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Structural characterization and molecular identification of arbuscular mycorrhiza morphotypes of *Alzatea verticillata* (Alzateaceae), a prominent tree in the tropical mountain rain forest of South Ecuador

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Abstract The vast majority of the highly diverse trees in the tropical mountain rain forest of South Ecuador form arbuscular mycorrhizas, and previous molecular investigations revealed a high diversity of fungi. In this study, we present a first trial to link fungal DNA-sequences with defined morphotypes characterized on the basis of partly new mycelial features obtained from field material of one tree species, *Alzatea verticillata*. Fine roots were halved lengthwise to study the mycelium anatomy on one half and to obtain fungal nuclear rDNA coding for the small subunit rRNA of *Glomeromycota* from the other half. Light microscopy revealed conspicuously large amounts of mycelium attaching to the surface of the rootlets. The mycelium formed fine- or large-branched appressoria-like plates, vesicles of regular or irregular shape, and very fine, multibranched structures ensheathed by septate hyphae. These previously undescribed features of the supraradical mycelia combined with intraradical mycelium structures were used for distinguishing of four main morphogroups and subordinate 14 morphotypes. DNA sequences of *Glomus* group A, *Acaulospora* and *Gigaspora*, were obtained and linked to three morphogroups. Two sequence types within *Glomus* group A could be tentatively associated to subordinate morphotypes.

Keywords *Alzatea verticillata* · Arbuscular mycorrhiza morphotypes · Fine and medium endophyte · nucSSU · Supraradical mycelium

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Introduction

Arbuscular mycorrhizas (AM) are formed by more than 80% of land plants including most tropical tree species (Alexander and Lee 2005). Based on DNA sequence types, tropical trees in the lowland forest of Panama (Husband et al. 2002), the dry African mountain forest (Wubet et al. 2006), and the tropical mountain rain forest of southern Ecuador (Kottke et al. 2007a, b) were found to harbor an exceptional richness in AM fungi (*Glomeromycota*; Schüßler et al. 2001). DNA sequences were mostly not identical with those species described so far. Light microscopic investigations carried out on mycorrhizas sampled in the tropical mountain rain forest of South Ecuador yielded new insights on structural features of the associated fungi. A new type of AM displaying intercellular hyphal characters reminiscent of Hartig net structures was described for *Alzatea verticillata* Ruiz and Pavon (Alzateaceae) by us recently (Beck et al. 2005). Prominent supraradical mycelia displaying distinctive characters not described so far were observed on AM sampled from several tree species in the mountain rain forest of South Ecuador. Large amounts of mycelium attaching to the root surface was not considered in previous studies, probably because it was not observed on mycorrhizas sampled in temperate climate areas. Alexander (1989), in a survey on mycorrhizas in tropical forests, mentioned AM with surfaces obscured by hyphae and hyphal fans, but no further details were given. We

presumed that a combination of distinctive characters of the supraradical mycelium with intraradical mycelial structures might probably be suited for morphotyping of AM, and distinguished morphotypes could probably be linked to specific DNA sequences. Morphological characterization of ectomycorrhizas by mycelial characters and identification of fungi by DNA sequencing carried out in parallel was successfully applied in field studies (Haug 2002; Haug et al. 2005). To test our assumption, we performed comparative microscopic studies of a number of AM of *A. verticillata* sampled in the tropical mountain rain forest in South Ecuador. In parallel, DNA was extracted from the individual other halves of the mycorrhizas and fungal nuclear rDNA coding for the small subunit rRNA (nucSSU) of *Glomeromycota* sequenced.

Materials and methods

Investigation site

The study area is situated at 1,820 to 2,400 m a.s.l. on the eastern slope of the Cordillera El Consuelo, which is part of the eastern chain of the Andes in South Ecuador. The protected forest belongs to the Reserva Biológica San Francisco, bordering the Podocarpus National Park, half way between Loja and Zamora, Loja-Chinchipe province (3° 58′S, 79°04′W). The mountain ridges and the steep slopes are covered by a primary tropical mountain rain forest extraordinarily rich in tree species (Homeier 2004, Homeier et al. 2007). *A. verticillata* (Alzateaceae) is one of the few frequent trees occurring scattered along the mountain ridge between 1,900 and 2,000 m a.s.l., forming 15- to 20-m-tall trees accounting for 15% of stem diameter in this part of the forest (Homeier 2004), and giving name to the Alzateetum verticillatae (Bussmann 2002). The trees form stilt roots. Following these roots, mycorrhizas can easily be sampled from the top soil layer. *A. verticillata* and the trees investigated for comparison of mycorrhizal features root in a 30- to 50-cm-deep, pure organic layer, which is structurally and chemically rather homogeneous throughout this forest type. Chemical soil analyses showed low nutrient availability especially concerning phosphate (Wilcke et al. 2002), a finding that correlates well with the high mycorrhization rate by *Glomeromycota* (Kottke et al. 2004, 2007a, b).

Sampling of mycorrhizas

Fine roots were sampled at 1,950 to 2,180 m a.s.l. from 22 individuals of *A. verticillata* in April 2001 and August 2003 and 2004. Trees had been identified and labeled by J. Homeier (Homeier 2004). Clusters of fine roots, two to six per individual tree, were sampled from the upper humus layer (2- to 10-cm depth) by following the stilt roots. The roots were cleaned in tap water and processed on the day of sampling. A large number of undamaged 0.5- to 2.5-cm-long mycorrhizal systems were selected using a compound microscope and were conserved in 50% ethanol for anatomical studies of mycorrhizal features. A smaller number of the fine roots were halved longitudinally, one root half was conserved in 50% ethanol for microscopic studies, the other root half was dried at 60°C for DNA extraction (Fig. S1).

Staining and preparation of individual mycorrhizas for microscopic investigation

As the roots of *A. verticillata* were frequently dark pigmented, observation of the stained mycelium was only possible after clearing. The last-order fine roots (diameter ≤1.5 mm and up to 4 cm in length) were cut into 1.5- to 2.5-cm pieces, cleared in 10% KOH for 1 to 3 days at 65°C in a water bath, time depending on clearing success. Still dark roots were additionally treated by 15% H_2O_2 up to 15 min at room temperature. Roots were then washed twice in tap water, acidified by 10% HCl for 2 min, and stained in 0.05% methyl blue in 90% lactic acid for 2 to 4 h at 65°C (Grace and Stribley 1991, modified). Less dark colored roots treated only for 1 day with KOH gave better staining of the intraradical mycelia of morphogroups II, III, and IV than those treated for 3 days (Tables 1, 2 and 3), but staining intensity of the supraradical mycelium was not influenced.

Using a compound microscope at magnification 20- to 60-fold, the roots were first observed for stained mycelium covering the surface (Fig. S2a). The tissue layers of these roots were then carefully separated from each other by the use of very fine, sharp needles. The epidermal and hypodermal layers, tightly connected in *A. verticillata* roots, were separated from the inner tissue layers. Parts of this two-layered outer tissue were turned around to observe parts from the outer and other parts from the inner side. Care was taken to preserve hyphal entry points and supraradical mycelia of 3- to 5-mm^2-large undisturbed areas. The loosely connected thin-walled cells of the inner cortical layers were separated and placed beside each other as well. Care was taken that cell layers did not obscure the microscopic view on the intraradical hyphae. Mycelia were observed at magnifications 160-, 400-, and 1,000-fold. Photographs were taken by a digital camera (COOLPIX 995). Drawings were carried out by pencil and with the help of a *camera lucida*. Drawings were later scanned and contrast improved using Photoshop.

Altogether, 362 mycorrhizas of a total length of 4 m of *A. verticillata* were studied microscopically. The investi-

gated roots were deposited on slides in lactic acid 90% as vouchers.

Terminology to define structures

The hyphae adhering to the root surface were termed *supraradical mycelium* (Fig. S2a) to distinguish these structures from the extraradical mycelium spreading in the soil (Beck et al. 2005). The supraradical mycelium forms thick- or thin-walled swellings of regular or irregular form, termed *supraradical vesicles* (Figs. S2g and S3d), to distinguish them from the intraradical vesicles. Finest-branched structures formed by the supraradical mycelium often ensheathed by brownish, septate hyphae were termed *fine multibranched structures* (Figs. S2h–k and S3d, e). The supraradical mycelium forms distinct, branched adhering plates, termed *appressoria-like plates* (Figs. S2b–f and S3a–c). The term *besom-like branching* was given for hyphae that branch off from one point at the supraradical mycelium (Figs. 13a and S2a). Finger-like branched hyphae between the inner cortical layers were termed *intercellular appressoria* (Figs. 7, 8 and 9 below; Beck et al. 2005). The term *H-junction* was used for hyphal connections between hyphae in the intercellular space (Figs. 11, 12, 13, 14, 15, 16 and 17). Abbott and Robson (1979) distinguished between H-, Y-, and S-junctions. We observed Y-junctions always together with H-junctions and therefore noted only the latter term. S-junctions, defined by Abbott and Robson (1979) as swellings of interconnecting hyphae within the intercellular space, were termed *broadened* hyphal junctions. The term *peg-like processes* was first used by Merryweather and Fitter (1998) to describe very short branches of intraradical hyphae. We used this term for very short branches of the intraradical hyphae and for the supraradical mycelium, including angular projections, which may be residues of collapsed hyphae (Butler 1939; Mosse 1959; Nicolson 1959).

Defining and presenting AM morphotypes

We defined a morphotype when the mycelium could be observed from outside the root via the entry point up to intraradical colonization to ensure that a fungus formed both supraradical and intraradical structures, including arbuscules. A morphotype was defined when hyphal characteristics in specific combination of features were observed repeatedly within one mycorrhiza and mostly in a number of mycorrhizas (5–36). Some rare morphotypes (e.g., IV.7), however, showed such distinct characters that we easily separated them from other morphotypes. In case of transitions among hyphal features, the variations were given (e.g., in morphotype II.1 and IV.3). Distinguishing characters of morphogroups and morphotypes were compiled in Tables 1, 2 and 3, and a list of the features used is given in Table S1. The morphotypes are illustrated by drawings at similar magnification (Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 and 17). Supraradical mycelium structures are shown on top, coils in outer cortex in the middle, and hyphae in the inner cortex, including arbuscules, at the bottom of each drawing. Root cells were not displayed because this would have obscured the drawings.

Processing of fungal DNA sequences

We chose 47 dried, 1.5- to 2.5-cm-long fine roots from which microscopic studies of the respective other root half confirmed colonization and distinguishable morphotypes (Fig. S1). DNA was isolated from the dried mycorrhizal samples using the DNAeasy Plant Mini Kit (Qiagen, Hilden, Germany). Part of the nucSSU of the AMF was amplified by the polymerase chain reaction (PCR), and a nested PCR was carried out (electronic appendix Table S3). The PCR reaction was optimized by adding 0.2 µl of 1% BSA (bovine serum albumine; Sigma) to the reaction volume. Primers and PCR-design are given in the electronic appendix (Tables S2 and S3). PCR products were purified using the QIAquick PCR purification kit (Qiagen). If direct sequencing failed, products were cloned using the pCR 2.1-Topo vector system (Invitrogen). Inserts were reamplified from clones using the M13 forward and reverse primers by picking 4–12 bacterial clones with a toothpick and placing them directly into the PCR reaction mixture and were subsequently sequenced. Cycle sequencing was conducted following the protocols given in Haug et al. (2005). Sequences were screened for possible chimerical origin using the program Pintail (Ashelford et al. 2005) and blasting parts of the sequences. Sequences were deposited at the National Centre for Biotechnology Information (NCBI, GenBank; http://www.ncbi.nlm.nih.gov).

Phylogenetic analysis

Sequence length was variable according to different primer combinations. The alignment and the phylogenetic calculation were done on the basis of the shortest sequence length of 738 bp.

Sequence similarities were determined using the BLAST (Basic Local Alignment Search Tool) sequence similarity search tool (Altschul et al. 1997) provided by NCBI (http://www.ncbi.nlm.nih.gov). Only one sequence was included in the final tree when several inserts of a cloned PCR product were very similar and appeared together in a terminal cluster.

SSU sequences were aligned with BLAST matches of ≥98% identity and with some representative sequences of identified *Glomeromycota* from the GenBank. We included the closest BLAST matches for the unknown fungus, which

Table 1 Characteristics and frequency of AM morphogroup I and II and subordinate morphotypes associated with *A. verticillata*

	Morphogroup I			Morphogroup II		
	Uniform faint hyphae with lobed vesicles; Figs. 1, 2 and 3	Morphotype I.1; Fig. 2	Morphotype I.2; Fig. 3	Large hyphae with auxiliary cells; Figs. 4, 5 and 6	Morphotype II.1: two variations (var1, var2); Figs. 4 and 6	Morphotype II.2; Figs. 5 and 6
Supraradical mycelium						
Staining intensity	Uniform faint, rarely uniform medium, never granular	See I	See I	Dark, medium or faint and granular	See II	Uniform dark/ medium or faint and granular
Diameter of last branches	Mostly <4.5 µm	Inconspicuous hyphae <4.5 µm, swollen parts 12–18 µm	<4.5 µm	4.5–12 µm, rarely up to 18 µm	Var1: 4.5–18 µm; var2: 4.5–10 µm; var1, 2: equal hyphal width at long area	10–20 µm
Hyphal wall	Medium staining hyphae with 0,8–1,2 µm distinctly lined cell wall	See I	See I	Often multilayered cell wall	See II	4–7 µm, distinctly lined, multilayered
Branched appressoria-like plates (bap) entry points (ep)	bap frequently, wide and fine branched; ep below simple appressoria or below wider bap	See I	See I	bap frequent; ep below simple or slightly irregular plates	See II	See II
Shape; besom-like branching (blb)	Sometimes blb with thick or finest branches of 1 µm in diameter	Additional swollen hyphae with coral-like branching at branching angles 45°–70°	See I	blb observed only in morphotype II.1 var1	Var1: branching base broadened, sometimes blb; var2: no blb, branching base not broadened	Often irregular outline with small ripples; no blb observed
Supraradical vesicles; frequency; staining intensity; shape	Few; uniform faint; mostly regular shape, rarely irregular lobes	Few; uniform faint; regular shape	Few; uniform faint; regular shape	–	–	–
Auxilliary cells	–	–	–	In groups, with concavities or spines or 1 spine on concavity	See II	?
Fine, multibranched structures	Frequent	See I	See I	–	–	–
Hyphal coils in outer cortical layer below entry point						
Width	6–12 µm	8–12 µm	7–9 µm	5–12 µm	see II	8–12 µm

Staining Intensity	Faint or medium	Faint	Medium	Faint or medium	Faint or medium; var1: if irregularly branched, dark with hyaline outer layer	Faint or medium
Shape	Regular width, no branches	See I	See I	Regular width, no branches, rarely irregular with few branches	Var1: no branches, some with few branches and irregular; var2: branchless	Regular width, no branches
Frequency	Frequently many, rarely few coils	Few	Numerous	Few	See II	See II
Intercellular hyphae in inner cortical layers						
Width	4–8 μm	See I	See I	4–8 (10) μm	See II	6–10 μm
Staining intensity*	Faint or medium	See I	Medium or faint	Medium (faint to very faint)	See II	See II
H-junctions	Few or missing	See I	See I	Few or missing	See II	See II
Branching base of junctions	Not broadened	See I	See I	Not broadened	See II	See II
Shape; peg-like processes (pp) and further features	Smooth, no or few pp	See I	See I	Knobby, with broad pp, rarely intercellular appressoria	See II; broad intercellular appressoria only in var1	See II
Vesicles						
Staining intensity	Faint or medium	See I	Medium	–	–	–
Frequency	Numerous, few or missing	Few	Numerous	–	–	–
Location in inner cortex and position at entry point (ep)	Inter- or intracellular, distributed along root, rarely numerous near ep	Inter- or intracellular, not numerous near ep	Inter- or intracellular, often numerous near ep	–	–	–
Size	50–180 μm	50–100 μm	Often 100–180 μm	–	–	–
Shape	Regular oval or rectangular or irregularly lobed	Regular or with small lobes	Irregularly lobed, large lobes enter cells	–	–	–
Vesicle wall	≤2 μm	See I	See I	–	–	–
Arbuscules	Branches tapering progressively, often colonizing entire cell	See I	See I	Trunk curved, branches taper abruptly, not colonizing entire cell	See II	See II
Colonization pattern	Mostly patchy, rarely continuous	Patchy	Patchy or continuous	Continuous	See II	See II
Observed in number of roots	169	8	17	51	Var1: 36; var2: 10	5

*In brackets: staining intensity after 2–3 days KOH treatment

Table 2 Characteristics and frequency of AM morphogroup III and the three subordinate morphotypes associated with *A. verticillata*

	Morphogroup III			
	Fine and medium hyphae; Figs. 7, 8 and 9	Morphotype III.1 fine hyphae; Fig. 7	Morphotype III.2 medium hyphae; Fig. 8	Morphotype III.3 medium hyphae; Fig. 9
Supraradical mycelium				
Staining Intensity	Basal hyphae dark, often with hyaline outer layer, last branches dark, medium or faint and granular	See III	See III	See III
Diameter of last branches	Frequently 1–2 µm	<1 µm	1–2 µm	Frequently 2–4.5 µm
Hyphal wall	Often with hyaline outer layer	See III	See III	See III
Branched appressoria-like plates (bap) and entry points (ep)	bap frequent, with wide and fine branches, dark blue with hyaline outer layer or medium or faint and granular; ep below simple appressoria or next to bap	See III	8 of 15 mycorrhizas with many 1-µm fine bap, some wider bap	See III; bap frequently about 100 µm, mostly with wider branches
Shape; peg-like processes (pp)	Highly branched, sometimes besom-like branching, often irregular outline; pp numerous	See III	See III	See III; rounded, knobby shape
Supraradical vesicles: frequency; staining intensity; shape	Mostly frequent; dark, often with hyaline outer layer, medium or faint and granular; regular or irregular shape	See III	See III	See III
Auxiliary cells	–	–	–	–
Fine, multibranched structures	–	–	–	–
Hyphal coils in outer cortical layer below entry point				
Width	1.5–5 µm	1.5–3 µm	3–5 µm	3–5 µm
Staining Intensity	Dark with hyaline outer layer	See III	See III	See III
Shape	Irregular knobby with many short swollen branches or hyphal swellings	See III; many prominent intercalary or lateral swellings ≤9 µm	See III; hyphal swellings slight	See III; hyphal swellings slight
Frequency	Few or numerous	Few	Few	Numerous
Intercellular hyphae in inner cortical layers				
Width	0.8–3 µm	0.8–1.5 µm	1–3 µm	1–3 µm
Staining Intensity*	Dark or medium (medium to faint)	Very dark (medium)	See III	See III
H-junctions	Numerous	See III	See III	See III
Branching base of junctions	Slightly broadened	See III	See III	See III
Shape; Intercellular appressoria (ia); peg-like processes (pp)	Irregular, fine fan-shaped hyphal junctions, sometimes connected by a broadened base; ia fan-shaped or ctenoid or with flat lobes; pp frequent or rare	See III; ia frequent, mostly fan-shaped, rarely ctenoid; pp frequent	See III; ia frequent, mostly of ctenoid form, rarely fan-shaped; pp rare	See III; ia rare; pp frequent
Vesicles	–	–	–	–
Arbuscules	Branches tapering progressively, not colonizing entire cell	See III	See III	See III
Colonization pattern	Continuous	See III	See III	See III
Observed in number of roots	113	11	15	15

*In brackets: staining intensity after 2–3 days KOH treatment

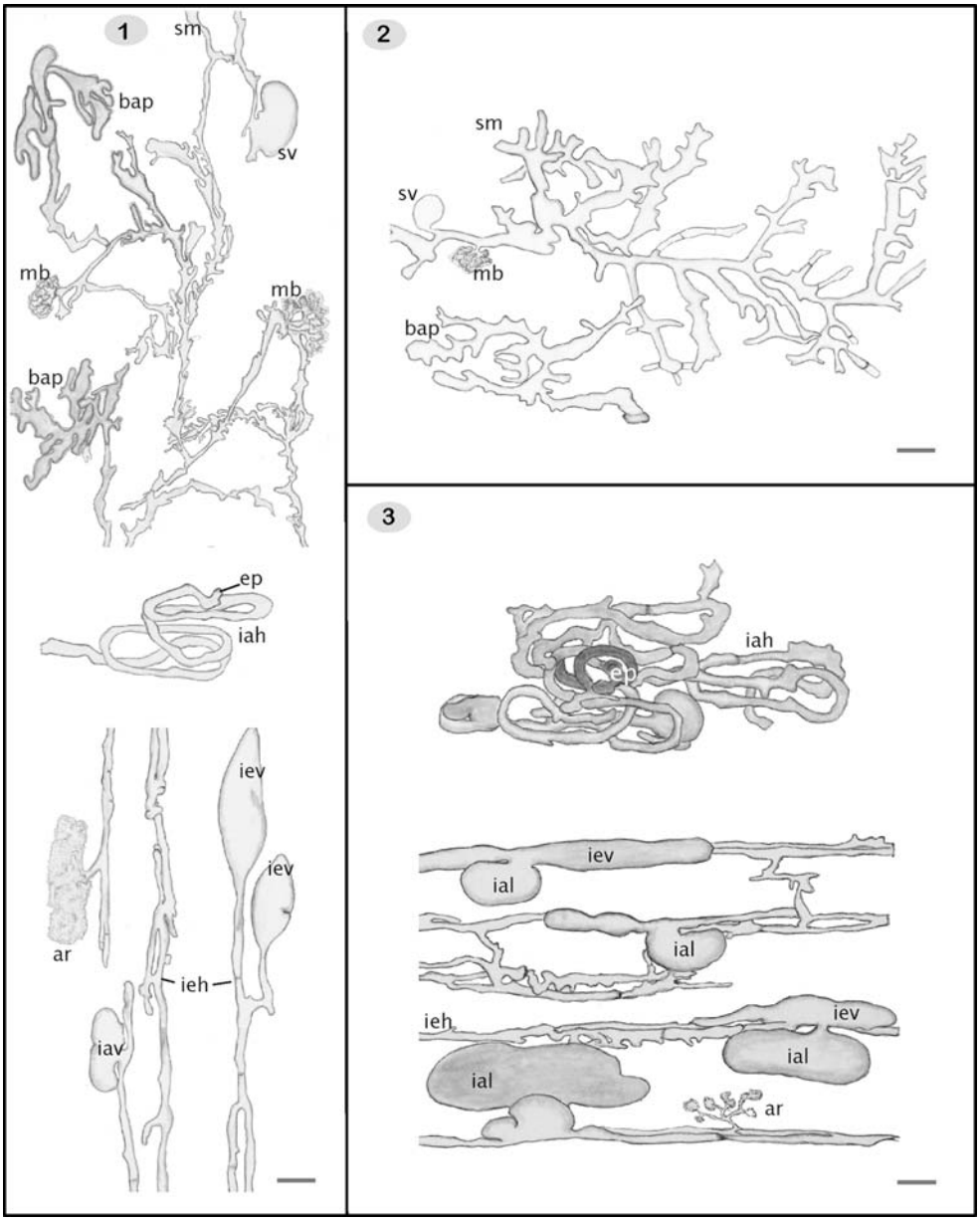
Table 3 Characteristics and frequency of AM morphogroup IV and the seven subordinate morphotypes associated with *A. verticillata*

	Morphogroup IV					
	H-branching hyphae, Figs. 10, 11, 12, 13, 14, 15, 16 and 17	Morphotype IV.1, Figs. 10 and 11; morphotype IV.2, Figs. 10 and 12	Morphotype IV.3, two variations (var1, var2), Fig. 13a,b	Morphotype IV.4, Fig. 14	Morphotype IV.5, Fig. 15; Morphotype IV.6, Fig. 16	Morphotype IV.7, Fig. 17
Supraradical mycelium						
Staining intensity	Basal hyphae dark or medium, finer hyphae mostly medium or faint and granular	Basal and last branches dark, some with fainter outer layer, rarely medium or granular faint	Basal hyphae always dark, last branches dark, medium or granular faint	Basal hyphae dark, uniform medium or uniform faint	Basal hyphae and last branches medium or faint, some medium with fainter outer layer	Basal hyphae medium, last branches medium or granular faint
Diameter of last branches	0.8–4.5 µm	Frequently 4.5 µm, rarely 2–3 µm, display often same width	2–4 µm	3–4 µm	1–4 µm	3–4 µm
Hyphal wall of last faint branch	≤1.2 µm	<1 µm	<1 µm	Up to 1.2 µm	<1 µm	<1 µm
Branched appressoria-like plates (bap), entry points (ep)	bap often formed; ep below simple appressoria or next to bap	bap large, thick-walled, besom-like-branched or thick fingers	Var1: small bap; var2: no bap;	bap large, thick-walled, besom-like-branched or thick fingers	Small bap	No bap observed in the one mycorrhiza
Shape; peg-like processes (pp) besom-like branching (blb)	blb in 50% of the mycorrhizas, pp frequent	blb frequent, pp frequent, hyphal tips broad, not cuspidate;regular hyphal width over long distance	Rigid; pp frequent; var1: blb and parallel running hyphae frequent; irregular outline, pp in series	blb frequent, pp cuspidate or broad and rounded, outline frequently irregular.	blb frequent, pp cuspidate, frequent	No blb observed; cuspidate and rounded pp
Supraradical vesicles: frequency; staining intensity; shape	Often numerous; dark, medium granular; different staining layers; regular or irregular	Few or missing; dark; regular shape	Numerous; dark, some with fainter outer layer; regular shape	Rare; uniform medium; slightly irregular shape	Frequent; medium or faint, some with different staining layers; irregular with pronounced lobes	Rare; medium granular; slightly irregular shape
Auxiliary cells	–	–	–	–	–	–
Fine, multibranched structures (mb)	Mostly frequent	IV.1: rare; IV.2: not observed	see IV	Rarely observed	see IV	Rarely observed
Hyphal coils in outer cortical layer below entry point						
Width	4–8 µm	IV.1: 6–9 µm; IV.2: 4–6 µm	4–5 µm	4–5 µm	IV.5: 4–5 µm; IV.6: 4–7 µm	5–7 µm
Staining intensity	Medium to faint	See IV	See IV	See IV	See IV	See IV
Shape; peg-like processes (pp)	No branches, regular width or slightly irregular, rarely pp	IV.1: slightly irregular, tiny pp; IV.2: regular or slightly irregular	Regular, rarely with tiny pp	Regular or slightly irregular	IV.5: regular or slightly irregular; IV.6: slightly irregular with pp	Regular or slightly irregular
Frequency	Mostly few, rarely numerous	IV.1: numerous; IV.2: few	Few	Few	IV.5: few; IV.6: numerous	Few
Intercellular hyphae in inner cortical layers						
Width	1–6 µm, broadened hyphal junctions up to 12 µm	IV.1: 3–8, up to 20 µm; IV.2: 4–7 µm	4–7 µm, rarely 1–2 µm, broad hyphal junctions up to 12 µm	2–5 µm	IV.5: 1–3 µm; IV.6: 1–5 µm	3–7 µm; swollen areas up to15 µm, junctions 1–2 µm

Table 3 (continued)

	Morphogroup IV H-branching hyphae, Figs. 10, 11, 12, 13, 14, 15, 16 and 17	Morphotype IV.1, Figs. 10 and 11; morphotype IV.2, Figs. 10 and 12	Morphotype IV.3, two variations (var1, var2), Fig. 13a,b	Morphotype IV.4, Fig. 14	Morphotype IV.5, Fig. 15; Morphotype IV.6, Fig. 16	Morphotype IV.7, Fig. 17
Staining intensity*	Medium to faint (often faint)	Medium (medium to faint)	Medium (medium to faint)	Medium to faint (faint)	Medium to faint (faint)	(Faint)
H-junctions	Numerous	See IV	See IV	See IV	See IV	See IV
Branching base of junctions	Broadened or not broadened	IV.1: broadened up to 20 µm; IV.2: not broadened	Broadened up to 15 µm	Not broadened	IV.5: sparsely broadened, IV.6: broadened up to 18 µm	Broadened up to 20 µm
Shape; peg-like processes (pp); intercellular appressoria (ia)	pp frequent, tiny, broad rounded or cuspidate	IV.1: irreg. outline, ia; broad and tiny pp; IV.2: broad pp, no ia	Tiny and broad cuspidate pp	pp frequent, cuspidate and rounded	Cuspidate pp	pp, 15-µm broad swellings, 1 µm fine junctions
Vesicles						
Staining intensity*	Dark, medium or faint	Dark to medium (medium)	Dark to medium (medium)	Medium to faint (faint)	Medium to faint (faint)	(Faint)
Frequency	Often numerous	Numerous	Numerous	Scattered	Scattered	Scattered
Location in inner cortex and position at entry point (ep)	Mostly intercellular, not numerous near ep	See IV	See IV	See IV	See IV	See IV
Size (length; width only given when striking)	35–150 µm	IV.1: 40–105 µm large; IV.2: 35–75 µm	35–80 µm	35–75 µm	IV.5: 35–150 µm long, 15 µm wide; IV.6: 50–90 µm long, > 25 µm wide	35–75 µm
Shape	Mostly regular oval, rarely irregular and with small lobes	IV.1: regular or irregular lobed; IV.2: regular oval, not lobed	Regular oval, not lobed	Regular oval, not lobed	IV.5: irregular longish, not lobed; IV.6: regular longish, not lobed	Oval, slightly irregular, small lobes
Vesicle wall	Frequently >2 µm	See IV	Frequently up to 5–7 µm	See IV	See IV	See IV
Arbuscules	Branches taper progressively, rarely colonize entire cell	See IV	See IV	See IV	IV.5: see IV; IV.6: see IV, colonize entire cell	See IV
Colonization pattern	Continuous	Continuous	Continuous	Continuous	Continuous	Continuous
Observed in number of roots	189	IV.1: 13; IV.2: 14	29	5	IV.5: 7; IV.6: 11	1

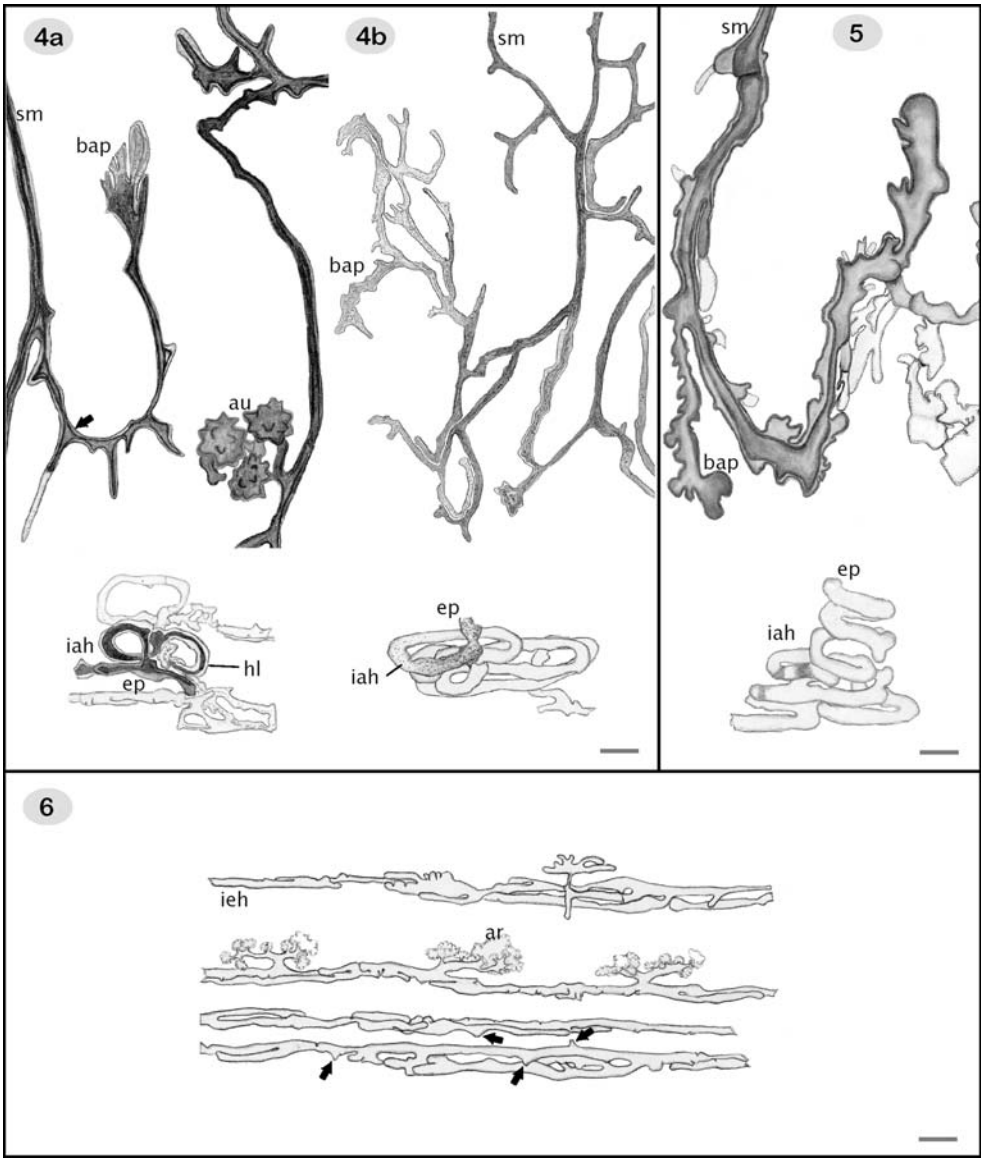
*In brackets: staining intensity after 2–3 days KOH treatment



Figs. 1, 2 and 3 Mycelial structures of AM morphogroup I.
Fig. 1 *Above*: supraradical mycelium (*sm*) uniformly faintly staining with branched appressoria-like plates (*bap*), supraradical vesicles (*sv*) and fine, multibranched structures (*mb*); medium staining hyphae with thicker walls; *middle*: unbranched, intracellular hyphal coils (*iah*) in outer cortical layer below entry point (*ep*); *bottom*: intercellular hyphae (*ieh*) in inner cortical layer smooth, forming lobed or regular oval intercellular (*iev*) or intracellular (*iav*) vesicles and arbuscules (*ar*)
Fig. 2 Morphotype I.1: Supraradical mycelium with coral-like swollen hyphae up to 20 μm in diameter
Fig. 3 Morphotype I.2; *above*: medium staining, unbranched intracellular hyphae colonizing many cells of the outer cortical layer; *bottom*: intercellular hyphae between the inner cortical layers with large intercellular vesicles connected to intracellular lobes (*ial*).
Scale bars 25 μm

were sequences of *Endogone* (96% identity) and Spicellomycetales (94–95% identity). Some further related fungi were also included for an approximate classification of the unknown fungus. Calculations including *Umbelopsis*, *Phycomyces*, and *Rhizopus*, which are further members of the Mucormycotina (James et al. 2006), yielded the same topology. Sequence alignments were done with MAFFT (Katoh et al. 2005). To estimate phylogenetic relationships, the alignment was analyzed using heuristic maximum likelihood (ML) as implemented in the PHYML software, version 2.4.4 (Guindon and Gascuel 2003), starting from a BIONJ tree (Gascuel 1997), with a general time-reversible model of nucleotide substitution and additionally assuming a percentage of invariant sites and gamma-distributed substitution rates at the remaining sites (GTR + I + G). The gamma distribution was approximated with four discrete rate categories. All model parameters were estimated using ML. Branch support was inferred from 1,000 replicates of nonparametric bootstrapping (Felsenstein 1985), with model parameters estimated via ML individually for each bootstrapped alignment. Neighbor-joining analyses (Saitou and Nei 1987) using the BIONJ modification (Gascuel 1997) yielded the same tree topology and only minor differences in bootstrap support (Fig. 18).

Glomus sequences, which clustered together and showed sequence similarities higher than 99%, were regarded as one sequence type. The classification of fungi in the basal taxa and the determination of *Rozella* as outgroup were done in respect to the newest results concerning fungi phylogeny (James et al. 2006).

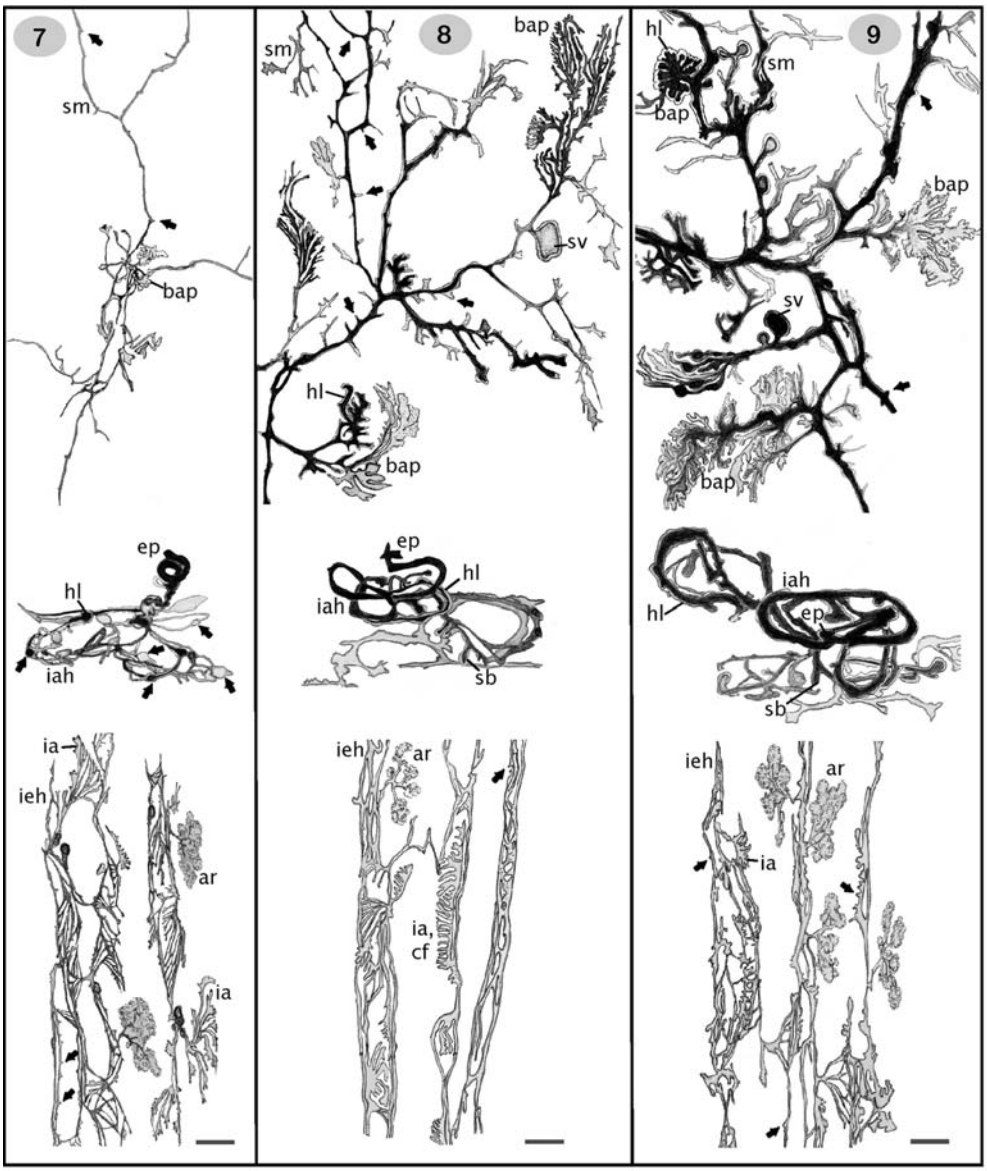


Figs. 4, 5 and 6 Mycelial structures of AM morphogroup II. **Figs. 4a and b** Two variations of morphotype II.1; *above*: terminal branches of supraradical mycelium (*sm*) not finer than 4.5 μm in diameter, medium to dark staining, with auxiliary cells (*au*), branched appressoria-like plates (*bap*), hyphal branching base broadened (**a**, *arrow*) or not (**b**) *bottom*: intracellular hyphal coils (*iah*) below entry point (*ep*) colonizing the outer cortical layer; coils with irregular outline, staining dark with hyaline outer layer (*hl*) at base and fainter distantly (**a**) or smooth, unbranched (**b**) **Fig. 5** Morphotype II.2; *above*: terminal branches of supraradical mycelium 10–20 μm in diameter, dark, medium, or hyaline staining, with irregular outline, branched appressoria-like plates (*bap*) and several thick wall layers; *bottom*: regular, unbranched intracellular hyphal coils in outer cortical layer below entry point **Fig. 6** Intercellular hyphae (*ieh*) in inner cortical layers formed by all morphotypes of morphogroup II with broad peg-like processes (*arrows*), arbuscules (*ar*) with branches tapering abruptly from broad trunk; no vesicles formed. Scale bars 25 μm

Results

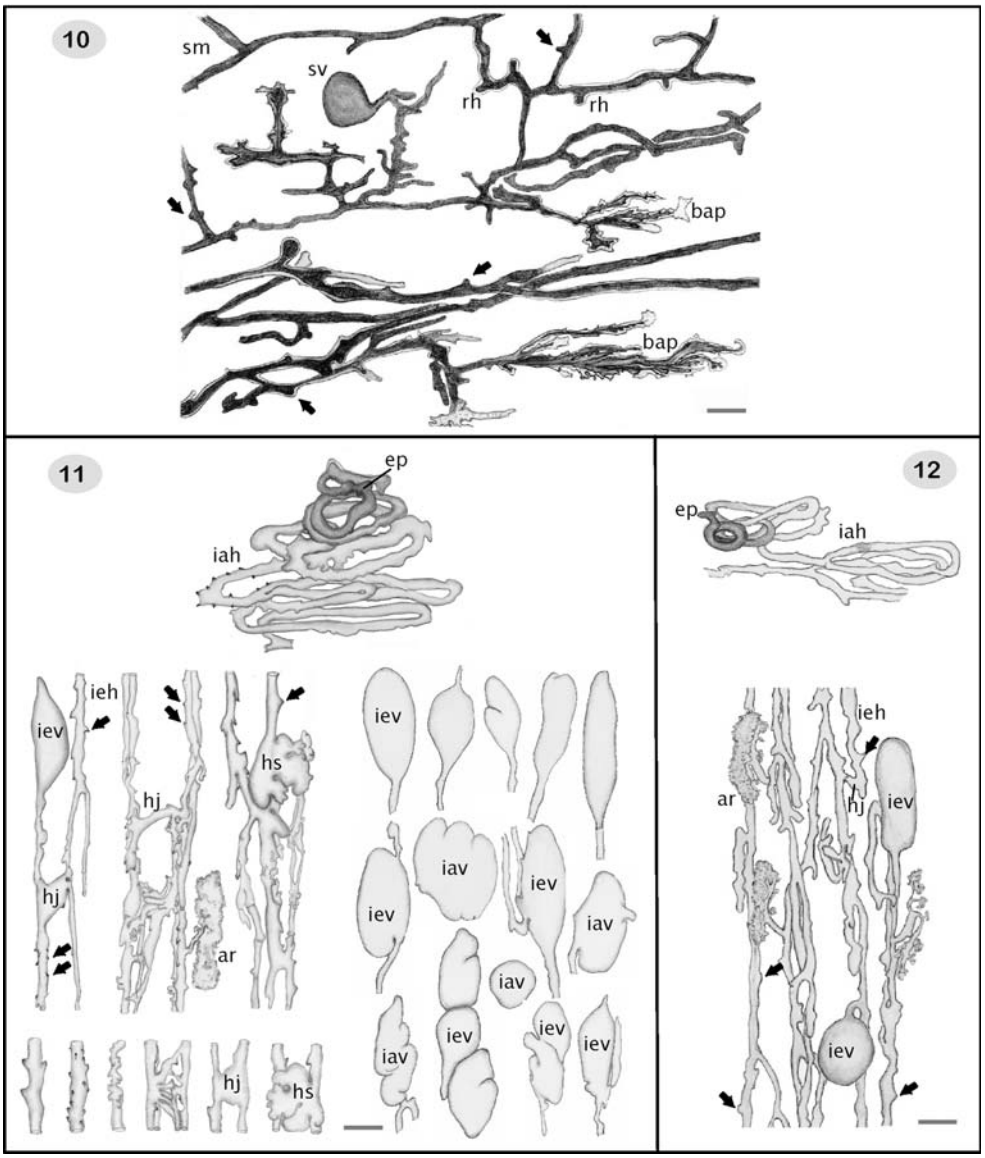
Description of the AM morphogroups and morphotypes

Light microscopy of the cleared and stained mycorrhizas revealed conspicuously large amounts of mycelium attaching to the surface of rootlets of *A. verticillata* (Fig. S2). Main characters of the supraradical mycelium are displayed in Figs. S2b–k and S3a–e. Irregularly branched appressoria-like plates with fine, dark-staining fingers (Figs. S2b and S3b), wider, faintly staining fingers (Figs. S2c and S3c), dark-staining fingers with prominent hyaline outer layer (Fig. S2d), uniformly faintly staining (Figs. S2e and S3a), medium staining (Fig. S2f), and fine, multibranched structures often ensheathed by brown septate hyphae (Figs. S2h–k and S3d,e) were observed. Frequently, the supraradical mycelium covered large areas of the rootlets without or with rare intraradical colonization. Following the hyphae, entry points were found, mostly at unbranched or slightly irregular appressoria, less frequently close to the branched appressoria-like plates. Supraradical vesicles of regular shape or with irregular lobes of different staining intensity attached to the root surface (Figs. S2g and S3d). The fungal features were similar in young and older roots of *A. verticillata*. The comparative microscopic studies yielded four AM morphogroups (I–IV) based on the supraradical and intraradical hyphal structures of 362 mycorrhizas of *A. verticillata* (Tables 1, 2 and 3). Within these four morphogroups, 197 individual mycorrhizas of *A. verticillata* were compiled into 14 morphotypes (Tables 1, 2 and 3), whereas the other 165 individual mycorrhizas were associated to the morphogroups only, but not distinguished as specific morphotypes. Numbers of incidence are given for each morphogroup and morphotype in Tables 1, 2 and 3 (bottom line).



Figs. 7, 8 and 9 Mycelial structures of the three AM morphotypes of morphogroup III.
Fig. 7 Morphotype III.1; *above*: supraradical mycelium (*sm*) fine, with peg-like processes (*arrows*) and small branched appressoria-like plates (*bap*); *middle*: intracellular hyphal coils (*iah*) in outer cortical layer below entry point (*ep*) dark staining with hyaline outer layer (*hl*), with frequent intercalary swellings (*arrows*); *bottom*: intercellular hyphae (*ieh*) 0,8–1,5 μm wide, with intercellular appressoria (*ia*), fine hyphal junctions, peg-like processes (*arrows*), and arbuscules (*ar*)
Fig. 8 Morphotype III.2; *above*: supraradical mycelium (*sm*) with frequent up to 1 μm fine, and some wider branched appressoria-like plates and with supraradical vesicles (*sv*); *middle*: hyphal coils with short swollen branches (*sb*); *bottom*: intercellular hyphae in inner cortical layer form many intercellular appressoria of ctenoid form (*cf*), fine hyphal junctions, arbuscules, and few peg-like processes (*arrow*)
Fig. 9 Morphotype III.3; supraradical mycelium (*above*) and intraradical mycelium (*middle and bottom*) with roundish knobs and peg-like processes (*arrows*), supraradical, branched appressoria-like plates frequent and often large, faintly staining or dark staining with hyaline outer layer.
Scale bars 25 μm

Main distinguishing characters of morphogroups are shortly presented while details of the morphotypes are given in Tables 1, 2 and 3 and by the illustrations with extensive legends. The most prominent feature of morphogroup I (Figs. 1, 2, 3 and S2e) was the uniformly faint, rarely medium-staining supraradical mycelium, hyphae displaying 0.8–1.2 μm thickened and distinctly lined cell walls. Supraradical, finest, multibranched structures, often ensheathed by brownish septate hyphae, were found in this morphogroup but else only in morphogroup IV. The most conspicuous concordant feature of the mycorrhizas of morphogroup II (Figs. 4, 5 and 6) was the rather wide (>4.5 up to 20 μm) predominantly dark-staining supraradical mycelium, forming auxiliary cells, displaying neither fine, multiple-branched structures nor supraradical or intraradical vesicles. Concordant features of morphogroup III (Figs. 7, 8 and 9) were the dark-staining supraradical mycelium with frequent 1-μm-wide terminal branches and large, branched appressoria-like plates. The dark-staining, knobby, irregular-branched coils below the entry point, and the <3-μm-fine, intercellular hyphae forming no vesicles but intercellular appressoria and many fine hyphal junctions were likewise striking and suitable to distinguish morphogroup III from the other morphogroups. Morphogroup IV (Figs. 10, 11, 12, 13, 14, 15, 16 and 17) was mainly distinguished by the intercellular hyphae forming many H-junctions. Supraradical hyphae displayed branched appressoria-like plates, supraradical vesicles of regular- or irregular-lobed form and fine, multibranched structures, often ensheathed by brown septate hyphae.



Figs. 10, 11 and 12 Mycelial structures of AM morphotypes IV.1 and IV.2.
Fig. 10 Supraradical mycelium (*sm*) of morphotypes IV.1 and IV.2, hyphae predominantly dark staining with branched appressoria-like plates (*bap*), broad peg-like processes (*arrows*), rounded hyphal tips (*rh*) regular hyphal width over long distance and regular supraradical vesicles (*sv*)
Fig. 11 Intraradical fungal structures of morphotype IV.1; *above*: intracellular hyphal coils (*iah*) below entry point (*ep*) irregular with peg-like processes (*arrows*); *bottom*: intercellular hyphae (*ieh*) in inner cortical layer with knobby irregular outline, wide and small peg-like processes (*arrows*), up to 30 μm large irregular hyphal swellings (*hs*), commonly broadened or fan-shaped H-junctions (*hj*), and arbuscules (*ar*); vesicles numerous, intercellular (*iev*) or intracellular (*iav*), regular oval or with small or pronounced lobes
Fig. 12 Intraradical fungal structures of morphotype IV.2; *above*: Coils below entry point unbranched regular; *bottom*: H-junctions of intercellular hyphae not broadened, peg-like processes broad, vesicles without lobes.
Scale bars 25 μm

Colonization by several fungi and incidence of morphogroups

A considerable amount of roots was colonized by hyphae of two up to four morphogroups. Seventeen out of 362 root pieces (4.7%) were not colonized by AMF. Morphogroup IV was most frequently observed (52%), followed by morphogroup I (47%), morphogroup III (31%), and morphogroup II (14%).

Sequencing and phylogenetic analysis of mycorrhiza-associated *Glomeromycota*

We obtained 26 sequences of *Glomeromycota* from 20 mycorrhizas. Up to three sequences were obtained from one mycorrhiza (Table 4). Nineteen sequences of *Glomus* group A clustered in five sequence types (*Glomus* 1 to 5; Fig. 18). One additional sequence of *Glomus* group A was obtained. Five sequences clustered with *Acaulospora* and two sequences with *Gigaspora* (Table 4 and Fig. 18). Sequences belonging to *Glomus* 1 were obtained from one *A. verticillata* and previously from two individuals of *Graffenrieda emarginata* and one *Hyeronima moritziana* (Kottke et al. 2007a). The sequences belonging to *Glomus* 2 were obtained from two individuals of *A. verticillata*, one sequence previously from *H. moritziana*. The three identical sequences of *Glomus* 3 were obtained from two individuals of *A. verticillata*. Very similar sequences were previously obtained from *G. emarginata*, *H. moritziana*, and *Clusia elliptica*. Sequences of *Glomus* 4 and the single sequence were new to science. *Glomus* 5 includes one sequence from *G. emarginata* and one from *Podocarpus*

Figs. 13 and 14 Mycelial structures of AM morphotypes IV.3 and IV.4.
Figs. 13a and b Morphotype IV.3; *above*: supraradical mycelium (*sm*) predominantly dark stained, besom-like branched (*blb*) and parallel running hyphae present (**a**) or not (**b**), supraradical vesicles (*sv*) regular; fine, multibranched structures (*mb*) formed; *middle*: intracellular hyphal coils (*iah*) below entry point (*ep*) regular; *bottom*: H-junctions (*hj*) of intercellular hyphae (*ieh*) in inner cortical layer broadened, peg-like processes tapering in a fine tip (*arrows*); vesicles (*iev*) without lobes
Fig. 14 Morphotype IV.4; *above*: supraradical mycelium with thick walls, staining uniformly medium or faintly; fine, multibranched structures formed; *middle*: hyphal coils below entry point sparsely irregular; *bottom*: H-junctions in inner cortical layer sparsely or not broadened, vesicles regular. Scale bars 25 μm

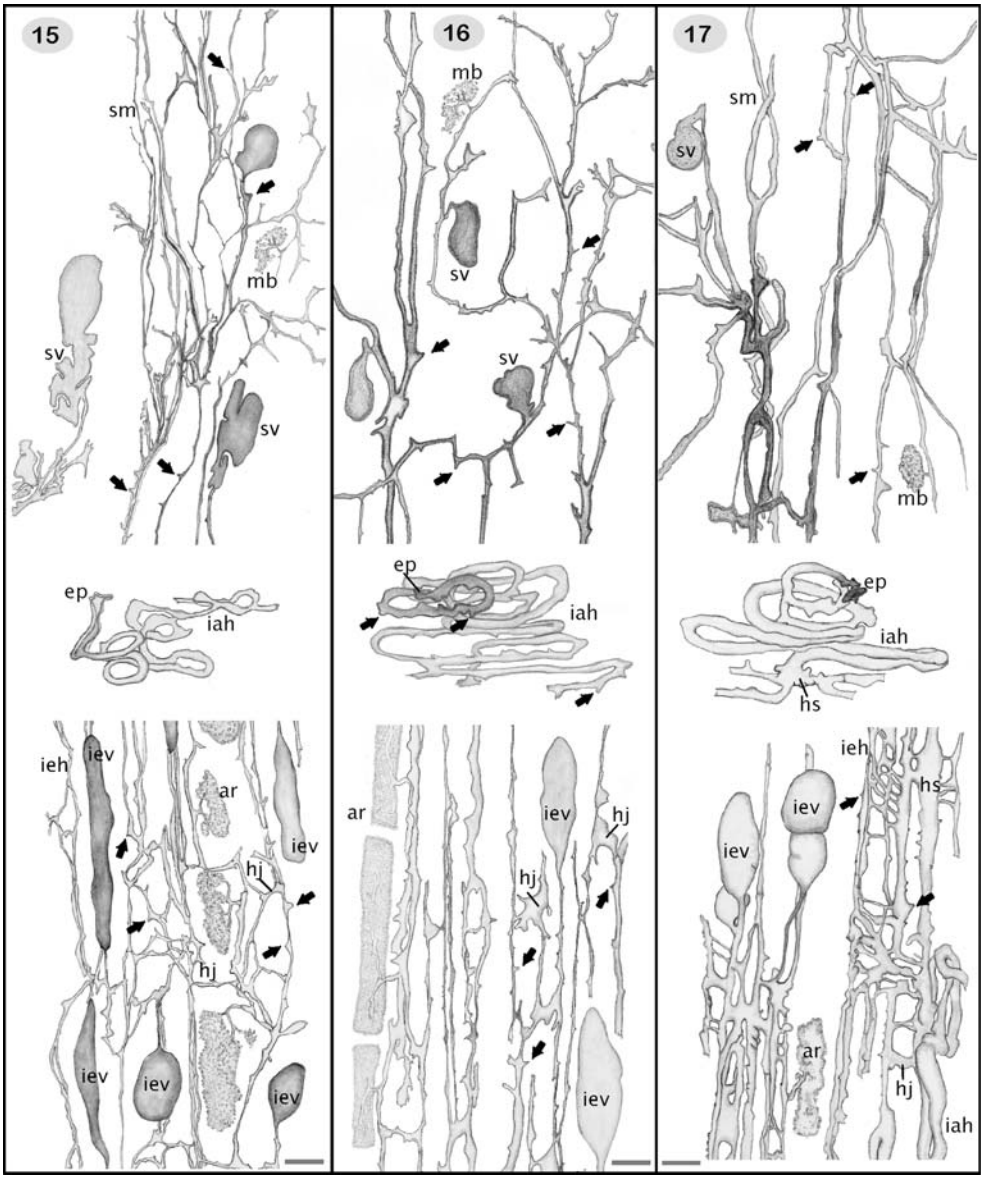
oleifolius. All the mentioned tree species were sampled in the same forest (Kottke et al. 2007a).

Nine identical or nearly identical sequences (1-bp difference) of an unknown fungus were obtained from nine roots of two *A. verticillata* individuals clustering as a sister group of *Endogone* (Fig. 18; bootstrap value 83%).

Discussion

Documenting and defining AM morphotypes

The investigation of AM formed with *A. verticillata* in the neotropical mountain rain forest revealed a high structural diversity of mycelia, not shown before. One reason of the rareness of investigations on mycelial structures of AM fungi is the adequate documentation of three-dimensional structures (Beck et al. 2005). In this study, we used drawings at the same scale of magnification and partitioning in three spatial levels, supraradical structures, hyphae in outer cortical layer below the entry point, and hyphae in the inner cortical layer for characterization of the morphogroups and morphotypes. Another problem, frequently questioned, is the potential influence of root or soil conditions on the appearance of the hyphal structures (Morton 1988; Brundrett and Kendrick 1990). However, statements presuming high host-plant-dependent morphology of AMF considered *Arum*- and *Paris*-type differences only but neglected further hyphal characteristics (Ahulu et al. 2007). Other studies pointed to the influence of soil



Figs. 15, 16 and 17 Mycelial structures of AM morphotypes IV.5, IV.6 and IV.7.
Fig. 15 Morphotype IV.5; *above*: supraradical mycelium (*sm*) sometimes with a diameter of 1 μm, supraradical vesicles (*sv*) staining uniformly, some with many prominent lobes; fine, multibranched structures (*mb*) formed; *middle*: intracellular hyphae (*iah*) below entry point (*ep*) sparsely irregular; *bottom*: intercellular hyphae (*ieh*) in inner cortical layer 1–3 μm in diameter with 15 μm wide, irregular longish intercellular vesicles (*iev*), H-junctions (*hj*) sparsely or not broadened, peg-like processes (*arrows*) taper in a fine tip
Fig. 16 Morphotype IV.6: differs from morphotype IV.5 by intercellular hyphae (*bottom*) with a diameter of 1–5 μm, H-junctions up to 18 μm broad, vesicles longish, about 30 μm thick
Fig. 17 Morphotype IV.7; *above*: supraradical mycelium without striking features; *middle*: intracellular hyphae below entry point with up to 15 μm thick hyphal swellings (*hs*); *bottom*: intercellular hyphae in inner cortical layer with many very fine parallel anastomoses and large hyphal swellings near the coils, H-junctions broadened.
Scale bars 25 μm

conditions on the quantity of hyphal growth and branching frequency of AMF (Mosse 1959; Bago et al. 2004), but an influence of soil on the structural features used in our study were never documented to our knowledge. Our observations support Abbott and Robson (1979) who found no obvious changes in development and shape of the fungal structures culturing *Glomus* sp. with three different host plants, *Trifolium subterraneum*, *Erodium botrys*, *and Lolium rigidum*, at different P-supply. The characteristics of the distinguished morphotypes in our study were observed on one host plant (*A. verticillata*) sampled in the pure organic top soil layer of one forest type (forest type II according to Homeier 2004; macrophyll ridge forest of Paulsch et al. 2007). Chemical analyses did not show significant differences of soil parameters (Wilcke et al. 2002). We frequently discerned two, three, or even four morphotypes colonizing the same root, and we found the morphotypes repeatedly on several roots of the same tree species. It is, therefore, rather unlikely that the observed features depended on root morphology. The structural characterization of the 14 morphotypes concerned one tree species. A more restricted study on mycorrhizas of *P. oleifolius*, *Critoniopsis floribunda*, *Micropholis guyanensis*, *Tabebuia chrysantha*, *Nectandra acutifolia*, and 13 melastomataceen trees collected in the same mountain rain forest verified the four morphogroups (Beck 2002; Beck unpublished). All the investigated trees formed *Arum*-type mycorrhizas (Gallaud 1905). It remains, so far, unknown if similar morphotypes as described here can be formed with *Paris*-type mycorrhizas. We would, however, at least expect identical characteristics of the supraradical mycelium for *Arum*- and *Paris*-type mycorrhizas.

Table 4 Numbers of mycorrhizas of *A. verticillata*, accession numbers and length of sequences, sequence types according to phylogenetic analysis (Fig. 18), and morphogroups and morphotypes according to Tables 1, 2 and 3; numbering of mycorrhizas see S1

Number of mycorrhiza	*Acaulospora* sequences	*Gigaspora* sequences	*Glomus* group A sequences; sequence types in bold	Morphogroup I	Morphogroup II	Morphogroup III	Morphogroup IV
4.2_1			EF447220 (1,129 bp) ***Glomus 4***			III.3	IV.1
4.2_3			EF447221 (1,129 bp) ***Glomus4***				IV.1
4.2_4			EF447222 (1,402 bp) ***Glomus 4*** EF447223 (1,130 bp) ***Glomus 2***	I			IV.1
4.4_4			EF447227 (751 bp) ***Glomus 4***				IV.1
4.4_5			EF447228 (738 bp) ***Glomus 4***				IV.1
4.4_9			EF447229 (750 bp) ***Glomus 4***			III.3	IV.1
4.4_13			EF447224 (1129 bp) ***Glomus 4***	I		III.3	IV.1
4.4_14			EF447225 (1130 bp) ***Glomus 4***			III.3	IV.1
4.4_15			EF44722 (1,129 bp) ***Glomus 4***			III.3	IV.1
5.1_3	EF447241_K15c1 (1,132 bp)	EF447242_K15c2 (908 bp)		I	II.1		
5.1_5			EF447230 (1,079 bp) ***Glomus 3***	I			IV.6, IV
5.1_6			EF447231 (1,005 bp) ***Glomus 1***	I	II.1	III.1	IV
5.1_7			EF447232 (1,079 bp) ***Glomus 3***	I			IV.6, IV
5.1_8	EF447239_K10c4 (1,132 bp)			I	II.1	III.1	IV
5.2_2			EF447233 (1,130 bp) ***Glomus*** 2EF447234 (1,128 bp) ***Glomus 1***	I		III.3	
5.2_3	EF447243_K16c1 (1,132 bp)	EF447240_K12c1 (1126 bp)		I	II.1	III.3	
5.2_7			EF447235 (1,008 bp) ***Glomus 2***		II.1	III.1, III.3	
5.4_1	EF447244_5.4.1 (750 bp)			I	II.1	III.3	
7.1_3	EF447245_7.1.3c (911 bp)		EF447236 (1043 bp) ***Glomus*** 3EF447237 (750 bp) ***Glomus 5***	I			IV.6
8.2_10			EF447238 (1,027 bp) single sequence	I			IV.3

Comparison of the AM morphogroups with mycelial structures of known AMF taxa

Features of morphogroup I (uniformly faintly staining hyphae and lobed vesicles)

Lobed vesicles are a characteristic feature of *Acaulospora* species (Morton and Redecker 2001). Abbott (1982) found vesicles of up to 60 μm in length with lobes extending in adjacent cells in mycorrhizas of *Acaulospora laevis*. Similar lobed vesicles in morphotype I.2 reached 180 μm in length. Faintly staining supraradical mycelium was reported up to now in connection with the extraradical hyphal dimorphism for the thin walled and temporary hyphae (Mosse 1959; Nicolson 1959). We observed basal and finer hyphae of the supraradical mycelium of morphogroup I staining faintly. Faintly staining AMF were also reported from *Paraglomeraceae* and *Archaeosporaceae* (Morton and Redecker 2001), but colonization by these groups was not supported by our molecular findings. Arbuscules colonizing the entire cell were observed, so far, with *Acaulospora* and *Glomus* species (Merryweather and Fitter 1998). Patchy colonization was reported for *Acaulospora* and *Glomus* species, *Archaeosporaceae*, and *Paraglomeraceae* (Morton and Redecker 2001).

Our molecular results supported *Acaulosporaceae* as forming mycorrhizas of morphogroup I as all the five roots from which we obtained sequences of *Acaulospora* were colonized by morphogroup I mycelia in the corresponding root halves (Fig. 18 and Table 4). Four of the five roots displayed also hyphae of morphogroup II (most likely *Gigasporaceae*, see below), and from two of these (5.1_3 and 5.2_3), we obtained additionally sequences of *Gigaspora*. From mycorrhiza 7.1_3, we obtained a sequence of *Acaulospora*, and this mycorrhiza and all the further investigated 14 mycorrhizas of the same root system (7.2) were colonized by morphogroup I but not by morphogroup II. From mycorrhiza 7.1_3, we obtained additionally to the *Acaulospora* sequence two sequences of *Glomus* group A. The mycorrhiza was colonized by morphogroup I and by morphogroup IV hyphae (see below). The molecular data thus support our distinction of the morphogroups.

Features of morphogroup II (large hyphae, auxiliary cells, no vesicles)

Auxiliary cells, lack of intraradical vesicles, intercellular hyphae with irregular, knobby outline, peg-like processes, and arbuscules with a long curved trunk and branches tapering abruptly are considered as specific characteristics

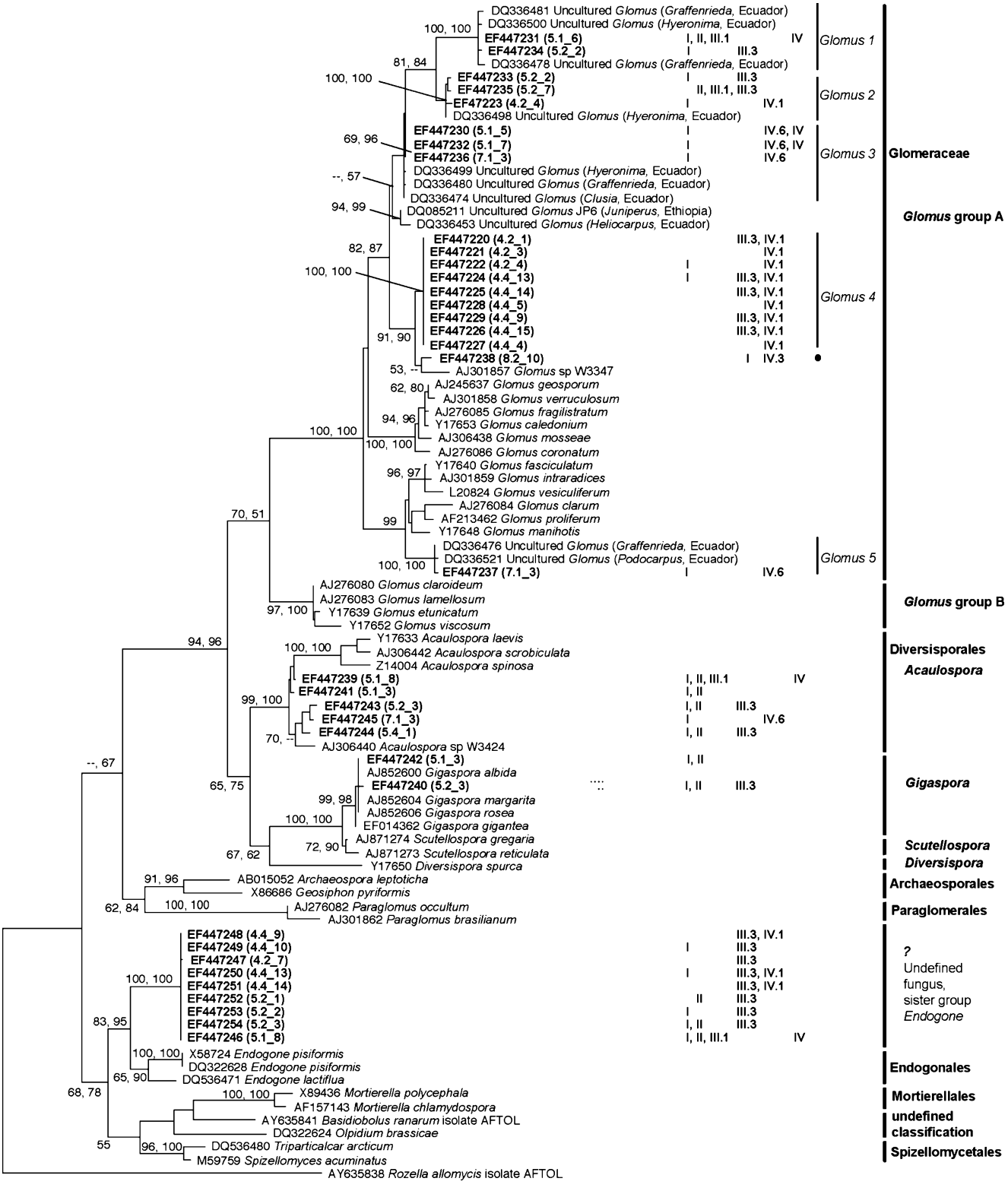


Fig. 18 ML-tree based on alignment of partial (738 bp) nucSSU rDNA sequences obtained from *A. verticillata* mycorrhizas (printed in bold) together with GenBank sequences of closest BLAST matches (≥98% identity), identified AMF species, *Endogone*, *Mortierella*, and few further fungi formerly related to *Zygomycetes*. The tree was rooted with *Rozella allomycis*. Bootstrap values of ML (first number) and BIONJ (second number) exceeding 50% are given. Number of mycorrhizas is given in brackets and morphogroups and morphotypes in roman numbers for *A. verticillata*. Host species and locations are added for closest *Glomerales* sequences

of the *Gigasporaceae* (INVAM http://www.invam.caf.wvu.edu/fungi/taxonomy/genuskey.htm). All characteristic features of the *Gigasporaceae* were found in morphotype II.1. We compiled morphotype II.2 together with morphotype II.1 in one morphogroup because of the wide supraradical hyphae and the missing vesicles, although auxiliary cells were not found. As auxiliary cells were also only rarely present in the mycorrhizas of morphotype II.1, it is not unlikely that they were lost during preparation of samples.

We obtained two *Gigaspora* sequences from two roots (5.1_3 and 5.2_3), which were colonized by morphotype II.1. Both roots displayed also hyphae of morphogroup I, and we additionally obtained sequences of *Acaulospora*, as mentioned above. One of the two roots was also colonized by morphogroup III, but we did not obtain further sequences. The findings, to our opinion, indicate that we could discern the morphogroups I and II, Acaulosporaceae and *Gigasporaceae*, respectively, correctly and find the corresponding sequences in the field material.

Features of morphogroup III (fine hyphae, no vesicles)

Morphotype III.1 with 0.8- to 1.5-μm-wide hyphae may belong to the group termed "fine endophytes" in literature, characterized by very fine hyphae, knobby intercalary swellings, no vesicles, and fine, fan-shaped branching (Hall 1977; Abbott 1982; Beck et al. 2005). Morphotypes III.2 and III.3 with 1- to 3-μm-wide intraradical hyphae may be related to mycorrhizas termed *medium* endophytes (Abbott 1982), which colonized the roots of subterranean clover grown in field soils from South to West Australia. External hyphae of the latter displayed a thick amorphous layer, intraradical hyphae often had a translucent outer layer, and no vesicles were found. Gianinazzi-Pearson et al. (1981), by using ultrastructure and cytochemistry, showed that fine endophytes (<2 μm) colonizing raspberry displayed a two-layered cell wall. We observed a fainter-staining outer layer of the supra- and intraradical hyphae in both the fine and the medium endophyte-like mycorrhizas of morphogroup III. The fine endophytes reported in the literature displayed both smooth hyphal surface and rough hyphal surface with angular projections (Merryweather and Fitter 1998; Nicolson and Schenck 1979; Thippayarugs et al. 1999). No sequences are available of the so-called fine and medium endophytes, and taxonomical situation is not known.

Features of morphogroup IV (hyphae forming H-junctions and vesicles)

Mycorrhizas of morphogroup IV may belong to the *Glomerales* (*Glomus* group A and B; Schüßler et al. 2001) forming H-junctions. Broadened hyphal junctions were previously observed on *Glomus* species (*Glomus monosporus*, *Glomus caledonius*; Abbott and Robson 1979; Abbott 1982) and also observed in some morphotypes of morphogroup IV. Descriptions or illustrations of *Glomus* species forming peg-like processes are given in Abbott and Robson (1979), Brundrett et al. (1996), and Merryweather and Fitter (1998). The features "hyphal basis at the entry point constricted or not" described by Abbott (1982) and "vesicle vacuole number and size" (Merryweather and Fitter 1998) could not be used in our observations because the differences were too variable in our field material.

Morphotype IV.1 can be related to *Glomus* 4. The nine identical or nearly identical sequences (1-bp difference) of sequence type *Glomus* 4 were obtained from nine individual mycorrhizas from tree 4, all well colonized by morphotype IV.1 in their corresponding root halves. Two roots were additionally colonized by morphogroup I, but this morphogroup was linked to *Acaulospora* sequences (see above). Five of the nine roots displayed also hyphae of morphotype III.3. Three sequences obtained from three mycorrhizas originating from two trees clustered in sequence type *Glomus* 3. All three mycorrhizas showed extensive colonization in the corresponding root halves by morphotype IV.6. All three mycorrhizas displayed hyphae of morphogroup I, and one (7.1_3) yielded a sequence of *Acaulospora* additionally (see above). The roots 5.1_5 and 5.1_7 displayed a further mycelium of morphogroup IV, differing from IV.6 but not classified as a morphotype, as colonization was too sparse. This mycelium was not found on the third mycorrhiza, 7.1_3, and in none of the further 14 investigated mycorrhizas of this root system. Therefore, it is unlikely that this mycelium constitutes *Glomus* 3. Root 7.1_3 yielded a further sequence clustering in *Glomus* 5, which could not be associated to a morphotype. The single sequence, which we obtained from root 8.2_10, is only weakly related to morphotype IV.3, as no repetition was obtained.

The undefined fungus clustering as a sister group of *Endogone* was amplified frequently in combination with the diverse morphogroups. We consider this fungus as a contaminant and not as an AMF.

The supraradical mycelium

The AMF mycelium spreading in the soil and attaching to the root surface was already mentioned by Peyronel (1924) and termed extraradical mycelium by Butler (1939). Nicolson (1959) and Mosse (1959) observed extensive AMF mycelium on roots sampled in the field and described hyphal dimorphism with ephemeral, often septate thin-walled hyphae and permanent thick-walled hyphae with angular projections. However, further morphological characterization and definitive naming of the supraradical mycelium to distinguish it from the mycelium spreading

in the soil was not performed before. Bradbury et al. (1993) observed large misshapen appressoria formed by *Glomus versiforme* and *Glomus intraradices* on the root surface of nonnodulating mutants of alfalfa, whereas intraradical colonization failed. However, intraradical colonization was common in the roots studied here. The reason for the enlargement of the fungal surface by a wide extension and by branched, appressoria-like plates as observed in our field material is unrevealed. The structures could point to a nutrient exchange already at the root surface. Requena et al. (2003, 2005) found an indication of sugar uptake at the appressorial stage of AM in axenic conditions. Studies proving plant-induced AMF hyphal growth by host plant specific signals were carried out with few herbaceous plant species up to now (Giovannetti et al. 1993, 1996; Nagahashi and Douds 2004; Vierheilig 2004; Scervino et al. 2005; Akiyama et al. 2005). The question therefore is open if the intense hyphal growth on the root surface is dependent on host specific signals of the trees, on the soil type of this forest, or on the AMF species predominantly new to science.

Conclusions

The results obtained by comparative microscopic studies of mycorrhizas of *A. verticillata* sampled in the tropical mountain rain forest of southern Ecuador revealed fungus-specific characters as useful for morphotype and morphogroup distinctions of arbuscular mycorrhizas. DNA sequencing of *Glomeromycota* from the same material supported morphotyping, although an unambiguous relation was not always possible due to co-colonization of roots by several mycorrhizal fungi. DNA sequences had their closest matches with sequences previously obtained from other trees in the same tropical forest or where new to science, a finding that correlates well with, so far, undescribed structures of mycelia. Thus, a multitude of new taxa of *Glomeromycota* most likely specific for this forest type is to be expected.

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