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Ambispora granatensis, a new arbuscular mycorrhizal fungus, associated with *Asparagus officinalis* in Andalucía (Spain)

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Abstract: A new dimorphic fungal species in the arbuscular mycorrhiza-forming Glomeromycota, *Ambispora granatensis*, was isolated from an agricultural site in the province of Granada (Andalucía, Spain) growing in the rhizosphere of *Asparagus officinalis*. It was propagated in pot cultures with *Trifolium pratense* and *Sorghum vulgare*. The fungus also colonized Ri T-DNA transformed *Daucus carota* roots but did not form spores in these root organ cultures. The spores of the acaulosporoid morph are 90–150 μm diam and hyaline to white to pale yellow. They have three walls and a papillae-like rough irregular surface on the outer surface of the outer wall. The irregular surface might become difficult to detect within a few hours in lactic acid-based mountings but are clearly visible in water. The structural central wall layer of the outer wall is only 0.8–1.5 μm thick. The glomoid spores are formed singly or in small, loose spore clusters of 2–10 spores. They are hyaline to pale yellow, (25)40–70 μm diam and have a bilayered spore wall without ornamentation. Nearly full length sequences of the 18S and the ITS regions of the ribosomal gene place the new fungus in a separate clade next to *Ambispora fennica* and *Ambispora gerdemannii*. The acaulosporoid spores of the new fungus can be distinguished easily from all other spores in genus *Ambispora* by the conspicuous thin outer wall.

Key words: arbuscular mycorrhiza, *Archaeospora*, bimorphism, dimorphism, germination, Glomeromycetes

INTRODUCTION

At present about 220 arbuscular mycorrhizal (AM) fungal species have been described in the Glomeromycota. The majority of these species belongs to Glomerales and Diversisporales (Schüßler et al. 2001), while only nine and three species respectively belong to Archaeosporales and Paraglomerales (Spain et al. 2006, Renker et al. 2007, Walker 2008, Goto et al. 2008). However recent molecular studies on the diversity of AM fungi strongly suggest that the Glomeromycota comprise many more species than are known so far (e.g. Hijri et al. 2006, Öpik et al. 2006). The large amount of environmental sequences that have been deposited in the public databases (e.g. EMBL) supports this assumption for all four glomeromycotan orders. Most of these environmental sequences cannot confidently be attributed to a described AM fungal species. On the other hand many undescribed AM fungi have been found with morphological identification tools, but due to insufficient numbers of specimens or unclear morphological features, these fungi could not be analyzed further and published (e.g. Oehl et al. 2009, 2010).

Several AM fungi have been grown successfully in pure pot cultures on many host plants for several decades (e.g. Mosse 1953, Gerdemann and Trappe 1974). This is easy for a large number of common AM fungi like *Glomus mosseae* and *Glomus intraradices* but difficult for many others, especially AM fungi that might have specific niches (e.g. Oehl and Sieverding 2004; Oehl et al. 2005, 2006; Redecker et al. 2007; Tchabi et al. 2009; Palenzuela et al. 2008, 2010).

Spores of a new AM fungal species belonging to the Archaeosporales were isolated repeatedly from the rhizosphere of 4–8 y old mono-crop *Asparagus officinalis* L. plants in the province of Granada (Andalucía, Spain). Pure cultures were established successfully in pot cultures after multiple spore inoculation of the host plants *Trifolium pratense* and *Sorghum vulgare*. Glomoid spores were formed in the pot cultures where only spores of the acaulosporoid morph were introduced, proving the bimorphic nature of this species. The fungus is described here under the epithet *Ambispora granatensis* based on morphological analyses of the two spore types, the mycorrhiza and mycelia structures, and on molecular analyses of the 18S and the ITS of the ribosomal gene.

MATERIALS AND METHODS

Soil sampling.—At several dates between 2004 and 2009 soil samples were taken with a shovel in a conventionally managed agricultural soil from the rhizosphere of *Aspara-*

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gus officinalis L. The site is in the community of Lachar (Granada, Andalucía, Spain) at 590 m (37°10′N, 03°49′W). Soil pH (H_2O) was 8.1, and plant available phosphorus was 26 mg kg^{-1}.

AM fungal pot cultures.—To cultivate the new AM fungus under reproducible conditions multiple acaulosporoid spores (20 spores per pot) were extracted directly from field samples and inoculated into the rhizosphere of 1 wk old *Trifolium pratense* and *Sorghum vulgare* plantlets. The fungus and its hosts were grown in 350 mL pots with a mixture of autoclaved field soil, vermiculite and sand (2 : 1 : 1) as potting substrate. Two (of 10) pure cultures of the new fungus were established successfully. Plants were grown in a greenhouse with 16 h photoperiod, 25/18 C day/night and 60% relative humidity. The pots were irrigated three times per week and fertilized every 4 wk with Long Ashton nutrient solution (Hewitt 1966). Subsamples of these cultures were used for spore analyses and analyses of the mycorrhizal and mycelia structures. Selected colonized roots were cut into 3 cm pieces for observation of the mycorrhizal structures after clearing and staining with trypan blue (Brundrett et al. 1994).

AM fungal monoxenic cultures.—The dual culture was initiated by placing a surface-sterilized acaulosporoid spore of the new species and about 0.5 cm long, actively growing root tip piece of a sterile *Daucus carota* Ri T-DNA transformed root (kindly provided by Y. Piché, Université Laval, Québec, Canada) in a Petri dish containing M medium (Becard and Fortin 1988). The culture was incubated in the dark at 24 C for > 24 mo. Plates initially were observed once a week until spore germination and then every 3 d until the hyphae made contact with the root. Mycorrhizal roots then were transferred every 6 mo to new Petri dishes containing young carrot roots and fresh M medium. Mycelia growth was checked every 2 wk under the stereomicroscope, but spores never formed in this culture.

Morphological analyses.—Spores were extracted from field soils and pot culture substrates by wet-sieving and sucrose centrifugation (Brundrett et al. 1994). About 100 acaulosporoid and 30 glomoid spores were examined. Spore formation characteristics and morphology of spores, sporiferous saccules, pedicels, subtending hyphae and the 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 adopted from Spain et al. (2006) and Goto et al. (2008) recently developed for *Ambispora* species (Spain et al. 2006, Walker 2008). Photographs were taken with a digital camera (Nikon DS-Fi1) on a compound microscope (Nikon eclipse 50i) 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 (herbarium of the University of Granada, Spain).

Molecular analyses.—Crude extracts from five single acaulosporoid spores were prepared as described by Ferrol et al. (2004) and used as template for the polymerase chain reaction. PCRs 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 with 1 μM concentration for each primer. Nearly full length SSU (18S) rDNA gene fragments were amplified by nested PCR as described in Schwarzott and Schüßler (2001). The ITS rDNA regions also were amplified by nested PCR using in the first PCR the universal eukaryotic primers SSU-Glom1 (Renker et al. 2003) and LSU-Glom1b (Walker et al. 2007), and the ITS1 and ITS4 (White et al. 1990) primers in the second run. Cycling parameters for the amplification of the DNA from ITS region were as described by Walker et al. (2007).

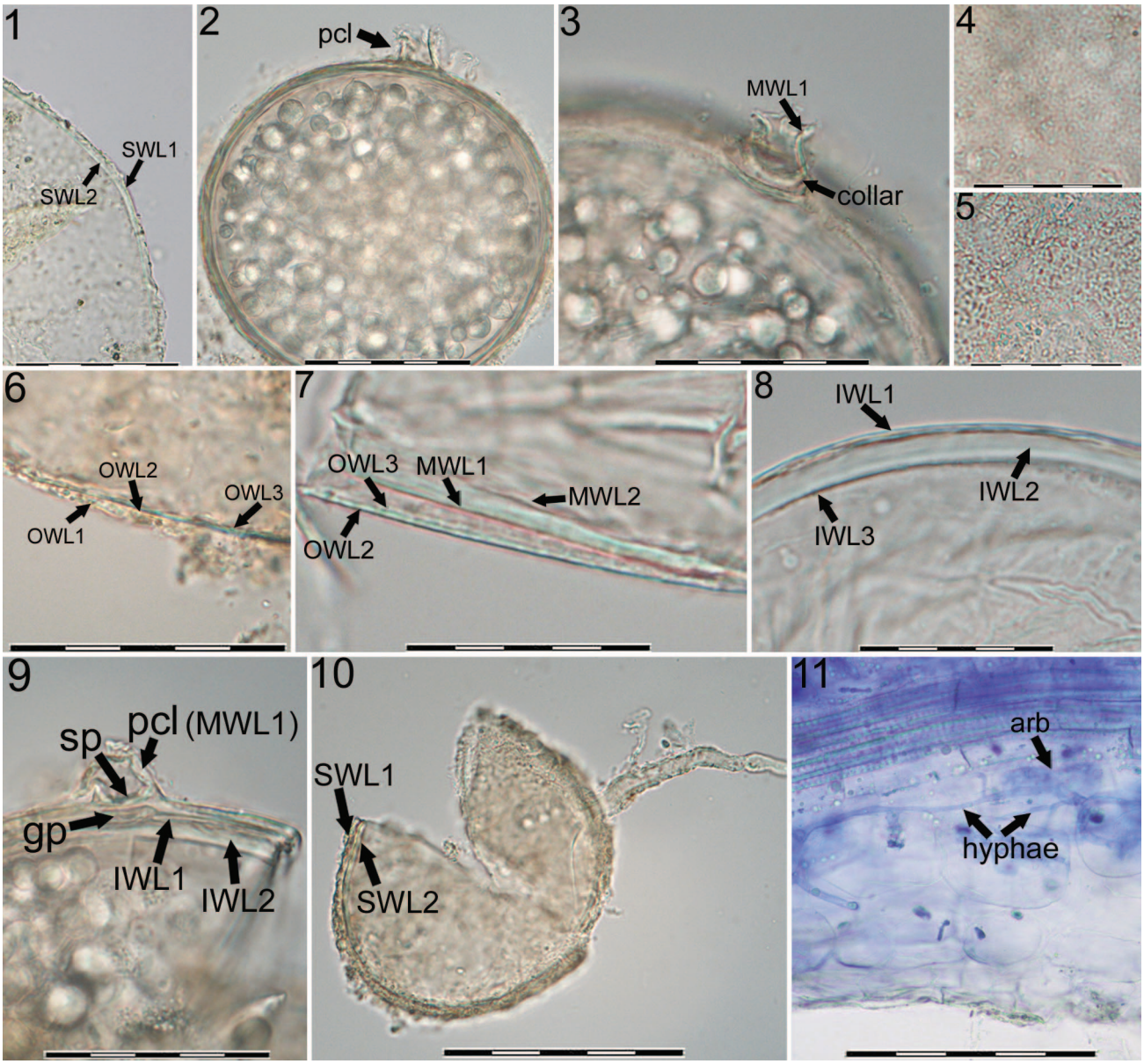
PCR products from the second round of amplifications were separated electrophoretically on 1.2% agarose, stained with ethidium bromide and viewed by UV illumination. The expected band was excised with a scalpel, isolated from the gel with the QIAEX II Gel Extraction kit (QIAGEN, USA) following the manufacturer's protocol, cloned into the pCR2.1 vector (Invitrogen, Carlsbard, California), and transformed into one shot TOP10 chemically competent *Escherichia coli* cells. After plasmid isolation from transformed cells cloned DNA fragments were sequenced with vector primers (White et al. 1990) in both directions. Nucleotide sequences were determined with *Taq* polymerase cycle sequencing and an automated DNA sequencer (Perkin-Elmer ABI Prism 373). Sequence data were compared to gene libraries (EMBL and GenBank) with BLAST programs (Altschul et al. 1990). Sequences for the 18S rRNA and ITS regions obtained in this study were deposited in the EMBL database under accession numbers FN820276–FN820282 and FN820272–FN820275 respectively. The 18S rDNA alignment comprised all archaeosporan near full length sequences available in the public databases, four individual sequences of *Ambispora granatensis* and sequences from members of *Paraglomerales* were used as outgroups. The ITS1, 5.8S and ITS2 alignments consisted of six individual and one consensus sequence of *A. granatensis*. Multiple sequence alignments of gene sequences were carried out with Clustal W 1.5 (Thompson et al. 1994). The Kimura two-parameter method was used to estimate distances, and the phylogenetic analyses for the 18S rDNA and ITS regions were performed by neighbor joining and parsimony with PHYLIP (Felstein 1993). The relative support of the different nodes was determined based on 1000 bootstrap trees. The phylogenetic trees were drawn with TreeView.

TAXONOMY

Ambispora granatensis sp. nov. J. Palenzuela, N. Ferrol & Oehl FIGS. 1–11

MYCOBANK MB513528

Sporocarpia ignota. Sporae dimorphae; sporae acaulosporoideae hyalinae vel albae vel raro flavoalbae, globosae



FIGS. 1–11. *Ambispora granatensis*. 1. Sporiferous saccule wall of acaulosporoid morph consisting of two layers (SWL1 and SWL2); bar = 75 µm. 2, 3. Spores with pedicel (pcl) formed by the evanescent outer wall and by the semi-persistent outer layer of the middle wall (MWL1), and with collar formed by the outer wall (FIG. 3). 4, 5. Papillae-like rough irregular outer spore surface in planar view; specimen mounted in water (FIG. 4) and in PVLG (FIG. 5) respectively; bars = 50 µm. 6. Three-layered OW (OWL1–3); bar = 50 µm. 7. Bilayered MW (MWL1–2); bar = 100 µm. 8. Three-layered inner wall (IWL1–3); bar = 20 µm. 9. Pedicel (pcl) with septum (sp) formed by MWL1; the IW shows a germination pore (gp) beneath the septum; bar = 50 µm. 10. Bilayered glomoid spores with evanescent light creamy outer layer (SWL1) and hyaline to subhyaline, persistent inner layer (SWL2); bar = 50 µm. 11. Mycorrhizal structures within *Trifolium pratense* with hyphae and an arbuscule (arb) staining pale blue in trypan blue; bars = 100 µm.

(92–145 µm diam) vel ovales (90–135 × 100–150 µm), tribus tunicis stratis pluribus, formatae appendice lateraliter ex hypha terminata in sacculo sporangifero; tunica exterior tunica mediaque coniuncta appendicis et hyphae; tunica exterior stratis tribus, hyalina vel alba vel albo-flava, 1.2–1.9 µm crassa; tunica media duobus stratis hyalinis, 2.4–4.0 µm crassa; tunica interior de novo formans stratis tribus, 2.5–4.9 µm crassa; stratum exterior tunicae exterioris, papillis irregularibus subtilibus; stratum secundum, 0.8–1.5 µm crassum, stratum interior tunicae exterioris subtile. Stratum medium tunicae exterioris et stratum exterior tunicae interioris flava colorantes in solutione Melzeri; tunica media porum sporarum acaulosporoidearum occludens. Sporae glomoideae hyalinae vel subhyalinae vel albae-flavae, (25–)40–70 µm, stratis duobus, in totum 2.0–3.5 µm crassis; porum sporae glomoideae generaliter apertae.

Formans structuras mycorrhizarum vesiculae-arbuscularum; structurae colorantes pallido-caeruleae cum trypan blue. Holotypus: 55-5501 (Z + ZT: ZT Myc 1626).

Type. Holotype 55-5501 (Z + ZT: ZT Myc 1626) isolated from the rhizosphere of *Asparagus officinalis* (community Lachar, province of Granada, Spain) at about 590 m (37°10′N, 03°49′W). Further type specimens from field samples and from pure cultures propagated on *Trifolium pratense* and *Sorghum vulgare* (55-5502, 55-5503, 55-5504, 55-5505, 55-5506): deposited at Z + ZT (ZT Myc 1626); isotypes 55-5511 and 55-5512 deposited at OSC (OSC No. 134,712); isotypes 55-5513, 55-5514, 55-5515, 55-5516, 55-5517 deposited at GDA-GDAC.

Etymology. *granatensis* referring to the province Granada (Andalucía, Spain) where the new species was found.

Spore formation. Sporocarp formation is unknown. The species is dimorphic differentiating acaulosporoid and glomoid spores, both having spore wall organizations that are typical for *Ambispora*. Acaulosporoid spores (FIGS. 1–9) form singly in soils on a short pedicel that branches laterally from the neck of a sporiferous saccule. Glomoid spores (FIG. 10) are formed singly or in clusters.

Sporiferous saccules are hyaline to subhyaline, globose to subglobose, 125–185 μm diam, and are formed terminally on mycelia hyphae. The saccule wall is bilayered (FIG. 1), consisting of a subhyaline to light yellow or light creamy, rapidly degrading, evanescent outer layer (SWL1; 0.5–1.2 μm thick) and a hyaline to subhyaline, semipersistent inner layer (SWL2; 1.1–2.5 μm thick). The hyphal neck of the saccule is 18–26 μm wide at the spore base and tapers to 5–10 μm within 200–350 μm. The sporiferous saccule often collapses and generally detaches from the mature spore while the pedicel often persists on the spore.

Acaulosporoid spores form singly on a short hyphal pedicel (FIGS. 2, 3) that branch from the saccule neck about 60–160 μm from the saccule terminus. The spores are globose (92–145 μm diam) to subglobose to oval, 90–135 × 100–150 μm, hyaline to white to white-yellow in water and generally become ocher-yellow with age and also when mounted in PVLG. They are white when the outer wall has sloughed from the spore. The spores have three walls (FIGS. 6–8): a three-layered outer wall (OW), a bilayered middle wall (MW) and a three-layered inner wall (IW).

Outer wall generally consists of three layers (FIGS. 6, 7). The outer layer (OWL1) is hyaline to white to white yellow in water, becoming ocher-yellow with age and also when mounted in PVLG. It is evanescent and has a papillae-like rough irregular surface (FIGS. 4, 5). The irregular surface is easily detectable in spores mounted in water but often disappears in lactic acid-based mounting media after a few hours. The structural second layer OWL2 apparently has no laminae, is only 0.8–1.5 μm thick and can appear slightly undulate. It generally stains bright yellow in Melzer's reagent but the staining diminishes within several months. The third layer OWL3 is generally difficult to detect (FIGS. 6, 7) because it is < 0.6 μm thick, tightly adhered to OWL2 and often is hidden further by the outer layer of the middle wall (MWL1). The OW is continuous with the undifferentiated outer wall layer of the pedicel and with SWL1 of the saccule wall.

Middle wall is bilayered, hyaline and in total 2.4–4.0 μm thick (FIG. 6). MWL1 is 1.5–3.0 μm thick and continuous with the persistent inner layer of the pedicel (FIG. 3) and with SWL2 of the saccule wall (FIG. 1). Sometimes one to two septa arise in the pedicel from MWL1 (FIG. 9). MWL2 is 1.0–2.0 μm thick, tightly adhered to MWL1 and forms the final pore closure of the pedicel at the spore base by continuous wall material. Under slight pressure on the cover slide both layers easily disaggregate into several to many small (about 5–15 × 5–10 μm), irregular pieces, indicating the fragile nature of the middle wall.

Inner wall is hyaline, in total 2.4–4.9 μm thick, and has three wall layers that generally are easily detectable (FIGS. 8). The IW forms de novo, presumably after the spore pore has been closed by the inner layer of the middle wall (MWL2). The outer layer IWL1 is 0.5–1.2 μm (FIG. 8), tightly adhered to the central layer IWL2 and regularly stains pale to bright yellow in Melzer's reagent. The staining reaction of IWL1 may disappear within several months. Second layer IWL2 is finely laminate and 2.0–3.5 μm thick (FIG. 8). Innermost layer IWL3 is 0.5–0.9 μm thick and also tightly adhered to IWL2 (FIG. 8). IWL3 sometimes separates slightly from IWL2 and often shows several thin folds, especially when pressure is applied to the cover slip. The IW functions as germinal wall as the germ structure, and subsequently germ tubes emerge from this wall through an initial germ pore (gp).

Pedicel of acaulosporoid spores is 11–25 μm long and 13–21 μm wide at the spore base tapering to 7–14 μm from the spore base (FIGS. 2, 3, 9). Sometimes two wall layers on the pedicel were observed that are continuous with the OW and MWL1 of the spore wall and with SWL1 and SWL2 of the saccule wall, but often the OW sloughs. In other cases the pedicel disappears from the spore base leaving a collar formed by the OW (FIG. 2). When the OW and pedicel are missing on the spore, a cicatrix-like ring structure formed by MWL1 remains at the point where the pedicel was attached. The OW, if present

on the pedicel, is 1.2–2.5 µm thick at the spore base tapering to 0.5–1.0 µm. The MW of the pedicel is 1.9–3.0 µm thick at the spore base tapering to 0.9–2.0 µm.

Germination of acaulosporoid spores was observed on several spores isolated from field soil samples in springtime. The main germ tube generally grows straight through the pedicel and the collar of the OW if they remain present on the germinating spores. After careful separation of the pedicel and the middle wall from the inner wall of ungerminated spores, a circular structure was detected on the IW of several spores directly beneath the pedicel, showing several thin folds on IWL1. An initial germ pore-like structure was detected only once beneath IWL2. The lobed germination structure (gs) emerges in germinating spores consisting of a few (3–6), 5–15 µm long lobes and 1–5 germ tubes arising between the lobes.

Glomoid spores are hyaline to subhyaline and have two wall layers that are continuous with the two wall layers of the mycelia hyphae (FIG. 10). They are about (25–)40–70 µm diam, and their wall is 2.0–3.5 µm thick. The outer layer (SWL1) is evanescent, hyaline to subhyaline in water, and 0.7–1.4 µm thick. It may become light yellow to light creamy with age and when mounted in PVLG. The inner layer (SWL2) is persistent, hyaline to subhyaline, 1.2–2.1 µm thick, and finely laminate. The cylindrical to slightly funnel-shaped subtending hyphae are 3.5–6.1 µm wide at the spore base, where rarely a thin septum was observed but generally the spore pore appeared to be open. Germinating glomoid spores were not found.

Mycelia hyphae are 3.1–7.9 µm diam. They consist of a thin outer layer, which is often undetectable in PVLG, and an inner structural layer. The layers are continuous with the wall layers of the glomoid morph and the sporiferous saccules (SWL1 and SWL2 respectively) and with the OW and MWL1 of the acaulosporoid morph. The outer layer is hyaline to subhyaline to yellow-white, evanescent and 0.1–0.5 µm thick; the inner layer is hyaline (to rarely light yellow in PVLG), semipersistent to persistent and (0.5–)1.0–1.8 µm thick.

Formation of vesicular arbuscular mycorrhiza with mycorrhizal structures that stain pale blue in trypan blue (FIG. 11).

Distribution. So far *A. granatensis* has been isolated only from a single agricultural site (community of Lachar, province of Granada, southern Spain) from the rhizosphere of 4–8 y old *Asparagus officinalis* plants together with the AM fungi *Glomus coronatum*, *G. mosseae*, *G. constrictum*, *G. claroideum* and *G. intraradices*.

Molecular analyses.—Nearly full length 18S and ITS sequences of the ribosomal gene were obtained from single spores of *A. granatensis*. Given that for both regions the neighbor joining and parsimony trees presented similar topologies, only the neighbor joining trees are presented (FIGS. 12, 13). The topologies are largely in agreement with those published previously (e.g. Walker et al. 2007). The phylogenetic analyses show that the sequence of *A. granatensis* clusters within the *Ambispora clade* next to *A. gerdemannii* and *A. fennica*.

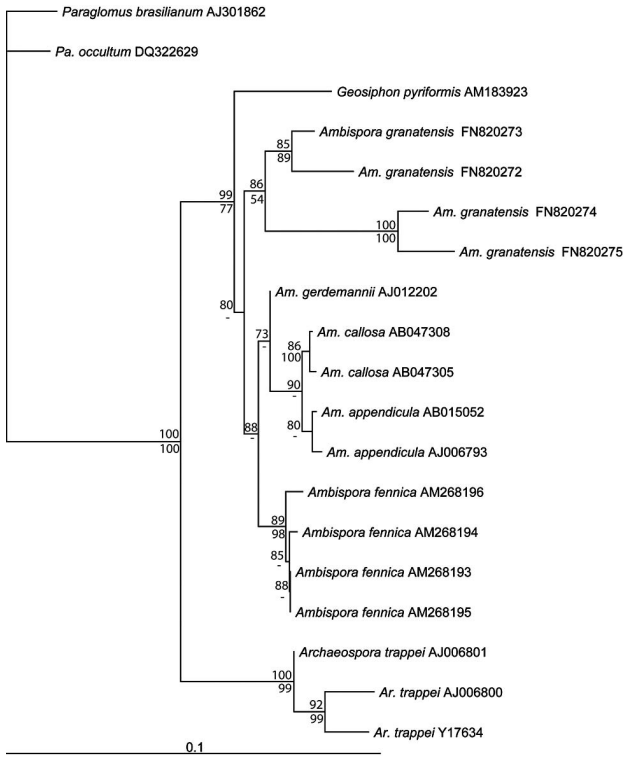


FIG. 12. Phylogenetic tree of the Archaeosporales obtained by neighbor joining analysis of nearly full length 18S sequences of the ribosomal gene. Sequences from members of *Paraglomerales* were used as outgroups. The new sequences obtained in this study are indicated in boldface. Sequences are labeled with database accession numbers. Bootstrap values (in %) above branches are from the neighbor joining analysis (1000 bootstraps) and under branches from maximum parsimony analyses (1000 bootstraps). Note: *Ambispora appendicula* and *Am. gerdemannii* are the former *Archaeospora leptoticha* and *Ar. gerdemannii* respectively (Spain et al. 2006, Walker 2008).

DISCUSSION

The morphological characteristics of the new fungus clearly suggested that it belongs to genus *Ambispora*. This finding was confirmed by phylogenetic analyses of sequences from the 18S rDNA gene and the ITS regions: *A. granatensis* clustered within the *Ambispora clade* (Spain et al. 2006, Walker et al. 2007), next to *A. gerdemannii* and *A. fennica*.

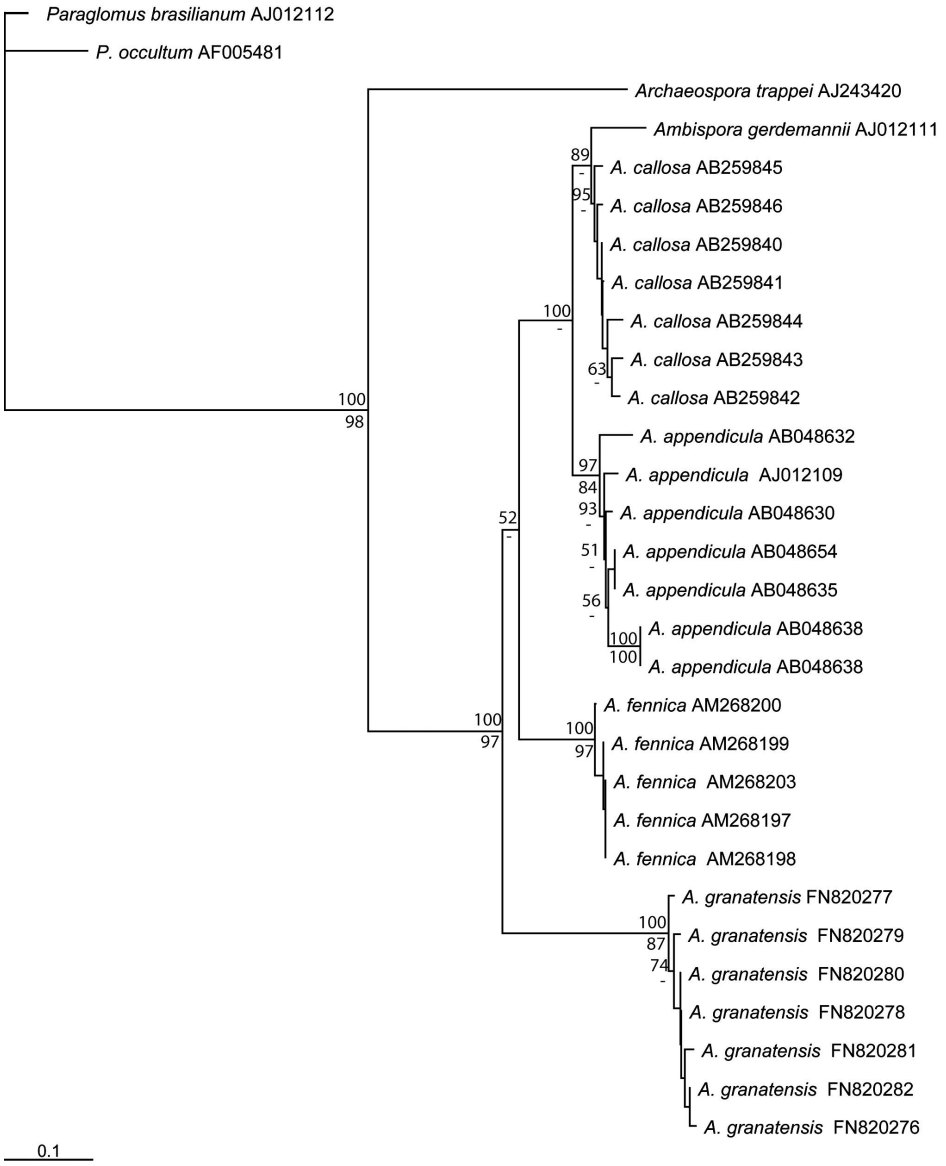


FIG. 13. Phylogenetic tree of the Archaeosporales obtained by neighbor joining analysis of the ITS sequence regions of the ribosomal gene. Sequences from members of *Paraglomerales* were used as outgroups. The new sequences obtained in this study are indicated in boldface. Sequences are labeled with database accession numbers. Bootstrap values (%) above branches are from the neighbor joining analysis (1000 bootstraps) and under branches from maximum parsimony analyses (1000 bootstraps). Note: *Ambispora appendicula* and *Am. gerdemannii* are the former *Archaeospora leptoticha* and *Ar. gerdemannii* respectively (Spain et al. 2006, Walker 2008).

Ambispora granatensis can be distinguished easily from all other known *Ambispora* species through analyses of the acaulosporoid morph because the outer spore wall of *A. granatensis* is significantly thinner than the outer wall of all other known species in *Ambispora*. In addition the acaulosporoid spores of *A. granatensis* possess a papillae-like rough irregular surface that is observed in specimens mounted in water but may disappear in PVLG within a few hours. So far only two *Ambispora* species, *A. jimgerdemanni* (Nicolson and Schenck 1979, Spain et al. 2006, Walker 2008) and *A. brasiliensis* (Goto et al. 2008), lack a smooth outer wall. However these species have clearly visible ornamentations that are either cerebriform (*A. jimgerdemannii*) or pustulate (*A. brasiliensis*). The other *Ambispora* species with acaulosporoid spore formation, *A. appendicula*, *A. gerdemannii* and *A. fennica*, have smooth outer walls (Spain et al. 2006, Walker et al. 2007).

The outer spore wall (especially the most prominent, structural, laminate layer of OW) is persistent in species of Glomerales and Diversisporales, while it often is semipersistent to evanescent in Archaeospor-

ales (Spain et al. 2006). For species of genus *Ambispora* it was often observed that the complete OW can disappear from the acaulosporoid spore (Spain et al. 2006, Goto et al. 2008). This is especially true for the thin OW of *A. granatensis*, which might degrade in the field within a few weeks.

Remarkably in *A. granatensis* the middle wall is fragile also, that is semipersistent to evanescent. Moreover the MW generally disaggregates immediately into several small irregular pieces when the spores are crushed under the cover slides. In most other *Ambispora* species, such as *A. appendicula* and *A. jimgerdemannii*, the MW is persistent and rigid and does not disintegrate into small pieces under strong pressure (Spain et al. 2006). Even in the small spores of *A. brasiliensis* the MW is persistent, although it is much thinner than the MW of *A. granatensis* (Goto et al. 2008). In *A. gerdemannii* and *A. fennica* the rigid MW splits into regular, often polygonal pieces with sharp sides (plate-like structures) under strong pressure on the cover slide (Morton and Redecker 2001, Spain et al. 2006, Walker et al. 2007).

The glomoid spores of *A. granatensis* are generally smaller than the glomoid spores of most other *Ambispora* species, with the exception of *A. brasiliensis* that might regularly have smaller glomoid spores (Goto et al. 2008). However at present morphological characters of glomoid spores cannot be used to identify *Ambispora* species (Goto et al. 2008).

Hitherto *Ambispora granatensis* has been found only in a single asparagus field in southern Spain. The specific multiannual cultivation of asparagus and the particular life strategy and harvest of this perennial cash crop might explain the abundant and consistent sporulation of *A. granatensis* during the harvest of the below-ground asparagus sprouts in springtime. It will be interesting to determine whether this fungus has a specific niche in European asparagus fields or whether its occurrence might be restricted to the warmest areas of the continent.

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