pyrola chlorantha Sw.	Forest	France	–	Selosse et al. 2007

MC mycorrhiza class, *ECM* ectomycorrhiza, *OM* orchid mycorrhiza, *ERM* ericoid mycorrhiza, *JM* jungermannioid mycorrhiza, *EEM* ectendomycorrhiza, – means information has not been provided in the original papers

ECM (host species with *) have been described morphologically and anatomically, other ECM are only identified by molecular identification *cina incrustans* on *Picea abies* and a *Scbacinoid* sp. ECM on *Tilia* as well as by Azul et al. (2006) who published a description of a sebacinoid ECM on *Quercus suber* under the name *Quercirhiza dendrohyphidiomorpha* (* in Table 1).

During the study of the ectomycorrhizal diversity on Chinese pine (*Pinus tabulaeformis* Carr.), two sebacinoid ECM have been found. The aim of our work is to characterize these two anatomotypes morpho-anatomically, to identify them by molecular analyses of nLSU and internal transcribed spacer (ITS) sequence and to compare them in detail with previously published descriptions. This is the first report of sebacinoid ECM on Chinese pine.

Materials and methods

Sampling and morpho-anatomical characterization

Soil samples were collected in pure Chinese pine forests at Helan Mountain (Yinchuan City, Ningxia Hui Nationality Autonomous Region, China) and at Heilihe National Reserve (Chi Feng City, Inner Mongolia Autonomous Region, China) throughout 2 years. ECM systems were assigned to anatomotypes and described according to Agerer (1987–2008, 1991). Anatomical studies are based on at least five ECM systems for each anatomotype. Drawings were performed with the aid of a Normarski interference contrast microscope (Standard 14, ZEISS West Germany) connected with a drawing tube. All drawings were made at a magnification of ×1,000. Reference specimens of the mycorrhizae are deposited in M (for herbarium abbreviation see Thiers 2009).

DNA extraction, PCR, and sequencing

One unramified end, previously fixed in CTAB, from each of the two morphotypes was used for DNA extraction following careful microscopical examination in order to ensure that the isolated DNA originated from the respective anatomotype. DNA of ECM was extracted using the DNAeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The nLSU and ITS region were amplified using the PCR primer pairs LROR and LR5 (Moncalvo et al. 2000) as well as ITS1F and ITS4 (Gardes and Bruns 1993; white et al. 1990), respectively. The obtained PCR product was purified using the QIAquick protocol (Qiagen, Hilden, Germany) and fragments were sequenced applying the PCR primers. Sequencing was performed by the sequencing service of the Department Biology I (Ludwig-Maximilians-Universität, München) using BigDye Terminator Ready Reaction Cycles Sequencing Kit v3.1.

Sequence alignments and molecular analyses

Using the obtained nLSU sequence of the two ECM as query megablast searches (Zhang et al. 2000) were performed in GenBank (http://www.ncbi.nlm.nih.gov/). The 100 most similar sequences each were downloaded. Duplicates, i.e., identical sequences found as closest matches of different query sequences, were omitted. Using the software BioEdit v7.0.5 (Hall 2005) the sequences were automatically aligned. The alignment was revised manually and columns not alignable with certainty were excluded from the following analyses. For further molecular phylogenetic analyses, 47 unique nLSU sequences were retained. RAxML Web-Servers (the CIPRES Portal v1.14 at the San Diego Supercomputing Center http://8ball.sdsc.edu:8889/cipres-web/Home.do, Stamatakis 2006; Stamatakis et al. 2008) was used for calculation of the most likely trees and the bootstrap support values (500 replicates). The GTRGAMMAI model of substitution with default was applied for nLSU analyses with maximum likelihood as optimality criterion. The most parsimonious trees were searched by executing batch files generated by PAUPRat (Sikes and Lewis 2001) in PAUP* v4.0 (Swofford 2003), with weighting mode set to multiplicative. ITS sequence comparisons were performed in GenBank using megablast and in UNITE (Kõljalg et al. 2005, http://unite.ut.ee/) using BlastN.

Results

Morpho-anatomical descriptions

Pinirhiza multifurcata

Morphological characters (Fig. 1a) Mycorrhizal systems unramified to dichotomous, with 0–2(3) orders of ramification, solitary or in small numbers, main axis 0.35–0.4 mm diam., hydrophilic, of short distance exploration type. *Unramified ends* straight or sometimes bent, cylindric, not inflated, (0.9)2.5(3.8) mm long, 0.35–0.4 mm diam., grayish orange-brown, very tips whitish, older parts dark brown to black, mantle opaque to semitransparent, not carbonizing, surface not smooth, bumpy, and loosely hairy. *Emanating hyphae* moderately frequent to frequent, not specifically distributed. *Cystidia* not distinct under stereoscope magnification. *Rhizomorphs* not found. *Sclerotia* not observed.

Anatomical characters of mantle in plan views (Figs. 2a–c) Plectenchymatous throughout, hyphae in all layers colorless and clampless. *Outer mantle layers* (Fig. 2a) plectenchymatous with mutiply branched and irregularly inflated hyphae in a matrix (mantle type E/C, according to Agerer 1987–

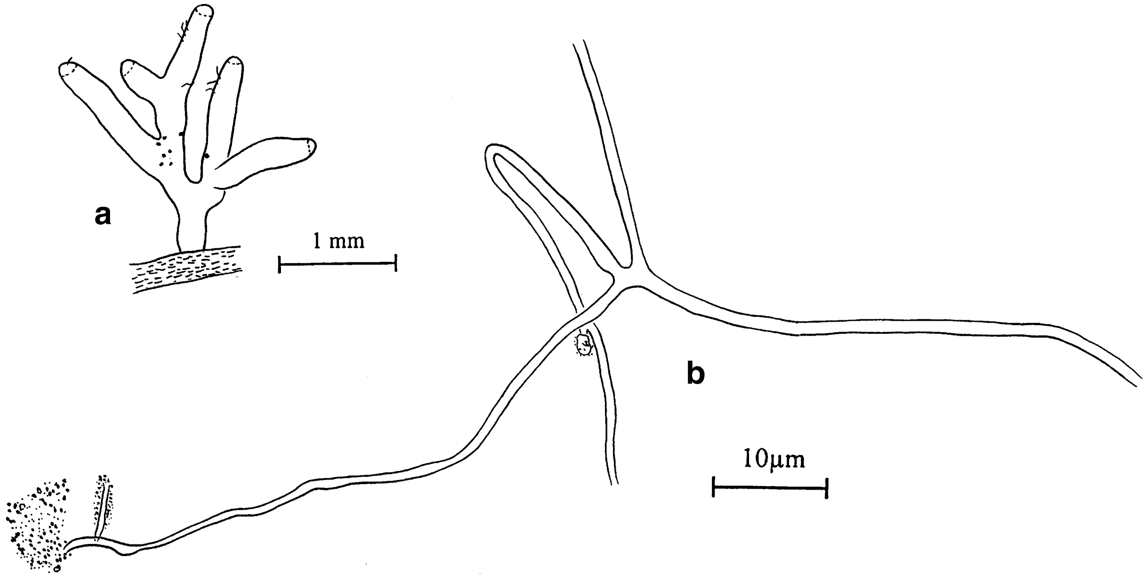

Fig. 1 *Pinirhiza multifurcata*. **a** Habit of ectomycorrhiza, surface with infrequent emanating elements. **b** Emanating hyphae with trifurcate ramification

2008, 1991, Agerer and Rambold 2004–2009), hyphae 2.5–5 μm diam. with up to 8 μm thick cells, cell walls 0.3–0.5 μm, surface smooth; in few hyphae septa with a large pore discernible; surface of mantle with many soil particles. *Middle mantle layer* (Fig. 2b) plectenchymatous, areas with longer hyphae intermixed with few lobed, multiply branched ones, 2–3.5(7) μm diam., cell walls 0.3 μm, in few hyphae septa with a large pore discernible. *Inner mantle layers* (Fig. 2c) densely plectenchymatous, hyphae frequently ramified and irregular in diam., some inflated, bent or curved; hyphal cells 2–4.5 μm wide, walls 0.3 μm. *Very tip* like remaining parts of the mantle.

Anatomical characters of emanating elements (Figs. 1b, 3) *Rhizomorphs* lacking. *Emanating hyphae* (Figs. 1b, 3) similar to cystidia (s. below), infrequent to frequent,

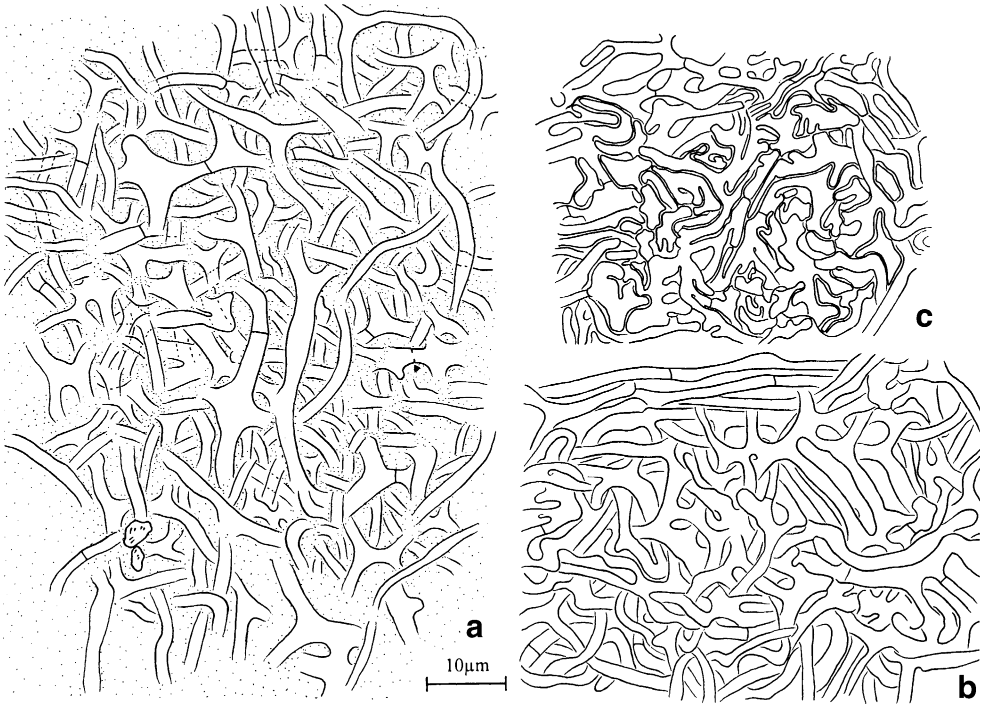

Fig. 2 *Pinirhiza multifurcata*. **a** Plan view of outer mantle layer with some multiply branched and irregularly inflated hyphal cells in a gelatinous matrix, septa with big pore in few hyphae discernible (*arrowhead*). **b** Plan view of middle mantle layer with multi-ramified hyphae. **c** Plan view of inner mantle layer

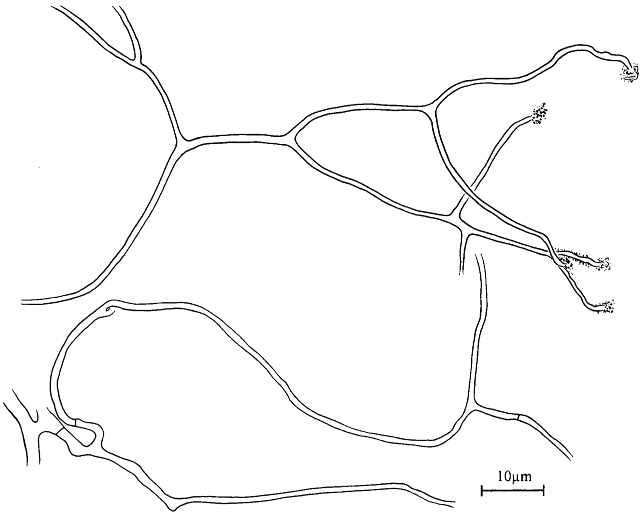

Fig. 3 *Pinirhiza multifurcata*. Emanating hyphae originated from the multiply ramified hyphae of outer mantle, with simple septa, and frequently ramified, Y-shaped emanating hyphae with triangular inflation at ramification points without septa

colorless, surface smooth, mostly straight, cylindrical or occasionally with elbow-like structures or irregular inflations, not constricted at septa, septa simple, very infrequent, clamps lacking, and septa slightly thinner than hyphal walls; most hyphae without septa; ramifications frequent, with 1–2 side branches, occasionally polytomies with three branches (Fig. 1b); ramification rectangular to Y-shaped with triangular inflations at points of ramification; hyphal dimensions very variable, (1)1.5–2.5(3.5) µm diam., slightly thick-walled, cell walls 0.3–0.8 µm; and no simple emanating hyphae found. *Cystidia* lacking, but emanating hyphae might be considered as cystidia due to their extraordinary type of ramification, their rather thick walls in comparison to their diam., and the almost lacking septa.

Color reactions with different reagents (preparations of mantle) Melzer's reagent: emanating hyphae (cystidia) dextrinoid; lactic acid: n. r.; KOH: n. r.; $FeSO_4$: n. r.

Anatomical characters of longitudinal section Mantle plectenchymatous, 5–10 µm wide. Mantle of very tip plectenchymatous, 5–10 µm wide. Tannin cells in 1 row, irregularly tangentially cylindrical. Cortical cells in 2–3 rows, radially oval to roundish, and 2–3 rows with Hartig net. Hartig net around tannin cells and cortical cells in 1–2 rows, palmetti-like in plan view, lobes 1–2 (3.5) µm broad.

Reference specimen The mycorrhiza was collected in a pure *P. tabulaeformis* forest (altitude, 1,700–2,300 m; precipitation, 200–400 mm/year, calcareous soil) at Helan Mountain, Suyukou National Reserve located in Yinchuan City, Ningxia Hui National Autonomous Region, China, myc. exc. and isol. by Jie Wei, 20.08.2007, JW 54a (in M). GenBank sequence accession numbers: GU269908 (nLSU), GU269910 (ITS).

Pinirhiza nondextrinoidea

Morphological characters (Fig. 4a) Mycorrhizal systems dichotomous, with 0–3 orders of ramification, in small numbers, main axis 0.4–0.45 mm diam., hydrophilic, of short distance exploration type or of the smooth subtype of medium distance exploration type. *Unramified ends* straight, cylindric, not inflated, 0.4–0.6 mm long, 0.35–0.4 mm diam., young parts cinnamon–brownish, older parts black, mantle surface with many soil particles, densely wooly. *Emanating hyphae* abundant, not specifically distributed. *Cystidia* not distinct under stereoscope magnification. *Rhizomorphs* infrequent, yellowish, 0.04–0.07 mm diam., no side branches observed, margin densely wooly. *Sclerotia* not observed.

Anatomical characters of mantle in plan views (Figs. 5a, b) Plectenchymatous throughout, hyphae in all layers colorless and clampless. *Outer mantle layers* (Fig. 5a) plectenchymatous with squarrosely branched hyphae and with a matrix (mantle type E/C, according to Agerer 1987–2008, 1991; Agerer and Rambold 2004–2009), emanating hyphae originating from these squarrosely branched hyphae; hyphae 1.5–3 µm diam., cell walls 0.3 µm; all hyphae with simple septa, in few hyphae septa with a large pore discernible (Fig. 5a); hyphae smooth; mantle surface covered by many soil particles. *Inner mantle layers* (Fig. 5b) densely plectenchymatous, hyphae frequently ramified, 2–3 µm diam., cell walls 0.3 µm, with simple septa, surface smooth. *Very tip* similar to remaining parts of the mantle.

Anatomical characters of emanating elements (Figs. 4b–f) Rhizomorphs (Figs. 4e, f) infrequent, with gelatinous matrix, not differentiated (type A/B according to Agerer 1987–2008, 1991; Agerer and Rambold 2004–2009); surface of rhizomorphs with many soil particles; in diameter and features all hyphae similar to emanating hyphae, very thin, 1–2 µm diam., thick-walled, cell wall 0.3–0.5 µm, colorless and clampless; most hyphae with a smooth surface. *Emanating hyphae* (Fig. 4b–d) abundant, colorless, thick-walled, thin, of variable diam., 1–2 µm, cell walls 0.3–0.5 µm; hyphae mostly straight, cylindrical, not constricted at septa, with infrequent, simple septa, septa as thick as hyphal walls; occasionally irregular inflations and elbow-like structures present; ramifications infrequent, Y-shaped or nearly rectangular, with triangular inflations at the points of ramification; surface of most hyphae smooth and gelatinous, with some soil particles, small crystals present on few hyphae; only few hyphae with ramified ends observed. *Cystidia* lacking.

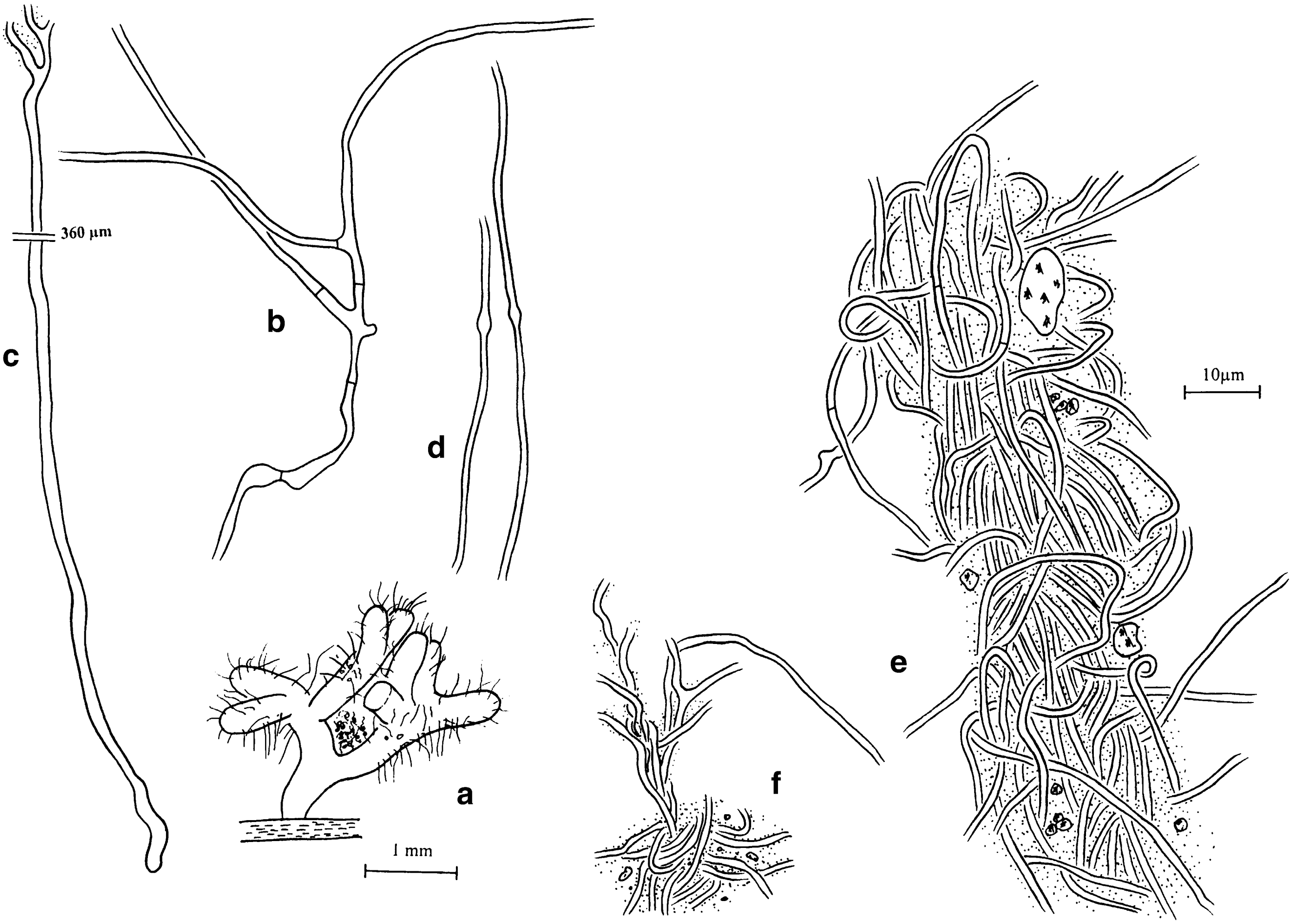

Fig. 4 *Pinirhiza nondextrinoidea*. **a** Habit of ectomycorrhiza, surface of mantle densely wooly. **b** Emanating hyphae with Y-shaped ramification and inflated at ramified points, septa simple. **c** Emanating hyphae with a ramified end. **d** Emanating hyphae with inflations. **e** Rhizomorph composed of uniform hyphae with gelatinous matrix covered with many soil particles. **f** Some inner hyphae of rhizomorph having the same diameter as remaining hyphae

Color reactions with different reagents (preparations of mantle) Melzer's reagent: n.r.; lactic acid: n. r.; KOH: n. r.; $FeSO_4$: n. r.

Anatomical characters of longitudinal section Mantle plectenchymatous, 9–22 µm wide. Mantle of very tip plectenchymatous, 15–20 µm wide. Tannin cells in 1–2 rows, oval to irregularly tangentially cylindrical. Cortical cells in 2–3 rows, oval to roundish to sometimes tangentially elongated, and 2–3 rows with Hartig net. Hartig net around tannin cells and cortical cells in 1–2 rows, at places reaching the endodermis, globular protrusions occasionally observed in cortical cells, palmetti-like in plan view, lobes 1–2.5 µm broad.

Reference specimen The mycorrhiza was collected in a pure *P. tabulaeformis* stand (altitude, 700–800 m; precipitation, ca. 500–600 mm/year, calcareous soil) in Heilihe National Reserve, Chi Feng City, Inner Mongolia, China, myc. exc. and isol. by Jie Wei, 08.09.2008, JW 185b (in M). GenBank sequence accession numbers: GU269909 (nLSU), GU269911 (ITS).

Sequence analyses

Topology of nLSU RAxML and PAUPRat trees

The likelihood of the most likely tree found was −2,976.528891, and the substitution rates estimated by RAxML were A↔C, 1.467049; A↔G, 5.056997; A↔T, 0.706806; C↔G, 0.641633; C↔T, 14.877343; and G↔T, 1. Of the 569 reliably alignable positions of the nLSU Alignment, 215 were variable, and 166 were parsimony informative. The 50% Majority Rule Consensus Tree was calculated of the 8944 most parsimonious trees.

The topologies of both the RAxML (Fig. 6) and PAUPRat trees (Fig. 7 as supplementary file) as generated by analyses of nLSU are largely concordant. The RAxML

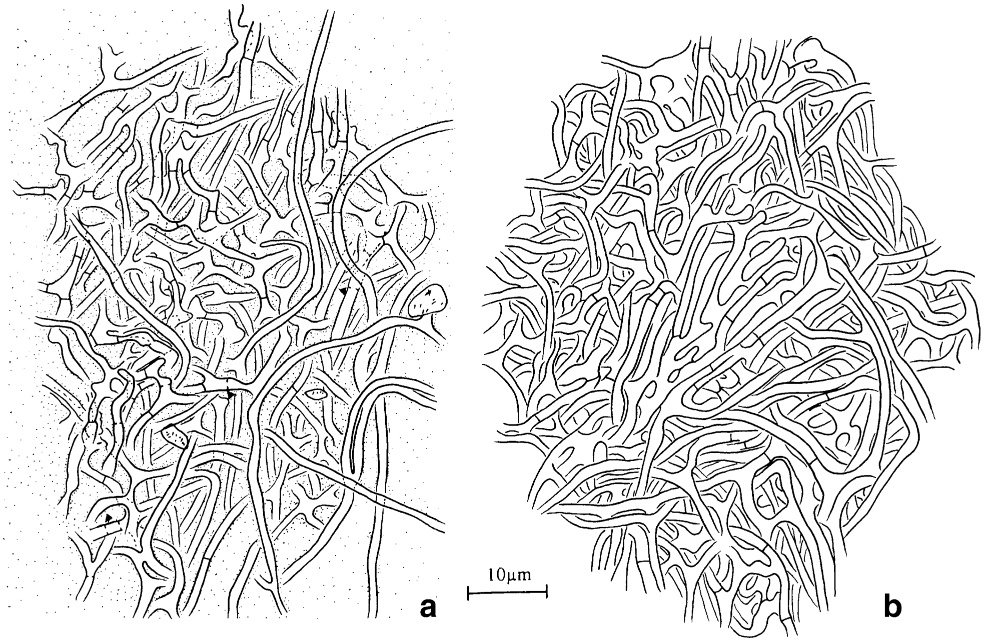

Fig. 5 *Pinirhiza nondextrinoidea*. **a** Outer mantle layer with squarrosely ramified hyphae in a gelatinous matrix gluing with some soil particles, some emanating hyphae originated from these squarrosely ramified hyphae, some septa with a large pore discernible (arrow heads). **b** Plectenchymatous inner mantle layer

tree indicates that the genus *Sebacina* is polyphyletic. It could be basically splitted into two clades I and II, with 97% and 100% BS support, respectively. Clade I comprises the *Sebacina*/*Tremellodendron* complex which includes mainly sebacinoid OM such as with *Neottia nidus-avis*, *Hexalectris spicata*, and *Epipactis helleborine*, few sebacinoid ECM, the *S. incrustans* (type species)—a ECM fungus (Urban et al. 2003; Tedersoo et al. 2006), as well as two *Tremellodendron* species and one ERM with 95% BS, in addition two *Craterocolla cerasi* isolates and three *Chaetospermum camelliae* isolates. Two *C. cerasi* isolates form a sister clade to the *Sebacina*/*Tremellodendron* complex with moderate support (54% BS), three *C. camelliae* isolates are again a sister clade to the *Sebacina*/*Tremellodendron* complex and two *C. cerasi* isolates receiving moderate support (66% BS). Clade II comprises *Sebacina vermifera* as well as some ERM, *Pyriformospora indica* and a *Rhizoctonia* sp. Two *Tremella* species represent the outgroup.

Molecular-phylogenetic position of P. multifurcata and P. nondextrinoidea

The RAxML tree (Fig. 6) shows that *P. multifurcata* and *P. nondextrinoidea* are nested in the *Sebacina–Tremellodendron* complex in clade I. They are located in different subclades, both of which received no significant bootstrap support, however. *P. multifurcata* is very close to an ECM of *Dryas octopetala* (AY452681) with 99% BS support. *P. nondextrinoidea* clusters with an ECM of Sebacinaceae (AM161532) with (55% BS).

ITS sequence comparisons of P. multifurcata and P. nondextrinoidea with sequences obtained from GenBank and UNITE

Molecular phylogenetic analyses of ITS sequences are not meaningful because only two fully identified sequences occurred among the first 100 most similar sequences each obtained in megablast searches in GenBank with our ECM sequences as query. Those identified sequences were not the most similar entities compared to our ECM sequences, however. The first 100 matches in GenBank have at least 89% query coverage and at least 90% maximum identity with the sequences of *P. multifurcata* and *P. nondextrinoidea* and belong nearly exclusively to the order Sebacinales (except for a *Tomentella* ECM (EU668944) and few unclassified, uncultured ECM) including mostly uncultured sebacinoid ECM, sebacinoid OM, and few Sebacinales isolates.

The best matches regarding the sequence of *P. multifurcata* are two uncultured sebacinoid ECM (EF218817 and EU645627) and two sebacinoid OM (AF440651 and AF440656) with 97% identity and 100% query coverage each. The only identified species among the 100 most similar sequences is *Sebacina* aff. *epigaea* (AF490393) that shows 95% maximum identity and 95% query coverage. The sequence of *P. nondextrinoidea* is most similar to three uncultured sebacinoid ECM (FJ210755, FJ196964, and AM161532) with 96% identity and at least 98% query coverage. The two to species level identified isolates among its 100 best matches represent *Sebacina epigaea* (AJ966754) and *S.* aff. *epigaea* (AF490393) that show 95%

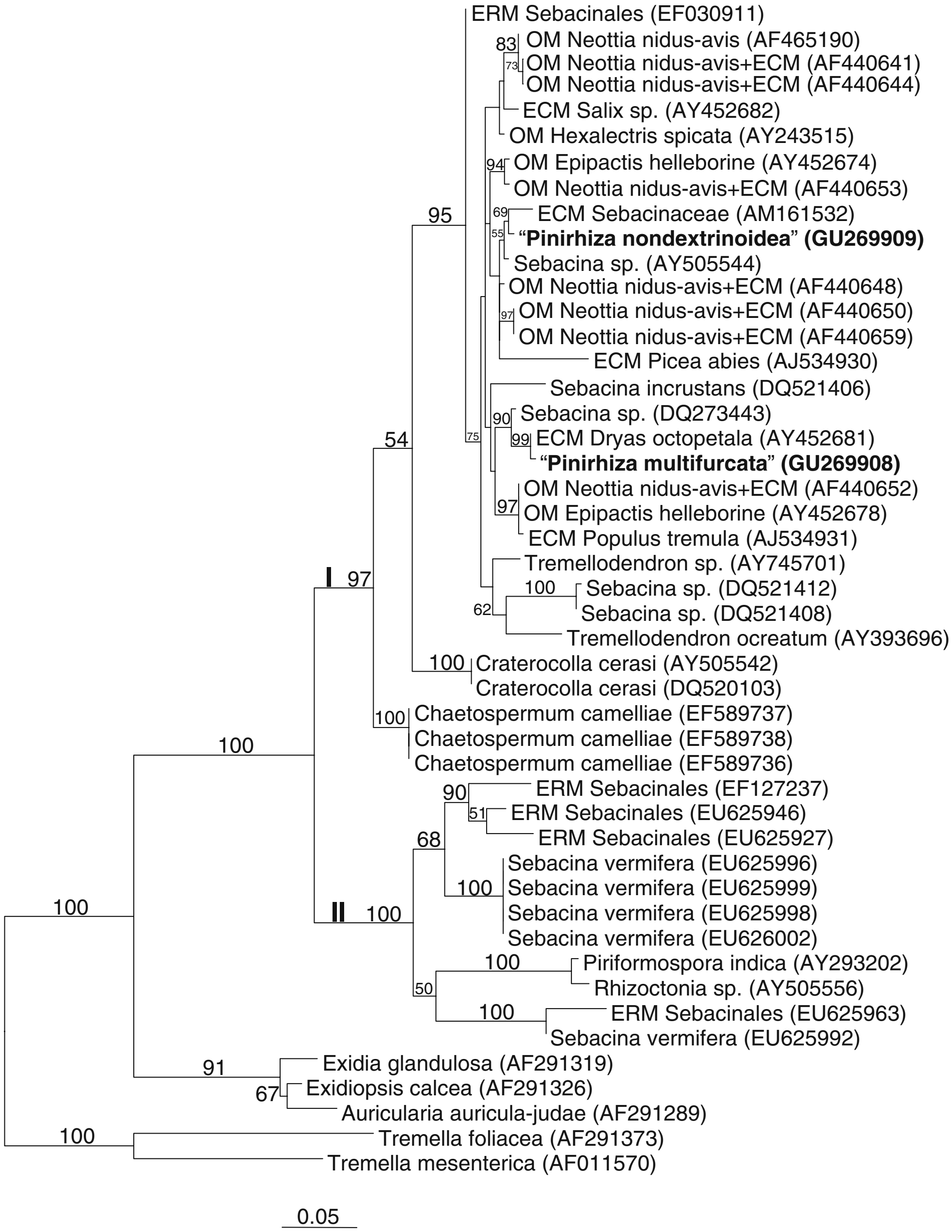

Fig. 6 Molecular-phylogenetic-placement of *Pinirhiza multifurcata* and *Pinirhiza nondextrinoidea* among selected Sebacinales inferred by the RAxML analysis of nLSU sequences. Bootstrap support values above 50% are noted above or left of the respective branches. GenBank accession numbers are given in parentheses following the species names

maximum identity and 90% query coverage, and 91% maximum identity and 100% query coverage, respectively.

Sequences obtained from UNITE using BlastN belong to different genera such as *Sebacina*, *Tomentella*, *Mycena*, *Amanita*, *Cortinarius*, and *Russula*. The sequence of *P. multifurcata* is most similar to two *Sebacina* spp. from Denmark (UDB00073 and UDB00074) with 89% (685 score, 0 *E* value) and 88% (626 score, e-180 *E* value) similarity, respectively. The similarity scores of sequences from other genera are even worse (at most 270 score) compared with the sequence of *P. multifurcata*. The sequence of *P. nondextrinoidea* is most similar to that of *S. epigaea* from Estonia (UDB000975) with 95% (832 score, 0 *E* value) similarity, while sequences of members of other genera have clearly lower (at most 268 score) similarity values.

Discussion

Morpho-anatomical features

P. multifurcata and *P. nondextrinoidea* share a number of features. They are hydrophilic; all hyphae have simple septa and are colorless, multiply ramified hyphae are present at least in the outer mantle. The outer mantle layer and its surface show a gelatinous matrix. The emanating hyphae are thick-walled, variable in diameter (1–3.5 µm in *P. multifurcata* and 1–2 µm in *P. nondextrinoidea*), infrequently simple septate, and have triangular inflations at their points of ramification. Nevertheless, *P. multifurcata* and *P. nondextrinoidea* can easily be distinguished from each other. *P. multifurcata* is grayish orange-brown with a bumpy surface that is loosely covered by emanating hyphae, whereas *P. nondextrinoidea* is cinnamon-like ochre brown, has an even surface, and a densely wooly coverage of emanating hyphae. Anatomically, *P. multifurcata* differs from *P. nondextrinoidea* in having thick cells in outer and middle mantle layers and by the lack of rhizomorphs. Emanating hyphae of *P. multifurcata* ramify frequently bifurcately and range from 1 to 3.5 µm in diameter, whereas those of *P. nondextrinoidea* have infrequent ramifications and are between 1 and 2 µm wide. In addition, exclusively, the emanating hyphae of *P. multifurcata* are dextrinoid.

P. multifurcata and *P. nondextrinoidea* are similar to three sebacinoid ECM that have been described in detail: *S. incrustans* (Pers.) Tul. and C. Tul. on *P. abies*, *Sebacinoid* sp. on *Tilia* sp. (Urban et al. 2003), and *Q. dendrohyphidiomorpha* on *Q. suber* (Azul et al. 2006). They can be separated according to their anatomical features, however. *P. multifurcata* differs from the ECM of *Sebacinoid* sp. in the lack of a pseudoparenchymatous outer mantle, in mostly bifurcate ramifications in emanating hyphae rather than polytomies as in the latter, and thinner cell walls of emanating hyphae (*P. multifurcata*: 0.3–0.5 µm, exceptionally up to 0.8 µm; *Sebacinoid* sp.: up to 1.3 µm). From the *S. incrustans* ECM *P. multifurcata* differs by the lack of a superficial hyphal net of thick-walled, lobed, and frequently branched hyphae, from *Q. dendrohyphidiomorpha* in having a plectenchymatous rather than a pseudoparenchymatous middle mantle layer.

P. nondextrinoidea can be distinguished from all these other sebacinoid ECM by the presence of rhizomorphs. Furthermore, it differs from the ECM of *S. incrustans* by thinner-walled (0.3–0.5 µm versus 0.8 µm) emanating hyphae and the lack of a superficial net consisting of frequently branched thick cells (2.5–6 µm) that are present in *S. incrustans* ECM. It differs from ECM of the *Sebacinoid* sp. by lacking the pseudoparenchymatous outer mantle and rather thin-walled emanating hyphae (0.3–0.5 µm versus up to 1.3 µm). In addition, the emanating hyphae of *P. nondextrinoidea* are not dextrinoid unlike the slightly dextrinoid ones in *Q. dendrohyphidiomorpha*. This chemical reaction was not checked in *S. incrustans* ECM and *Sebacinoid* sp. ECM (Urban et al. 2003).

Concerning the emanating hyphae which are frequently ramified and clampless, *P. multifurcata*, *S. incrustans* ECM, ECM of *Sebacinoid* sp., and *Quercirhiza dendrohyphidiomorpha* are similar to two *Trichophaea* ECM, *Pinirhiza trichophaeoides* (Wei et al. 2009) and *Quercirhiza quadratum* (Águeda et al. 2008). With the exception of the ECM of *Sebacinoid* sp. that forms a pseudoparenchymatous outer mantle with epidermoid cell, the sebacinoid ECM have plectenchymatous outer mantles, *Trichophaea* ECM have angular cells in the outer mantle. Emanating hyphae of these sebacinoid ECM are colorless, generally smooth, and mostly with Y-shaped or trifurcate ramifications and at least those of *P. multifurcata* and *Quercirhiza dendrohyphidiomorpha* are dextrinoid. The emanating hyphae of the two *Trichophaea* ECM are partly warty, brownish, have mostly rectangular ramifications, and are not dextrinoid.

Molecular-phylogenetic analyses

The two well supported clades of Sebacinales in the RAxML tree generated by the analysis of nLSU (Fig. 6) concur with the results of Weiß and Oberwinkler (2001), Urban et al. (2003), Weiß et al. (2004), and Selosse et al. (2009). *P. multifurcata* and *P. nondextrinoidea* are members of the Sebacinales, because both clusters in the *Sebacina*/*Tremellodendron* complex in clade I with good support (95% BS), and they appear as two different species according to the RAxML tree.

Our two *Pinirhiza* collections do not belong to the ERM fungi complex (at most 88% identity and 100% query coverage compared with the sequences of *Pinirhiza* spp.) in the nLSU tree (clade II). This corresponds well with the results of ITS-sequence comparisons in which no sequences of *S. vermifera*/ERM fungi were among the most similar sequences obtained by megablast search using our sequences as queries.

Due to the lack of species names of included sequences and the insufficient BS support of the clades *P. multifurcata* and *P. nondextrinoidea* are positioned in, the assignment of the two *Pinirhiza* isolates described here to fungal genera or species is impossible to date, however.

Although the sequence of *P. multifurcata* is very similar to a sequence of a Sebacinaceae ECM of *D. octopetala* (99% BS), it keeps unknown according to our present knowledge whether both fungal partners of *P. multifurcata* and of *D. octopetala* ECM are the same species. *P. nondextrinoidea* is placed in a very weakly supported clade (55% BS) with a sebacinoid ECM on *Fagus sylvatica*. As identification to species-level is impossible, the two ECM for the present are named with artificial binomina as has frequently been done for unidentified, comprehensively described ECM since Gronbach and Agerer (1986).

The presence of two ECM of Sebacinales in China support the suggestion that the Sebacinales have a wide geographical distribution and host spectrum (Weiß et al. 2004).

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