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ARTICLE

Septoglomus altomontanum, a new arbuscular mycorrhizal fungus from mountainous and alpine areas in Andalucía (southern Spain)

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Abstract: A new arbuscular mycorrhizal (AM) fungus was found in Sierra Nevada National Park of Andalucía (Southern Spain). It forms intraradical hyphae, vesicles and arbuscles, typical characteristics of *Glomeromycetes*. The spores are dark reddish brown to dark reddish black, 132–205 µm diam, and are formed on pigmented subtending hyphae whose pores are regularly closed by a thick septum at the spore base but without support of introverted wall thickening. Phylogenetic analyses on concatenate sequences of the partial SSU, ITS region and the partial LSU of the rDNA confirm the new species, described here as *Septoglomus altomontanum*, in a monophyletic clade next to *S. africanum*. An identification key to all *Septoglomus* species described is given. The new fungus can unequivocally be distinguished from all other *Septoglomus* species by the combination of spore size, colour and spore wall structure, and especially by the shape and colour of the subtending hyphae. *Septoglomus altomontanum* has so far been found only in soils with pH 5.9–6.7, located in mountainous and alpine altitudes (1800–3100 m asl) of Sierra Nevada which is well known for a high degree of plant endemism. While it is a frequent fungus in this area, it has so far not been found in lower altitudes in Andalucía.

Key words:
biodiversity
conservation biology
DNA phylogeny
Glomeromycota
systematic

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INTRODUCTION

Arbuscular mycorrhizal (AM) fungi (*Glomeromycota*) may be important for the productivity and plant species diversity of grasslands (van der Heijden *et al*. 1998). This might be especially true for high mountainous and alpine grasslands, where the vegetation often grows on relatively young, less weathered soils. In recent years, a high diversity of AM fungi was found in mountainous and alpine areas worldwide (e.g. Castillo *et al*. 2006, Oehl *et al*. 2011a, Gai *et al*. 2012) and several new fungi have been reported from such regions (Oehl & Sieverding 2004, Oehl *et al*. 2006, 2011e, 2012, Palenzuela *et al*. 2008, 2010, 2013).

Classification and systematics of AM fungi have substantially changed in recent years (Schüßler *et al*. 2001, Schüßler & Walker 2010, Oehl *et al*. 2011b, Stürmer 2012). According to Oehl *et al*. (2011b, c) and Goto *et al*. (2012), there are currently three classes, 15 families and 31 genera in the phylum *Glomeromycota*. Several genera have substantially increased in the number of known species over the past years, such as *Acaulospora*, *Ambispora*, *Diversispora*, *Racocetra,* and *Septoglomus* (e.g. Gamper *et al*. 2009, Estrada *et al*. 2011, Oehl *et al*. 2011f, 2012, Lin & Yen 2011, Goto *et al*. 2011, 2013, Palenzuela *et al*. 2011, Błaszkowski *et al*. 2013) that currently include approximately 10–40 species each. Here, we report a new AM fungus from high altitudes of Sierra Nevada National Park with distinctive morphology and molecular phylogeny.

MATERIAL AND METHODS

Study sites and study plants

At 27 sites of the Sierra Nevada National Park, Granada, Spain, the mycorrhizal status of 34 flowering plant and fern species and the AM fungi present as spores in their habitat soils were investigated (Palenzuela *et al*. 2010, Azcón-Aguilar *et al.* 2012). The 34 flowering plant and fern species investigated are categorized as either endemic to the Sierra Nevada or threatened with extinction (Blanca *et al*. 1999, 2000, 2002). Soil samples were taken between November 2006 and October 2008 in the rhizosphere of these 34 plant species, as described in Palenzuela *et al*. (2010) and Azcón-Aguilar *et al.* (2012).

AM fungal trap cultures

Pot cultures, often called trap or bait cultures, were established to cultivate the new fungus. The pots were cylindrical, 1500

mL capacity (12 cm diam) and filled with natural soil collected from around the plants in the field and, when possible, the native plants. The pots were irrigated three times per week and fertilized every 4 wk with Long-Aston nutrient solution (Hewitt 1966). The cultures have been maintained in the greenhouse of the Estación Experimental del Zaidín (EEZ, Granada) for more than 3 years. Single species cultures of the new fungus were established with *Trifolium pratense* and *Sorghum vulgare* in 350 mL pots, as described in Palenzuela *et al.* (2010), by adding to each 10–20 spores isolated from the trap cultures. Spores isolated from the trap cultures were stratified for 2 wk at 4 °C before inoculation. Single species cultures have been maintained in EEZ since 2008.

Morphological analyses

AM fungal spores were separated from the soil samples by a wet sieving process (Sieverding 1991). The morphological spore characteristics and their subcellular structures were described from specimen mounted in: (1) polyvinyl alcohol-lactic acid-glycerol (PVLG; Koske & Tessier 1983); (2) a mixture of PVLG and Melzer's reagent (Brundrett *et al*. 1994); (3) a mixture of lactic acid to water at 1:1; (4) Melzer's reagent; and (5) water (Spain 1990). The spore wall structure terminology follows Oehl *et al*. (2005, 2011b) for species with glomoid spores. Photographs (Fig. 1) were taken with a Nikon DS-Fi1 digital camera, on a compound microscope (Nikon eclipse 50i). Specimens mounted in PVLG and in PVLG+Melzer's mixtures were deposited in Z+ZT (ETH Zurich, Switzerland), GDA-GDAC (University of Granada, Spain), and URM (Federal University of Pernambuco, Recife, Brazil).

Molecular analyses

Five spores isolated from the single species culture were surface-sterilized with chloramine T (2 %) and streptomycin (0.02 %) (Mosse 1962) and crushed with a sterile disposable micropestle in 23 µL milli-Q water. Direct PCR of the crude extracts was obtained in an automated thermal cycler (Gene Amp PCR System 2400, Perkin-Elmer, Foster City, CA) with a pureTaq Ready-To-Go PCR Bead (Amersham Biosciences Europe, Germany) following the manufacturer's instructions with 0.4 µM concentration of each primer. A two-step PCR amplified the partial SSU, ITS region and the partial LSU of the rDNA using the SSUmAf/LSUmAr and SSUmCf/LSUmBr primers consecutively (Krüger *et al*. 2009). Part of the second PCR products were analysed by electrophoresis in a 1.2 % agarose gel stained with Gel Red™ (Biotium Inc., Hayward, CA) and viewed by UV illumination. The amplicons of expected size were purified using the GFX PCR DNA kit and Gel Band Purification Illustra, cloned into the PCR2.1 vector (Invitrogen, Carlsbad, CA), 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 in both directions by Taq polymerase cycle sequencing on an automated DNA sequencer (Perkin-Elmer ABI Prism 373). Sequence data were compared to sequences in public databases (EMBL and GenBank) using BLASTn (Altschul *et al.* 1990). The new sequences were deposited in the EMBL database under the accession numbers HF674438–HF674440.

Phylogenetic analyses

The phylogeny was reconstructed by concatenate analyses of the partial SSU, ITS region and the partial LSU of the rDNA. The AM fungal sequences obtained were aligned with other glomeromycotan sequences from GenBank in ClustalX (Larkin *et al.* 2007) and edited with BioEdit (Hall 1999). Only species with at least the ITS and partial LSU rDNA sequences were considered for the phylogeny. In some cases two separated sequences from ITS region and partial LSU rDNA were put together for the analyses (sequences of *S. deserticola, S. furcatum, S. fuscum* and *S. xanthium*). *Claroideoglomus claroideum* and *C. etunicatum* were included as outgroup. Prior to the phylogenetic analysis, the model of nucleotide substitution was estimated using Topali v. 2.5 (Milne *et al*. 2004). Bayesian (two runs over 1×10^6 generations with a burn in value of 2500) and maximum likelihood (1000 bootstrap) analyses were performed in MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003) and PhyML (Guindon & Gascuel 2003), respectively, launched from Topali 2.5, using the GTR + G model.

TAXONOMY

Septoglomus altomontanum Palenz., Oehl, Azcón-Aguilar & G.A.Silva, **sp. nov.**
MycoBank MB803242
(Fig. 1A–I)

Etymology: Latin, referring to the high altitudes where the fungus was found in Sierra Nevada National Park of Andalucía in Spain (1800–2500 m asl).

Diagnosis: The new species differs from *Septoglomus constrictum* in the shape and colour of the subtending hyphae. Subtending hyphae regularly wider at the spore base and 20–35 µm from the base, than 5–20 µm from the base, and lighter in colour (dark yellow-brown to reddish brown) than the spores, that are 137–175(–208) × 125–170(–204) µm diam, dark reddish brown to dark reddish black.

Type: **Spain**: *Andalucía*: Sierra Nevada National Park. Soil sample from grassland growing in the rhizosphere of *Ophioglossum vulgatum* (endangered in Sierra Nevada), and plants like *Holcus lanatus*, *Trifolium repens*, *Mentha suaveolens,* and *Carum verticillatum*, 37°00' N; 3°22' W, 1980 m asl, 30 July 2007, *J. Palenzuela* [propagated on *Sorghum vulgare* and *Trifolium pratense*] (ZT Myc 30432 – holotype[1]; ZT Myc 30433, GDA-GDAC[2], and URM 85581[3] – isotypes).

Other specimens examined: **Spain**: *Andalucía*: Sierra Nevada National Park, from soil samples originating from seven other grasslands (Table 1), 37°00'–37°07'N 2°51'–3°26' W, 1800–3100 m asl, Nov. 2006 – Oct. 2008, mainly associated with the endemic

[1]Deposited at Z + ZT, the common mycological herbarium of the University of Zurich and ETH Zurich, Switzerland.
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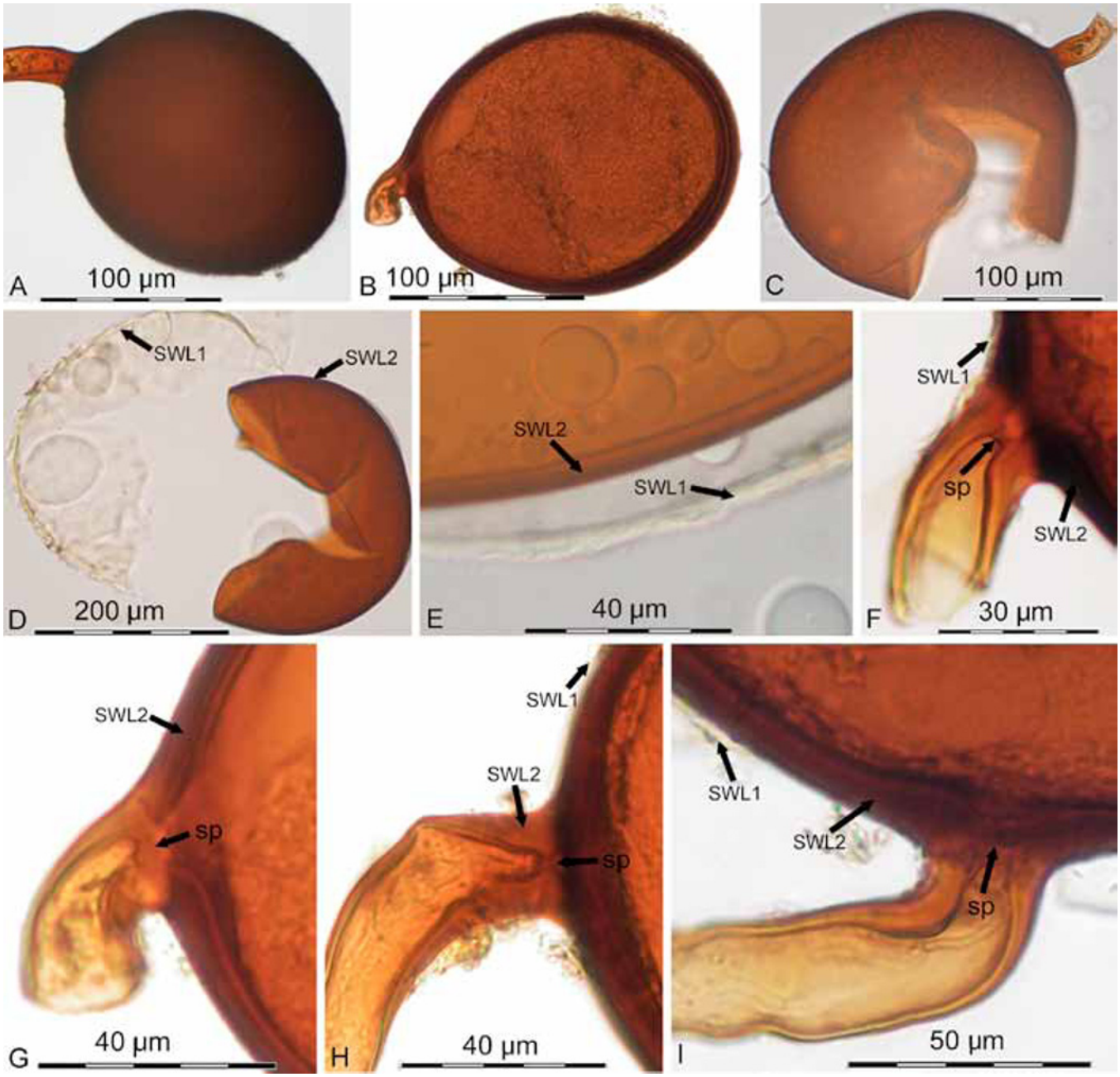

Fig. 1. A–I. *Septoglomus altomontanum* (ZT Myc 30432 and 30433). Spores are dark reddish-black (**A**) to dark reddish brown (**B, C**), often oval to ellipsoid, with two wall layers (SWL1 and SWL2) (**D, E**). Subtending hyphae regularly lighter in colour than spores (**F, G**), and cylindrical (**A, C, F**), and frequently recurved (**B, G–I**). They are regularly widest at spore base and at some distance from the spores, while they are about 3–5 µm thinner in between and taper to 8–13 µm at further distances (approx. 35–130 µm) from the spore base. The pores at the spore base are generally closed by a thick septum (sp) (**F, G–I**).

Narcissus nevadensis among other plant species (ZT Myc 30434 deposited in Z+ZT, GDA-GDAC).

Description: *Spores* formed singly in soils and rarely within roots, oval, ovoid to elliptical to rarely subglobose to globose, 137–175(–208) × 125–170(–204) µm, dark reddish brown to reddish black, with one bi-layered wall (SW). *Spore wall* dark reddish brown to reddish black, 6.5–9.0 µm thick; outer wall layer (SWL1) subhyaline to dark yellow, smooth, 2.5–3.0 µm thick; inner layer (SWL2) dark reddish brown to reddish black, smooth, laminate, 4.0–8.0 µm thick; the layers not staining in Melzer's reagent. *Subtending hyphae* regularly slightly lighter in colour (dark yellow-brown to reddish brown) than the spores, cylindrical to sometimes somewhat funnel-shaped, often curved; often widest at the spore base and 20–35 µm from the spores, and there (15–)20–25(–31) µm wide; thinner and about (12–)18–23 µm between, i.e. 5–20 µm from the spores; subtending hyphae tapering to 8–13 µm further from the spore base (approx. 70–130 µm); the two spore wall layers continuing in the subtending hyphae, and are 2.0–3.0 and 4.0–7.5 µm thick at the spore base, respectively, tapering to 0.5–1.0 and 2.0–4.5 µm within the first 15–25 µm from the base, and to 0.5–1.0 and 1.5–2.5 µm at further distances towards the hyaline hyphal wall. *Spore pore* generally closed by a

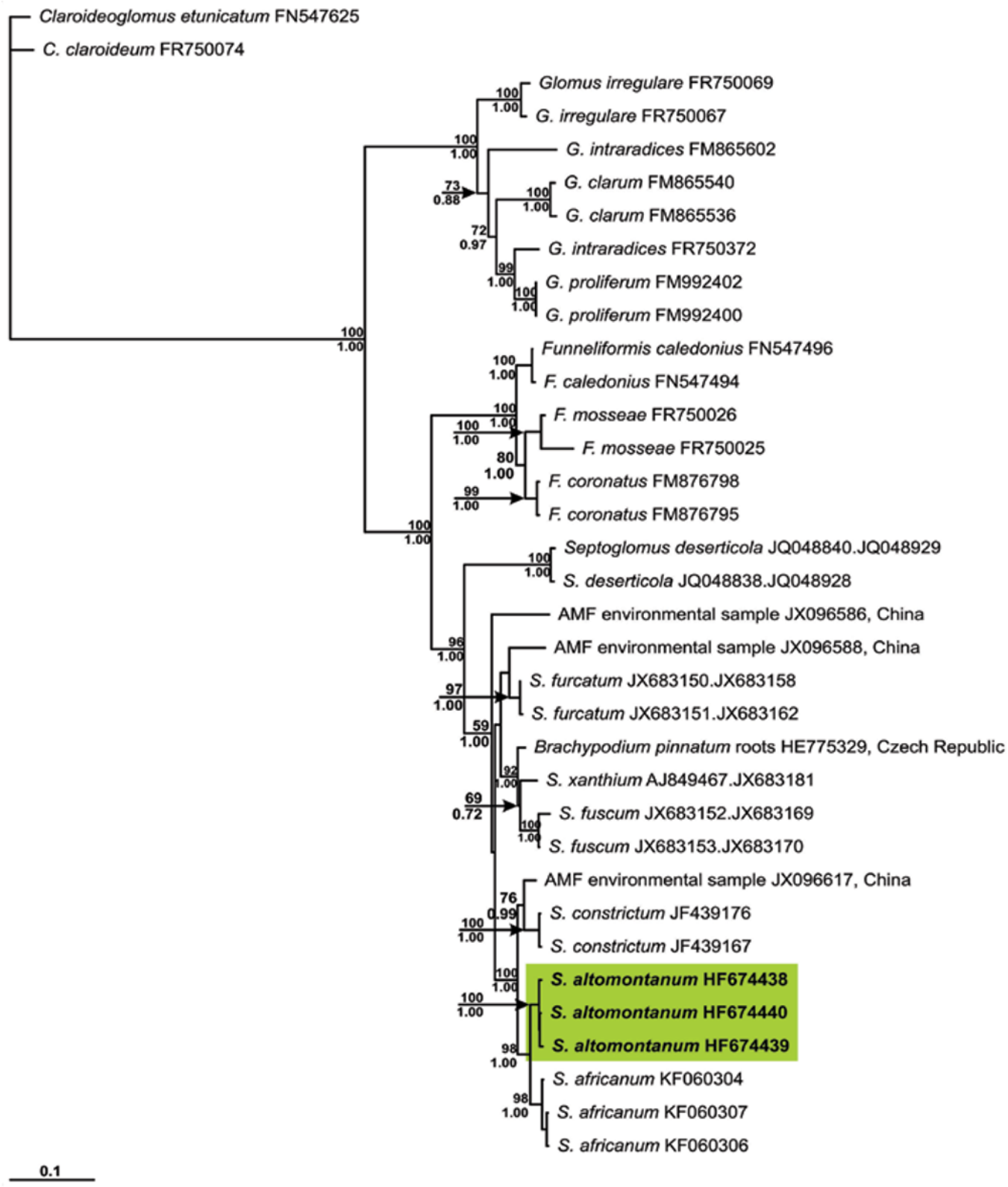

Fig. 2. Phylogenetic tree of *Glomeraceae* obtained by analysis from rDNA sequences (partial SSU, ITS region and partial LSU). Sequences are labelled with their database accession numbers (in some cases with two numbers, one from ITS and other from partial LSU sequences, respectively). Support values (from top) are from maximum likelihood (ML) and bayesian analyses, respectively. Only bootstrap values of at least 50 % are shown. Sequences obtained in this study are in boldface. The tree was rooted by *Claroideoglomus claroideum* and *C. etunicatum*.

broad bridging septum arising from SWL2 at a short distance from the spore base. *Septum* concolourous with SWL2 of the spore wall, when formed at the spore base, concolourous with the lighter coloured subtending hyphae when formed a short distance from the spore. *Mycorrhizal structures* (arbuscles, vesicles and hyphae) blue to dark blue with trypan blue.

Table 1. Soil and geographical parameters at sites of *Septoglomus altomontanum* isolation in grasslands of the Sierra Nevada National Park (Andalucía, Spain).

pH (H_2O)	C_{org} g/kg	N g/kg	Available P mg/kg	Altitude (m asl)	Latitude	Longitude	Sampling time	Plant species investigated
6.5	125.5	1.1	0.4	1980	37º00'N	3º22'W	July 2007	*Ophioglossum vulgatum*
6.7	56.8	0.5	2.5	2430	37º03'N	3º24'W	Nov. 2006	*Salix hastata* subsp. *sierrae nevadae*
6.0	87.7	0.7	1.6	1896	37º07'N	3º22'W	June 2007	*Sorbus hybrida*
6.5	89.3	0.7	0.9	2500	37º05'N	3º18'W	Oct. 2008	*Alchemilla fontqueri*
6.9	63.7	0.6	1.0	2000	37º05'N	2º51'W	Aug. 2008	*Gentiana sierrae*
6.7	89.6	0.6	1.6	2000	37º05'N	2º51'W	Aug. 2008	*Pinguicula grandiflora*
5.9	19.4	0.2	0.2	2250	37º06'N	3º23'W	July 2008	*Pinguicula nevadensis*
6.0	26.5	0.2	0.6	3100	37º03'N	3º21'W	July 2007	*Artemisia granatensis*
6.4	170.1	1.3	1.0	1800	37º07'N	3º26'W	Mar. 2007	*Narcissus nevadensis*

Molecular analyses: Phylogenetic analyses on sequences of the partial SSU, ITS region and the partial LSU of the rDNA reveal that the sequences of the new species group in a separate clade within *Septoglomus* (Fig. 2). The sequences of the new species are most similar to those of *S. africanum*. No environmental sequences deposited in the GenBank correspond to the new fungus in the BLASTn analysis.

Distribution: The new fungus was detected in eight of 27 collection sites, all in mountainous and alpine altitudes (1800–3100 m asl) of the Sierra Nevada National Park, and in soils of pH 5.9–6.7 in the rhizospheric soils of eight endangered plant species (Table 1). In some of the sampled sites, the main plant species investigated was not colonised by mycorrhizal fungi. This was the case with *Pinguicola grandiflora* and *P. nevadensis,* both insectivorous plants in *Lentibulariaceae*. However, the fungus was detected in the surrounding soil, probably associated to neighbour plant species. The fungus has so far not been found in lower altitudes in Andalucía.

Key to the species in *Septoglomus*

The following key to all known species of the genus is adapted from that in Oehl *et al.* (2011d).

1 Spores pale yellow to brownish yellow to ochre 2
Spores brown, orange brown, dark brown, dark reddish black to black 3

2(1) Spores pale yellow to brownish yellow, 80–110 × 90–140 µm, bi-layered; SWL1 hyaline and semi-permanent, with blister-like outgrowths; SWL2 laminate, smooth, pale yellow to brownish yellow, (1.0–)1.7(–2.7) µm. Subtending hyphae cylindrical to slightly funnel-shaped at the spore base (Oehl *et al.* 2011d) ..... **S. africanum**
Spores light yellow to ochre, 20–55 × 45–100 µm, triple-layered: SWL1 semi-permanent, hyaline to light yellow; SWL2 rigid, permanent, hyaline; SWL3 laminate, light yellow to yellow ochre, (0.5–)1.5(–2.0) µm; subtending hyphae cylindrical, rarely constricted at the spore base (Oehl *et al.* 2011d) ..... **S. xanthium**

3(1) Spore regularly < 100 µm 4
Spore regularly > 100 µm 5

4(3) Spores reddish brown, globose to subglobose, (47–)54–115 × (37–)52–102 µm, bi-layered; SWL1 hyaline to subhyaline, evanescent; SWL2 reddish brown, laminate, 1.5–4.0 µm; subtending hyphae cylindrical to slightly funnel-shaped at the spore base (Oehl *et al.* 2011d) ..... **S. deserticola**
Spores brownish orange to dark brown, 21–50 × 23–60 µm, bi-layered; SWL1 semi-persistent, semi-flexible, orange-white to golden yellow, rarely hyaline; SWL2 brownish orange to dark brown, (2.0–)4.0(–7.0) µm; subtending hyphae cylindrical to funnel-shaped, sometimes slightly constricted at the spore base (Błaszkowski *et al.* 2013) **S. fuscum**

5(3) Spores with two wall layers 6
Spores with three wall layers 7

6(5) Spores dark brown to black, 150–330 µm, bi-layered; SWL1 evanescent to semi-permanent, sub-hyaline to dark yellow; SWL2 dark brown to black, laminate, 7–15 µm; subtending hyphae constricted to rarely cylindrical at the spore base, concolourous with the SWL2 (Oehl *et al.* 2011d) ..... **S. constrictum**
Spores dark reddish brown to reddish black, 137–175(–208) × 125–170(–204) µm, bi-layered; SWL1 semi-permanent, sub-hyaline to dark yellow; SWL2, laminate, 4.0–8.0 µm. Subtending hyphae regularly wider at the spore base and

in 20–35 µm distances from the spore base than in 5–20 µm distances; colour change between spores (dark reddish brown to reddish black) and subtending hyphae (dark yellow brown to reddish brown) ..... **S. altomontanum**

7(5) Spores reddish brown to dark brown, 108–127 × 135–160 µm, triple-layered; SWL1 semi-permanent, hyaline to light orange; SWL2 semi-permanent, hyaline to golden yellow middle layer; SWL3 laminate, smooth, reddish brown to dark brown, (5.5–)7.5(–11.5) µm; subtending hyphae cylindrical to slightly funnel-shaped, sometimes slightly constricted at the spore base (Błaszkowski *et al.* 2013) ..... **S. furcatum**

Spores yellow-brown to orange-brown, (243–)265–375(–400) µm, triple-layered; SWL1 sub-hyaline to light yellow; SWL2 yellow brown, unit; SWL3 orange-brown to dark red-brown, laminate, 12.8–19.2 µm. Subtending hyphae cylindrical to constricted, to rarely funnel-shaped at the spore base (Goto *et al.* 2013) ..... **S. titan**

DISCUSSION

The new fungus, *Septoglomus altomontanum* can easily be distinguished from all other *Septoglomus* species by the combination of spore size, colour, spore wall structure, and especially the shape and colour of the subtending hyphae, as well as by molecular phylogenetics (Fig. 2). *Septoglomus deserticola*, *S. xanthium*, *S. africanum*, and *S. fuscum* (Trappe *et al*. 1984, Błaszkowski *et al*. 2004, 2010, 2013) have substantially smaller spores and thinner subtending hyphae than *S. altomontanum*, while *S. titan* (Goto *et al*. 2013) has substantially larger and thicker-walled spores. *Septoglomus altomontanum* can easily be differentiated from *S. furcatum* and *S. constrictum* by the characteristic colour change between spore and subtending hyphae at the spore base, and by the shape of the subtending hyphae which are regularly constricted just beyond the spore base (Trappe 1977, Błaszkowski *et al*. 2013) in the former two species, while in *S. altomontanum*, they are regularly widest at the spore base and at some distance from the spores, but about 3–5 µm thinner in between. Phylogenetically, *S. altomontanum* separates well from all other *Septoglomus* species, forming a monophyletic clade next to *S. africanum*.

To our knowledge, the new fungus has not been found thus far from other regions in Andalucía. Our data suggest that the fungus is widespread in mountainous and alpine altitudes of the Sierra Nevada National Park with a quite narrow pH range.

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