

## *Otospora bareai*, a new fungal species in the Glomeromycetes from a dolomitic shrub land in Sierra de Baza National Park (Granada, Spain)

Javier Palenzuela

Nuria Ferrol<sup>1</sup>

*Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain*

Thomas Boller

*Zurich-Basel Plant Science Center, Institute of Botany, University of Basel, Hebelstrasse 1, CH-4056 Basel, Switzerland*

Concepción Azcón-Aguilar

*Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain*

Fritz Oehl

*Zurich-Basel Plant Science Center, Institute of Botany, University of Basel, Hebelstrasse 1, CH-4056 Basel, Switzerland*

**Abstract:** A new fungal species of the Glomeromycetes was isolated from the rhizosphere of *Pterocephalus spathulatus* and *Thymus granatensis*, two rare endemic plants growing on dolomite in the Sierra de Baza (Granada, southern Spain). The fungus was propagated in pot cultures of *Sorghum vulgare* and *Trifolium pratense* for 4 y and it is described here on the basis of the spores found in nature and formed in pot cultures. Its brown spores (140–210 µm diam) form laterally on a persistent, brown stalk (=neck) of a sporiferous saccule. They have two walls without ornamentation: a brown, three- to four-layered outer wall and a hyaline two- to three-layered inner wall. The unique combination of spore formation and spore wall structure does not fit with any of the known fungal genera. Spore formation is similar to that of *Acaulospora* spp. and *Archaeospora trappei*, but *Acaulospora* spp. has three spore walls with a characteristic “beaded” wall, and the outer wall of *Ar. trappei* is simple, thin, hyaline and only bilayered. Spore wall structure of the new fungus is similar to that of *Entrophospora infrequens*, however this fungus forms its spores internally, inside the hyphal stalk of the sporiferous saccule. Molecular analyses of the small subunit of the ribosomal gene phylogenetically place the new fungus next to *Diversispora spurca*, which forms one-walled glomoid spores (i.e. terminally on

hyphae). Based on these analyses we place the new fungus into a new genus in the family Diversisporaceae under the epithet *Otospora bareai*.

**Key words:** arbuscular mycorrhiza, endangered plant species, endemism, Glomeromycota

### INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) of the Glomeromycota (Schüssler et al 2001) play an important role in maintaining the diversity and functioning of natural ecosystems (van der Heijden et al 1998) as well as in the productivity of different agro-ecosystems (Oehl et al 2003). This has led to an increased awareness of the diversity of AMF and to an interest in identifying and naming new species, particularly in habitats where so-called “specialist” AMF occur (Oehl et al 2005a, b, 2006) or in habitats of increased (socio-)ecological value, that is habitats of high biodiversity level, increased presence of rare, endangered plant species (Fuchs and Haselwandter 2004) or rare habitats themselves (Mota et al 2002).

The southern Iberian Peninsula is rich in rare endemic plants (Melendo et al 2003). We discovered a new fungal species from the rhizosphere of the two endangered, endemic plants *Pterocephalus spathulatus* and *Thymus granatensis* in a study on the diversity of AMF in the rhizosphere of endangered and endemic plant species in a dolomitic shrub land from Sierra de Baza Natural Park (Granada, Spain). Spores of this new fungal species could easily be distinguished and separated under the dissecting microscope from spores of other species because of the typical laterally adherent pigmented, persistent stalks (=necks) of the sporiferous saccule. Under the compound microscope, spores showed two distinct walls, each with a multiple-layered structure. The unique combination of acaulosporoid spore formation and spore wall structure indicated that the fungus may represent a new genus in the Glomeromycetes. This was confirmed by molecular analyses on the small subunit of the ribosomal gene. The fungus phylogenetically was not related to any other known species with acaulosporoid-type spore formation but was closest to *Diversispora spurca* (C.M. Pfeiff., C. Walker & Bloss) C. Walker & A. Schüssler, which has glomoid-type spore formation (Walker and Schüssler 2004). Consequently we placed the new fungus in a new genus of the Glomeromycetes and

provide here a formal description of the new genus *Otospora* and its type species, *Otospora bareai*.

#### MATERIALS AND METHODS

*Soil sampling.*—At several dates between 2002 and 2006, soil samples were taken with a shovel in dolomitic soils (pH 8.1) from the rhizosphere of two rare endemic plant species *Pterocephalus spathulatus* (Lag.) Coulter (a perennial herb of the family Dipsacaceae) and *Thymus granatensis* Boiss. (a perennial herb of the family Lamiaceae). The site is located in a dolomitic shrub land in Sierra de Baza Natural Park (Granada, Andalucía, Spain) at 1600 m a.s.l. (2°97'W, 37°37'N).

*AMF pot cultures.*—To cultivate the new fungus under reproducible conditions, pot cultures were established with cylindrical 1500 mL pots (12 cm diam) filled with soil from the field site thoroughly mixed (1:1) with the potting substrate (a 1:1 mixture of vermiculite and sand, pH 8.2). As host plants *Sorghum vulgare* and *Trifolium pratense* s.l. were used. The pots were irrigated 3×/wk and fertilized every 4 wk with Long Ashton nutrient solution (Hewitt 1952). For 4 y, the new fungus frequently produced spores in the pot cultures. Pure pot cultures of the new species were attempted unsuccessfully several times with spores from the trap cultures and *S. vulgare*, *T. pratense* and *T. granatensis* as host plants. The growing substrate was a mixture of sterile field soil/sepiolite/vermiculite (2/1/1, v/v/v). Single or multiple (about 10), apparently healthy and viable spores, previously incubated at 4 C for 2 wk, were used as inoculum. Spore viability was checked by observation of the spore cell contents (especially of the lipids and content consistency in general) under 100–400× magnification.

*Morphological analyses.*—Spore formation characteristics and morphology of spores, sporiferous saccules, saccule stalks (=necks) and their mycelial hyphae attached were observed on specimens mounted in polyvinyl alcohol-lactic acid-glycerol (PVLG, Koske and Tessier 1983) in a mixture of PVLG and Melzer's reagent (Brundrett et al 1994) and in water (Spain 1990). The terminology of the spore wall structure is basically as suggested by INVAM (International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi, see homepage: www.invam.caf.wvu.edu), with slight modifications described recently (Oehl et al 2006, Spain et al 2006). Photographs were taken with a digital camera (Olympus DP70-CU) on a compound microscope (Zeiss, Axioplan) up to 400× magnification. Specimens mounted in PVLG and the mixture of PVLG and Melzer's reagent were deposited at Z+ZT (common mycological herbarium of the University and ETH of Zurich, Switzerland), OSC (mycological herbarium of the Oregon State University, Corvallis, USA) and at GDA-GDAC (mycological herbarium of the University of Granada, Spain).

*Molecular analyses.*—Crude DNA extracts from single spores were prepared as described by Ferrol et al (2004). Roots from the trap cultures were ground in liquid nitrogen

with a pestle and a mortar, and DNA was extracted with a DNeasy Plant Mini kit (QIAGEN) according to the manufacturer's instructions. PCR were performed in an automated thermal cycler (Gene Amp PCR System 2400, Perkin-Elmer, Foster City, California) with a puReTaq Ready-To-Go PCR Bead (Amersham Biosciences Europe GmbH, Germany) following manufacturer's instructions and 1 μM each primer. For amplifying the NS31-AM1 region of the small ribosomal gene a two-step polymerase chain reaction was conducted. The first round of amplification was performed with the universal eukaryote primers NS31 and NS41 (Simon et al 1992) and the second one with the universal primer NS31 and the AM fungal specific primer AM1 (5'-GTTTCCCGTAAAGGCGGCGAA-3') (Helgason et al 1998). Root and spore DNA also were used as templates for PCR amplification of the 3' end of the small subunit and the ITS region by using the new specific primer GLOC1335, especially designed for species phylogenetically closely related to *Diversispora* spp., together with the fungal specific primer ITS4i (Redecker et al 2007). Cycling parameters were 3 min at 95 C, followed by 35 cycles of 45 s at 95 C, 50 s at 55 C and 90 s at 72 C. The program was concluded by a final extension phase of 10 min at 72 C.

PCR products were separated electrophoretically on 1.6% agarose, stained with ethidium bromide, viewed by UV illumination and the expected bands were excised with a scalpel. The amplified DNA was isolated from the gel with the QIAEX II Gel Extraction kit (QIAGEN, USA) following the manufacturer's protocol. Nucleotide sequences were determined with *Taq* polymerase cycle sequencing and an automated DNA sequencer (Perkin-Elmer ABI Prism 373). DNA fragments were sequenced in both strands. Sequence data were compared to gene libraries (EMBL and GenBank) with BLAST programs (Altschul et al 1990). The new sequences were deposited in the EMBL database under accession numbers AM400229 and AM905318. The 18S rRNA NS31-AM1 region of the new fungus was aligned with other Glomeromycotan sequences available in the public databases. Multiple sequence alignments of gene sequences were carried out with Clustal W (version 1.5, Thompson et al 1994). The Kimura two-parameter method was used to estimate distances, and the phylogenetic analyses were performed by neighbor joining and maximum parsimony methods with PHYLIP (Felstein 1993). A sequence of *Mortierella polycephala* was used as outgroup. The relative support of the different groups was determined based on 1000 bootstrap trees. Phylogenetic trees were drawn with Treeview.

#### TAXONOMIC ANALYSIS

***Otospora* gen. nov.** Oehl, J. Palenzuela & N. Ferrol  
Type species: *Otospora bareai* J. Palenzuela, N. Ferrol & Oehl

Sporae singulatim lateraliterque efformatae in distantia ad sacculum sporiferum terminalem vel intercalarem. Sporae globosae vel subglobosae cum tunicis duabus: tunica exterior et tunica interior. Pauciores strata exteriores sporarum coniuncta tunica hyphae et sacculi. Strata

interiores tunicae exterioris persistentes. Tunica interior sporae subtiliter laminata et perpetua, sine strato granulato.

Formation of sporocarps unknown. Spores formed in a short distance to a terminal or intercalary formed sporiferous saccule by swelling laterally on the hyphal stalk (=neck) of the saccule with two spore walls, an outer and an inner wall. Most layers of the outer wall are continuous with the wall of the sporiferous saccule. The inner layers of the outer wall are persistent. One to several septa are formed in the hyphal stalk during spore formation; at the beginning of spore formation, the content of the sporiferous saccule is separated from the hypha by septa at some distance of the terminus and the not yet developed spore; at later developmental stages additional septa in the stalk, positioned between the saccule terminus and the developing spore, may separate the collapsing saccule terminus from the spore. A final plug-like septum usually closes the pore at the spore base. The inner wall forms de novo during spore formation and consists of a thick, finely laminate layer that might have each one thin layer adherent on its outer and its inner surface. None of the layers of the inner wall have a beaded appearance. Formation of vesicular-arbuscular mycorrhizae unknown.

*Etymology.* Greek: *oto-* (ωτος = ear), *-spora* (σπορα = seed, spore) referring to the pigmented, tangential-lateral ear-like stalk at the spore base that persists on the spore of the type species after the terminus of the sporiferous saccule has collapsed and detached from the saccule stalk.

*Commentary.* The principal characters that differentiate *Otospora* from all other species in the Glomeromycetes forming spores laterally on (i.e. acaulosporoid) or within (i.e. entrophosporoid) a stalk of a sporiferous saccule are summarized (TABLE I). In brief the new genus shares acaulosporoid type of spore formation with the genera *Acaulospora* Gerd. & Trappe (Gerdemann and Trappe 1974) and with the bimorphic genera *Archaeospora* J.B. Morton & D. Redecker emend. Spain (Morton and Redecker 2001, Spain 2003) and *Appendicispora* Spain, Oehl & Sieverd. (Spain et al 2006). However acaulosporoid spores of *Acaulospora* and *Appendicispora* species have a more complex spore wall structure than *Otospora*; that is they have three walls (Stürmer and Morton 1999, Oehl et al 2006, Spain et al 2006). Furthermore acaulosporoid spores of *Acaulospora* have a "beaded" ornamentation on the surface of the inner wall (Morton and Benny 1990) and acaulosporoid spores of *Appendicispora* spp. do not directly arise laterally on the hyphal stalk of the sporiferous saccule but form on a pedicel, which branches laterally from the stalk. Only acaulosporoid spores of *Archaeospora* have two walls as in *Otospora*. However *Archaeospora* spores have a

simply structured outer wall being thin, only bilayered, hyaline and sometimes evanescent (TABLE I; Spain 2003, Hafeel 2004, Spain et al 2006).

***Otospora bareai* sp. nov.** J. Palenzuela, N. Ferrol & Oehl  
FIGS. 1–11

*Sporocarpia* ignota. Sacculus sporifer subhyalinus vel pallido-luteus vel luteus, globosus (150–210 µm diam) vel subglobosus (140–190 × 150–215 µm diam) et formationi sporae praecedens. Sporae singulae lateraliter formatae ad hypham in 30–90 µm distantia ad sacculum terminalem, flavae-brunneae vel fulvae-brunneae vel brunneae, globosae (150–200 µm diam) vel subglobosae (145–185 × 175–210 µm diam) vel ovoideae vel ellipsoideae vel irregulares. Sporae non colorantes reagente Melzeri et tunicis duabus: tunica exterior et interior. Tunica exterior in totum 5–12 µm crassa, stratis quatuoribus: stratum exterior hyalinum, tenue et evanescent; stratum secundum laminatum vel unitum, flavum vel fulvum (2.0–)3.0–4.5(–6.5) µm crassum, semi-persistens; stratum tertium laminatum, fulvum-brunneum vel brunneum et (2.5–)4.0–6.0 µm crassum et persistens; stratum interior (flavum vel) fulvum vel brunneum, subtile ad 1.5 µm crassum et persistens. Tunica interior de novo formans, hyalina stratis (duobus vel) ternibus, in totum 2.5–4.5 µm crassa. Stratum exterior tunicae interioris subtile ad invisibile; secundum stratum tunicae interioris subtiliter laminatum, 2.0–3.5 µm crassum; stratum interior tunicae interioris subtile ad 0.8 µm crassum. Hypha sacculi sporiferis fulva vel brunnea et lateraliter persistens ad sporam maturam. Septum strati tertioris et strati quatuoris tunicae exterioris porum sporae occludens. Holotypus hic designatus 91–9101: Z+ZT (ZT Myc 160).

*Sporocarps* not found in field samples or pot cultures.

*Sporiferous saccule* (FIGS. 1–3) is subhyaline to light yellow (to yellow), saccule terminus usually globose (ca. 150–210 µm diam) to subglobose (140–190 × 150–215 µm), with three wall layers (owl1–3) that are in total 2.0–3.5 µm thick (FIG. 2), formed at the end of a hyphal stalk at 30–90 µm from the spore that arises thereafter (FIG. 3). The two outer wall layers (owl1, owl2) are hyaline, evanescent and each 0.4–1.0 µm thick; the innermost wall layer (owl 3) is subhyaline to light yellow, 1.8–2.7 µm thick (FIG. 2). Only the globose terminus of the sporiferous saccule collapses at the stalk (FIGS. 4, 5) after the spore wall has formed and usually is rapidly detached from the stalk of mature spores in soil samples or pot cultures, while the stalk generally persists on the spore (FIGS. 6, 7).

*Stalk* (=neck) of the sporiferous saccule is yellow-brown to brown, in total 150–450 µm long and arising from a mycelium hypha; 25–40 µm wide at the base of the saccule terminus, tapering to 15–30 µm at the area where the spore arises laterally on the stalk and finally tapering to 5–12 µm toward the hypha (FIGS. 4, 5). Three wall layers of the sporiferous saccule

TABLE I. Principal morphological spore characteristics separating *Otospora* gen. nov. from all other genera with spore formation beneath a terminal or intercalary sporiferous saccule

	<i>Otospora</i> gen. nov.		<i>Archaeospora</i>	<i>Appendicispora</i>	<i>Acaulospora</i>	<i>Kuklospora</i>	<i>Intraspora</i>	<i>Entrophospora</i>
Spore dimorphism	Unknown	Yes	Yes	Yes	Unknown	Unknown	Unknown	Unknown
Spore formation type(s)	Acaulosporoid <sup>a</sup> :	Acaulosporoid & glomoid <sup>b</sup>	Acaulosporoid & glomoid <sup>b</sup>	Acaulosporoid & glomoid	Acaulosporoid	Entrophosporoid <sup>c</sup>	Entrophosporoid	Entrophosporoid
Presence of pedicel on spore base	No	No	No	Yes	No	No	No	No
Persisting stalk on the spore	Yes, tangential-laterally <sup>d</sup>	No	No	No, but a pedicel branching from the stalk often persists on the spore base, resembling a subtending hypha	No	No	No	Yes, vertically from the spore base <sup>e</sup> , resembling a subtending hypha
Number of walls	2 (ow, iw)	2 (ow, iw)	2 (ow, iw)	3 (ow, mw, iw)	3 (ow, mw, iw)	3 (ow, mw, iw)	2 (ow, iw)	2 (ow, iw)
Number of walls formed after closure of spore pore	1 (iw)	1 (iw)	1 (iw)	1 (iw)	2 (mw, iw)	2 (mw, iw)	1 (iw)	1 (iw)
Number of wall layers on ow	4	2	2	3	3(-4)	3	2	4
Known ornamentation types of ow	Unknown	Unknown	Unknown	Cerebriform	Cerebriform, pits, reticula, projections	Pits	Unknown	Warts, pits in the tips of knobby projections
Known ornamentation types on mw	—	—	—	Alveolate	Unknown	Unknown	—	—
Known ornamentation types on iw	Unknown	Unknown	Unknown	Unknown	Characteristic 'beaded' iw11	Characteristic 'beaded' iw11	Unknown	Unknown
Spore pore closed by	Septum of owl3 and by owl4	Septum arising from owl2	Septum arising from owl2	Septum arising from mw11 and by mw12	Laminae arising from owl2 and by owl3	Laminae arising from owl2 and by owl3	Septum arising from owl2	Plug of wall material from owl3 and by owl4

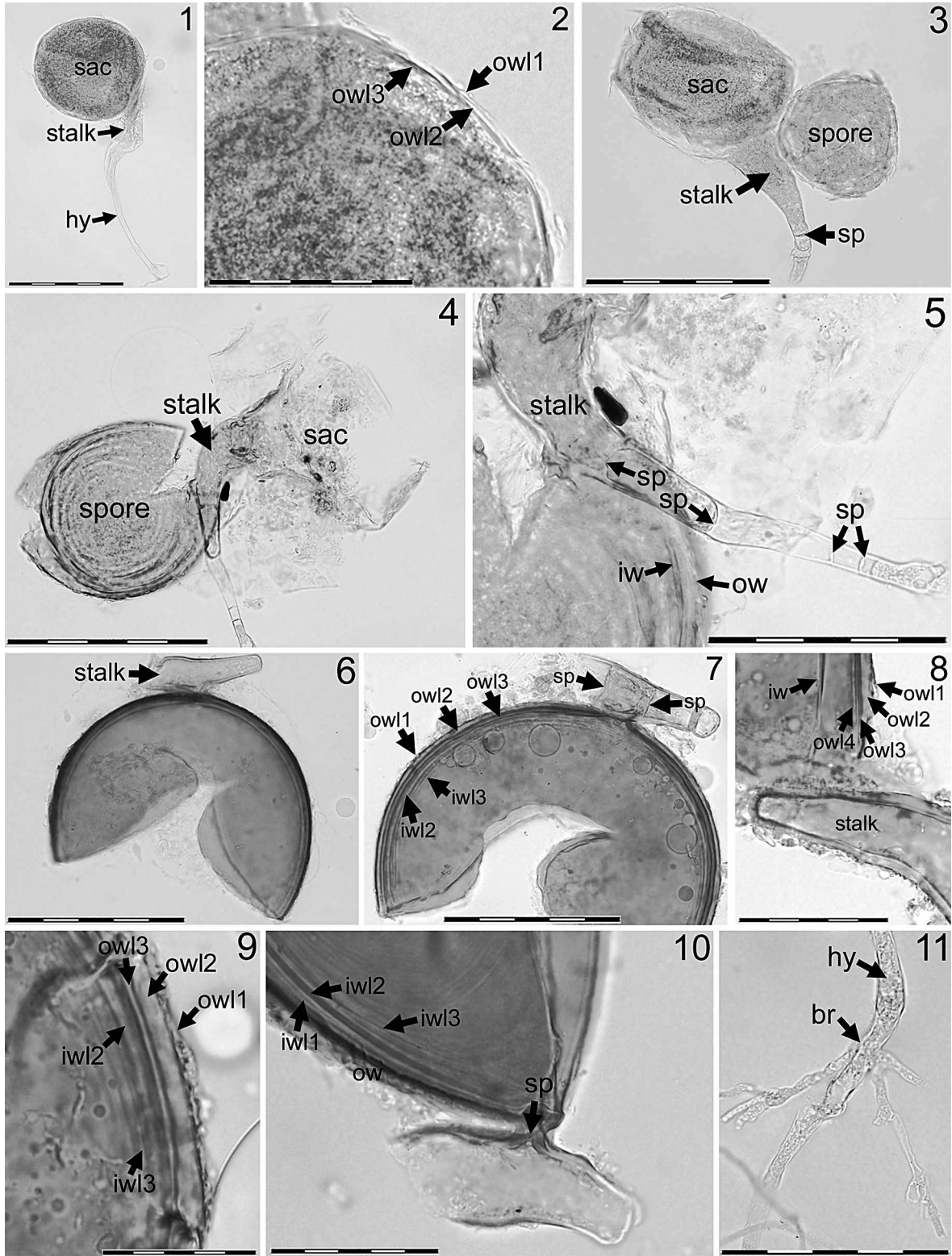
<sup>a</sup> Spore formation laterally on the stalk of a sporiferous saccule.

<sup>b</sup> Spore formation terminally on mycelia hyphae.

<sup>c</sup> Spore formation within the stalk of a sporiferous saccule.

<sup>d</sup> Known for the type species *O. bareai*.

<sup>e</sup> Known for the type species *E. infrequens*; abbreviations of spore walls and spore wall layers: outer wall (ow), middle wall (mw) and inner wall (iw) with 1-4 layers (e.g. owl3, mw12).



FIGS. 1-11. *Otospora bareai*. 1. Terminal sporiferous saccule (sac) with hyphal stalk (=neck) formed on mycelial hypha (hy); bar = 200  $\mu$ m. 2. Wall of sporiferous saccule with three wall layers (owl1-3); bar = 50  $\mu$ m. 3. Young, immature spore (spore) forming laterally on the stalk of a fully developed terminal saccule (sac). A septum (sp) formed in the hyphal stalk distal to the saccule (sac) and arising spore; bar = 200  $\mu$ m. 4. Mature, crushed spore formed on the persistent and pigmented stalk of a collapsing sporiferous saccule (sac); bar = 200  $\mu$ m. 5. Mature, crushed spore having outer wall (ow) and inner wall

continue over the total length of the stalk with 2.5–4.5  $\mu\text{m}$  at the base of the saccule terminus and at the area where the spore arises, tapering to 1.5–3.0  $\mu\text{m}$  toward the mycelium hypha. The outer layer is hyaline, thin (0.4–1.0  $\mu\text{m}$ ) and evanescent; middle layer is subhyaline to light yellow and 1.5–2.5  $\mu\text{m}$  thick at the base of the saccule terminus and at the area where the spore arises, tapering to 0.5–1.0  $\mu\text{m}$  toward the mycelium hypha; this layer is more resistant on the stalk than the outer layer but is often missing on mature spores too; inner layer is yellow-brown to brown, 2.0–3.5  $\mu\text{m}$  thick at the saccule base and at the area where the spore arises (FIGS. 4–8), tapering to 0.5–1.5  $\mu\text{m}$  toward the mycelium hypha (FIGS. 4, 5). One to several septa arising from the brown-pigmented third wall layer separate the contents of the sporiferous saccule from the hypha distal to the area where the spore thereafter may arise (FIGS. 3, 5). One to three additional septa may arise from the brown-pigmented wall layer separating the spore contents from the collapsing saccule (FIG. 7) at a later stage of spore formation. Finally an often plug-like, additional septum regularly arises at the spore base that separates the saccule stalk from the developing spore. The thick-walled saccule stalk generally persists on the spore resembling a tangential-lateral, inflated, single-branched subtending hypha (FIGS. 4–8).

*Spores* found singly in soils or in pot cultures, formed laterally on the hyphal stalk of a sporiferous saccule (FIGS. 4, 5). The spores are yellow-brown to brown, globose (150–200  $\mu\text{m}$ ) to subglobose (145–185  $\times$  175–210  $\mu\text{m}$ ) to rarely ovoid or irregular and consist of a yellow-brown to brown outer wall and a hyaline inner wall.

*Outer spore wall* with four layers (owl1–4; FIGS. 7–9), in total 5–12  $\mu\text{m}$  thick: outer layer owl1 hyaline, thin (0.5–1.0  $\mu\text{m}$ ) and evanescent; owl2 subhyaline to light yellow, (2.0–)3.0–4.5(–6.5)  $\mu\text{m}$  thick, semipersistent and sometimes slightly expanding in lactic acid based mountants; owl3 yellow-brown to brown (2.5–)4.0–6.0  $\mu\text{m}$  thick, laminated and tightly adherent to owl2;

owl4 concolorous with owl3, 0.5–1.5  $\mu\text{m}$  thick and, as tightly adherent to owl3, often difficult to observe. The three outer wall layers are continuous with the wall layers of the stalk of the sporiferous saccule.

*Inner wall* forms de novo during spore formation and consists of three hyaline layers (iwl1–3; FIGS. 7–10). Outer layer iwl1 is thin and usually hard to detect in PVLG based mountants because it usually is tightly adherent to iwl2; iwl2 is 2.0–3.5  $\mu\text{m}$  thick and finely laminate; iwl3 is 0.5–0.8  $\mu\text{m}$  thick and sometimes difficult to observe, especially when tightly adherent to iwl2; sometimes it readily separates from iwl2 and then is clearly visible forming often several folds (FIG. 10).

*Pore* at the spore base is (3–)6–11(–14)  $\mu\text{m}$  diam; usually occluded by a plug-like septum, which is 1.5–3.5  $\mu\text{m}$  thick and arising directly at the spore base from the laminate owl3 (FIG. 10) and adherent thin layer owl4. The pore at the spore base rarely appears to be open. Then the septa of the saccule stalk—proximal and distal to the terminus of the sporiferous saccule and next to the area where the spore has arisen—might separate the spore contents from the saccule stalk (FIG. 7). In plan view the spore pore resembles a ring or a cicatrix, being connected to the persistent saccule stalk.

*Mycelial hyphae* (FIG. 11) are hyaline, 1.2–3.0  $\mu\text{m}$  thick with two visible layers when recently formed; outer hyphal layer evanescent; inner hyphal layer persistent and continuous with owl3 of the saccule stalk and the spore.

*Formation of arbuscular mycorrhizae* so far unknown but assumed from spore ontogeny and morphology, from molecular phylogeny placing the fungus within the Diversisporales and from its molecular detection in the roots.

*Etymology.* *bareai*, in honor of Prof. José-Miguel Barea, Spanish pioneer researcher on arbuscular mycorrhizal fungi.

*Type.* Isolated from the pot culture rhizospheres of *Sorghum vulgare* and *Trifolium pratense* s.l. inoculated with soil taken from an endangered plant community of dolomitic endemisms located in the Sierra de Baza

←

(iw). Several septa (sp) have separated the contents of the hyphal mycelia from the contents of sporiferous saccule and the spore; bar = 100  $\mu\text{m}$ . 6. Crushed spore with persistent saccule stalk after both the terminus of the saccule and the hyphal mycelia have already detached; bar = 150  $\mu\text{m}$ . 7. Crushed spore showing outer and inner spore wall, each with several layers (owl1-3 and iwl2-3). Septa (sp) in the saccule stalk proximal and distal to the detached terminal saccule are also indicated; bar = 100  $\mu\text{m}$ . 8. Detail of wall structure: four-layered outer wall (owl1-4) and inner wall (iw), in the region where the stalk persists; bar = 50  $\mu\text{m}$ . 9. Outer and inner walls; owl2 slightly expanded in PVLG after light pressure on the cover slide; bar = 40  $\mu\text{m}$ . 10. Outer wall (ow) and three-layered inner wall (iwl1-3) in the region where the stalk persists. Inner wall layer iwl3 showing several thin folds. The septum (sp) closing the pore at the spore base arises from the brown laminate layer owl3; bar = 50  $\mu\text{m}$ . 11. Mycelial hypha (hy) with multiple branching (br) near a sporiferous saccule (which would be found in the lower left, outside the margins of this figure); bar = 100  $\mu\text{m}$ .

(Granada, Spain) at 1600 m a.s.l. (2°97'W, 37°37'). Holotype and type deposited at Z+ZT (accession number ZT Myc 160); isotypes deposited at OSC (OSC #134502) and GDA-GDAC.

*Distribution.* So far, *O. bareai* is known only from the type location in the Sierra de Baza National Park (dolomitic shrub land, Granada, Spain) detected in the rhizosphere of *Pterocephalus spathulatus* and *Thymus granatensis* on dolomite.

*Molecular analyses.*—Sequences of approximately 550 bp corresponding to the NS31-AM1 region of the 18S ribosomal gene were obtained from five single spores. All five sequences were identical. To examine evolutionary relationships among *O. bareai* and other species in the Glomeromycetes, phylogenetic trees were generated from multiple aligned sequences by using evolutionary parsimony and neighbor joining methods. Because both analyses produced trees with basically the same topology, only the neighbor joining tree is presented (FIG. 12). This topology is largely in agreement with those published by Schüssler et al (2001) and Redecker et al (2007). The phylogenetic analyses suggest that the sequence of *O. bareai* forms a distinct sister clade to *Diversispora spurca* and *Glomus versiforme* sequences of the *Glomus* group C sensu Schüssler (Schüssler et al 2001, Schwarzott et al 2001) of which so far only the type species *Diversispora spurca* recently was transferred to a new genus *Diversispora* of family Diversisporaceae (Walker and Schüssler 2004). The position of *O. bareai* close to the *D. spurca* clade was confirmed by PCR amplification and sequencing using the new primer designed for species related to *Diversispora* spp. (Redecker et al 2007) and DNA extracted from newly formed spores. By using this primer we finally were able to prove the presence of *O. bareai* in the roots, sampled from the pot cultures.

#### DISCUSSION

Under the compound microscope, spores of the new genus *Otophora* easily can be distinguished from spores of all other genera in the Glomeromycetes by its unique combination of type of spore formation and spore wall structure. Acaulosporoid spore formation and formation of two spore walls (ow and iw) is shared only with *Archaeospora* (TABLE I). However spores of *Ar. trappei*, the single species currently placed in the genus *Archaeospora*, are small (<100 µm) and have a simple-structured outer wall, which is not multiple layered, laminated, pigmented or persistent as in *Otophora bareai* (FIGS. 7–9).

Molecular analyses confirm our morphological observations (FIG. 12). *Otophora* phylogenetically is

clearly distant from all species with acaulosporoid spore formation, above all from the ancestral lineages of *Archaeospora* and *Appendicispora* (Redecker et al 2000, Morton and Redecker 2001, Spain et al 2006) but also from the species of *Acaulospora* (Redecker et al 2000, Oehl et al 2006). The data reveal that *O. bareai* phylogenetically is closest to *Diversispora spurca* (FIG. 12), which forms spores in a different manner, that is terminally on a mycelial hypha (=glomoid spore formation). Thus we propose to place the new genus *Otophora* with its type species *O. bareai* into family Diversisporaceae. Remarkably, the new genus represents a second lineage with acaulosporoid spores in the Diversisporales. It also is a further example of a lineage with acaulosporoid spores closely related to glomoid spores (FIG. 12).

Among the species that form their spores in a short distance to a terminal saccule, *O. bareai* shares the multiple-layered outer wall in combination with a single, bi- to three-layered inner wall only with the two species of the genus *Entrophospora* (I.R. Hall) R.N. Ames & R.W. Schneid. emend. Oehl & Sieverd.: *E. infrequens* (I.R. Hall) R.N. Ames & R.W. Schneid. emend. Oehl & Sieverd. (Hall 1977, Ames and Schneider 1979, Sieverding and Oehl 2006) and *E. baltica* Blas., Madej & Tadych (Blaszkowski et al 1998). However in the later genus type of spore formation is entrophosporoid (i.e. intrahyphally in the hyphal stalk of a sporiferous saccule). Due to the high similarities in spore wall composition between *O. bareai* and especially *E. infrequens*, which is the type species of *Entrophospora*, we considered the possibility of placing *Otophora* in family Entrophosporaceae. This would be justified because two other AMF families exist containing each two so-called “sister genera” differentiated by acaulosporoid or entrophosporoid spore formation but having strong similarities in spore wall composition. Species of *Archaeospora* and *Intraspora* Oehl & Sieverd. of the Archaeosporaceae have biwalled, hyaline and small spores, and *Acaulospora* and *Kuklospora* Oehl & Sieverd. of the Acaulosporaceae have three walls including a characteristic “beaded” inner wall (Sieverding and Oehl 2006). However the phylogenetic position of both *Entrophospora* species has not been clarified (Blaszkowski et al 1998, Millner et al 2001, Rodriguez et al 2001, Wubet et al 2003, Sieverding and Oehl 2006). We suggest therefore that no new genus should be included in the Entrophosporaceae, at least as long as the phylogenetic position of *E. infrequens* is not clear.

Under the dissecting microscope, a few AMF species could be confused with *O. bareai* because of similar spore size and color and lateral spore formation in a short distance of a sporiferous saccule.

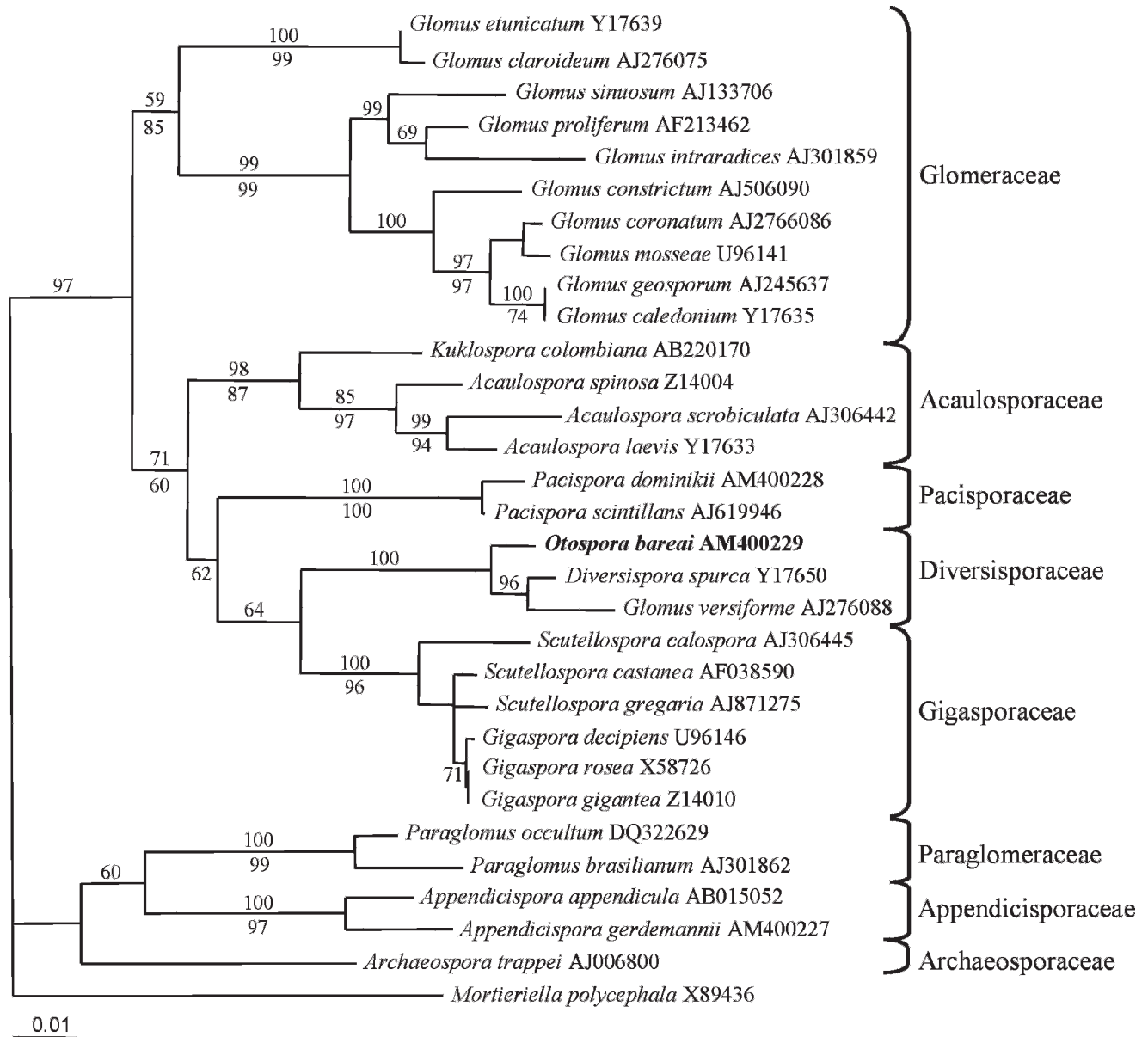


FIG. 12. Phylogenetic tree of the Glomeromycota obtained by neighbor joining analysis of partial subunit sequences of their ribosomal genes. The new sequence obtained in this study is indicated in bold. Sequences are labeled with their database accession numbers. Bootstrap values (in %) above branches are from the neighbor joining analysis (1000 bootstraps) and under branches from the Maximum Parsimony analyses (1000 bootstraps). Only topologies with bootstrap support of at least 60% are shown. The boxes to the right show the glomeromycotan families and delimitations of the phylotypes. Note: *Kuklospora colombiana* is the former *Entrophospora colombiana* (Sieverding and Oehl 2006); *Appendicispora appendicula* and *Ap. gerdemannii* are the former *Archaeospora leptoticha* and *Ar. gerdemannii*, respectively (Spain et al 2006).

These are *Acaulospora laevis* Gerd. & Trappe (Gerde-mann and Trappe 1974), *A. thomii* Blaszkowski (Blaszkowski 1988), *A. capsicula* Blaszkowski (Blaszkowski 1990) and *A. colossica* P.A. Schultz, Bever & J.B. Morton (Schultz et al 1999). However spores of these species can be distinguished easily from *O. bareai* under the compound microscope by the number and characteristics of the inner walls (TABLE I). Moreover the four *Acaulospora* spp. mentioned never have a pigmented

hyphal stalk persisting on the spore. Spores of the new species also could be confused under low magnification with some *Glomus* Tul. & C. Tul. (Gerde-mann and Trappe 1974), or *Pacispora* Sieverd. & Oehl (Oehl and Sieverding 2004) species. However such species generally have either a cylindrical, funnel-shaped or constricted hyphal attachment (so called subtending hypha) but not a tangential-laterally hyphal stalk, and beside *Pacispora robigina*



Sieverd. & Oehl (Oehl and Sieverding 2004) they lack a separate, hyaline inner wall.

Despite several attempts in 4 y we have not been able to grow the new fungus in pure culture (i.e. without other AMF) on any host plant. Thus we could not prove the arbuscular mycorrhizal nature of the fungus. However spore morphology and the phylogenetic position (FIG. 12) in the Diversisporales suggest that the fungus forms typical arbuscular mycorrhizae. All genera in the Diversisporales and all species closely related to *Diversispora* (*D. spurca*, *G. versiforme* and *G. aurantium*), as well as all AM fungal genera with acaulosporoid or entrophosporoid spore formation that include species with pigmented spores >100 µm, form typical arbuscular mycorrhizae (Morton and Benny 1990, Morton and Redecker 2001, Oehl and Sieverding 2004, Sieverding and Oehl 2006, Spain et al 2006). Moreover by using a new primer designed for species related to Diversisporaceae (Redecker et al 2007) we proved the presence of *O. bareai* in roots collected from the trap culture plants confirming our assumption. However it still remains to be shown conclusively whether *O. bareai* is indeed an AM fungal species or if it is associated with other rhizosphere organisms or might even be a saprophytic fungus, thus far unknown in the Glomeromycetes.

*Otospora bareai* thus far is known only from dolomitic soils of the Sierra de Baza in Andalucía growing in the rhizosphere of *Pteroccephalus spathulatus* and *Thymus granatensis*. Thus far morphological or molecular studies or so called “ecological sequences” obtained from molecular analyses and deposited in public databases have failed to suggest that *O. bareai* is present at any place elsewhere around the globe. Therefore it might represent a special, rare or even endemic species and the hypothesis might be considered that it is above all if it is an AMF species important for the presence of rare endemic plant species of the Sierra de Baza in southern Spain. Several AM fungal species accompanying *O. bareai* at its type location (e.g. *Glomus mosseae*, *G. constrictum*, *G. coronatum*, *G. etunicatum*, *Pacispora dominikii* and *Appendicispora gerdemanni*) have a broad distribution pattern in regularly managed Mediterranean land use systems, on other continents with similar climates or even in completely different climates and different soils around the globe (e.g. Calvente et al 2004, Ferrol et al 2004, Spain et al 2006, Bashan et al 2007). It will be interesting to examine the geographical distribution of *O. bareai* in more detail.

#### ACKNOWLEDGMENTS

This research was supported by the Spanish CICYT project REN2003-968. We cordially thank Ewald Sieverding (Insti-

tute for Plant Production and Agroecology in the Tropics and Subtropics, University of Hohenheim, Germany) for his valuable comments on the manuscript.

#### LITERATURE CITED

- Altschul SF, Gish W, Miller W, Myers EW, Lipton DJ. 1990. Basic local alignment search tool. *J Mol Biol* 215:403–410.
- Ames RN, Linderman RG. 1976. *Acaulospora trappei* sp. nov. *Mycotaxon* 3:565–569.
- , Schneider RW. 1979. *Entrophospora*, a new genus in the Endogonaceae. *Mycotaxon* 2:347–352.
- Bashan Y, Khaosad T, Salazar BG, Ocampo JA, Wiemken A, Oehl F, Vierheilig H. 2007. Mycorrhizal characterization of the boojum tree, *Fouquieria columnaris*, an endemic ancient tree from the Baja California Peninsula, Mexico. *Trees: Structure Function* 21:329–335.
- Blaszkowski J. 1988. Four new species of Endogonaceae (Zycomycotina). *Karstenia* 23:409–418.
- . 1990. Polish Endogonaceae VII. *Acaulospora capsicula* sp. nov. *Mycologia* 82:794–798.
- , Madej T, Tadych M. 1998. *Entrophospora baltica* sp. nov. and *Glomus fuegianum*, two species in the Glomales from Poland. *Mycotaxon* 68:165–184.
- Brundrett M, Melville L, Peterson L. 1994. Practical methods in mycorrhizal research. University of Guelph, Guelph, Ontario, Canada: Mycologue Publications.
- Calvente R, Cano C, Ferrol N, Azcón-Aguilar C, Barea JM. 2004. Analysing natural diversity of arbuscular mycorrhizal fungi in olive tree (*Olea europaea* L.) plantations and assessment of the effectiveness of native fungal isolates as inoculants for commercial cultivars of olive plantlets. *Appl Soil Ecol* 26:11–19.
- Felstein J. 1993. PHYLIP, version 3.5, Department of Genetics, University of Washington, USA.
- Ferrol N, Calvente R, Cano C, Barea JM, Azcón-Aguilar C. 2004. Analysing arbuscular mycorrhizal fungal diversity in shrub-associated resource-islands from a desertification-threatened semiarid Mediterranean ecosystem. *Appl Soil Ecol* 25:123–133.
- Fuchs B, Haselwandter K. 2004. Red list plants: colonization by arbuscular mycorrhizal fungi and dark septate endophytes. *Mycorrhiza* 14:277–281.
- Gerdemann JW, Trappe JM. 1974. The Endogonaceae in the Pacific Northwest. *Mycologia Memoir* 5. 76 p.
- Hafeel KM. 2004. Spore ontogeny of the arbuscular mycorrhizal fungus *Archaeospora trappei* (Ames & Schneider) Morton & Redecker (Archaeosporaceae). *Mycorrhiza* 14:213–219.
- Hall IR. 1977. Species and mycorrhizal infections of New Zealand Endogonaceae. *Trans Brit Mycol Res* 341:356.
- Helgason T, Daniell TJ, Husband R, Fitter A, Young P. 1998. Ploughing up the wood-wide web? *Nature* 394:431.
- Hewitt EJ. 1952. Sand and water culture methods used in the study of plant nutrition. Farnham Royal, Commonwealth Agricultural Bureau Bucks. Technical Communication 22.
- Koske RE, Tessier B. 1983. A convenient, permanent slide mounting medium. *Mycol Soc Am Newsl* 34:59.

- Melendo M, Giménez E, Cano E, Gómez-Mercado F, Valle F. 2003. The endemic flora in the south of the Iberian Peninsula: taxonomic composition, biological spectrum, pollination, reproductive mode and dispersal. *Flora* 198:260–276.
- Millner PD, Mulbry WW, Reynolds SL. 2001. Taxon-specific oligonucleotide primers for detection of *Glomus etunicatum*. *Mycorrhiza* 10:259–265.
- Morton JB, Benny GL. 1990. Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): a new Glomales, two new suborders, Glomineae and Gigasporineae, and two new families, Acaulosporaceae and Gigasporaceae, with an emendation of Glomaceae. *Mycotaxon* 37:471–491.
- , Redecker D. 2001. Two new families of Glomales, Archaeosporaceae and Paraglomaceae, with two new genera *Archaeospora* and *Paraglomus*, based on concordant molecular and morphological characters. *Mycologia* 93:181–195.
- Mota JF, Pérez-García FJ, Jiménez ML, Amate JJ, Peñas J. 2002. Phytogeographical relationships among high mountain areas in the Baetic Ranges (South Spain). *Global Ecol Biogeogr* 11:497–504.
- Oehl F, Redecker D, Sieverding E. 2005a. *Glomus badium*, a new sporocarpic mycorrhizal fungal species from European grasslands with higher soil pH. *J Appl Bot & Food Qual* 79:38–43.
- , Sieverding E. 2004. *Pacispora*, a new vesicular arbuscular mycorrhizal fungal genus in the Glomeromycetes. *J Appl Bot & Food Qual* 78:72–82.
- , ——, Ineichen K, Mäder P, Boller T, Wiemken A. 2003. Impact of land use intensity on the species diversity of arbuscular mycorrhizal fungi in agroecosystems of Central Europe. *Appl Environ Microbiol* 69:2616–2624.
- , ——, ——, Ris EA, Boller T, Wiemken A. 2005b. Community structure of arbuscular mycorrhizal fungi at different soil depths in extensively and intensively managed agroecosystems. *New Phytol* 165:273–283.
- , Sýkorová Z, Redecker D, Wiemken A, Sieverding E. 2006. *Acaulospora alpina*, a new arbuscular mycorrhizal fungal species characteristic for high mountainous and alpine regions of the Swiss Alps. *Mycologia* 98:286–294.
- Redecker D, Morton JB, Bruns TD. 2000. Ancestral lineages of arbuscular mycorrhizal fungi (Glomales). *Mol Phylogenet Evol* 14:297–301.
- , Raab P, Oehl F, Camacho FJ, Courtecuisse R. 2007. A novel clade of sporocarps-forming species of glomeromycotan fungi in the *Diversisporales* lineage. *Mycol Progress* 6:35–44.
- Rodriguez A, Dougall T, Dodd JC, Clapp JP. 2001. The large subunit ribosomal RNA genes of *Entrophospora infrequens* comprise sequences related to two different glomeralean families. *New Phytol* 152:159–167.
- Schüßler A, Schwarzott D, Walker C. 2001. A new fungal phylum, the Glomeromycota: phylogeny and evolution. *Mycol Res* 105:1413–1421.
- Schultz PA, Bever JD, Morton JB. 1999. *Acaulospora colossica* sp nov from an old field in North Carolina and morphological comparisons with similar species *A-laevis* and *O-koskei*. *Mycologia* 91:676–683.
- Schwarzott D, Walker C, Schüßler A. 2001. *Glomus*, the largest genus of the arbuscular mycorrhizal fungi (Glomales), is non-monophyletic. *Mol Phylogenet Evol* 21:190–197.
- Simon L, Lalonde M, Bruns TD. 1992. Specific amplification of 18S fungal ribosomal genes from VA endomycorrhizal fungi colonising roots. *Appl Environ Microbiol* 58:291–295.
- Spain JL. 1990. Arguments for diagnoses based on unaltered wall structures. *Mycotaxon* 38:71–76.
- . 2003. Emendation of *Archaeospora* and of its type species, *Archaeospora trappei*. *Mycotaxon* 87:109–112.
- , Sieverding E, Oehl F. 2006. *Appendicispora*, a new genus in the arbuscular mycorrhizal-forming Glomeromycetes, with a discussion of the genus *Archaeospora*. *Mycotaxon* 97:163–182.
- Stürmer SL, Morton JB. 1999. Taxonomic reinterpretation of morphological characters in Acaulosporaceae based on developmental patterns. *Mycologia* 91:849–857.
- Thompson JD, Higgins DG, Gibson TJ. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acid Res* 22:4673–4680.
- van der Heijden MGA, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, Wiemken A, Sanders IR. 1998. Mycorrhizal diversity determines plant diversity, ecosystem variability and productivity. *Nature* 396:69–72.
- Walker C, Schüßler A. 2004. Nomenclatural clarifications and new taxa in the Glomeromycota. *Mycol Res* 108:979–982.
- Wubet T, Weiss M, Kottke I, Oberwinkler F. 2003. Morphology and molecular diversity of arbuscular mycorrhizal fungi in wild and cultivated yew (*Taxus baccata*). *Can J Bot* 81:255–266.