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# *Glomus drummondii* and *G. walkeri*, two new species of arbuscular mycorrhizal fungi (*Glomeromycota*)

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ABSTRACT

Two new ectocarpic arbuscular mycorrhizal fungal species, *Glomus drummondii* and *G. walkeri* (*Glomeromycota*), found in maritime sand dunes of northern Poland and those adjacent to the Mediterranean Sea are described and illustrated. Mature spores of *G. drummondii* are pastel yellow to maize yellow, globose to subglobose, (58–)71(–85) μm diam, or ovoid, 50–80 × 63–98 μm. Their wall consists of three layers: an evanescent, hyaline, short-lived outermost layer, a laminate, smooth, pastel yellow to maize yellow middle layer, and a flexible, smooth, hyaline innermost layer. Spores of *G. walkeri* are white to pale yellow, globose to subglobose, (55–)81(–95) μm diam, or ovoid, 60–90 × 75–115 μm, and have a spore wall composed of three layers: a semi-permanent, hyaline outermost layer, a laminate, smooth, white to pale yellow middle layer, and a flexible, smooth, hyaline innermost layer. In Melzer's reagent, only the inner- and outermost layers stain reddish white to greyish rose in *G. drummondii* and *G. walkeri*, respectively. Both species form vesicular–arbuscular mycorrhizae in one-species cultures with *Plantago lanceolata* as the host plant. Phylogenetic analyses of the ITS and parts of the LSU of the nrDNA of spores placed both species in *Glomus* Group B *sensu* Schüßler *et al.* [Schüßler A, Schwarzott D, Walker C, 2001. A new fungal phylum, the *Glomeromycota*: phylogeny and evolution. *Mycolological Research* 105: 1413-1421.]



## Introduction

Investigations of trap cultures established with soils collected under plants colonizing maritime sand dunes of northern Poland and those of the Mediterranean Sea revealed two undescribed species of arbuscular fungi of the genus *Glomus*. These fungi are described here as *G. drummondii* and *G. walkeri*.

## Materials and methods

### *Establishment of trap cultures and one-species cultures*

Collection of soil samples, establishment of trap and one-species pot cultures, as well as growth conditions were as described previously (Błaszkowski & Tadych 1997). Briefly, rhizosphere soils and roots of sampled plants were collected

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from a depth of 5–30 cm using a small garden shovel. In the laboratory, about 100 g sub-samples were used from each sample to isolate spores of arbuscular mycorrhizal fungal species, while the rest of each sample was either air-dried for two weeks and subsequently refrigerated at 4 °C or directly used to establish trap cultures. Trap cultures were established to obtain a great number of living spores and to initiate sporulation of species that were present but not sporulating in the field collections. The growing substrate of the trap cultures was the field-collected material mixed with an autoclaved coarse-grained sand coming from maritime dunes adjacent to Świnoujście (pH 6.7; 12 mg $l^{-1}$ phosphorus and 26 mg $l^{-1}$ potassium, respectively; Błaszkowski 1995). The mixtures were placed into 9 × 12.5 cm plastic pots (500 $cm^3$) and densely seeded with *Plantago lanceolata*. Plants were grown in a glasshouse at 15–30 °C with supplemental 8–16 h lighting provided by one SON-T AGRO sodium lamp (Philips Lighting, Gostynin Poland) placed 1 m above pots. The maximum light intensity was 180 μE $m^{-2}s^{-1}$ at pot level. Plants were watered two to three times a week. No fertilizer was applied during the growing period. Trap cultures were first harvested three months after plant germination and then monthly until the fifth to the seventh month of cultivation. Spores were extracted by wet sieving and decanting (Gerdemann & Nicolson 1963). Presence of mycorrhizae was determined following clearing and staining of roots (Phillips & Hayman 1970) modified as follows: tissue acidification with 20 % hydrochloric acid instead of 1 %, and trypan blue concentration 0.1 % instead of 0.05 % (Koske, pers. comm.).

One-species pot cultures were established from about 50–100 spores newly formed in the trap cultures. Before inoculation, the spores were stored in water at 4 °C for 24 h. After removal of soils debris, spores were collected in a pipette and transferred onto a compact layer of mycorrhizae-free roots of 10–14 d old seedlings of *P. lanceolata* placed at the bottom of a hole *ca* 1 cm wide and 4 cm deep formed in a wetted growing medium filling 8 cm plastic pots (250 $cm^3$). The growing medium was an autoclaved sand of maritime dunes adjacent to Świnoujście with the chemical properties listed above. Subsequently, the spores were covered with another layer of roots attached to four to six additional host plants, and the roots and sandwiched spores were buried in the growing medium. Finally, three to six seeds of *P. lanceolata* were placed onto the surface of the growing substrate and covered with a thin layer of autoclaved sand. The cultures were harvested after four to eight months and spores were extracted.

### Microscopy

Morphological properties of spores and their wall structures were determined based on observation of at least 100 spores mounted in polyvinyl alcohol/lactic acid/glycerol (PVLG; Omar *et al.* 1979) and a mixture of PVLG and Melzer's reagent (1:1, v/v). Spores at all stages of development 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. These were examined under an Olympus BX 50 compound microscope equipped with Nomarski differential interference contrast optics. Microphotographs were recorded on a Sony 3CDD colour video camera coupled to the microscope.

Terminology of spore structure is that suggested by Stürmer and Morton (1997) and Walker (1983). Spore colour was examined under a dissecting microscope on fresh specimens immersed in water. Colour names are from Kornerup and Wanscher (1983). Nomenclature of fungi and plants is that of Walker and Trappe (1993) and Mirek *et al.* (1995), respectively. The authors of the fungal names are those presented at the Index Fungorum website: http://www.indexfungorum.org/AuthorsOfFungalNames.htm. Specimens were mounted in PVLG on slides and deposited in the Department of Plant Pathology, University of Agriculture, Szczecin, Poland, and in the herbarium at Oregon State University (OSC) in Corvallis, Oregon, USA. Colour microphotographs of spores and mycorrhizae of *G. drummondii* and *G. walkeri* can be viewed at the URL http://www.agro.ar.szczecin.pl/~jblaszkowski/.

### PCR amplification

To amplify DNA from single spores, these were separated in a drop of sterile water. The water was removed before spores were crushed, mixed with 8 μl of the PCR-Mix using a pipette and used directly for PCR.

Amplification of the analysed ITS and LSU nu-rDNA region by PCR was performed on a Hybaid. OmniGene TR3 CM220 Thermo Cycler (MWG-Biotech, Ebersberg, Germany) in a total volume of 50 μl containing 2 U *Taq* DNA polymerase (Promega, Heidelberg, Germany), 5 μl of 10× *Taq* polymerase reaction buffer (Promega), 4 μl 25 mm magnesium chloride, 10 nmol of each dNTP (MBI-Fermentas, St Leon-Rot, Germany), 50 pmol of each of the two primers, and 1 μl of the DNA extract. The reactions were performed as hot-start PCR with 10 min initial denaturation at 94 °C before adding the *Taq* polymerase at 80 °C. The PCR program comprised 32 cycles (40 s at 94 °C, 30 s at 54 °C, 40 s at 72 °C). A final elongation of 10 min at 72 °C followed the last cycle. SSU-Glom1 (ATT ACG TCC CTG CCC TTT GTA CA) and LSU-Glom1 (CTT CAA TCG TTT CCC TTT CA) previously described by Renker *et al.* (2003) were used as primers to amplify the target regions.

### Cloning, sequencing and sequence analyses

PCR products were cloned into the pCR4-Topo Vector following the manufacturer's protocol of the TOPO TA Cloning Kit (Invitrogen Life Technologies, Karlsruhe, Germany) and transformed into TOP10 chemically competent *Escherichia coli*. Sequencing was performed using a ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Warrington, UK) using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's instructions.

DNA sequences of the complete ITS and parts of the ribosomal SSU and LSU of *G. drummondii*, *G. walkeri*, and *G. diaphanum* were submitted to the EMBL database under the accession numbers given in Figs 1–2. The latter fungus was sequenced because of the high morphological similarity of its spores to those of *G. walkeri*. Spores of *G. diaphanum* were obtained from Joseph B. Morton (West Virginia University, USA; accession no. INVAM WV5498). Reference sequences to analyse the systematic position of the new sequences were taken from GenBank. First, a sequence data set of available

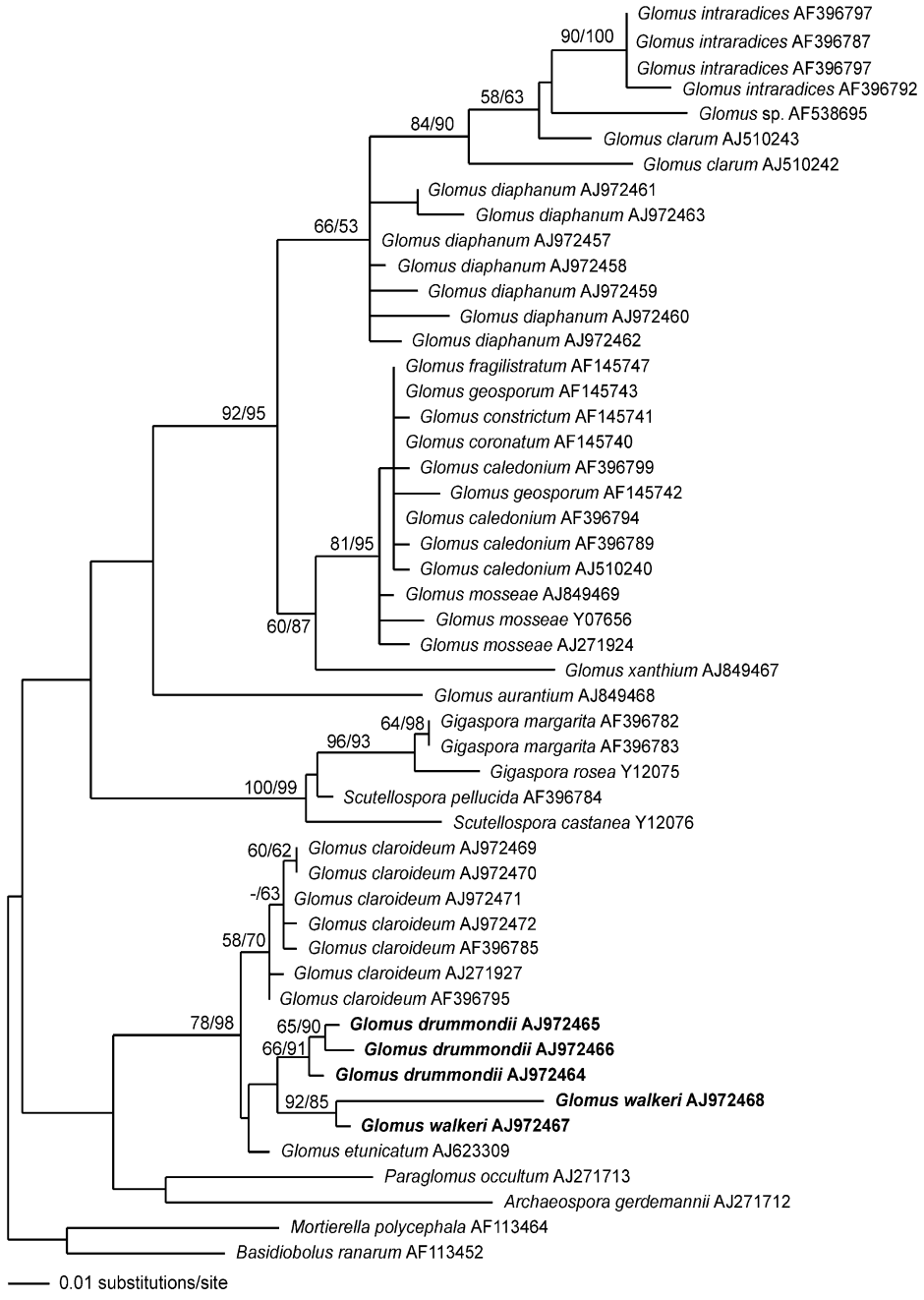


**Fig 1 – Maximum likelihood analysis of the *Glomeromycota* based on 50 LSU sequences of the nrDNA. [using the model of Tamura and Nei (1993) with gamma shape]. BS percentage values (>50 %) were determined for ML (using TrN+G; 200 resamplings) and NJ (using Kimura two-parameter; 1000 resamplings) analyses (first values above branches ML, second values NJ). Sequences of *Mortierella polycephala* and *Basidiobolus ranarum* were used as outgroup.**

LSU sequences was aligned to ensure the phylogentic position of the new species. In a second step, the full-length ITS sequence of *G. drummondii* and *G. walkeri* were aligned with those of other members of *Glomus* Group B *sensu* Schüßler *et al.* (2001) in order to specify their systematic position. Sequences were aligned manually in BioEdit version 5.0.9 (Hall 1999). For the LSU, a first alignment of 362 bp was obtained. Afterwards ambiguously aligned positions were excluded from the phylogenetic analyses, resulting in an alignment of 297 putative homologous sites. For the ITS, a first alignment of 588 bp was reduced to 450 putative homologous sites. The final alignments are available in TreeBASE (http://www.treebase.org/treebase/).

Phylogenetic trees were inferred using distance, MP, and ML criteria as implemented in PAUP 4.0b10 (Swofford 2003). To decide on the evolutionary model that best fitted the

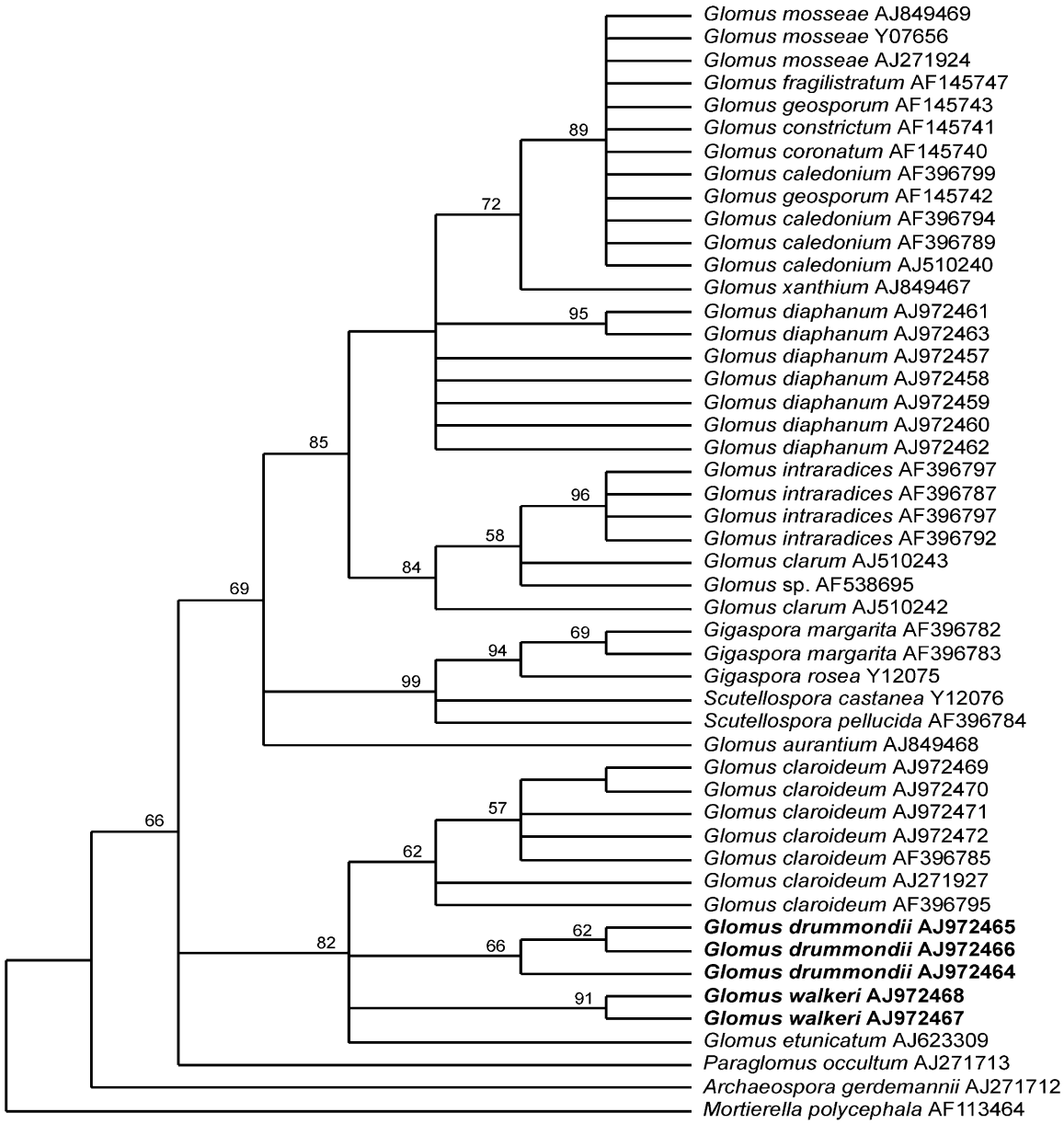


**Fig 2 – Strict consensus tree of the *Glomeromycota* based on 50 LSU sequences of the nrDNA. Phylogenetic positions of the newly described *Glomus drummondii* and *G. walkeri* are given in bold. Numbers above branches give BS support derived from 1000 replicates. A sequence of *Mortierella polycephala* was used as outgroup.**

data, the program Modeltest 3.06 (Posada & Crandall 1998) was used. Based on the results of the test, the model selected by the hierarchical likelihood ratio test (hLRT) for both datasets was the TrN model (Tamura & Nei 1993) with a gamma shape parameter (G) for among site variation (TrN+G). The ML method (with the TrN+G model) was used for phylogenetic analyses (Fig 1). MP analyses were performed using the heuristic search mode with 10 random-addition sequence replicates, tree bisection–reconnection branch swapping, MULTrees option on, and collapse zero-length branches off. All characters were treated as unordered and equally weighted. Strict consensus trees were calculated including all MP trees (Figs 2–3). The confidence of branching was assessed using 200 BS resamplings in ML analyses and 1000 BS resamplings in MP (heuristic search with TBR swapping) and distance (NJ method using the Kimura two-parameter model) analyses.

The data set used to reconstruct the LSU MP tree (Fig 2) contained 297 characters of which 163 were constant, 61 parsimony uninformative, and 73 parsimony informative. The heuristic search recovered 468 equally most parsimonious trees with a length of 257 steps, a CI of 0.67, a RI of 0.87, and a rescaled CI (RC) of 0.58. The data set used to reconstruct the ITS MP tree (Fig 3) contained 452 characters of which 352 were constant, 50 parsimony uninformative, and 50 parsimony informative. The Branch-and-Bound-Search recovered one most parsimonious tree with a length of 132 steps, a CI of 0.87, an RI of 0.86, and a rescaled RC of 0.75.

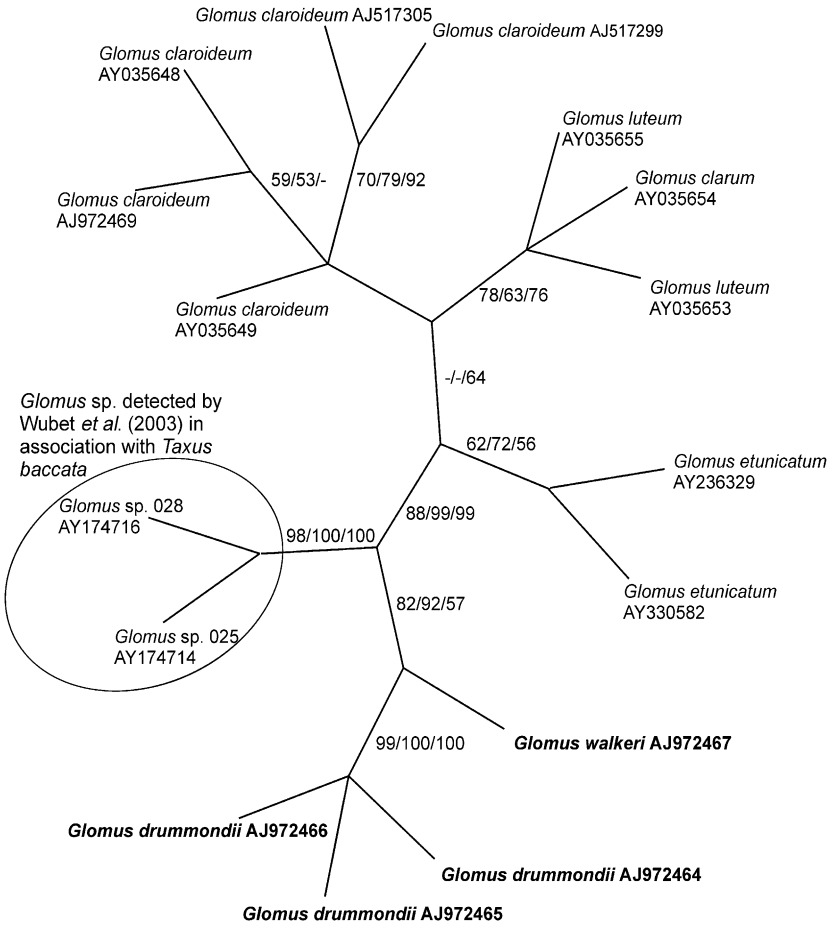


**Fig 3 – Strict consensus network of the *Glomus* Group B *sensu* Schüßler *et al.* (2001) based on 16 ITS sequences of the nrDNA. Phylogenetic positions of the newly described *G. drummondii* and *G. walkeri* are given in bold. Bootsrap percentage values (>50 %) were determined for ML (using TrN+G; 200 resamplings), MP (1000 resamplings), and NJ (using Kimura two-parameter; 1000 resamplings) analyses (first values above branches ML, second values MP, third values NJ).**

## Taxonomy

**Glomus drummondii** Błaszk. & C. Renker, **sp. nov.**

**Figs 4–5**

*Etym*: '*Drummondii*', referring to the plant species *Oenothera drummondii* under which this fungal species was first found.

*Sporocarpia ignota. Sporae singulae in solo efformatae; sub-luteae vel maydi-flavae; globosae vel subglobosae; (58–)71(–58) μm diam; aliquando ovoideae; 50–80 × 63–98 μm. Tunica sporae e stratis tribus (strati 1–3); strato '1' caduco, hyalino, 0.5-1 μm crasso; strato '2' laminato, glabro, sub-luteo vel maydi-flavo, (1.5–)3.5(–5.5) μm crasso; strato '3' elastico, glabro, hyalino, (0.5–)1(–1.5) μm crasso. Hypha subtendens sub-luteo vel maydi-flavo; recta vel recurva; cylindrica, raro coliga; (4–)6(–9.5) μm lata ad basim sporae; pariete sub-luteo vel maydi-flavo; (1–)2(–3) μm crasso, stratis 1–3 sporae continuo. Porus e septo continuo strati 3 sporae efformata. Mycorrhizas vesicular-arbusculares formans.*

*Typus*: **Poland**: Szczecin, infra *P. lanceolata*, 18 May 2005, *J. Błaszkowski*, 2578 (DPP[1] Holotypus).

*Sporocarps* unknown. *Spores* occur singly in the soil (Fig 4A); origin blastically at the tip of extraradical hyphae of mycorrhizal roots. Spores pastel yellow (3A4) to maize yellow (4A6); globose to subglobose; (58–)71(–85) μm diam; sometimes ovoid; 50–80 × 63–98 μm; with a single subtending hypha (Fig 4A and F). *Spore wall structure* (Fig 4B–F) consists of three layers (layers 1–3) in two groups (groups A and B). Outermost layer 1 in group A evanescent, hyaline, 0.5–1 μm thick (Fig 4B–C), short-lived, usually completely sloughed in mature spores. Layer 2 of group A laminate, smooth, pastel yellow (3A4) to maize yellow (4A6), (1.5–)3.5(–5.5) μm thick (Fig 4B–F). Layer 3 of group B flexible, smooth, hyaline, (0.5–)0.9(–1.2) μm thick (Fig 4B–F), easily separating from layer 2 except for its small protrusion, the outer surface of which is usually attached to the inner surface of subtending hyphal wall layer 2 close at the spore base to form a curved septum in the lumen of the subtending hypha. Only layer 3 stains reddish white (7A2) to greyish rose (12B3) in Melzer's reagent (Fig 4E). *Subtending hypha* hyaline to maize yellow (4A6); straight or recurvate; cylindrical or slightly flared, rarely constricted; (4–)6(–9.5) μm wide at the spore base (Fig 4F). *Wall of subtending hypha* hyaline to

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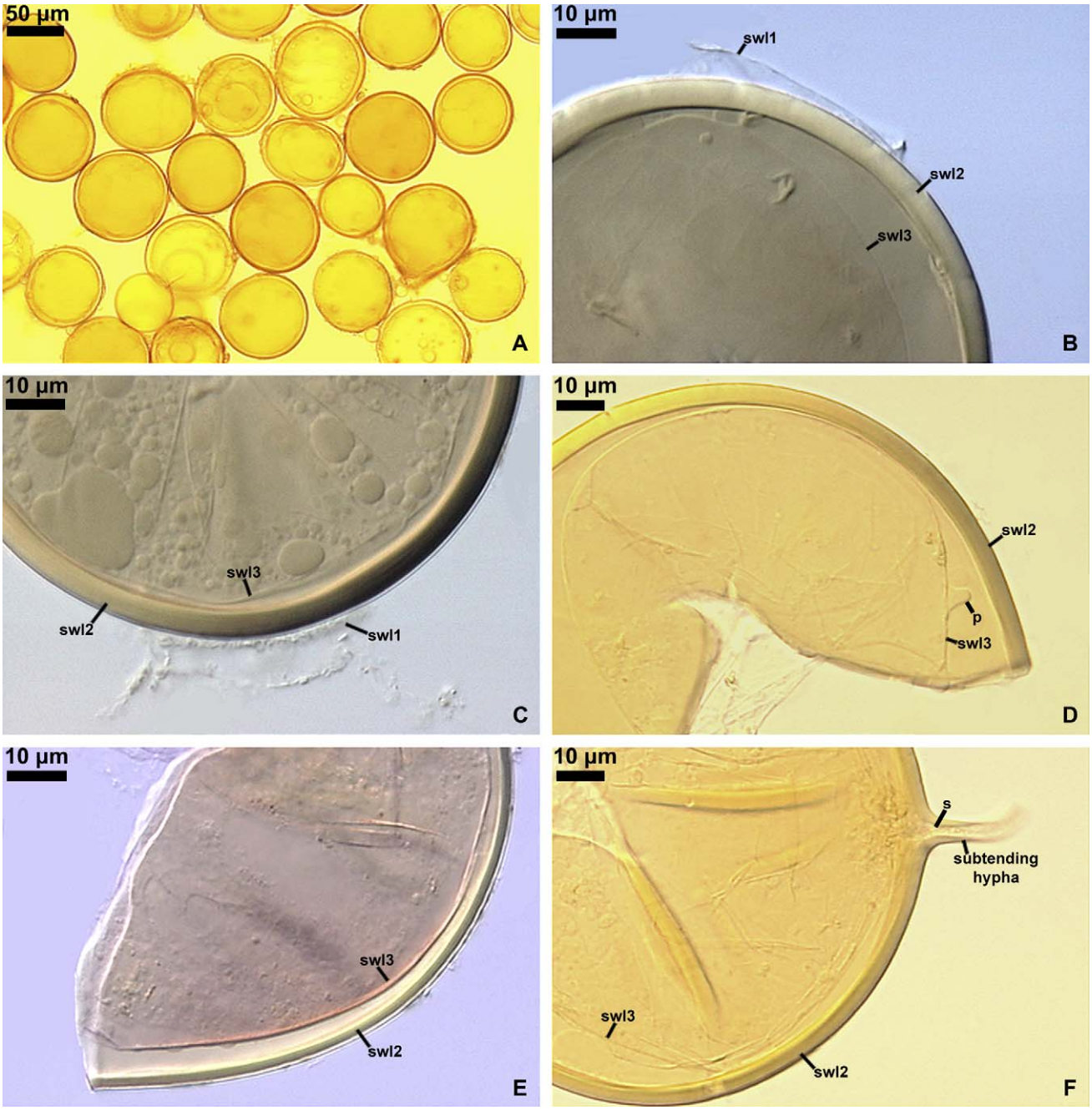


**Fig 4 – Spores of *Glomus drummondii*. A. Mature spores. B. Spore wall layers 1 (swl1) and 3 (swl3) separated from the laminate spore wall layer 2 (swl2). C. Highly deteriorated spore wall layer 1 (swl1) and adherent spore wall layers 2 (swl2) and 3 (swl3). D. Spore wall layer 3 (swl3) with a small protrusion (p) separated from the lumen of subtending hypha and spore wall layer 2 (swl2); spore wall layer 1 is completely sloughed in this spore. E. Spore wall layer 3 (swl3) stained in Melzer's reagent and spore wall layer 2 (swl2). F. Flared subtending hypha occluded by a curved septum formed by spore wall layer 3 (swl3); spore wall layer 2 (swl2) is also indicated. A, intact spores in water. B–D,F, spores crushed in PVLG. E, spore crushed in PVLG + Melzer's reagent. A, bright-field microscopy; B–F, differential interference microscopy.**

maize yellow (4A6); (1–)2(–3.5) µm thick at the spore base; continuous with spore wall layers 1 and 2 (Fig 4F); layers 1 extends up to 5.0 below the spore base; layer 1 usually completely sloughed in mature spores. *Pore* occluded by a septum, (0.5–)2(–4.5) µm wide, continuous with spore wall layer 3, positioned up to 5.9 µm below the spore base (Figs 4D, F and 6).

*Other material examined*. **Spain**: Loret de Mar (41°42′N, 2°51′E), Costa Brava, from the root zone of *Oenothera drummondii*, 22 Aug. 1999, *J. Błaszkowski*, (DPP); Majorca, El Arenal (39°31′N, 2°45′E), under *O. drummondii*, 22 Aug. 2001, *J. Błaszkowski*, (DPP); Cape Salinas (36°19′N, 3°2′E), among roots of *Ammophila arenaria*, 24 Aug. 2001, *J. Błaszkowski*, (DPP). **Poland**: Szczecin, under pot-cultured *P. lanceolata*, 18 May 2005, *J. Błaszkowski*, 2575-2577 and 2579-2596

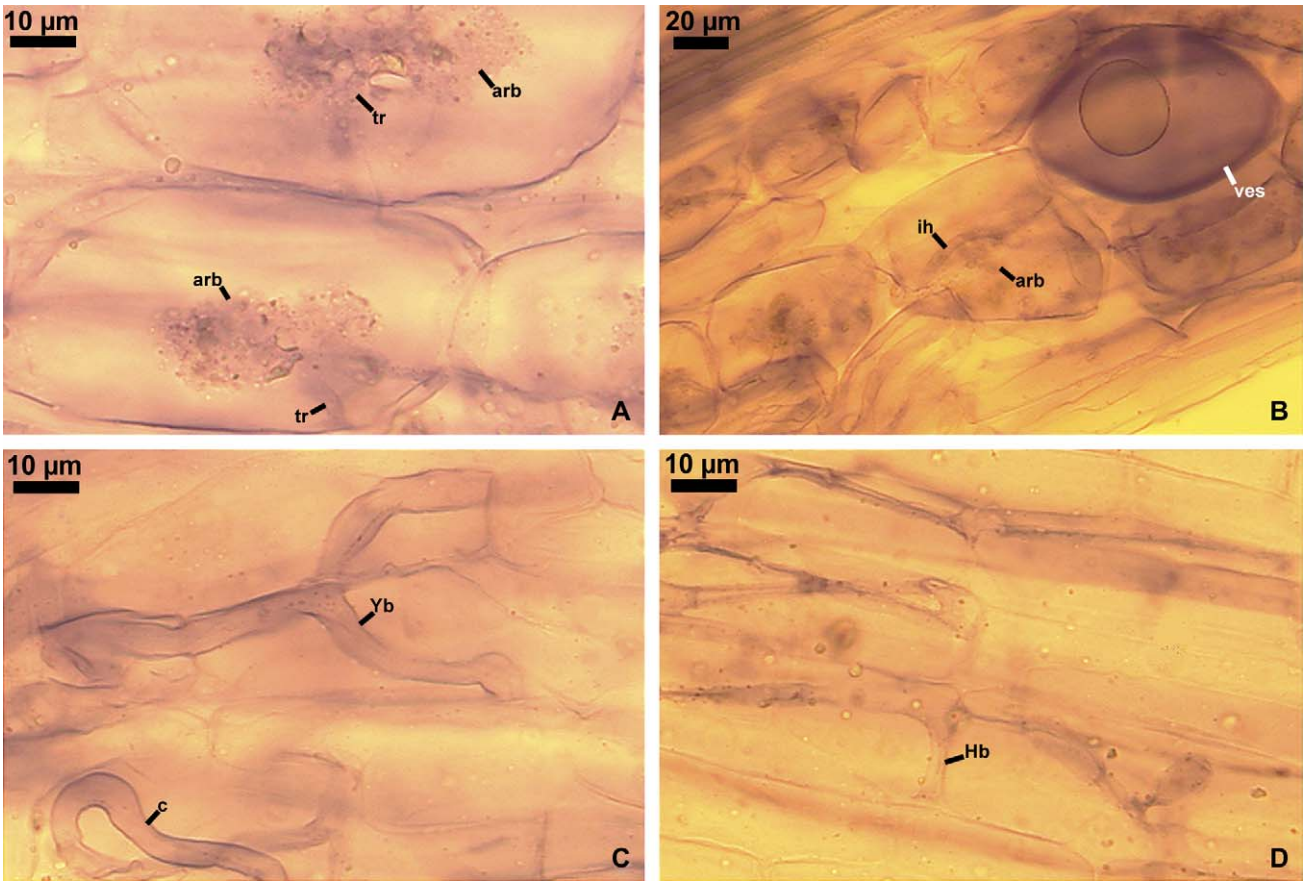


**Fig 5 – Mycorrhizae of *Glomus drummondii* in roots of *Plantago lanceolata* stained in 0.1 % trypan blue (mycorrhizae in PVLG viewed using bright-field microscopy). A. Arbuscules (arb) with trunks (tr). B. Arbuscules (arb), vesicles (ves), and intraradical hyphae (ih). C. Y-shaped branch (Yb) and coil (c). D. H-shaped branch (Hb).**

(DPP; and two slides at OSC); Jurata (N54°38′, E18°41′), around roots of *A. arenaria*, 5 Sep. 2000, *J. Błaszkowski*, (DPP); Jastrzębia Góra (54°50′N, 18°18′E), under *A. arenaria*, 26 Aug. 2000, *J. Błaszkowski*, (DPP); Osłonino (54°42′N, 18°28′E), from the root zone of *Juncus conglomerates*, 1 Sep. 2000, *J. Błaszkowski*, (DPP); Hel (54°36′N, 18°49′E), among roots of *A. arenaria*, 26 Aug. 2000, *J. Błaszkowski*, (DPP). **Portugal**: Faro (37°1′N, 7°56′W), under *Zea mays*, 1 Dec. 2000, *J. Błaszkowski*, (DPP). **Turkey**: Near Karabucak-Tuzla (36°43′N, 34°59′E), in the rhizosphere of *A. arenaria*, 10 June 2001, *J. Błaszkowski*, (DPP). **Greece**: *Cyprus*: near Larnaca (34°55′N, 33°38′E), under *O. drummondii*, 23 Oct. 2003, *J. Błaszkowski*,(DPP).

*Distribution and habitat*: *Glomus drummondii* was found for the first time in a trap culture with a root–rhizosphere soil mixture of *Oenothera drummondii* colonizing dunes of the Mediterranean Sea located near Loret de Mar, Costa Brava, Spain, in August 1999. Subsequently, this fungus was isolated from trap cultures with root fragments and rhizosphere soils of *A. arenaria* (4 cultures) of the Baltic Sea dunes adjacent to Jurata, Jastrzębia Góra, and Hel, as well as of *J. conglomeratus* (one culture) inhabiting a salt march located in Osłonino, *ca* 100 m from the bank of the Puck Bay (all the sites are located in northern Poland and were sampled from 29 August to 5 September of 2000); *Z. mays* (one culture) cultivated near Faro, Portugal, in December 2000; *O. drummondii* (eight cultures) growing along the coast of the Mediterranean Sea extending near El Arenal and *A. arenaria* (nine cultures) colonizing mobile dunes of Cape Salinas, Majorca, Spain, in August 2001; *A. arenaria* (seven cultures) growing in sandy dunes of the Mediterranean Sea located near Karabucak-Tuzla, Turkey, in June 2001; and *O. drummondii* (one culture) colonizing sandy areas extending along the bank of the Mediterranean Sea adjacent to Larnaca, Cyprus, in October 2003.

The occurrence of arbuscular fungi in any field-collected root–rhizosphere soil sample of the plant species listed above was not determined. The only species of arbuscular fungi isolated together with *G. drummondii* from the cultures representing *O. drummondii* growing in the Loret de Mar dunes and *Z. mays* cultivated in Portugal was *G. mosseae*. Apart from spores of *G. drummondii*, the trap cultures with Polish soils contained spores of *Acaulospora mellea*. The fungi co-occurring with *G. drummondii* in Majorca's cultures included *A. paulinae*, *Archaeospora trappei*, *Diversispora spurca*, *Entrophospora infrequens*, *G. aurantium*, *G. constrictum*, *G. coronatum*, *G. fasciculatum*, *G. mosseae*, *G. xanthium*, *Pacispora franciscana*, *P. scintillans* and *Scutellospora calospora*. The cultures from Turkey also contained *G. arenarium*, *G. constrictum*, *G. coronatum*, *G. deserticola*, *G. intraradices*, *P. scintillans* and an undescribed *Glomus 138*, and that from Cyprus still hosted *Ar. trappei*, *G. coronatum*, and *G. geosporum*.

*Mycorrhizal associations*: The presence of mycorrhizae in field-collected root fragments of the plant species sampled was not determined.

In one-species cultures with *P. lanceolata* as the host plant, mycorrhizae of *G. drummondii* comprised arbuscules, vesicles, as well as intra- and extraradical hyphae (Fig 5). Arbuscules were numerous and evenly distributed along the root fragments examined. They consisted of short trunks grown from parent hyphae and numerous branches with very fine tips (Fig 5A). Vesicles were not numerous and occurred singly or in aggregates widely dispersed along the roots. They were ellipsoid to prolate; 17.5–90.0 × 30.0–150.0 μm (Fig 5B). Intraradical hyphae grew along the root axis, were (2.5–)5.3(–7.8) μm wide, straight or slightly curved, and sometimes formed Y- or H-shaped branches and coils (Fig 5B–D). The coils usually were ellipsoid; 24.0–37.5 × 48.0–90.0 μm; rarely circular; 35.0–40.5 × 35.0–40.5 μm; when seen in a plane view. Extraradical hyphae were (3.0–)4.9(–6.8) μm and occurred in low abundances. In 0.1 % trypan blue, arbuscules stained pale violet (16A3) to reddish violet (16C7), vesicles violet white (16A2) to greyish violet (16C4), intraradical hyphae pale violet (16A3) to reddish violet (16B6), coils violet white (15A2) to lilac (16B3), and extraradical hyphae pastel violet (16A4) to greyish violet (16C5).

*Phylogenetic position*: Phylogenetic analyses of LSU sequences placed *G. drummondii* in *Glomus* Group B *sensu* Schüßler *et al.* (2001; Figs 1 and 2). ITS sequences data placed *G. drummondii* sister to *G. walkeri* Błaszk. & C. Renker, a species described below (Fig 3). This clade again is placed sister to species belonging to *Glomus* Group B (i.e., *Glomus claroideum*, *G. clarum*, *G. etunicatum*, and *G. luteum*) and two recently published sequences derived from roots of *Taxus baccata* L. (Wubet *et al.* 2003). From the original *Glomus* Group B, we were able to include reference sequences of *G. claroideum* N.C. Schenck & S.M. Sm., *G. clarum* T.H. Nicolson & N.C. Schenck, *G. etunicatum* W.N. Becker & Gerd., and *G. luteum* L.J. Kenn *et al.* in the ITS analysis; all these sequences clustered together, while sequences of the newly described *G. drummondii* grouped distant, indicating its distinct phylogenetic position (Fig 2).

*Discussion*: The morphological characters most distinguishing *G. drummondii* are its small and yellow-coloured spores with their thin, colourless, flexible innermost wall layer intensively staining in Melzer's reagent (Fig 4).

When observed under a dissecting microscope, spores of *G. drummondii* may be confused with small-spored isolates of *G. arenarium*, *G. claroideum*, *G. etunicatum*, *G. insculptum*, *G. lamellosum*, *G. luteum*, and *G. pustulatum* because of their similar appearance and pigmentation.

The first property differentiating *G. drummondii* from all but one of the species listed above is size of their spores. The mean diameter of *G. drummondii* spores overlaps with that of spores of only *G. insculptum* (Błaszkowski *et al.* 2004). In the other species, only their smallest spores are more or less within the upper diameter range of spores of *G. drummondii*.

Additionally, mature spores of *G. drummondii*, being pastel yellow to maize yellow in colour, are lighter than mature spores of *G. arenarium* (orange to raw umber; Błaszkowski 2003; Błaszkowski *et al.* 2001), *G. etunicatum* (orange to red brown; Morton 2002), *G. insculptum* (yellowish white to golden yellow; Błaszkowski *et al.* 2004), *G. luteum* (pale yellow to dark yellow with a brownish tint; Kennedy *et al.* 1999; Morton 2002), and *G. pustulatum* (deep orange to orange brown; Błaszkowski 1994; Koske *et al.* 1986).

The highest differences between the fungi compared here reside in phenotypic and biochemical properties, as well as in the number of wall components of their spores. Although *G. claroideum*, *G. lamellosum*, *G. luteum*, and *G. pustulatum* have a thin, hyaline, flexible innermost layer resembling spore wall layer 3 of *G. drummondii* (Błaszkowski 2003; Kennedy *et al.* 1999; Koske *et al.* 1986; Stürmer & Morton 1997; Walker & Vestberg 1998; Fig 4B–F), this layer reacts with Melzer's reagent only in *G. drummondii* and *G. lamellosum*. However, the staining reaction is markedly more intensive in the former species [reddish white (7A2) to greyish rose (12B3)] than in the latter fungus [reddish white (7A2) to pastel red (9A4); Błaszkowski 2003]. Additionally, layer 3 of crushed spores of *G. drummondii* easily separates from the laminate layer 2 (Fig 4B, D–F), whereas that of *G. lamellosum* spores usually tightly adheres to this layer and, thereby, is difficult to see. Thus, although this layer in the two species is a component of the spore wall originated from and attached to the upper part of subtending hypha as Stürmer and Morton (1997) found, it is located in groups B and A in the former and the latter species, respectively, following the concept of Walker and Vestberg (1998). Another character readily separating *G. drummondii* and *G. lamellosum* is the relatively thick and long-lived (semipermanent) outermost layer of the latter fungus (*versus* a short-lived and thin layer in the former species; Fig 4C).

Spores of *G. pustulatum* also are three-layered, but the outermost wall layer, forming the spore surface, in this species is a permanent structure (*versus* a short-lived one in *G. drummondii*; Fig 4C). Moreover, it is ornamented with blister-, cup- or irregularly-shaped processes (Błaszkowski 2003; Koske *et al.* 1986; *versus* smooth or roughened in *G. drummondii* when not sloughed).

Another species producing small spores with a flexible innermost layer easily separating from their laminate layer is *G. proliferum* (Declerck *et al.* 2000). However, spores of *G. drummondii* are yellow-coloured, while those of *G. proliferum* are hyaline.

Apart from the biochemical properties of the flexible innermost spore wall layer discussed above, *G. drummondii*, *G. claroideum*, *G. luteum*, and *G. proliferum* also differ in the number and nature of the layers located above the structural, laminate wall layer of their spores. In contrast to the single, sloughing outer layer associated with the laminate wall layer of *G. drummondii* spores (Fig 4B–C), the laminate layer of *G. claroideum* and *G. luteum* is covered with two impermanent layers, of which the outer one stains pink to purplish red in Melzer's reagent (Błaszkowski 2003; Kennedy *et al.* 1999; Stürmer & Morton 1997; *versus* no reaction of layer 1 of *G. drummondii* in this reagent). The laminate layer of *G. proliferum* spores is overlaid with two permanent layers; both are non-reactive in Melzer's reagent (Declerck *et al.* 2000). *G. arenarium* and *G. etunicatum* diverge from *G. drummondii* mainly because of the lack of the flexible innermost layer of the last fungus.

The close relationship of *G. drummondii* with *G. claroideum*, *G. lamellosum*, and *G. luteum*, members of *Glomus* Group B *sensu* Schüßler *et al.* (2001), found based on comparison of their morphological and biochemical characters, was also

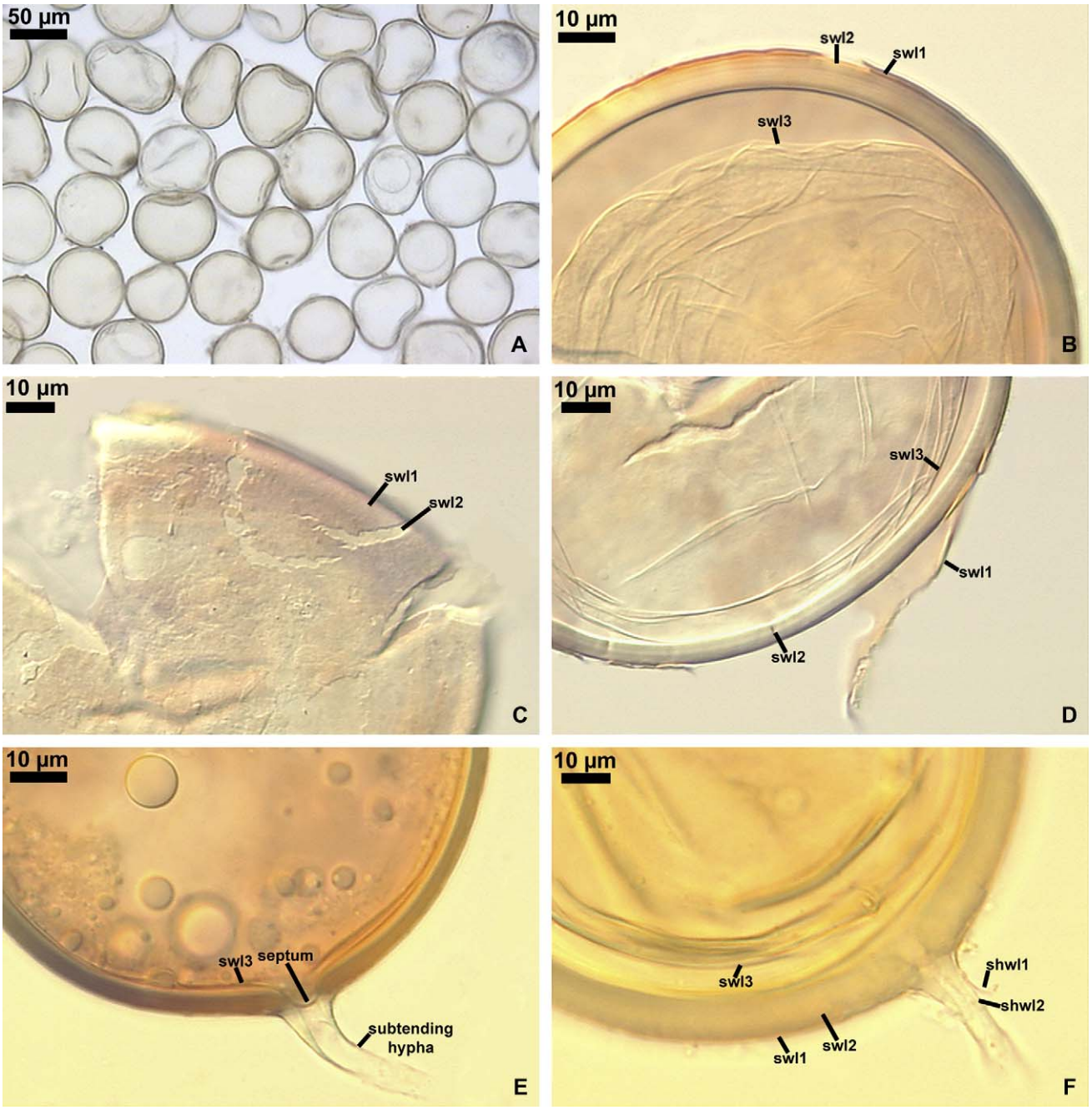


**Fig 6 – Spores of *Glomus walkeri*. A. Mature spores. B–D. Spore wall layers 1-3 (swl1-3). E. Curved septum of subtending hypha formed by spore wall layer 3 (swl3). F. Spore wall layers 1-3 (swl1-3) and subtending hyphal wall layers 1 (shwl1) and 2 (shwl2). A, intact spores in water. F, spore crushed in PVLG. B–E, spores crushed in PVLG + Melzer's reagent. A, bright field microscopy; B–F, differential interference microscopy.**

mirrored in the phylogenetic analyses performed (Figs 1 and 2). These placed *G. drummondii* in *Glomus* Group B and, thereby, in the vicinity of the three abovementioned *Glomus* spp.

**Glomus walkeri** Błaszk. & C. Renker, **sp. nov.**

Figs 6–7

*Etym*: in honour of Chris Walker for his contribution to knowledge of the nature and classification of arbuscular mycorrhizal fungi.

*Sporocarpia ignota. Sporae singulae in solo efformatae; albae vel sub-luteae; globosae vel subglobosae; (55–)81(–95) µm diam; aliquando ovoideae; 60–90 × 75–115 µm. Tunica sporae e stratis tribus (strati 1–3); strato '1' caduco, hyalino, 0.5–1 µm crasso; strato '2' laminato, glabro, albo vel sub-luteo, (1.5–)3.5(–5.5) µm crasso; strato '3' elastico, glabro, hyalino, (0.5–)1(–1.5) µm crasso. Hypha subtendens albo vel sub-luteo; recta vel recurva; cylindrica, raro coliga; (4–)6(–9.5) µm lata ad basim sporae; pariete albo vel sub-luteo; (1–)2(–3.5) µm crasso, stratis 1–3 sporae continuo. Porus e septo continuo strati 3 sporae efformata. Mycorrhizas vesicular–arbusculares formans.*

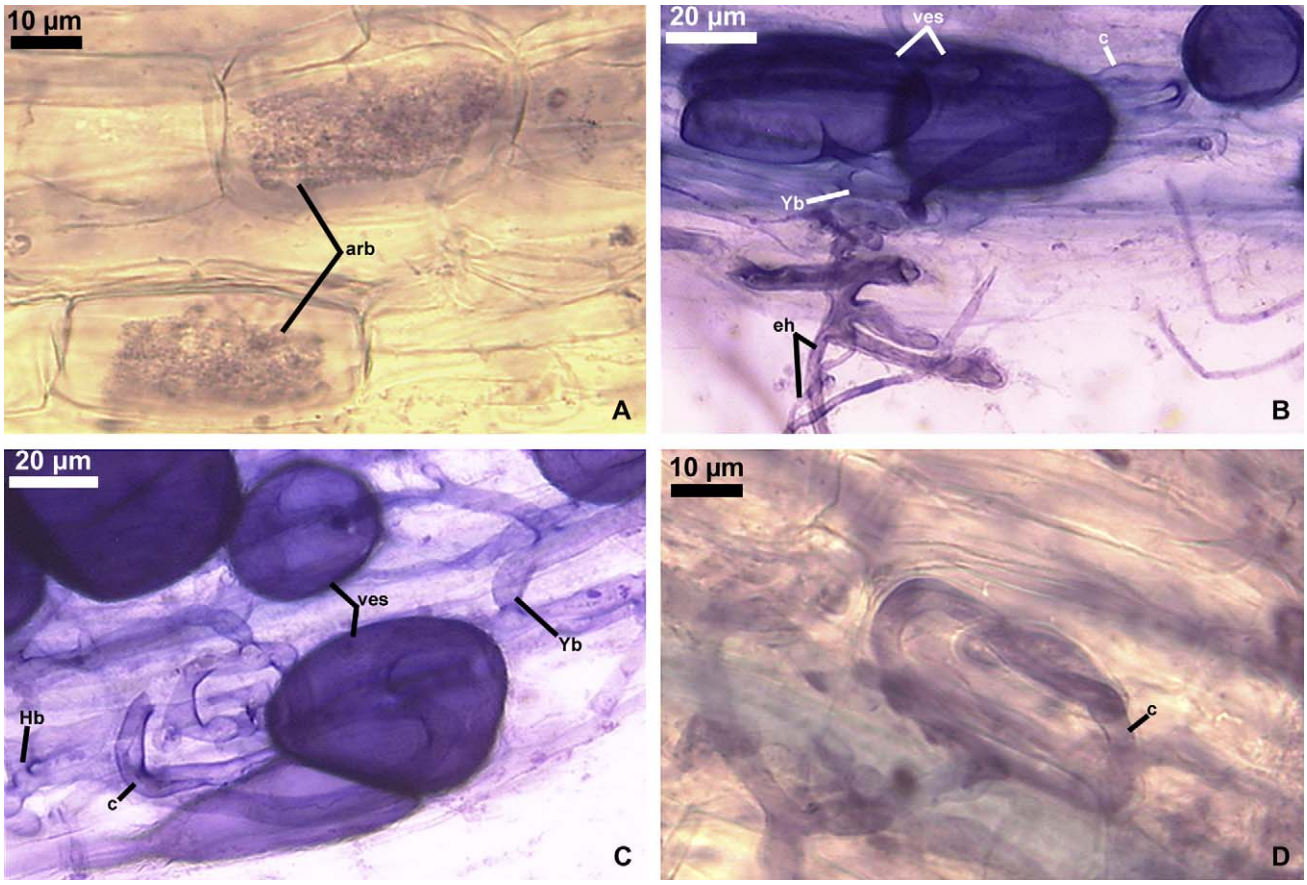


**Fig 7 – Mycorrhiza of *Glomus walkeri* in roots of *Plantago lanceolata* stained in 0.1 % trypan blue. (Mycorrhizae in PVLG.) A. Arbuscules (arb). B–C. Vesicles (ves), coils (c), Y-(Yb) and H-(Hb)shaped branches, and extraradical hyphae (e). D. Coil (c).**

*Typus*: **Poland**: Szczecin, infra *Plantago lanceolata*, 18 May 2005, *J. Błaszkowski*, *2609* (DPP - holotypes).

*Sporocarps* unknown. *Spores* occur singly in the soil (Fig 6A); origin blastically at the tip of extraradical hyphae of mycorrhizal roots. Spores white (4A1) to pale yellow (4A3); globose to subglobose; (55–)81(–95) µm diam; sometimes ovoid; 60–90 × 75–115 µm; with a single subtending hypha (Fig 6A, E, F). *Spore wall structure* (Fig 6B, D–F) consists of three layers (layers 1–3) in two groups (groups A and B). Outermost layer 1 in group A semi-permanent, hyaline, (0.5–)1.5(–1.5) µm thick (Fig 6B–D, F), deteriorating with age, but usually present in mature spores. Layer 2 of group A laminate, smooth, white (4A1) to pale yellow (4A3), (3.5–)5.5(–9.5) µm thick (Fig 6B, D–F). Layer 3 of group B flexible, smooth, hyaline, (0.5–)1(–1.5) µm thick (Fig 6, D–F), easily separating from layer 2 except for its small protrusion whose the outer surface is usually attached to the inner surface of subtending hyphal wall layer 2, close at the spore base to form a curved septum in the lumen of the subtending hypha (Fig 6E). Only layer 1 stains reddish white (8A2) to greyish rose (12B3) in Melzer's reagent (Fig 6 B–E). *Subtending hypha* white (4A1) to pale yellow (4A3); straight or recurvate; cylindrical or slightly flared, rarely funnel-shaped or constricted; (7.5–)9(–12.5) µm wide at the spore base (Fig 6E–F). *Wall of subtending hypha* white (4A1) to pale yellow (4A3); (1.5–)2(–2.5) µm thick at the spore base; continuous with spore wall layers 1 and 2 (Fig 6E–F); layer 1 extends up to 12 µm below the spore base. *Pore* occluded by a septum, (1.5–)3(–5) µm wide, continuous with spore wall layer 3, positioned up to 3.5 µm below the spore base (Fig 6E)

*Other collections examined*: **Israel**: near Tel-Aviv (32°4′, 34°46′E), around the roots of *Oenothera drummondii*, 15 June 2000, *J. Błaszkowski*, (DPP). **Spain**: *Majorca*: near Cape Salinas, under *A. arenaria*, 22 Aug. 2001, *J. Błaszkowski*, (DPP). **Italy**: near Calambrone (43°35′N, 10°18′E), from the rhizosphere of *A. arenaria*, 11 Oct. 2002, *J. Błaszkowski*, *unnumbered collection* (DPP). **Poland**: Szczecin, under pot-cultured *P. lanceolata*, 18 May 2005, *J. Błaszkowski*, *2597-2608* and *2610-2633* (DPP; and two slides at OSC).

*Distribution and habitat*: The first record of *Glomus walkeri* comes from a trap culture established from a root–rhizosphere mixture sampled under *O. drummondii* colonizing dunes of the Mediterranean Sea adjacent to Tel-Aviv in December 2000. One of each trap cultures harbouring spores of this new fungus represented the root zone of *A. arenaria* growing in the Mediterranean Sea dunes located near Cape Salinas, Majorca, Spain, in August 2001 and Calambrone, Italy, in October 2002.

The only species of arbuscular mycorrhizal fungi accompanying *G. walkeri* in Israeli cultures was *G. intraradices*, and the cultures containing root fragments and soils from Majorca and Calambrone also hosted *G. coronatum*.

*Mycorrhizal associations*: The presence of arbuscular mycorrhizae in root fragments taken from under the plant species sampled growing in the field was not determined.

In one-species cultures with the host plant *P. lanceolata*, mycorrhizae of *G. walkeri* consisted of arbuscules, vesicles, as well as intra- and extraradical hyphae (Fig 7). Arbuscules generally were not numerous and unevenly distributed along root fragments (Fig 7A). Vesicles occurred very abundantly and were evenly distributed along root fragments; they were mainly ellipsoid to elongate; 10–50 × 18–215 μm; rarely globose to subglobose; (30–)44(–65) μm diam (Fig 7B–C). Intraradical hyphae were (1–)4.5(–7.5) μm wide and grew parallel to the root axis. They were straight or slightly curved, sometimes formed Y- or H-shaped branches and coils (Fig 7B–D). The coils were 20–100 × 27–220 μm. Extraradical hyphae were (2–)3.5(–4.5) μm wide. Their abundance varied, depending on the root fragments examined. In 0.1 % trypan blue, arbuscules stained violet white (16A2) to greyish violet (16C6), vesicles pastel violet (16B4) to deep violet (16E8), intraradical hyphae violet white (16A2) to campanula violet (17C7), coils greyish violet (16B5–17C5), and extraradical hyphae pastel violet (16A4) to royal purple (16D8).

*Phylogenetic position*: Phylogenetic analysis of LSU placed *G. walkeri* sister to *G. drummondii*, a species described above, and separate from other known *Glomus* spp. sequences on a branch with high bootstrap support (Figs 1–2). In the ITS analysis, reference sequences of *G. claroideum*, *G. clarum*, *G. etunicatum*, and *G. luteum*, members of *Glomus* Group B, clustered together, while the sequence of the newly described *G. walkeri* grouped distant (Fig 3). ITS data again placed *G. walkeri* next to *G. drummondii* and two recently published sequences derived from roots of *T. baccata* (Wubet *et al.* 2003; Fig 3). Thus, phylogenetic analysis confirmed the distinctiveness of *G. walkeri* earlier determined based on observations of morphological and biochemical properties of components of its spores.

*Discussion*: *Glomus walkeri* is distinguished by its single, small and white to pale yellow spores with three-layered wall, of which the outermost one is a semi-permanent structure staining intensively in Melzer's reagent, and the innermost layer is smooth and easily separates from the structural laminate middle layer (Fig 6).

When observed under a dissecting microscope, spores of *G. walkeri* are similar in appearance to those of *D. spurca*, *G. diaphanum*, *G. eburneum*, *G. fasciculatum*, *G. gibbosum*, *G. intraradices*, *G. laccatum*, *P. franciscana*, and *Paraglomus occultum*. Another fungus forming spores resembling those of *G. walkeri* is *G. minutum*. However, most spores of *G. minutum* occur in aggregates associated with roots (*versus* only singly in the soil in *G. walkeri*) and only its largest spores attain the size of the smallest spores of *G. walkeri*. Additionally, spores of *G. minutum* are hyaline, whereas those of *G. walkeri* are white to pale yellow.

Examination under a compound microscope of spores crushed in PVLG and PVLG mixed with Melzer's reagent readily divides the species listed above into two distinct groups. Only *G. diaphanum*, *G. fasciculatum*, and *G. gibbosum* produce spores with an innermost flexible wall layer of identical properties to that of *G. walkeri* spores (Fig 6B, D–F; Błaszkowski 1988, 1997, 2003; Kennedy *et al.* 1999; Morton 2002; Morton & Redecker 2001; Morton & Walker 1984; Oehl & Sieverding 2004; Pfeiffer *et al.* 1996; Stürmer & Morton 1997; Walker 1982; Walker & Koske 1987). The differences between these species regard the number, as well as the phenotypic and biochemical properties of the other wall layers of their spores. First, although the innermost spore wall layer of *G. walkeri*, *G. diaphanum*, and *G. fasciculatum* is surrounded by only two layers, the outermost wall layer of *G. diaphanum* spores is mucilagenous and short-lived (Błaszkowski 2003; Morton 2002; Morton & Walker 1984; *versus* semi-permanent and usually present in mature spores of *G. walkeri*; Fig 6B–D), and the outermost spore wall layer of *G. fasciculatum* is persistent and does not deteriorate with age (Błaszkowski 2003; Walker & Koske 1987; *versus* it is more or less degraded in *G. walkeri* at maturity). Second, the middle laminate spore wall layer of *G. diaphanum* is consistently hyaline throughout the life cycle of the fungus (Morton & Walker 1984), whereas that of *G. walkeri* is white to pale yellow. Additionally, this layer in the former fungus is very brittle, which causes the spore to readily break apart or fragment (Błaszkowski, pers. observ.; Morton 2002), a phenomenon not observed in *G. walkeri*. Third, the outermost spore wall layer of *G. walkeri* stains markedly more intensively in Melzer's reagent (up to greyish rose) than that of *G. diaphanum* (light pink; Morton 2002) and *G. fasciculatum* (reddish white; Błaszkowski 2003). In *G. fasciculatum*, its laminate middle layer also reacts in Melzer's reagent (*versus* no reaction in *G. walkeri*). Fourth, spores of *G. walkeri* occur only singly in the soil (Fig 6A), whereas those of *G. fasciculatum* are frequently produced in sporocarps (Błaszkowski 2003; Walker & Koske 1987). Apart from single spores, *G. diaphanum* also produces spores in loose clusters (Morton & Walker 1984). Finally, *G. diaphanum* commonly forms abundant compact clusters of spores in the root cortex of its host plants (Morton & Walker 1984; *versus* no intraradical spores in the mycorrhizal roots of *G. walkeri* were found).

In spores of *G. gibbosum*, three layers lie over the innermost layer of their wall (Błaszkowski 1997, 2003; *versus* two layers in *G. walkeri*; Fig 6B–D, F). The laminate layer, similar to that of *G. walkeri*, is coated with a semi-flexible, permanent layer (*versus* semi-permanent in *G. walkeri*) associated with an evanescent outermost layer (*versus* no such layer in *G. walkeri*); both the layers easily separate from the laminate layer and balloon in lactic acid-based mountants (*versus* no ballooning in *G. walkeri*). Other differences between *G. walkeri* and *G. gibbosum* are the lack of reactivity of any spore wall layer of the latter fungus in Melzer's reagent (*versus* layer 1 of *G. walkeri* stains in this reagent; Fig 6B–D) and the occasional formation of its spores in sporocarps enclosed by a common hyphal mantle (Błaszkowski 1997, 2003; *versus* only single spores formed in the soil by *G. walkeri*).

Although *G. walkeri* is morphologically similar to *G. fasciculatum*, a member of *Glomus* group A *sensu* Schüßler *et al.* (2001), phylogenetic analyses clearly placed it in *Glomus* group B. In this group, species forming spores with a flexible innermost wall layer resembling that of *G. walkeri* are *G. claroideum*, *G. lamellosum*, and *G. luteum*. However, all these fungi produce larger and darker-coloured spores that are never white at maturity as *G. walkeri*. Additionally, the spore wall structure of *G. walkeri* and *G. lamellosum* is three-layered, whereas that of the other species consists of four layers. The spore wall layers of *G. walkeri* and *G. lamellosum* are phenotypically similar. However, compared with *G. lamellosum*, the outermost spore wall layer of *G. walkeri* is much thinner (0.5–1.5 μm *versus* 2–14 μm thick) and stains in Melzer's reagent (*versus* no reaction in *G. lamellosum*), and the innermost one is non-reactive in this

reagent (*versus* it occasionally stains; Błaszkowski 2003; Morton 2002).

Interestingly, phylogenetic analyses placed *G. diaphanum*, a species producing spores highly reminiscent of those of *G. walkeri*, in *Glomus* group A. Depending on the mode of phylogenetic analyses, *G. diaphanum* was either closer to the subgroup 'a' of *Glomus* group A, comprising *G. mosseae*, *G. caledonium*, *G. fragilistratum*, *G. geosporum*, *G. constrictum* and *G. xanthium* (Fig 2), or fell into the subgroup 'b' of *Glomus* group A *sensu* Schwarzott *et al.* (2001), which is represented by sequence data from the *G. intraradices*/*G. clarum* clade (Fig 1).

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