Mycologia, 102(3), 2010, pp. 624–632. DOI: 10.3852/09-145

Entrophospora nevadensis, a new arbuscular mycorrhizal fungus from Sierra Nevada National Park (southeastern Spain)

Javier Palenzuela
José Miguel Barea
Nuria Ferrol
Concepción Azcón-Aguilar[1]

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

Fritz Oehl

Agroscope Reckenholz-Tänikon Research Station ART, Ecological Farming Systems, Reckenholzstrasse 191, CH-8046 Zurich, Switzerland

Abstract: A new fungal species in the arbuscular mycorrhiza-forming Glomeromycetes, *Entrophospora nevadensis*, was isolated from soil near the roots of several endemic and endangered plant species (e.g. *Plantago nivalis* and *Alchemilla fontqueri*) growing in Sierra Nevada National Park (Granada, Andalucía, Spain). The fungus was propagated in trap cultures on *Plantago nivalis* and *Sorbus hybrida* and in pure cultures on *Trifolium pratense* and *Sorghum vulgare*. Spores are yellow brown to brown, 90–115 μm diam and form singly in soil, in the neck of adherent sporiferous saccules that form either terminally or intercalary on mycelial hyphae. Spores have two three-layered walls and conspicuous, 6–12 μm long, spiny, thorn-like projections on the outer wall consisting of hyaline to subhyaline, evanescent tips and yellow brown to brown, persistent bases. In aging spores these projections are usually shorter (1–2.8 μm) and dome-shaped or rounded, sometimes with a central pit on top where the evanescent tip has sloughed off. Molecular analysis with partial sequences of the 18S ribosomal gene places the fungus within the Diversisporales. The new fungus was found in soil near plants with different living strategies but growing in high altitude soils with acidic pH, high soil moisture and organic carbon content, and close to streams.

Key words: arbuscular mycorrhiza, Diversisporaceae, endangered plant species, endemism, Entrophosporaceae, Glomeromycota

Submitted 17 Jun 2009; accepted for publication 15 Sep 2009.
[1] Corresponding author. E-mail: conchi.azcon@eez.csic.es

INTRODUCTION

Sierra Nevada (the Spanish name for snowy range) is rugged and extensive, included in the Baetic Cordillera and located in southwestern Europe (Andalucía, provinces of Granada and Almería, Spain). Sierra Nevada contains the highest points of continental Spain with more than 20 peaks above 3000 m, which makes it the second highest mountain range in Europe after the Alps. It was designated a UNESCO Biosphere Reserve in 1986 and a National Park in 1999 in recognition of its exceptionally high plant and animal diversity. Floristic biodiversity in the Sierra Nevada is due to its geographic isolation and abruptness of ecological gradients with large altitudinal ranks thus creating a diversity of ecological niches (Blanca et al. 2002). Approximately 2100 plant species (more than 80 endemic species and some seriously threatened with extinction) occur in the park (Blanca et al. 2002). Several research programs are being developed to conserve threatened plant species in Sierra Nevada, but little effort so far has been directed to documenting or conserving mycorrhizal symbionts that co-exist with various plant communities in the region.

Arbuscular mycorrhizal (AM) symbiosis has played a key role in plant evolution on Earth (Simon et al. 1993, Redecker et al. 2000) and in the development and maintenance of plant diversity of terrestrial ecosystems (van der Heijden et al. 1998). Most plants depend on AM fungi to thrive, particularly in fragile and stressed environments (Haselwandter 1987), such as those in certain areas of Sierra Nevada. This has led to an increased awareness of the diversity of AM fungi and to an interest in identifying new species, particularly in habitats of increased ecological value, that is habitats of high biodiversity and an increased presence of rare or endangered plant species (Fuchs and Haselwandter 2004).

In a survey of AM fungi associated with endemic and endangered plant species in Sierra Nevada National Park, a new fungus was found close to several endemic plant species (i.e. *Plantago nivalis*, *Alchemilla fontqueri* and *Senecio elodes*) and a nonendemic tree (*Sorbus hybrida*). The fungus is characterized by a novel spore ornamentation type, unique within phylum Glomeromycota. It first was propagated in trap cultures on *P. nivalis* and subsequently in trap cultures of *Sorbus hybrida* and in pure cultures on *Trifolium pratense* and *Sorghum vulgare*. Molecular

analyses of the 18S ribosomal gene were performed to elucidate the phylogenetic position of the fungus. Due to its entrophosporoid spore formation and spore wall composition, which is similar to that of *Entrophospora infrequens* and *Entrophospora baltica*, the new fungus is described here under the epithet *Entrophospora nevadensis*.

MATERIALS AND METHODS

Study plants.—The mycorrhizal status and the AM fungi present as spores in soil were investigated in 34 plant species that are either endemic to the Sierra Nevada or threatened with extinction, according to the Red List of Endangered Plant Species of Andalucía (Blanca et al. 1999, 2000) and the compilation of Threatened and endemic flora of Sierra Nevada (Blanca et al. 2002). The new fungus hereafter described was found near four plant species, *Plantago nivalis* Boiss. (endemic), *Alchemilla fontqueri* Rothm. and *Senecio elodes* Boiss. (both endemic and critically endangered) and *Sorbus hybrida* L. (nonendemic but critically endangered in Sierra Nevada).

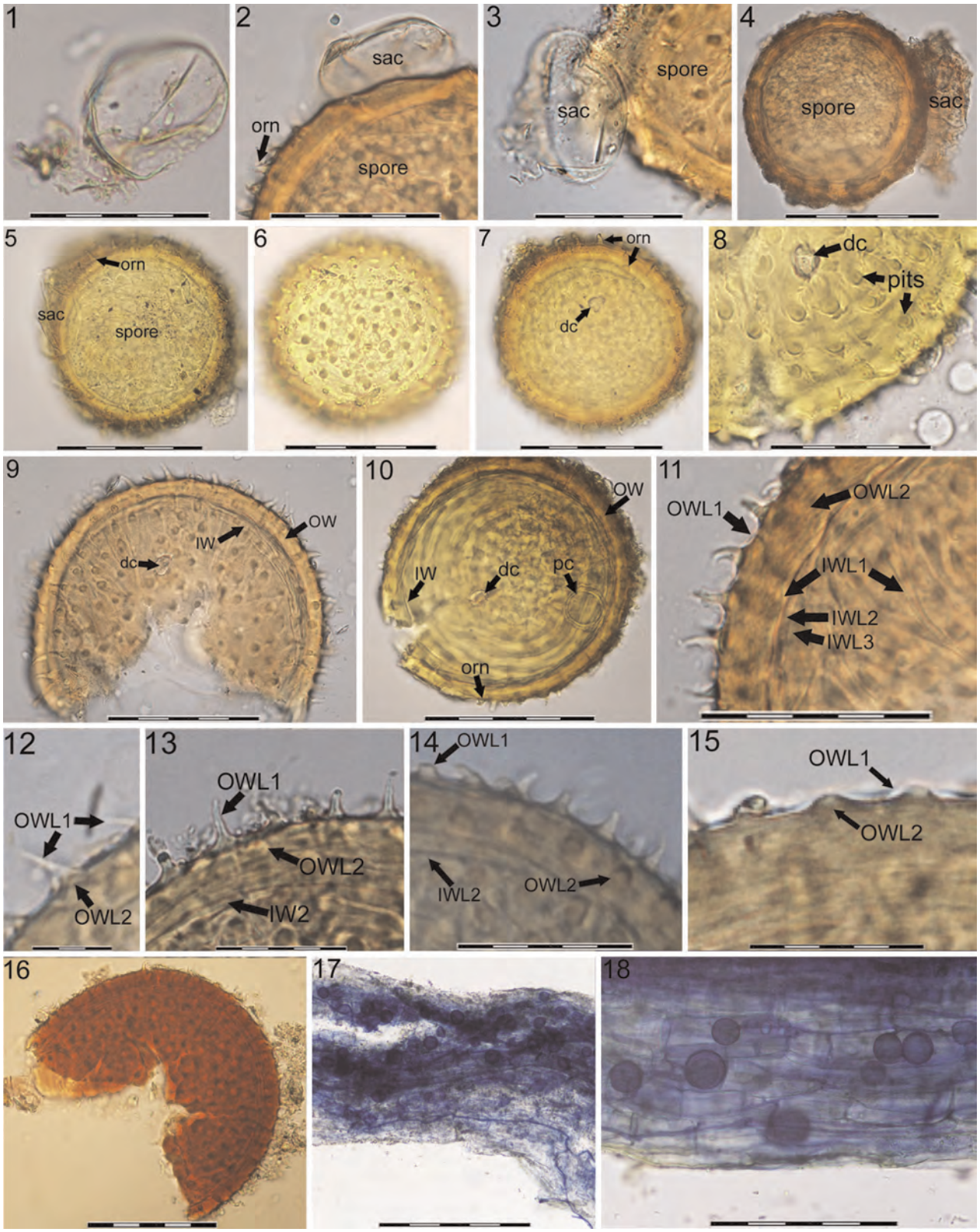
Plant and soil sampling.—For each plant species five intact plant individuals and the soil attached to the roots were collected at the sampling sites to establish AM fungal pot cultures. In addition three samples of the soil surrounding the roots of each plant (0.5–1 kg) were taken with a shovel from a depth of 5–25 cm and thoroughly mixed to prepare a composite soil sample per individual plant. These samples were used to isolate AM fungal spores and determine soil characteristics. These soil chemical properties were determined: soil pH (in a 1/2.5 w/v aqueous solution), the content of organic carbon (according to Yeomans and Bremmer 1989) and that of the major mineral nutrients including nitrogen (with an elemental analyzer), phosphorus, potassium and calcium (by ICP-OES, after acid digestion). The soil type was identified according to the World Reference Base for Soil Resources (FAO 2006). The new fungus was found in all five samples of *P. nivalis* and *S. elodes*, in two samples of *S. hybrida* and in one *A. fontcheri* sample.

AM fungal pot cultures.—To cultivate AM fungi pot cultures were established with the collected native plants in cylindrical 1500 mL pots (12 cm diam) filled with the soil originally around the plants in the field. The pots were irrigated three times per wk and fertilized every 4 wk with Long-Aston nutrient solution (Hewitt 1966). Pure cultures of *E. nevadensis* were established with *Trifolium pratense* and *Sorghum vulgare* in 350 mL pots containing a mixture (1 : 1) steam-sterilized soil from the *P. nivalis* samples and a potting substrate (1 : 1 mixture vermiculite and sand; pH 6.0) by adding 20 spores isolated from the trap cultures of *P. nivalis*. Spores isolated from the trap cultures were stratified 2 wk at 4 C before inoculation. Pure cultures were used repeatedly for spore analyses and mycorrhizal formation.

Morphological analyses.—Spore formation and the morphology of spores, sporiferous saccules and mycelial hyphae were observed on about 100 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 as suggested by INVAM (International Culture Collection of [Vesicular]-Arbuscular Mycorrhizal Fungi, www.invam.caf.wvu.edu) with slight modifications described recently for species in the Diversisporales (Oehl and Sieverding 2004, Oehl et al. 2006, Sieverding and Oehl 2006, Palenzuela et al. 2008). Photographs were taken with a digital camera (Nikon DS-Fi1) on a compound microscope (Nikon eclipse 50i) up to 400-fold magnification. Specimens mounted in PVLG and PVLG + Melzer's reagent were deposited at GDA-GDAC (herbarium of the University of Granada, Spain), Z+ZT (common mycological herbarium of University and ETH of Zurich, Switzerland) and at OSC (mycological herbarium of Oregon State University, Corvallis, Oregon).

Molecular analyses.—Crude DNA extracts were obtained by crushing surface-sterilized single spores with a sterile disposable micropestle in 40 µL milli-Q water, as described by Ferrol et al. (2004). Spores were sterilized with chloramine T (2%) and streptomycin (0.02%) (Mosse 1962). PCR reactions 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 each primer. A two-step PCR was conducted to amplify the NS31-AM1 region of the small subunit ribosomal gene. The first round of amplification was performed with the universal eukaryote primers NS31 and NS41 (White et al. 1990) and the second with the universal primer NS31 and the AM fungal specific primer AM1 (5′-GTTTCCCGTAAGGCGCCGAA-3′) (Helgason et al. 1998). 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 an extension phase of 10 min at 72 C.

PCR products were separated electrophoretically on 1.2% agarose gels, stained with ethidium bromide and viewed by UV illumination. The expected band was excised with a scalpel and 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 on 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 sequence was deposited in the EMBL database under accession number FN397100. The 18S rRNA NS31-AM1 region of the new fungus was aligned to other Glomeromycotan sequences available in the public databases. 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 genetic distances, and the phylogenetic analyses were performed by neighbor joining and maximum parsimony methods with PHYLIP (Felstein 1993). A sequence of *Mortierella*



FIGS. 1–18. *Entrophospora nevadensis*. 1–5. Sporiferous saccules (sac) are formed terminally or intercalary on mycelia hyphae and generally adhere tightly to ornamented (orn) spores; saccules rapidly decompose (4) or collapse (5) and slough off from the newly formed spores. Bars: 1–3 = 50 µm, 4–5 = 75 µm. 6–15. Spores have two three-layered walls (outer and inner wall; OWL1-3 and IWL1-3) and long spiny, thorn-like projections on the spore surface (orn). 6. Projections in planar

TABLE I. Plant species associated with *Entrophospora nevadensis* and geographical data of the collection sites

Plant species	Collection site	Altitude (m)	Latitude	Longitude	Sampling date
Plantago nivalis	Hygrophilic high mountain pastures in the upper course of the Dilar river	2600	37°03′N	3°23′West	Nov 2006
Alchemilla fontqueri	Alcazaba streams	2500	37°05′N	3°18′W	Oct 2008
Senecio elodes	Hygrophilic high mountain pastures in the upper course of the Poqueira river (Reguera fria)	3090	36°59′N	3°19′W	Jul 2008
Sorbus hybrida	Upper part of the San Juan ravine	1900	37°07′N	3°22′W	Jun 2007

polycephala was used as outgroup. The relative support of the different groups was determined based on 1000 bootstrap trees. The phylogenetic trees were drawn with Treeview and edited with Adobe Illustrator CS3.

TAXONOMY

Entrophospora nevadensis sp. nov. J. Palenzuela, N. Ferrol, Azcón-Aguilar & Oehl

MycoBank MB 513527

Sporocarpia ignota. Sporae singulatim efformatae in hyphae inflatae anguste adiaceta ad sacculos sporiferos terminales vel intercalares. Sacculi sporiferi hyalini vel subhyalini, subglobosi vel ovo similes (36–50 × 43–75 μm diam) et formationi sporarum praecedentes. Sporae entrophosporoideae flavo-brunneae vel brunneae, globosae vel subglobosae (90–115 μm), cum duabus tunicis stratis pluribus: tunica externa flavo-brunnea vel brunnea stratis tribus, in totum 4.9–10.5 μm crassa tunicaque interna hyalina stratis tribus in totum 2.9–4.5 μm crassa; tunica externa spinibus ornata altis 6.0–12(–16) μm et 2.4–3.6 μm latis ad basem tunicae; spinae efformatae ex stratibus duobus externibus tunicae externae, 4.8–12.0 μm in distantia; stratum externum tunicae externae hyalinum, evanescens, 0.6–1.8 μm crassum, pallide purpureum colorans in solutione Melzeri; stratum secundum flavo-brunneum vel brunneum, laminatum, stratum internum tunicae externae flavo-bruneum, subtile vel invisibile; stratum secundum et stratum internum rubeo-fusca at purpureo-fusca colorantes in solutione Melzeri; tunica interna non colorans in solutione Melzeri; stratum externum tunicae internae 0.8–1.2(1.5) μm crassum; stratum secundum 2.0–3.5 μm crassum, tenuiter laminatum; stratum internum 0.6–1.2 μm crassum. Formans structuras mycorrhizarum vesiculum-arbuscularum; structurae fungorum colorantes caeruleae cum ''trypan blue''. Holotypus: 76-7601 (Z+ZT: ZT Myc 1625).

Type. Holotype (76-7601) from pure culture, propagated on *Trifolium pratense* and *Sorghum vulgare* (deposited at Z+ZT: ZT Myc 1625); the fungus originally was isolated from the soil near *Plantago nivalis* (Sierra Nevada, Granada, Andalucía, Spain; 37°03′N, 3°23′W; 2600 m); isotype specimens (76-7602, 76-7603, 76-7604, 76-7605; deposited at Z+ZT: ZT Myc 1625); paratype specimens isolated from trap cultures on *P. nivalis* were deposited at GDA-GDAC (76-7613, 76-7614, 76-7615) and OSC (76-7611, 76-7612; OSC #134,711), and those from the soil around the roots of *Alchemilla fontqueri*, *Senecio elodes* and *Sorbus hybrida* (Sierra Nevada, Granada, Andalucía, Spain deposited at GDA-GDAC (76-7621, 76-7622, 76-7623, 76-7624).

Etymology. Latin: *nevadensis* referring to Sierra Nevada (Andalucía, Spain) where the new species was first found near *P. nivalis*.

Spore formation. Sporocarp formation is unknown. Spores are formed singly in soil, rarely in roots, entrophosporoid (i.e. in inflating hyphae) adhering tightly to terminal or intercalary sporiferous saccules.

Sporiferous saccules are hyaline to subhyaline, subglobose to oval, generally broader than long, 36–50 × 43–75 μm (FIGS. 1–3) and tightly adherent to the developing spore. They have a thin (< 0.6 μm), evanescent outer layer and a semipersistent, 1.5–2.0 μm thick, inner wall layer (FIGS. 1–3) that are continuous with the outer two layers of the spore wall.

←

view. Bar = 75 μm. 7–10. Spores formed intrahyphally have regularly two conspicuous cicatrices on the spore wall: a wide cicatrix proximal to the saccule (pc) and a small cicatrix distal to the saccule (dc). Bars: 7 = 75 μm, 8 = 35 μm, 9–10 = 75 μm. 11–15. The hyaline to subhyaline elongated parts of the projections are formed by outgrowths of the OWL1, have generally a hollow structure (13) and may rapidly break or slough from the spore surface (13–15). The projections of OWL2 are persistent and shallow, dome-shaped, convex, flat or sometimes may have a central pit on the tops (11–15). Bars: 11 = 40 μm, 12 = 15 μm, 13–14 = 25 μm, 15 = 15 μm. 16. Wall layers OWL2 and OWL3 stain reddish-brown in Melzer's reagent, while the outer layer OWL1 does not stain or stains slightly pinkish. Bar = 75 μm. 17–18. Mycorrhizal structures (hyphae, arbuscules and vesicles) stain blue to dark blue in trypan blue. Bars: 17 = 400 μm, 18 = 150 μm.

The saccules decompose rapidly (FIG. 4) and often collapse after spore wall differentiation (FIG. 5). Generally they are detached from mature spores in the soils (FIGS. 6–10). The hyphae of the saccule neck rarely remained attached to the spores. When present they were cylindrical, up to 90 µm long and 4.5–8.5 µm diam. Their walls also were continuous with the two outer layers of the spore wall and 1.2–1.6 µm thick.

Spores are formed in the inflating neck of closely adherent sporiferous saccules. They are yellow brown to brown, globose to subglobose and 90–115 µm diam. They have two walls: a conspicuously ornamented outer wall with thorn-like projections and a smooth inner wall (OW and IW, FIGS. 9–16).

Outer wall generally consists of three layers and is in total 4.9–10.5 µm thick (FIGS. 9–11). The outer layer (OWL1) is hyaline to subhyaline, 0.6–1.8 µm thick and evanescent. The second layer (OWL2) is brown-yellow to yellow-brown, darkening with age, laminate and 3.5–9.0 µm thick. The inner layer (OWL3) is brown-yellow to yellow-brown, generally up to 1.0 µm thick, and often difficult to detect because it adheres tightly to OWL2 and also because it was regularly hidden by the folds of IWL1, even in crushed spores. The outer surface of the OW has conspicuous, spiny to thorn-like, often curved projections (FIGS. 9–16). The projections are 6.0–12(–16) µm long and 2.4–3.6 µm wide at their bases tapering to 0.2–0.4 µm at their tips. They are outgrowths of OWL1 and OWL2. The hyaline to subhyaline OWL1 of the projections is evanescent to semipersistent and 1.0–1.7 µm thick at the spore surface tapering to up to 0.3 µm at the tips (FIGS. 12–15). The newly formed projections of OWL1 thus often are hollow toward the bases and often break at different lengths during aging (FIG. 13). The persistent bases of the projections are brown-yellow to yellow-brown and formed by OWL2. These bases are about 1.0–2.8 µm high, either dome-shaped to convex, planar, or may even have a central pit depression at the top (FIGS. 8–15). The projections are about 4.8–12.0 µm from each other. In Melzer's reagent OWL1 sometimes may stain pinkish, while OWL2 and OWL3 regularly stain reddish-brown to purple brown (FIG. 16).

Inner wall forms de novo after the spore pores has been closed on the OW. It is hyaline, 2.9–4.5 µm thick (FIGS. 9–11). It has three wall layers (FIG. 11). The outer layer (IWL1) is 0.8–1.5 µm, adhering tightly to the central layer IWL2 and often forming several conspicuous folds after spore crushing (FIG. 11). IWL2 is finely laminate, 2.0–3.5 µm and under pressure in PVLG may expand to 5.5 µm. IWL3 is 0.6–1.2 µm thick, usually adhering tightly to IWL2 but sometimes slightly separate. It forms several folds that are more difficult to observe than the folds of IWL1. Remarkably, although IWL2 is the most obvious layer of the IW in uncrushed spores, it appears sometimes to be hidden between the folds of IWL1 and IWL3 in crushed spores (FIG. 11).

Cicatrices. The globose to subglobose spore pore proximal to the sporiferous saccule (pc) is closed by OWL2 and OWL3 and is 13–19 µm diam leaving a cicatrix that forms a ring-structure on the spore base (FIG. 10). The globose to sometimes oval to irregular cicatrix distant from the sporiferous saccule (dc) also is formed and closed by OWL2 and OWL3. It is 6–8.5 µm diam (FIGS. 7–10). Both cicatrices are persistent on the spore (FIG. 10).

Germination. The germination tubes (one to rarely a few per spore) grow directly through the spore wall. A specific germination structure (germ trunks, lobes, orbs or shields) has not been observed so far.

Mycorrhizal formation. The new fungus forms typical vesicular-arbuscular mycorriza, with mycorrhizal structures (arbuscules, vesicles and hyphae) that stain blue to dark blue in trypan blue (FIGS. 17–18).

Distribution. E. nevadensis is known only from the soil around *Plantago nivalis*, *Alchemilla fontqueri*, *Senecio elodes* and *Sorbus hybrida*, all of them growing at high altitude in Sierra Nevada National Park (Granada, Andalucía, Spain). The characteristics of the sites, sampling dates, soil types and soil chemical characteristics are shown (TABLES I, II).

Molecular analyses.—Identical sequences of 551 bp corresponding to the NS31-AM1 region of the 18S ribosomal gene were obtained from the two single spores analyzed. To examine the evolutionary relationships among *E. nevadensis* and other species in the Glomeromycetes phylogenetic trees were generated from multiple aligned sequences with evolutionary parsimony and neighbor joining methods. Because both analyses produced trees with basically the same topology only the neighbor joining tree is presented (FIG. 19). This topology is largely in agreement with those of Schüßler et al. (2001), Redecker et al. (2007) and Palenzuela et al. (2008). The phylogenetic analyses suggest that *E. nevadensis* clusters within the *Diversispora* clade, that is *Glomus* group C *sensu* Schüßler (Schüßler et al. 2001, Schwarzott et al. 2001) and is closely related to *Diversispora celata* and *Glomus eburneum*. This clade so far comprises mainly species forming glomoid spores, for example the type species of *Diversispora*, *D. spurca* (Walker and Schüßler 2004), *Diversispora celata* (Gamper et al. 2009) and *Glomus versiforme* (FIG. 19). After *Otospora bareai* (Palenzuela et al. 2008) that forms acaulosporoid spores, *E. nevadensis* is the second species in this clade that does not form glomoid spores.

TABLE II. Characteristics of soils harboring *Entrophospora nevadensis*

Sampled plant species	Soil type	pH (H_2O)	Total content (g kg^{-1})				
			Organic C	N	P	K	Ca
Plantago nivalis	Haplic Gleysol	5.1	41.1	3.8	0.71	5.5	3.0
Alchemilla fontqueri	Gleyic Leptosol	6.5	89.3	7.1	0.61	4.6	10.7
Senecio elodes	Haplic Gleysol	5.8	118.3	9.2	0.69	4.3	3.5
Sorbus hybrida	Haplic & Endogleyic Regosol	6.0	132.0	6.8	0.19	6.4	3.5

DISCUSSION

The spores of the new fungus (entrophosporoid glomerospores, according to Goto and Maia 2006) can be distinguished easily from spores of all other species (presently > 210) known in the Glomeromycota due to their unique thorn-like projections on the outer spore surface. Moreover within the phylum only five other AMF species are known with entrophosporoid spore formation (i.e. with single spore formation within the stalk of sporiferous saccules, viz. *Kuklospora colombiana*, *K. kentinensis*, *Intraspora schenckii*, *E. infrequens* and *E. baltica*). The two *Kuklospora* species have three walls including a characteristic beaded inner, germinal wall, known for all species in the Acaulosporaceae (Sieverding and Oehl 2006), while the new fungus has two walls and the beaded wall is missing. The small, white to hyaline *Intraspora schenckii* spores have a simple-structured, thin and evanescent, hyaline outer spore wall and mycorrhizal structures in roots that may faintly stain with trypan blue (Sieverding and Oehl 2006). Only *E. infrequens* and *E. baltica* have bi-walled entrophosporoid spores similar to *E. nevadensis*, but in *E. infrequens* the ornamentation consists of dentate projections that are formed under a smooth spore surface (Hall 1977, Ames and Schneider 1979, Sieverding and Oehl 2006) while *E. baltica* has small, round warts on the spore surface that are covered by a hyphal mantle (Błaszkowski et al. 1998, Sieverding and Oehl 2006).

The molecular analyses of the NS31-AM1 region of the 18S ribosomal gene revealed that the fungus belongs to the Diversisporales and is closely related to *Diversispora celata*, *Glomus eburneum* and *Diversispora spurca* of glomoid spore formation and to *Otospora bareai* of acaulosporoid spore formation. Of interest, co-occurring acaulosporoid, entrophosporoid and glomoid spore formation of closely related species already was known in another glomeromycotan order, the Archaeosporales (Morton and Redecker 2001, Sieverding and Oehl 2006, Spain et al. 2006), and co-occurring acaulosporoid and entrophosporoid spore formation occurs also in another major clade of the Diversisporales, the Acaulosporaceae. Thus our observations confirm that co-occurring acaulosporoid and entrophosporoid spore formation is a highly convergent trait within the Glomeromycota. Moreover the results on *E. nevadensis* render the Diversisporaceae paraphyletic, confirming the findings on *O. bareai* (Palenzuela et al. 2008, Gamper et al. 2009). Further molecular analyses are needed (e.g. a full length analysis of all the ribosomal genes to elucidate the phylogenetic relationships among *E. nevadensis*, *O. bareai* and species with glomoid spore formation belonging to the *Diversispora* clade.

Before our findings here with *E. nevadensis* no data unequivocally confirmed the phylogenetic position of Entrophosporaceae (reviewed in Sieverding and Oehl 2006, Krüger et al. 2009). The molecular analyses of *E. nevadensis* suggest that the Entrophosporaceae indeed belongs to the Diversisporales as suggested by Sieverding and Oehl (2006). However reliable data on the type species of the genus, *E. infrequens*, are still lacking and there are some morphological differences among *E. infrequens* and *E. nevadensis* and *E. baltica* (e.g. in the structure of the spore walls, cicatrices, and sporiferous saccules) that might make it questionable whether the three species indeed belong in the same family. *Entrophospora nevadensis* is the second of these three species that has successfully been established in single species culture, but unfortunately the pure culture of *E. infrequens* has been lost (Sieverding and Toro 1985, Sieverding and Oehl 2006), preventing comparisons. Further morphological and molecular analyses of the three *Entrophospora* species are necessary to show the phylogenetic relationships among them.

After *Otospora bareai* (Palenzuela et al. 2008) *Entrophospora nevadensis* is the second novel fungus described from the Baetic Cordillera, which constitutes an important center of diversity and speciation in the Mediterranean Basin with exceptionally high floristic diversity (Medail and Quezel 1997, 1999). Like *O. bareai*, *E. nevadensis* initially was isolated from endemic plant species. However a more extensive survey of AM fungi associated with numerous plant species in Sierra Nevada let us isolate the fungus from the nonendemic, deciduous tree *S. hydrida*. It will be interesting to learn whether these fungi also are present elsewhere in southern Spain and around the globe.

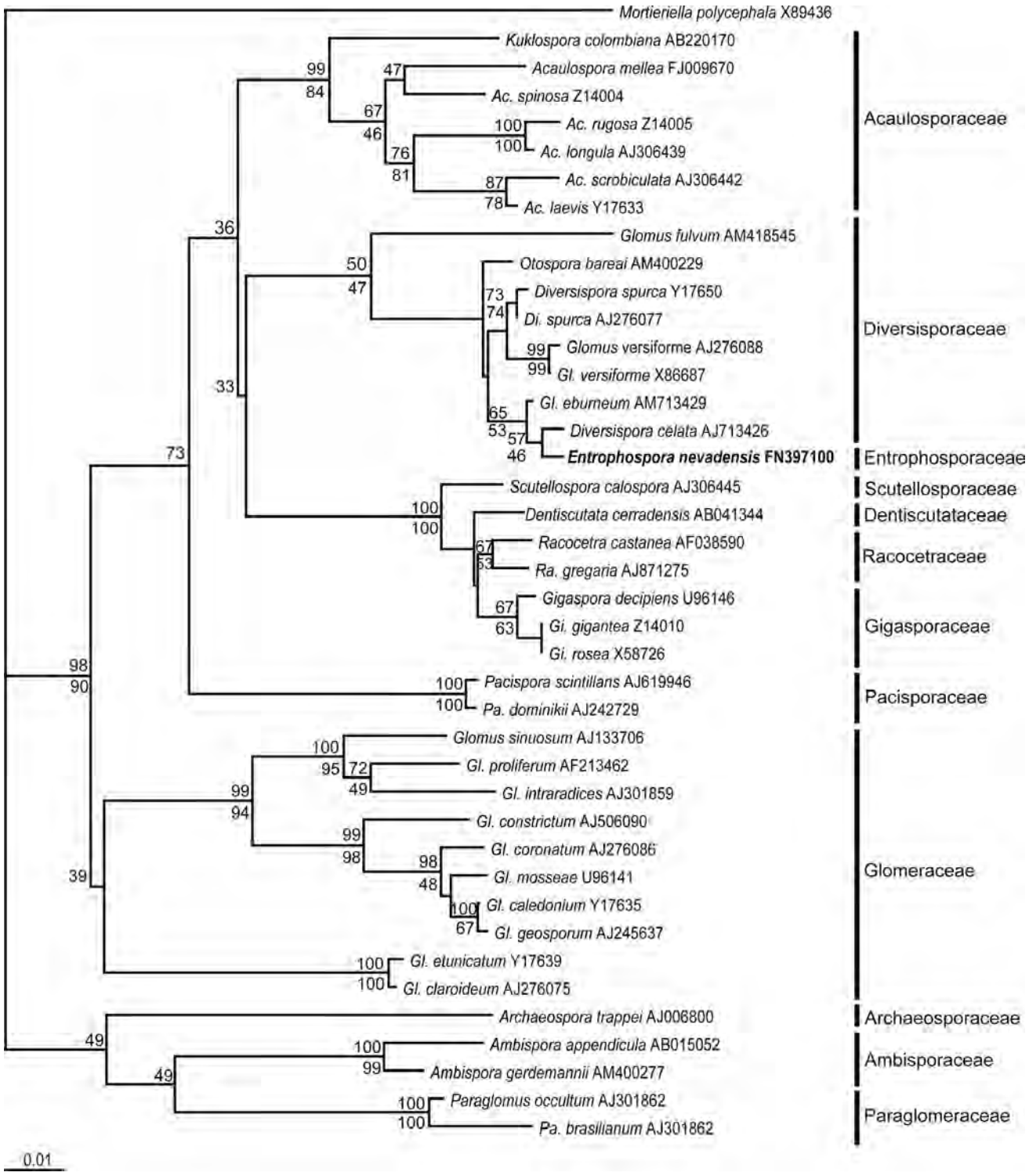


FIG. 19. Phylogenetic tree of the Glomeromycota obtained by neighbor joining analysis of the partial subunit sequence of the 18S ribosomal genes. The new sequence in this study is indicated in boldface. Sequences are labeled with database accession numbers. Bootstrap values (%) above branches are from neighbor joining analysis (1000 bootstraps) and under branches from maximum parsimony analyses (1000 bootstraps). The names to the right indicate the glomeromycotan families. Note: *Kuklospora colombiana* is the former *Entrophospora colombiana* (Sieverding and Oehl 2006); *Ambispora appendicula* and *Am. gerdemannii* are respectively the former *Archaeospora leptoticha* and *Ar. gerdemannii* (Spain et al. 2006, Walker 2008). Families Scutellosporaceae, Dentiscutataceae and Racocetraceae were described by Oehl et al. (2008).

Entrophospora nevadensis has been found near only four of the 34 studied plant species in Sierra Nevada (*P. nivalis*, *A. fontqueri*, *S. elodes* and *S. hybrida*). However although *E. nevadensis* is likely associated with all these plant species this fact has been confirmed so far only for *P. nivalis* and *S. hybrida* because the fungus was sporulating in the trap cultures in which these two plants were hosts. However this cannot be definitively concluded for *A. fontqueri* and *S. elodes* because in the sampling sites other plants species were growing close to them. Further studies with molecular techniques are required to determine the preferential hosts for *E. nevadensis* under natural conditions.

Remarkably the new fungus has always been found in high altitude soils, with acidic pH, high contents of organic carbon and high soil moisture because it was isolated from plants growing close to streams. These observations suggest that most probably edaphic and climatic conditions in Sierra Nevada National Park are responsible for the localized occurrence of this AM fungus. Further studies on the distribution of *E. nevadensis* will be necessary to confirm this hypothesis.

ACKNOWLEDGMENTS

The authors thank Dr Ewald Sieverding for corrections of the Latin diagnosis, Mario Ruiz Girela and Santiago Ferrón Moraleda for their invaluable help in sample collection and Ionomic Service of the Centro de Edafología y Biología Aplicada del Segura, CSIC (Murcia, Spain), for soil analyses. This study was financially supported by the Spanish Ministry of Environment (MMA-OAPN, project 70/2005) and the Junta de Andalucía (project P07-CVI-02952).

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, Schneider RW. 1979. *Entrophospora*, a new genus in the Endogonaceae. Mycotaxon 2:347–352.

Blanca G, Hernández Bermejo JE, Herrera CM, Molero Mesa J, Muñoz J, Valdés B. 1999. Libro rojo de la flora silvestre amenazada de Andalucía. Vol. I: Especies en peligro de extinción. Sevilla, Spain: Junta de Andalucía. 302 p.

———, ———, ———, ———, ———, ———. 2000. Libro rojo de la flora silvestre amenazada de Andalucía. Vol. II: Especies vulnerables. Sevilla, Spain: Junta de Andalucía. 375 p.

———, López Onieva MR, Lorite J, Matínez Lirola MJ, Molero Mesa J, Quintas S, Ruíz Girela M, Varo MA, Vidal S. 2002. Flora amenazada y endémica de Sierra Nevada. Granada, Spain: Univ Granada. 407 p.

Błaszkowski J, 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. Guelph, Ontario: Univ. Guelph, Mycologue Publications. 161 p.

FAO. 2006. World Reference Base for Soil Resources. World soil resources reports. Vol. 103. Food and Agricultural Organization of the United Nations (FAO) & International Soil Reference and Information Center (ISRIC) & International Society of Soil Science (ISSS), Rome, Italy. 88 p.

Felstein J. 1993. PHYLIP Version 3.5. Seattle: Department of Genetics, Univ Washington.

Ferrol N, Calvente R, Cano C, Barea JM, Azcón-Aguilar C. 2004. Analyzing arbuscular mycorrhizal fungal diversity in shrub-associated resource islands from a desertification-threatened semi-arid 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.

Gamper HA, Walker C, Schüßler A. 2009. *Diversispora celata* sp. nov.: molecular ecology and phylotaxonomy of an inconspicuous arbuscular mycorrhizal fungus. New Phytol 182:495–506.

Goto BT, Maia LC. 2006. Glomerospores: a new denomination for the spores of *Glomeromycota*, a group molecularly distinct from *Zygomycota*. Mycotaxon 96:29–132.

Hall IR. 1977. Species and mycorrhizal infections of New Zealand Endogonaceae. Trans Br Mycol Soc 68:341–356.

Haselwandter H. 1987. Mycorrhizal infection and its possible ecological significance in climatically and nutritionally stressed alpine plant-communities. Angew Bot 61:107–114.

Helgason T, Daniell TJ, Husband R, Fitter A, Young P. 1998. Plowing up the wood-wide web? Nature 394:431.

Hewitt EJ. 1966. Sand and water culture methods used in the study of plant nutrition. Farnhan Royal. Farnham, England: Commonwealth Agricultural Bureau. 547 p.

Koske RE, Tessier B. 1983. A convenient, permanent slide mounting medium. Mycol Soc Am Newslett 34:59.

Krüger M, Stockinger H, Krüger C, Schüßler A. 2009. DNA-based species level detection of *Glomeromycota*: one PCR primer set for all arbuscular mycorrhizal fungi. New Phytol 183:212–223.

Médail F, Quézel P. 1997. Hot-spots analysis for conservation of plant biodiversity in the Mediterranean Basin. Ann Missouri Bot Garden 84:112–127.

———, ———. 1999. Biodiversity hotspots in the Mediterranean Basin: setting global conservation priorities. Conserv Biol 13:1510–1513.

Morton JB, 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.

Mosse B. 1962. Establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. J Gen Microbiol 27: 509–520.

Oehl F, de Souza FA, Sieverding E. 2008. Revision of *Scutellospora* and description of five new genera and

three new families in the arbuscular mycorrhiza-forming *Glomeromycetes*. Mycotaxon 106:311–360.

———, Sieverding E. 2004. *Pacispora*, a new vesicular-arbuscular mycorrhizal fungal genus in the Glomeromycetes. J Appl Bot Food Qual 78:72–82.

———, 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.

Palenzuela J, Ferrol N, Boller T, Azcón-Aquilar C, Oehl F. 2008. *Otospora bareai*, a new fungal species in the *Glomeromycetes* from a dolomitic shrub-land in the Natural Park of Sierra de Baza (Granada, Spain). Mycologia 100:296–305.

Redecker D, Kodner R, Graham LE. 2000. Glomalean fungi from the Ordovician. Science 289:1920–1921.

———, Raab P, Oehl F, Camacho FJ, Courtecuisse R. 2007. A novel clade of sporocarp-forming species of glomeromycotan fungi in the Diversisporales lineage. Mycol Prog 6:35–44.

Schüßler A, Schwarzott D, Walker C. 2001. A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycol Res 105:1413–1421.

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.

Sieverding E, Oehl F. 2006. Revision of *Entrophospora* and description of *Kuklospora* and *Intraspora*, two new genera in the arbuscular mycorrhizal *Glomeromycetes*. J Appl Bot Food Qual 80:69–81.

———, Toro S. 1985. The genus *Entrophospora* in Colombia. In: Gianinazzi-Pearson V, Gianinazzi S, eds. Physiological and genetical aspects of Mycorrhizae. Proceedings of the 1st European Symposium on Mycorrhizae, Dijon, 1–5 Jul 1985. Versailles, France: INRA, Service des Publications, Versailles.

Simon L, Bousquet J, Levesque RC, Lalonde M. 1993. Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature 363: 67–69.

Spain JL. 1990. Arguments for diagnoses based on unaltered wall structures. Mycotaxon 38:71–76.

———, 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.

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 Acids 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. 2008. *Ambispora* and *Ambisporaceae* resurrected. Mycol Res 112:297–298.

———, Schüßler A. 2004. Nomenclatural clarifications and new taxa in *Glomeromycota*. Mycol Res 108:981–982.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, White TJ, eds. PCR protocols: a guide to methods and applications. San Diego: Academic Press. p 315–322.

Yeomans J, Bremmer JM. 1989. A rapid and precise method four routine determination of organic carbon in soil. Commun Soil Sci Plant Anal 19:1467–1476.