

Isolation and identification of desert habituated arbuscular mycorrhizal fungi newly reported from the Arabian Peninsula

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Abstract: Arbuscular mycorrhizal fungi (AMF) are known to facilitate the growth and vigour of many plants, particularly in arid ecosystems. In a survey of AMF in a date palm plantation and two natural sites of a desert in Oman, we generated many single spore-derived cultures of AMF. We identified a number of these isolates based on spore morphotyping and molecular phylogenetic analysis using the sequence of the LSU-rDNA. Here, we presented the characteristics of four species of AMF recovered, namely *Claroideoglossum drummondii*, *Diversispora aurantia*, *Diversispora spurca* and *Funnelformis africanum*. The four species have been described previously, but for the Arabian Peninsula they are reported here for the first time. Our endeavor of isolation and characterization of some AMF habituated to arid sites of Arabia represents a first step towards application for environmental conservation and sustainable agriculture in this region.

Keywords: arbuscular mycorrhizal fungi; biotechnology; isolation; characterization; desert ecosystem; Arabian Peninsula; Oman

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Arbuscular mycorrhizal fungi (AMF) are well known to convey multiple benefits for plant growth and health in stressful environments (Newsham et al., 1995; Smith and Read, 2008). Under arid conditions, for example, mycorrhizal plants were found to maintain higher drought tolerance (Augé, 2001) and to have better access to phosphorus than non-mycorrhizal ones (Neumann and George, 2004). AMF may also enhance soil aggregate stability (Rillig and Mummey, 2006), a feature of particular relevance for the sandy soils prone to erosion.

Under the extreme conditions of desert ecosystems, AMF are believed to play an important role for the

prospering of vegetation. Inoculation with AMF, for example, was found to improve water and nutrient uptake of desert succulents (Cui and Nobel, 1992), promote the desert tree *Prosopis cineraria* and thereby restore soil fertility by increasing soil organic carbon (Mathur and Vyas, 1995), enhance growth of the desert ephemeral plant *Plantago minuta* (Zhang et al., 2011) and the productivity of *Plantago ovata*, a cash crop in arid and semiarid regions (Mathur et al., 2006) and, furthermore, have the potential to contribute to conservation efforts for endangered tree species (Panwar and Vyas, 2002; Shen and Wang, 2011).

The diversity of AMF has been investigated in

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many desert ecosystems of the world (Kiran et al., 1989; Jacobson, 1997; Stutz et al., 2000; Błaszowski and Czerniawska, 2006; Uhlmann et al., 2006; Shi et al., 2007; Błaszowski, 2012). One of the areas that have been neglected with respect to AMF until recently is the Arabian Peninsula. Our morphological and molecular analyses revealed unique communities of AMF in this area (Al-Yahya'ei et al., 2011). This uniqueness may be attributed to the particularity of the studied ecosystems. Geological history and the influence of past climates of this area (Preusser et al., 2002) have played a major role in shaping its unique and harsh environmental settings (Fisher and Mambery, 1998; Glennie and Singhvi, 2002).

In the current study, we combined further biodiversity explorations with the establishment of a single-species culture collection of typical AMF of this region. Such a collection is crucially needed as a basis for various basic and applied researches. AMF can be applied as a biotechnological tool (Gianinazzi et al., 2002) to promote sustainable agriculture and environmental conservation. As recently summarized, strains of AMF isolated from such sites are likely well-adapted ecotypes, and they may have unique physiological capabilities to cope with the extreme conditions prevailing in the desert, such as long drought seasons leading to desiccation, extreme heat and low availability of soil nutrients during summer (Antunes et al., 2011). Therefore, these isolates might exhibit optimal performance in their original climate (Caravaca et al., 2003; Calvente et al., 2004; Marulanda et al., 2007).

Here, we used morphological and molecular identification techniques to characterize a part of the successfully cultured single-spore derived isolates of AMF, namely those which have obvious affinities to well-described species.

1 Materials and methods

1.1 Study area

The study area occurs in the northern territory of the Sultanate of Oman (Fig. 1a). The area is characterized by hyper-aridity (UNEP, 2006) with an annual rainfall which does not exceed 100 mm (Fisher and Mambery, 1998) and high summer temperatures exceeding 48°C (Glennie and Singhvi, 2002). The four species of AMF described here were recovered from one or more of

three distinct sites (i) a date palm (*Phoenix dactylifera*) plantation in the oasis of Al-Kamel (22°12'56"N, 59°12'9"E) (Fig. 1b), (ii) a natural, undisturbed site (22°14'11"N, 59°10'53"E) (Fig. 1c) with *Tetraena qatarensis* Beier & Thulin. (previously *Zygophyllum qatarense*), *Salvadora persica* Wall., *Prosopis cineraria* (L.) Druce, and an inter-plant area (IPA) where dispersed patches of dry grass were apparently growing after rainfall, and (iii) a sand dune site (21°52'39"N, 58°52'44"E) in the Al-Sharqiya Sands (Fig. 1d) with scattered *Heliotropium kotschy* Gürke. More detailed information about the characteristics of the three sites was given in Al-Yahya'ei et al. (2011).

1.2 Sampling, establishment and growth of trap and single-species cultures

Four replicate plots of 200 m² were randomly chosen at each site. From each plot, four plants of each species were selected for obtaining a pooled sample per plant species. The collection of soil and root samples and the procedure to establish the initial trap cultures have been described previously (Al-Yahya'ei et al., 2011).

The spores used for the morphological characterization were obtained from single-spore derived cultures established by the previously described pipette-tip procedure (Tchabi et al., 2010), starting with single spores isolated from the initial trap cultures. The cultures were maintained for six months using a consortium of the following host plants for AMF: *Allium porrum* L., *Hieracium pilosella* L. and *Plantago lanceolata* L.

1.3 Spore extraction

Spores were extracted by wet sieving and sucrose density gradient centrifugation, using a modification of the method of Daniels and Skipper (1982). For each of the single-spore derived cultures, around 10 mL of harvested substrate were well suspended in 20 mL of water in a 50 mL Falcon tube. A 25-mL sucrose solution (70%, v/w) was injected to the bottom of the tube, forming a stepped density gradient that was centrifuged at 900g for 2 min. Spores of AMF were collected from the interface of sucrose solution, washed with tap water on a 32-µm sieve for 2 min, and transferred to Petri dishes. Spores were then picked individually under a stereomicroscope and either examined morphologically or used to extract DNA for molecular analysis.

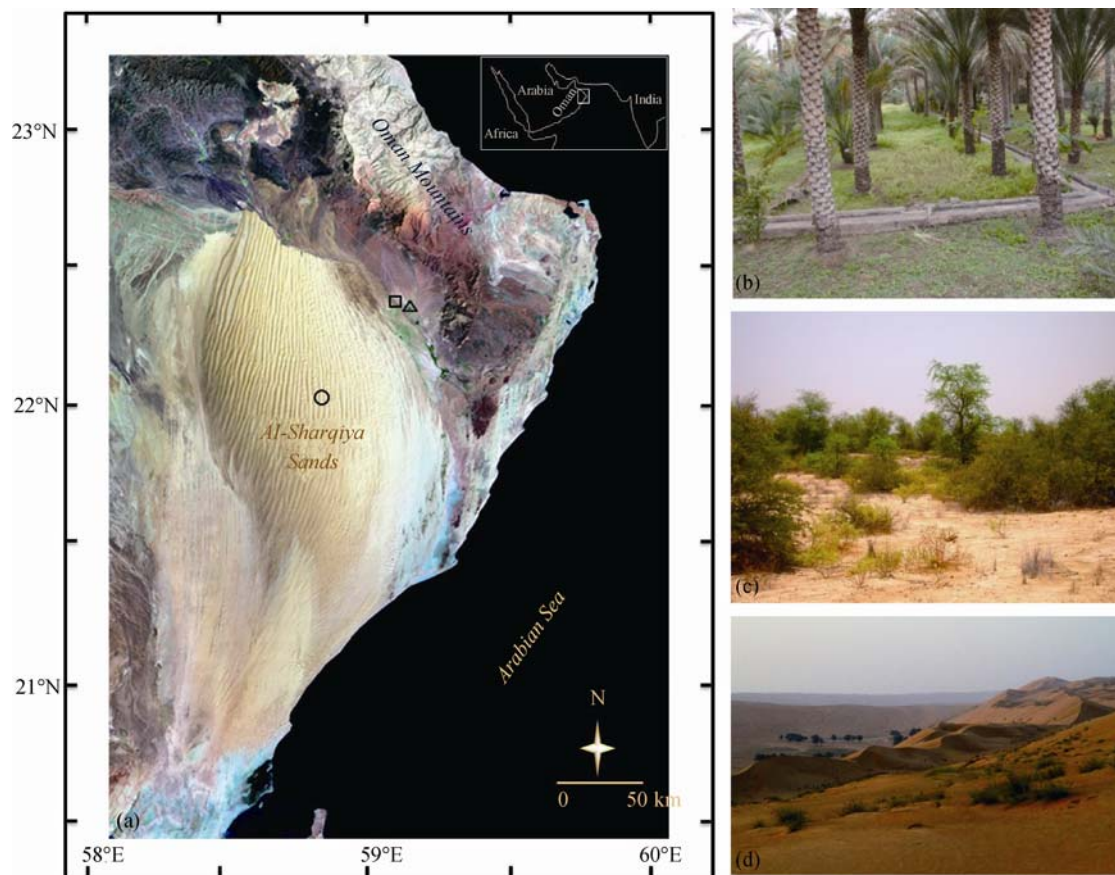


Fig. 1 The geography of the area and the explored sites. (a) Satellite image (Landsat TM RGB 742 courtesy of the U.S. Geological Survey) of Southern Arabia showing main geographical features of the area. Symbols correspond to sites depicted in (b)–(d). (b) Traditional date palm plantation (for site, see triangle on Fig.1a). (c) Natural undisturbed site (square on Fig.1a) with *Tetraena qatarensis*, *Salvadora persica* and *Prosopis cineraria* and interspersed patches of dry grasses. (d) Typical sand dune site in the region of Al-Sharqiya Sands (circle on Fig. 1a) where *Heliotropium kotschy* occurs.

1.4 Microscopy identification

We determined the morphological characteristics of spores including their wall structure based on examination of at least 100 spores mounted either in water, lactic acid, polyvinyl alcohol/lactic acid/glycerol (PVLG) (Omar et al., 1979) or in a mixture (1:1, v/v) of PVLG and Melzer's reagent (water, iodine, potassium iodide). Spores at all developmental stages were crushed to varying degrees by applying pressure to the cover slip and then stored at 65°C for 24 h to clear their contents from oil droplets. They were then examined under an Olympus BX 50 compound microscope equipped with Nomarski differential interference contrast optics. Microphotographs were recorded on a Sony 3-CDD color video camera coupled with the microscope.

Voucher specimens were mounted in PVLG and a

mixture of PVLG and Melzer's reagent (1:1, v/v) on slides and deposited in the Soil and Water Research Center, Ministry of Agriculture and Fisheries, Sultanate of Oman; Institute of Botany, University of Basel, Switzerland; Department of Plant Protection (DPP), West Pomeranian University of Technology, Szczecin, Poland; and in the herbarium at Oregon State University (OSC) in Corvallis, Oregon, USA.

1.5 Molecular analyses and phylogeny

DNA was extracted by crushing single spores with a pipette tip in a 0.2 mL PCR tube containing 2 µL of 0.25 M NaOH. The tubes were incubated in a T3 Thermocycler (Biometra GmbH, Goettingen, Germany) at 95°C for 2 min before 1 µL of 0.5 M Tris HCl (pH 8.0) and 2 µL of 0.25 M HCl were added and incubated again at 95°C for 2 min. In between incubations the tubes were kept on ice. The extracts were

either directly used as templates for polymerase chain reaction (PCR) or stored at -20°C . To obtain the LSU rDNA sequences, PCR was performed in a nested procedure with the SSUmAf-LSUmAr and the SSUmCf-LSUmBr primer pairs for the first and second nested PCR, respectively (Krüger et al., 2009). The final concentration of the master mix contained 0.6 U Taq polymerase (GE Healthcare, Glattbrugg, Switzerland), 2 mM MgCl_2 , 0.2 μM of each primer, 0.125 mM of each deoxynucleotide and 5 μg BSA (Bioconcept, Allschwil, Switzerland). Thermal cycling was done in the T3 Thermocycler with the following conditions for the first PCR: 3 min initial denaturation at 95°C ; 35 cycles of 30 s denaturation at 95°C , 90 s annealing at 60°C , 2 min elongation at 72°C and 10 min at 72°C for final elongation. The same conditions were used for the nested PCR except that the annealing temperature was 63°C . The PCR products were visualized on 1.5% agarose gels with $1\times$ TAE buffer and $1\times$ Midori green for staining (NIPPON Genetics EUROPE GmbH, Dueren, Germany). The PCR products with the expected-size bands of 1,500 bp were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturers' protocols. PCR products were cloned into pGEM-T Vector system (Promega, Madison Wisconsin) and the clones were transformed into competent JM109 *Escherichia coli* cells (Promega, Madison Wisconsin) following the manufacturers' protocols. Ten positive clones from each single spore were confirmed with direct colony PCR technique using the universal M13F and M13R vector primers and purified with ExoSAP-IT (GE Healthcare, Glattbrugg, Switzerland). For sequencing of amplified clones the BigDye Terminator Cycle Sequencing Kit and the ABI3500 were used (Applied Biosystems, Foster City, California). Sequencing primers for the respective samples were the universal vector primers mentioned above in addition to ITS4 and ITS3 primers (White et al., 1990). The electropherograms were processed and analyzed with ChromasPro Version 1.5 (<http://www.technelysium.com.au>).

The Glomeromycotan origin of the sequences was initially tested by BLAST search. Sequences from the present study were aligned in PAUP*4b10 (Swofford, 2001) to those sequences from GenBank which showed high similarity to them, in addition to all

available sequences for other species in their genera. The phylogenetic tree was inferred using maximum likelihood criteria as implemented in PAUP*. Sequences generated in this study were registered in GenBank under the accession numbers JQ287625 to JQ287641. The taxonomic nomenclature recently proposed by Redecker et al. (2013) was used for the species description of AMF.

2 Results and discussion

2.1 Taxonomy

Morphological and molecular phylogenetic analyses (Figs. 2 and 3) consistently confirmed the affiliations of the fungal strains recovered in one or more of the three desert sites studied and presented here, to four species of AMF. They were identified as *Claroideoglomus drummondii*, *Diversispora aurantia*, *Diversispora spurca* and *Funneliformis africanum*, clearly differing from each other and from other known species of AMF with glomoid spores and closely related in both morphology and molecular phylogeny.

Our two single spore-isolates of *Claroideoglomus drummondii* were clearly recognized morphologically by their yellow-colored, small spores and the innermost spore wall layer 3, which usually stains intensively in Melzer's reagent (Figs. 2a and b) (Błaszczkowski et al., 2006). *C. walkeri*, the closest molecular phylogenetic relative of *C. drummondii*, produces white or much lighter spores (Błaszczkowski et al., 2006). Sequences from spores from two cultures (F41 and F72) of our collection of *C. drummondii* were clustered as a sister group to both *C. drummondii* (96% sequence similarity) and *C. walkeri* (94% sequence similarity) with a clearly shorter distance to *C. drummondii*.

Two of our single spore isolates belonged to *Diversispora aurantia*. The most distinct morphological feature of this species is the deep orange to golden yellow color of its spores, their smooth surface and their relatively thick, coriaceous *sensu* Walker (1986), innermost spore wall layer 3 (Figs. 2c and d) (Błaszczkowski et al., 2004). These render the spores of *D. aurantia* unique among species of AMF with glomoid colored spores of a 3-layered spore wall, of which layer 3 is flexible to semi-flexible (Błaszczkowski, 2012). Sequences from two cultures (G5 and I76) of *D.*

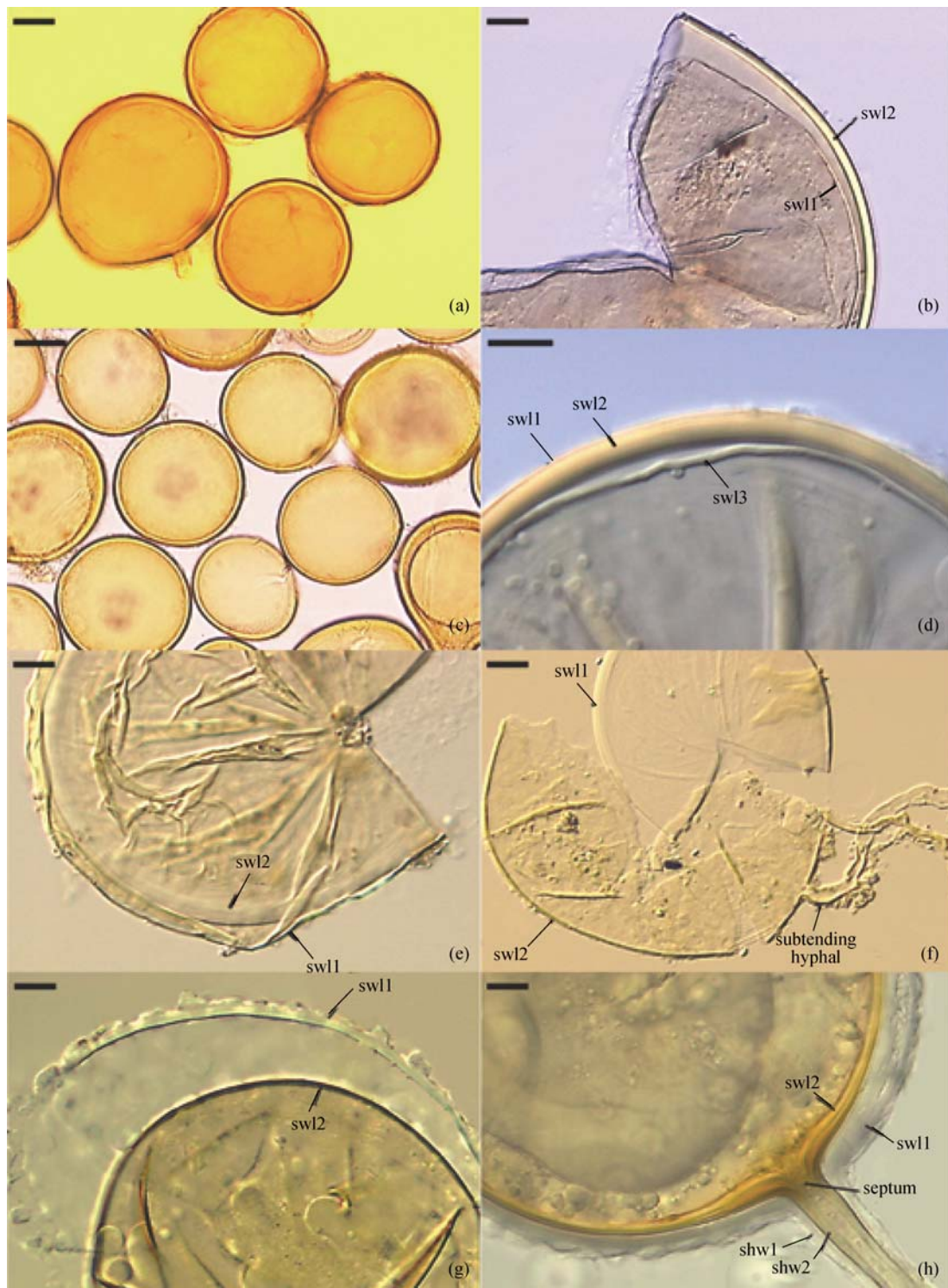


Fig. 2 Morphological characteristics of the spores. (a, b) *Claroideoglossum drummondii*. (a) Intact spores. (b) Laminated spore wall layer 2 (swl2) and flexible swl3 stained intensively in Melzer's reagent. Swl1 is completely sloughed in this spore. (c, d) *Diversispora aurantia*. (c) Intact orange spores. (d) Swl1-3; note the thick, coriaceous swl3 separated from laminated swl2. (e, f) *Diversispora spurca*. (e) Crushed spore with clearly separated swl1 and swl2. (f) Swl1 continuous with the main subtending hyphal wall layer 1 completely separated from swl2 in crushed spore. (g, h) *Funneliformis africanum*. (g) Separated swl1 and 2 in crushed spore. (h) Swl1 and 2 continuous with subtending hyphal wall layers (shw1) and 2 and septum in the lumen of subtending hypha; (g, h) note the much thicker swl1 than swl2. (a, c) Spores in lactic acid. (d, g, h) Spores in PVLG. (e, f) Spores in PVLG+Melzer's reagent. (a-h) differential interference microscopy. Scale bars: a, f=20 μm , c=50 μm , b, d, e, g, h=10 μm .

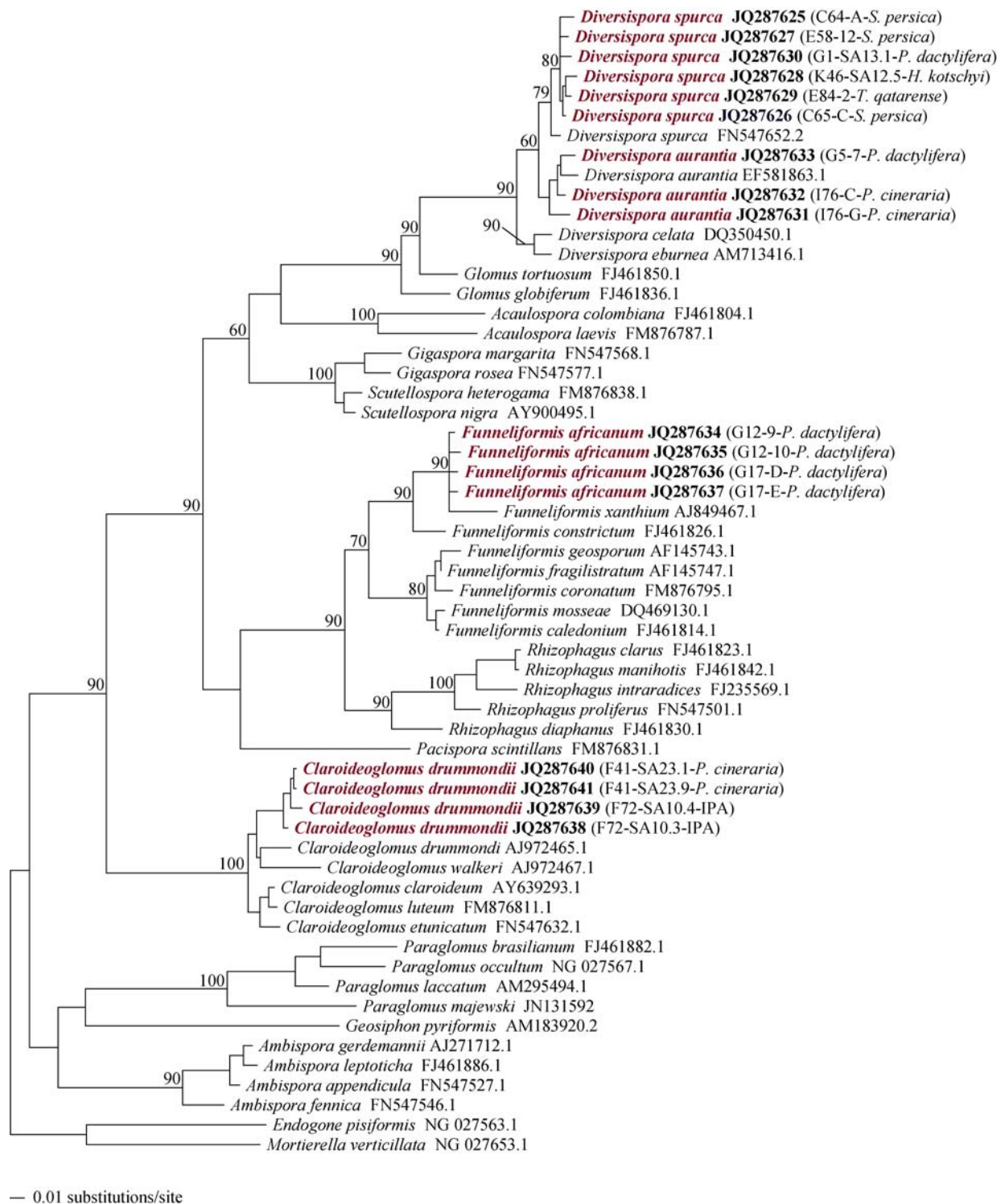


Fig. 3 Phylogenetic tree (maximum likelihood) was inferred from the partial LSU rDNA region (628 dataset characters), showing the positions of the four isolated species of AMF. The sequences obtained from these species are shown in coloured boldface followed by the GenBank accession number. The labels between the brackets indicate the isolate, clone code and either the likely host plant or the interspersed patch of dry grasses (IPA) from where the isolate was obtained. Values associated with branches are bootstrap values of maximum likelihood (100 replicates) as percentages. Only bootstraps above 50% are included.

aurantia cluster together with a sequence of this species (96% sequence similarity) (Fig. 3). *Diversispora spurca* spores are clearly distinct morphologically because their spore wall layer 1, which is loosely associated with a laminate spore wall layer 2, is continuous with subtending hyphal wall layer 1, being the main structural component of the subtending hyphal wall (Figs. 2e and f) (Pfeiffer et al., 1996; Kennedy et al., 1999; Błaszowski, 2003). Subtending hyphal wall layer 2, continuous with spore wall layer 2, is present only closely at the spore base and hence it barely touches layer 1 of the subtending hypha. Consequently, in crushed spores the subtending hypha usually detaches along with spore wall layer 1 from the structural spore wall layer 2, forming the main extraradical body of the fungus (Fig. 2f). This phenomenon does not occur in any of the two other known *Diversispora* spp. and other described species of AMF with glomoid spores (Błaszowski, 2012). Six different isolates (G64, E58, G1, K46, E84 and C65) showed these morphological characteristics. As it is shown in the phylogenetic tree (Fig. 3), they were all closest to *D. spurca* based on ribosomal LSU-based phylogeny (95% sequence similarity).

Two of our isolates belonged to *Funnelliformis africanum*. Among so far recognized species forming colored spores with two spore wall layers, *F. africanum* is unique, because only its structural laminate spore wall layer 2 is thinner than the sloughing spore wall layer 1, forming the spore surface (Figs. 2g and h) (Błaszowski et al., 2010). Its closest phylogenetic relative, as indicated in our analyses of sequences of the LSU rDNA region (Fig. 3), *F. xanthium*, differs clearly in spore color and size (much lighter and larger, respectively, in *F. africanum*), the number of spore wall layers (2 vs. 3 in *F. xanthium*), their phenotypic characters (e.g. layer 1, forming the spore surface, is much thicker and less persistent than that of *F. xanthium*), and features of the spore subtending hypha (Błaszowski et al., 2004, 2010). However, as results from phylogenetic analyses of sequences of the SSU region of rDNA (Błaszowski et al., 2010 and our data not shown), *F. africanum* is closest to *F. constrictum*, which also differs much in spore color, the phenotypic characters of spore wall components and features of the spore subtending hypha (Trappe, 1977; Błaszowski, 2003, 2012).

2.2 Global and local distribution

Based on the previous literature review, all the species described here, except for *D. aurantia*, have a worldwide distribution. They were recorded from different sites in Asia, Europe and the Americas (Trappe, 1977; Pfeiffer et al., 1996; Kennedy et al., 1999; Błaszowski, 2003, 2012; Błaszowski et al., 2006, 2010). The occurrence of *D. aurantia* is likely restricted to regions with warm climate. Of the 6,000 examined rhizosphere soils coming from different regions of the world, this species has never been found in its northern parts (Błaszowski et al., 2004; Błaszowski, 2012; Błaszowski, unpublished data). Pirozynski (1968) concluded that temperature is the major climatic factor determining the distribution and occurrence of fungi in general.

In this study, *D. aurantia* was recovered at two sites, the date palm plantation (*P. dactylifera*) and the natural undisturbed site (*P. cineraria*) while *F. africanum* and *C. drummondii* were recovered only at a single site, the date palm plantation and the undisturbed natural site (*P. cineraria* and IPA), respectively. *D. spurca* was, interestingly, recovered at all the three contrasting sites and was associated with the five different plants in these sites, making it a possibly generalist species in the area. This might be due to its ability to survive and function under contrasting soil conditions and different host plants in addition to its apparent ability to cope with the agricultural practices in the date palm plantation.

2.3 Importance of the isolated species

AMF are potential biotechnological tools (Azcón-Aguilar and Barea, 1997; Gianinazzi et al., 2002; Koltai, 2010) to be used as biofertilizers for sustainable agriculture (Siddiqui et al., 2008) and in re-vegetation programs, especially for endangered plants (Bothe et al., 2010) and desertified ecosystems (Requena et al., 2001). A high potential for success was obtained when AMF were introduced to sites resembling the site of their origin (Caravaca et al., 2003; Calvente et al., 2004; Marulanda et al., 2007; Shen and Wang, 2011).

It has been suggested that AMF of the same species originating from contrasting climatic conditions are functionally diverse suggesting ecotypic differentiation (Antunes et al., 2011) and that fungi exhibit opti-

mal performance within the range of temperature resembling their original climate (López-Gutiérrez et al., 2008). Such a population differentiation has also been found to result from salinity stress (Carvalho et al., 2004) and edaphic nutrient availability (Johnson et al., 2010). It is reasonable then to propose that our isolated species belong to populations exhibiting unique traits and physiological properties adapted to withstand the extreme desert conditions such as desiccation in long drought seasons, hot summer temperatures and low soil nutrients. Therefore, these species represent good candidates to be used as biofertilizers to enhance crop productivity and the fitness and survival of native plants in soil revegetation and reclamation measures under the environmental conditions prevailing in the Arabian Peninsula and, perhaps, other similar regions. However, functional studies under different arid land conditions and stresses are still needed to verify the physiological adaptability of our species to such conditions.

A prerequisite for the application of AMF as biofertilizers in agriculture and landscaping (Koltai, 2010) is the availability of mass production procedures for the selected strains of AMF and of suitable, preferentially locally available carriers (Sreenivasa and Bagyaraj, 1988; Barea et al., 1993; Kapulnik et al., 1994; Douds et al., 2006). Moreover, a strict quality control is essential including methods for ascertaining the absence of infestations with soil-borne pathogens and, preferentially, for assessing the performance of the inoculated AMF in the field.

In Oman, where we identified this collection of potentially desert-adapted strains of AMF, there is a great effort to preserve the country's natural vegetation (Patzelt et al., 2008; Pickering and Patzelt, 2008). Mycorrhizal biotechnology might be a crucial factor to overcome difficulties met in propagation and maintenance of some indigenous plants (Patzelt et al., 2008).

3 Conclusions

The four described species in this paper are recorded for the first time in the whole Arabian Peninsula and, thus, expand the species number currently known to occur in this arid region (Khalil, 1989; Al-Yahya'ei et al., 2011). Because some of our isolates come from extremely arid environments, these AMF may be an

asset to biodiversity conservation in desert areas, particularly in the Arabian Peninsula. They may also open the door for basic research regarding functional characterization, as well as for applied research in view of their use as biofertilizers to promote sustainable agriculture under arid conditions.

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