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Glomus aurantium and *G. xanthium*, new species in Glomeromycota

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Abstract–Two new ectocarpic arbuscular mycorrhizal fungal species, *Glomus aurantium* and *G. xanthium* (Glomeromycota), were discovered in the Mediterranean Sea dunes located in Israel, Greece, Italy, and Majorca, Spain. Mature spores of *G. aurantium* are deep orange, globose to subglobose, (70-)98(-120) μm diam, or ovoid, 80-120 x 110-150 μm. Their wall consists of a permanent, flexible to semiflexible, hyaline outermost layer, easily separating from a laminate, deep orange middle layer, and a flexible, hyaline innermost layer. Spores of *G. xanthium* usually are tightly adherent to roots and frequently occur within roots. They are light yellow to ochre, globose to subglobose, (23-)50(-70) μm diam or ovoid, 20-55 x 45-100 μm, and have a spore wall with a rigid, semi-permanent, hyaline to light yellow outermost layer adherent to a rigid, permanent, hyaline middle layer, and a laminate, light yellow to yellow ochre innermost layer. Both *G. aurantium* and *G. xanthium* formed vesicular-arbuscular mycorrhizae in one-species cultures with *Zea mays*. Analysis of ITS and LSU nuclear rDNA in spores placed *G. aurantium* sequences in the *G. versiforme* group, while *G. xanthium* sequences were aligned with those in *Glomus* Group A.

Key words–molecular phylogeny, nuclear ribosomal DNA, vesicular-arbuscular fungi

Introduction

The plant associations hosting exceptionally abundant and diverse populations of arbuscular mycorrhizal fungi (Glomeromycota) are those colonizing maritime sand dunes (Błaszkowski 1993; Koske 1987; Tadych & Błaszkowski 2000). Many of the described species of these fungi have been originally isolated from dunes (Błaszkowski 2003). Examination of pot trap cultures with rhizosphere soils of plants of coastal areas

of the Mediterranean Sea revealed two undescribed species of arbuscular fungi that probably prefer warm sites. They have not been found in ca. 3000 soil samples coming from different dune and non-dune soils of northern Europe (Błaszkowski 2003). These fungi are described here as *Glomus aurantium* and *G. xanthium* spp. nov. based on morphology, ontogenetic patterns of spore differentiation, and molecular properties of spores.

Materials and Methods

Establishment of trap cultures and one-species cultures. Collection of soil samples, establishment of trap and single-species pot cultures, as well as growth conditions generally were as those described previously (Błaszkowski & Tadych 1997). Briefly, rhizosphere soils and roots of sampled plants were collected from a depth of 5-30 cm using a small garden shovel. In the laboratory, about 100-g subsamples were taken from each sample to isolate spores of arbuscular mycorrhizal fungal species sporulating in the field. The remaining part of the sample was either air dried for 2 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 of different developmental stages 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 and 26 mg L^{-1} P and K, respectively; Błaszkowski 1995). The mixtures were placed in 9x12.5-cm plastic pots (500 cm^3) and densely seeded with *Plantago lanceolata* L. Plants were grown in a greenhouse at 15-30°C with supplemental 8-16-h lighting provided by one SON-T AGRO sodium lamp (Philips Lighting Poland S. A.) placed 1 m above pots. The maximum light intensity was 180 μE $m^{-2}s^{-1}$ at pot level. Plants were watered 2-3 times a week. No fertilizer was applied during the growing period. Trap cultures were harvested at approximately 1-month intervals, beginning three months and ending five to seven months after plant emergence. 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% HCl instead of 1%, and trypan blue concentration 0.1% instead of 0.05% (Koske, pers. comm.).

Single-species pot cultures were established from about 50 to 100 newly formed spores stored before inoculation 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 day 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 chemical properties listed above. Subsequently, the spores were covered with another layer of roots attached to 4-6 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 on the surface of the growing substrate and covered with a thin layer of autoclaved sand. The cultures were harvested after 4-8 months

and spores were extracted. The effectiveness of this method in establishing one-species cultures usually exceeded 90% (Błaszkowski et al. 2002).

Microscopical survey. Morphological properties of spores and their subcellular structures were determined based on at least 100 spores mounted in polyvinyl alcohol/lactic acid/glycerol (PVLG; Koske & Tessier 1983) 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 coverslip and then stored at 65°C for 24 h to clear their contents of 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 color video camera coupled to the microscope.

Terminology of spore structure is that suggested by Spain et al. (1989), Stürmer & Morton (1997), and Walker (1983). Spore color was examined under a dissecting microscope on fresh specimens immersed in water. Color names are from Kornerup & Wanscher (1983). Nomenclature of fungi and plants is that of Walker & Trappe (1993) and Mirek et al. (1995), respectively. The authors of the fungal names are as those presented at the URL web page http://www.indexfungorum.org/AuthorsOfFungalNames.htm. Specimens were mounted in PVLG on slides and deposited in the Department of Plant Pathology (DPP), University of Agriculture, Szczecin, Poland, and in the herbarium at Oregon State University (OSC) in Corvallis, Oregon, USA.

Color microphotographs of spores and mycorrhizae of *G. aurantium* and *G. xanthium* can be viewed at the URL http://www.agro.ar.szcecin.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, pipetted with 8 µl of the PCR-Mix and used directly for PCR.

Amplification of the analysed ITS and LSU nrDNA region by PCR was performed on a Hybaid Ltd. 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 10x Taq polymerase reaction buffer (Promega), 4 µl 25 mM $MgCl_2$, 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 (C TT 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 done using a LI-COR

DNA Sequencer Long Reader 4200 and the Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Little Chalfont, UK).

DNA sequences of the complete ITS and parts of the ribosomal small and large subunit were submitted to the EMBL database under the accession numbers given in Figs. 17 and 18 for *G. aurantium* and *G. xanthium*. Reference sequences to analyze the systematic position of the new sequences were taken from GenBank. In a first step, only the 5.8S subunit genes embedded between the ITS1 and ITS2 regions were aligned by hand to allow an analysis of the systematic position of *G. aurantium* and *G. xanthium* based on a large sequence data set. In a second step, the full-length ITS sequence of *G. xanthium* was aligned with other *Glomus* Group A *sensu* Schüßler et al. (2001) sequences to take a closer look at its systematic position. Furthermore a small sequence data set of available LSU sequences was aligned to ensure the phylogenetic position of the new species. An alignment of the full-length sequences of *G. aurantium* and *G. xanthium* was not possible due to a lack of comparable sequence data from other AMF species, but also due to the high variation within the ITS1 and ITS2 region when comparing sequences from different groups within the Glomeromycota.

Maximum parsimony analyses were performed with PAUP* 4.0b10 (Swofford 2003) 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. The confidence of branching was assessed using 1000 bootstrap resamplings.

The data set used to reconstruct the 5.8S phylogenetic tree (Fig. 17) contained 158 characters of which 83 were constant, 18 parsimony uninformative and 57 parsimony informative. The data set used to reconstruct the LSU tree (Fig. 18) contained 357 characters of which 195 were constant, 55 parsimony uninformative and 107 parsimony informative. The full length ITS tree containing *G. xanthium* (Fig. 35) has 572 characters of which 371 were constant, 74 parsimony uninformative and 127 parsimony informative.

Descriptions of the species

Glomus aurantium J. Błaszk., V. Blanke, C. Renker & F. Buscot, sp. nov.

Figs. 1-16

Sporocarpia ignota. Sporae singulae in solo efformatae; pallide luteae vel aurantiae vel ochraceae; globosae vel subglobosae; (70-)98(-120) µm diam; aliquando ovoideae; 80-120 x 110-150 µm. Tunica sporae e stratis tribus (strati 1-3); strato "1" elastico vel semielastico, glabro, hyalino, (0.7-)1.0(-1.5)µm crasso; strato "2" laminato, glabro, pallide luteo, intense aurantio vel aureo, (2.7-)5.8(-8.8) µm crasso; strato "3" elastico, glabro, hyalino, (0.5-)0.7(-1.0) µm crasso. Hypha subtendens pallide luteo, intense aurantio vel aureo; recta vel recurva; cylindrica, raro coliga; (0.5-)0.7(-1.0) µm lata ad basim sporae; pariete pallide luteo, intense aurantio vel aureo; (0.7-)2.0(-4.7) µm crasso, stratis 1-3 sporae continuo. Porus e septo continuo strati 3 sporae efformata. Mycorrhizas vesicular-arbusculares formans.

HOLOTYPUS. POLAND. Szczecin, infra *P. lanceolata*, 10 Jan. 1998, *Błaszkowski, J.*, *2444* (DPP).

Sporocarps unknown. *Spores* occur singly in the soil (Fig. 1); origin blastically at the tip of extraradical hyphae of mycorrhizal roots. Spores yellowish white (4A2) when young, deep orange (5A7-8) at maturity, to golden yellow (5B8) when older; globose to subglobose; (70-)98(-120) μm diam; sometimes ovoid; 80-120 x 110-150 μm; with a single subtending hypha (Figs. 1, 11, 12). *Subcellular structure of spores* (Figs. 2-12) consists of one wall including three layers (layers 1-3). Outermost layer 1 permanent, flexible to semiflexible, smooth, hyaline, (0.7-)1.0(-1.5) μm thick (Figs. 2-5, 7-12), sometimes ballooning, and then extending up to 30 μm from layer 1 in spores mounted in PVLG (Fig. 3). This layer frequently accumulates a granular material, up to 1.5-7.0 μm thick, composed of soil debris (Figs. 4, 5, 7, 10). Layer 2 laminate, smooth, yellowish white (4A2) to golden yellow (5B8), (2.7-)5.8(-8.8) μm thick (Figs. 2-12). Layer 3 flexible, smooth, hyaline, (0.5-)0.7(-1.3) μm thick (Figs. 2-12), usually tightly adherent to layer 2 and almost always inseparably attached to the inner surface of subtending hyphal wall layer 3, close at the spore base to form a curved septum in the lumen of the subtending hypha (Fig. 11). None of the spore wall layers reacts in Melzer's reagent. *Subtending hypha* yellowish white (4A2) to golden yellow (5B8), straight or recurvate; cylindrical or slightly flared, rarely constricted; (3.2-)7.1(-11.8) μm wide at the spore base (Figs. 11, 12). *Wall of subtending hypha* yellowish white (4A2) to golden yellow (5B8); (0.7-)2.0(-4.7) μm thick at the spore base; continuous with spore wall layers 1-3 (Fig. 11); layers 1 and 3 extend up to 10.0 and 2.0-3.4 μm, respectively, below the spore base. *Pore* occluded by a septum, (2.0-)2.9(-4.2) μm wide, continuous with spore wall layer 3 (Fig. 11) and occasionally also by a septum formed by a few innermost laminae of spore wall layer 2, positioned 3.0-6.5 μm below the spore base (Fig. 12).

Collections examined. HOLOTYPE. POLAND. Szczecin, from under pot-cultured *P. lanceolata*, 6 Jul. 2003, *Błaszkowski*, *J*., *2444* (DPP); isotypes: *Błaszkowski*, *J*., *2445-2459* (DPP) and two slides at OSC.

Other material examined. ISRAEL. Near Tel-Aviv (32º4'N, 34º46'E), from the root zone of *Cenothera drummondi* Hook, 16 Dec. 1997 and 15 June 2000 and *Ammophila arenaria* (L.) Link, 15 June 2000, *Błaszkowski*, *J., unnumbered collection* (DPP). SPAIN. Near Cape Salinas (36º19'N, 3º2'E) and Sóller (39º46'N, 2º40'E), Majorca, from around roots of *A. arenaria*, 15 Aug. 2001, *Błaszkowski*, *J*., unnumbered collection (DPP). ITALY. Near Calambrone (43º35'N, 10º18'E), from under *A. arenaria*, 11 Oct. 2002, *Błaszkowski*, *J*., unnumbered collection (DPP).

Etymology. *Aurantium*, referring to the orange-golden-colored spores.

Distribution and habitat. *Glomus aurantium* was discovered in a trap culture with a rhizosphere soil of *C. drummondi* colonizing dunes of the Mediterranean Sea adjacent to Tel-Aviv in December 1997. This fungus also sporulated in 60 other trap cultures with dune rhizosphere soils of *A. arenaria* and *C. drummondi* sampled near Tel-Aviv in 1997 (9 samples) and 2000 (51). Later, spores of *G. aurantium* were isolated from eight trap cultures established from soils collected under *A. arenaria* growing

near Cape Salinas and Sóller, Majorca, Spain, and seven trap cultures containing rhizosphere soil and root mixtures taken from under *A. arenaria* growing in dunes adjacent to Calambrone, Italy.

The arbuscular fungi accompanying *G. aurantium* in the field were *G. constrictum* Trappe and *G. coronatum* Giovann. The fungi co-occurring with *G. aurantium* in trap cultures with Israeli soils were *Acaulospora paulinae* Błaszk., *Archaeospora trappei* (R.N. Ames & Linderman) J.B. Morton & D. Redecker, *G. arenarium* Błaszk. et al., *G. constrictum*, *G. coronatum*, *G. corymbiforme* Błaszk., *G. dominikii* Błaszk., *G. gibbosum* Błaszk., *G. mosseae* (Nicol. & Gerd.) Gerd. & Trappe, *G. xanthium* Błaszk. et al., an undescribed *Glomus* sp., *S. calospora* (Nicol. & Gerd.) C. Walker & F.E. Sanders, and *S. persica* (Koske & C. Walker) C. Walker & F.E. Sanders. Apart from *G. aurantium*, the Majorca's cultures contained *G. constrictum*, *G. coronatum*, *G. dominikii*, *G. macrocarpum* Tul. & C. Tul., two undescribed *Glomus* spp., *S. calospora*, and *S. persica*. The cultures representing Italy still hosted *Ac. scrobiculata* Trappe, *Entrophospora schenckii* Sieverd. & S. Toro, *G. constrictum*, *G. dominikii*, two undescribed *Glomus* spp., *S. fulgida* Koske & C. Walker, and *S. persica*.

Mycorrhizal associations. *Glomus aurantium* was associated in the field with vesicular-arbuscular mycorrhizae of *A. arenaria* in Israel, Majorca, Spain, and Italy, as well as with those of *C. drummondi* growing in Israel.

In one-species cultures with *Z. mays* as the host plant, mycorrhizae of *G. aurantium* consisted of arbuscules, vesicles, as well as intra- and extraradical hyphae (Figs. 13-16). Arbuscules generally were numerous and evenly distributed along root fragments (Fig. 13). Vesicles occurred very abundantly and were globose to subglobose; (18-)32(-45) μm diam; sometimes ellipsoid; 20-40 x 55-110 μm (Fig. 14). Intraradical hyphae were (1.0-)4.8(-7.4) μm wide and grew parallel to the root axis (Fig. 15). They were straight or slightly curved, sometimes formed Y- or H-shaped branches and coils (Fig. 16). The coils were 12.5-50.0 x 22.5-140.0 μm. Extraradical hyphae were (2.5-)4.0(-4.7) μm wide. Their abundance varied, depending on the root fragments examined. In 0.1% trypan blue, arbuscules stained violet white (16A2) to lilac (16B4), vesicles violet white (19A2) to deep blue (19E6), intraradical hyphae violet white (16A2) to

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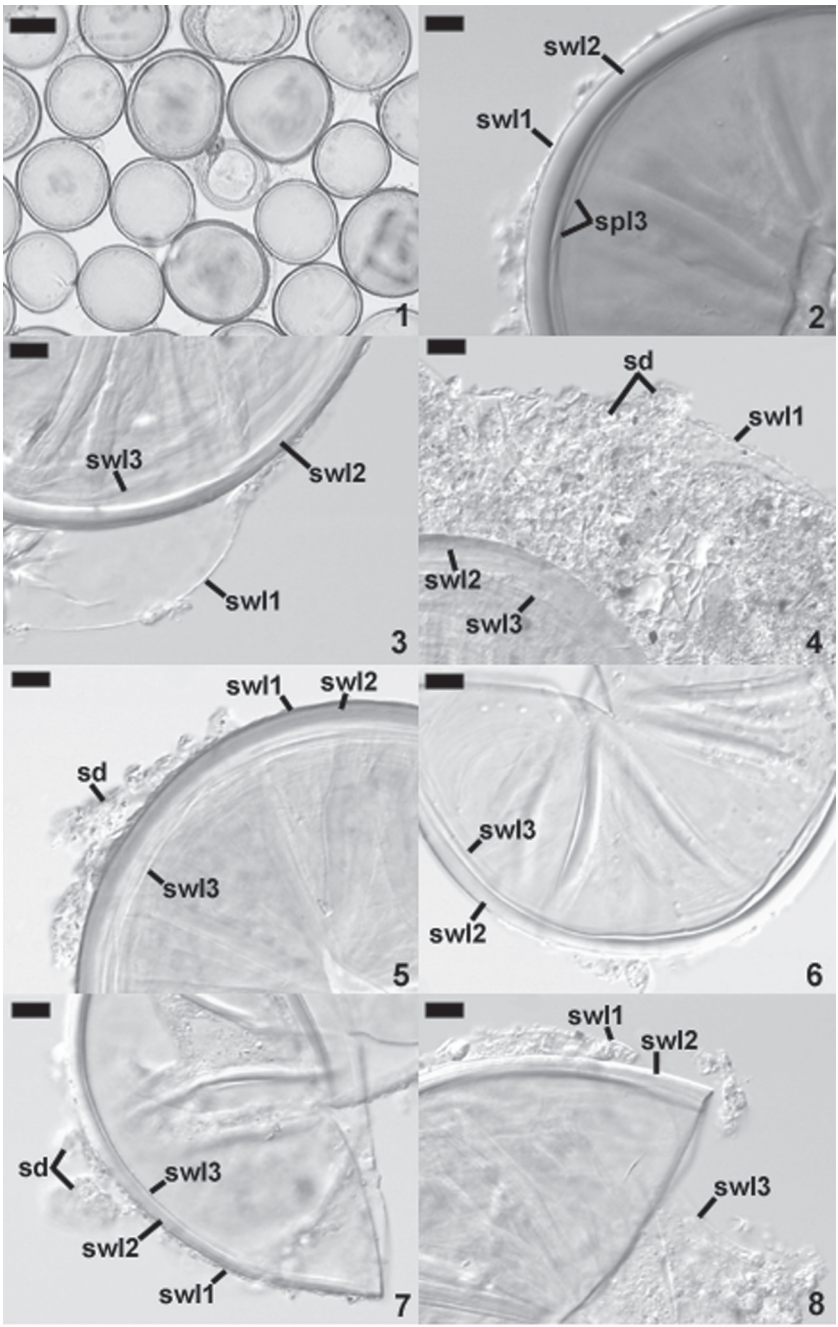
Figs. 1-16. Spores of *Glomus aurantium* and its mycorrhizae in roots of *Zea mays* stained in 0.1% trypan blue. **1.** Mature spores. 2. Adherent spore wall layers 1 (swl1) and 2 (swl2) and spore wall layer 3 (swl3) slightly separated from spore wall layer 2. **3.** Ballooned spore wall layer 1 (swl1) locally separated from spore wall layer 2 (swl2) and spore wall layer 3 (swl3). **4-10.** Spore wall layers (swl) 1-3 and soil debris (sd) accumulated by spore wall layer 1. **11.** Subtending hyphal wall layers 1 (shwl1) and 2 (shwl2) continuous with spore wall layers 1 (swl1) and 2 (swl2); curved septum formed by invaginated spore wall layer 3 (swl3) is visible. **12.** Spore wall layers (swl) 1-3 and subtending hypha with septum (s) continuous with the innermost laminae of spore wall layer 2. **13.** Arbuscules (arb) and arbuscule trunk (tr) developed from intraradical hypha (hyp). **14.** Vesicles (ves). **15.** Straight (shyp), H-(Hb), and Y-(Yb) branched intraradical hyphae. **16.** Coils. Fig. 1., intact spores in water. Fig. 2, spore crushed in PVLG. Figs. 3-12, spores crushed in PVLG+Melzer's reagent. Figs. 13-16, mycorrhizae in PVLG. Figs. 1 and 13-16, bright field microscopy; Figs. 2-12, differential interference contrast. Bars: Fig. 1=50 μm, Figs. 2-16=10 μm.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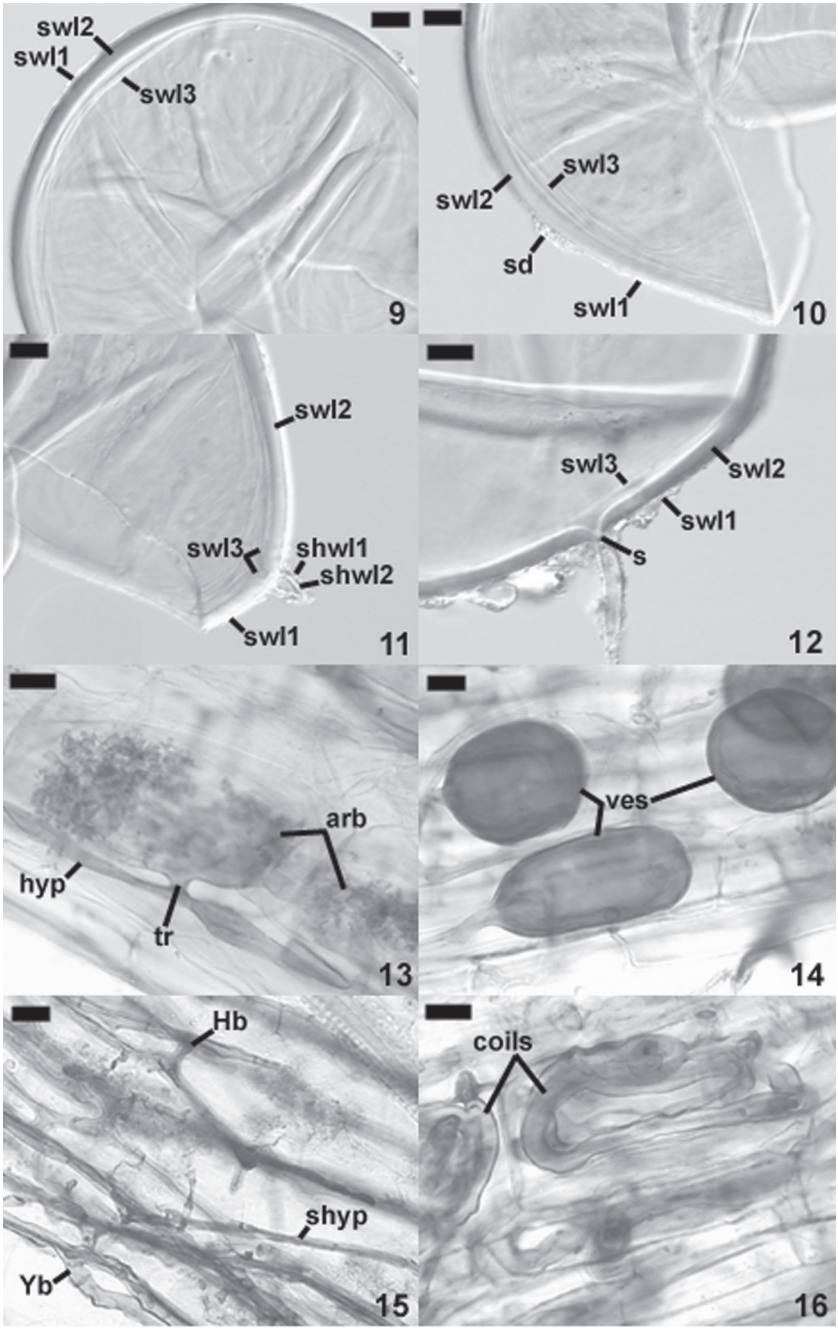
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reddish violet (16C8), coils pale violet (16A3) to reddish violet (16B6), and extraradical hyphae light lilac (16A5) to royal purple (16D8).

Phylogenetic position. The analysis of the 5.8S gene within the ITS region revealed a close relationship between *G. aurantium* and *G. versiforme* (P. Karsten) S.M. Berch (Fig. 17). In the LSU tree, *G. aurantium* is the terminal taxon of a not supported lineage placed sister to the *Glomus* Group A (Fig. 18). Comparing the full length ITS sequences of *G. aurantium* with sequences assigned to *G. versiforme* available in GenBank, the molecular similarity of the two fungi ranged from 89 to 92% (Table 1).

Table 1. Comparison of the *Glomus aurantium* ITS nrDNA sequence with GenBank sequences

Sequences from GenBank	**Identity** [%]	**# bp aligned** (with gaps)	**# variable positions**	**# of gaps**
Glomus versiforme AF246141	91	548	41	11
Glomus versiforme AF246142	89	550	49	11
Glomus versiforme AF246143	90	550	41	12
Glomus versiforme AJ504642	90	559	34	22
Glomus versiforme AJ504643	92	555	24	21
Glomus versiforme AJ504644	91	547	36	11
Uncultured *Glomus* AY236283	85	561	57	29

Column 2 - the identity of the *Glomus aurantium* ITS sequence (between the priming sites of the ITS1/ITS4 primer) with the sequence mentioned in column 1 (calculated as the value in column 3 minus the values in columns 4 and 5 divided by the value in column 3); column 3 - the number of base pairs of the paired sequence alignments between our sequences and the corresponding sequence of column 1; column 4 - the number of variable positions; column 5 - the number of gaps in these alignments

Discussion. *Glomus aurantium* most distinguishes its orange-colored spores and their wall structure. In the 3-layered spore wall, the structures most distinctive are the flexible to semi-flexible permanent outermost layer 1, which sometimes balloons in lactic acid-based mountants, and the flexible innermost layer 3. None of the three layers stains in Melzer's reagent.

When viewed under a dissecting microscope, spores of *G. aurantium* most resemble those of *G. pustulatum* Koske et al. and *G. versiforme* because of their orange pigmentation and a similar size range (Błaszkowski 2003; Koske et al. 1986; Morton 2000). Younger, yellow-colored spores of *G. aurantium* are also reminiscent of those of *G. claroideum* N.C. Schenck & S.M. Sm., *G. fasciculatum* (Thaxt.) Gerd. & Trappe emend. C. Walker & Koske, *G. lamellosum* Dalpé et al., and *G. luteum* L.J. Kenn. et al.

Examination of spores crushed in PVLG and PVLG mixed with Melzer's reagent under a compound microscope readily separates *G. aurantium* from the six species listed above. Although both *G. aurantium* and *G. pustulatum* produce spores with a wall consisting of three permanent layers, the outermost layer of the former species is smooth or covered with granular debris and that of *G. pustulatum* is ornamented with blistery, cup- or irregularly-shaped processes (Błaszkowski 2003; Koske et al. 1986).

The character most distinguishing *G. versiforme* from *G. aurantium* is the lack of the innermost flexible spore wall layer of the latter species (Błaszkowski 2003; Morton 2000). Additionally, the outermost spore wall layer in *G. aurantium* is permanent, and that of *G. versiforme* degrades with age.

The spore wall of *G. fasciculatum* and *G. lamellosum* also consists of three layers (Błaszkowski 2003; Błaszkowski et al. 2002; Dalpé et al. 1992; Walker & Koske 1987). Moreover, in *G. fasciculatum*, these layers are persistent and have similar phenotypic properties as those of *G. aurantium*. However, two outer spore wall layers of the former species stain red in Melzer's reagent, whereas all three wall layers of *G. aurantium* spores remain non-reactive in this reagent. Additionally, the outermost spore wall layer of *G. fasciculatum* does not accumulate debris as that of *G. aurantium* and spores of the former fungus frequently occur in aggregates (vs. only single spores in *G. aurantium*).

In contrast to the outermost persistent layer and the innermost in-amyloid layer of spore wall of *G. aurantium*, the outermost wall layer of spores of *G. lamellosum* degrades with age and their innermost layer stains pinkish in Melzer's reagent (Błaszkowski 2003; Błaszkowski et al. 2002; Dalpé et al. 1992; Morton 2000).

Glomus aurantium differs from *G. claroideum* and *G. luteum* in the number of spore wall layers, as well as in their phenotypic and biochemical properties. Spores of *G. claroideum* and *G. luteum* have a penultimate laminate wall layer and an innermost flexible wall layer of spores of *G. aurantium* (Błaszkowski et al. 2003; Kennedy et al. 1999; Schenck & Smith 1982; Stürmer & Morton 1997; Walker & Vestberg 1998). However, the laminate layer in *G. aurantium* is surrounded with only one permanent layer, whereas that of the former two species is covered with two layers, of which each degrades and sloughs with age. Finally, the outermost spore wall layer of *G. claroideum* and *G. luteum* stains pink to purplish red in Melzer's reagent, but remains non-reactive in *Gl. aurantium*.

The accumulation of soil debris on the surface of mature spores of *G. aurantium* is a property that also distinguishes spores of *G. viscosum* Nicol., another species producing spores of a similar structure of their wall and a size range (Morton 2000; Walker et al. 1995). However, spores of *G. viscosum* are much lighter-colored (subhyaline to pale yellow vs. yellowish white to golden yellow in *G. aurantium*) and frequently occur in aggregates (vs. only singly in *G. aurantium*).

The molecular analysis of the 5.8S gene confirmed a close relationship of *G. aurantium* with *G. versiforme* (Fig. 17, Table 1). Based on sequence data from the ribosomal small subunit (SSU), *G. etunicatum* W.N. Becker & Gerd. (isolate W3239/

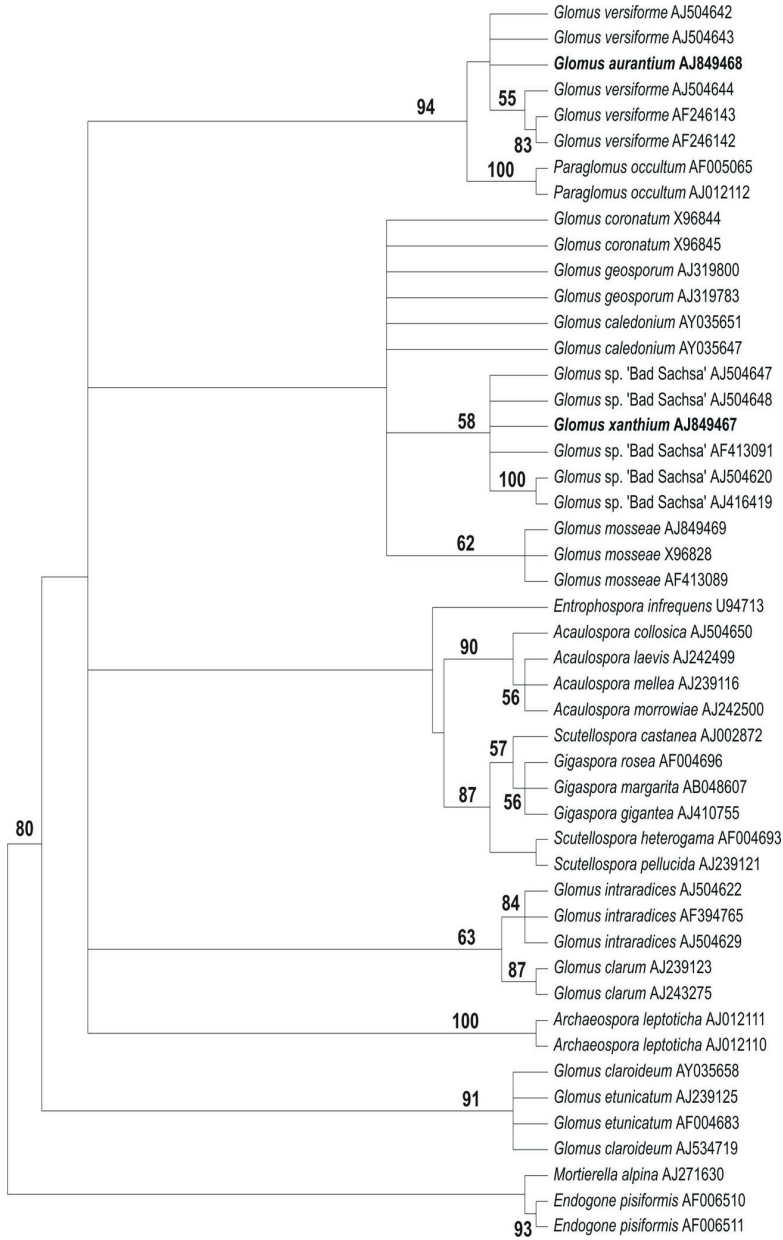

Fig. 17. Strict Consensus Tree of the Glomeromycota based on 49 5.8S sequences of the nrDNA. Phylogenetic positions of the newly described *Glomus aurantium* and *G. xanthium* are given in bold. Numbers above branches give bootstrap support derived from 1000 replicates. Sequences of *Mortierella alpine* and *Endogone pisiformis* were used as outgroups.

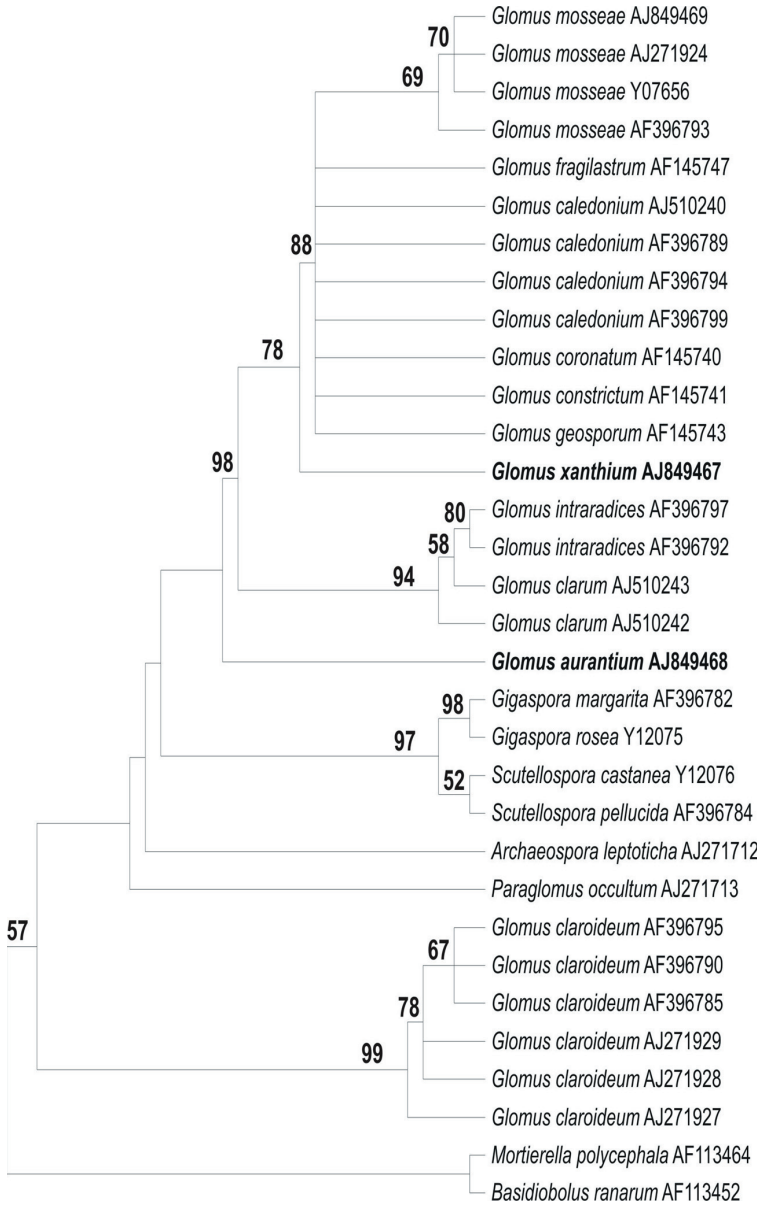

Fig. 18. Strict Consensus Tree of the Glomeromycota based on 34 partial 28S sequences (large subunit) of the nrDNA. Phylogenetic positions of the newly described *Glomus aurantium* and *G. xanthium* are in bold. Numbers above branches give bootstrap support derived from 1000 replicates. *Mortierella polycephala* and *Basidiobolus ranarum* sequences were used as outgroups.

Att382-16) and *G. spurcum* C.M. Pfeiff., C. Walker & Bloss emend. L.J. Kenn. et al. also clustered phylogenetically next to *G. versiforme* (Schüßler et al. 2001). However, Schüßler et al. (2001) showed some SSU sequences of *G. etunicatum* (isolate UT 316) that rather clustered in *Glomus* Group B, which was also the case for ITS sequences of *G. etunicatum* from GenBank used in our study (Fig. 17). Unfortunately, LSU sequence data of *G. versiforme*, *G. etunicatum*, and *G. spurcum* to further assess and confirm the phylogenetic position of *G. aurantium* are missing and therefore *G. aurantium* formed a single lineage in the LSU phylogenetic tree presented here (Fig. 18). Finally, comparing morphological properties of the four fungi listed above, only *G. aurantium* produces spores having an innermost flexible layer in their wall structure (Błaszkowski 2003; Morton 2000).

Glomus xanthium J. Błaszk., V. Blanke, C. Renker & F. Buscot, sp. nov.
Figs. 19-34

Sporocarpia ignota. Sporae singulae in solo efformatae; pallide luteae vel ochraceae; globosae vel subglobosae; (23-)50(-70) µm diam; aliquando ovoideae; 20-55 x 45-100 µm. Tunica sporae e stratis tribus (strati 1-3); strato "1" rigido, glabro, hyalino vel pallide luteo, (1.2-)1.8(-2.7) µm crasso; strato "2" semielastico, hyalino, (0.2-)0.8(-1.2) µm crasso; strato "3" laminato, glabro, pallide luteo vel ochraceo, (0.7-)1.6(-2.2) µm crasso. Hypha pallide lutea vel ochracea; recta vel recurva; cylindrica vel infundibuliforma, raro coliga; (2.7-)5.6(-9.1) µm lata ad basim sporae; pariete pallide luteo vel ochraceo; (0.5-)0.9(-1.5) µm crasso, stratis 1-3 sporae continuo. Porus e septo continuo strati 3 sporae efformata. Arbuscular mycorrhizae formans.

HOLOTYPUS. POLAND. Szczecin, infra_*Plantago lanceolata* L., 10 Jan. 1998, *Błaszkowski, J., 2202* (DPP).

Sporocarps unknown. Spores occur singly in the soil (Fig. 19) and usually are closely adherent to roots (Fig. 20), as well as frequently form within roots (Fig. 21); origin blastically at the tip of extraradical hyphae of mycorrhizal roots. *Spores* light yellow (4A4) to yellow ochre (5C7); globose to subglobose; (23-)50(-70) µm diam; sometimes ovoid; 20-55 x 45-100 µm; with a single subtending hypha (Figs. 19-24, 30). *Subcellular structure of spores* (Figs. 22-29) of one wall with three layers (layers 1-3). Outermost layer 1 rigid, smooth, hyaline, ca. 0.5 µm thick, continuous with a one-layered subtending hypha of the most juvenile spores, then darkening to light yellow (4A4) and thickening to (1.2-)1.8(-2.7) µm (Figs. 22-29). Layer 2 rigid, smooth, hyaline, (0.2-)0.8(-1.2) µm thick (Figs. 22-29), closely adherent to layer 1. In mature spores, layer 1 sometimes more or less deteriorates with age (Figs. 28 and 29), whereas layer 2 always remains intact. Layer 3 laminate, smooth, light yellow (4A4) to yellow ochre (5C7), (0.7-)1.6(-2.2) µm thick (Figs. 22-29). In crushed spores, layers 1 and 2 usually are adherent (Figs. 22-26, 28 and 29) or sometimes separate from each other (Fig. 27). However, the two layers easily separate from layer 3 (Figs. 22-29). None of the spore wall layers reacts in Melzer's reagent. The wall of youngest spores consists of layer 1 only. Then, layer 2 origins, and layer 3 begins to form after a full differentiation of layer 2. *Subtending_hypha* light yellow (4A3) to yellow ochre (5C7); straight or recurvate; cylindrical or flared (Figs. 24, 30), rarely constricted; (2.7-)5.6(-9.1) µm wide at the spore base. *Wall of subtending hypha* light yellow (4A4) to yellow ochre

(5C7); (0.5-)0.9(-1.5) μm thick at the spore base; continuous with spore wall layers 1—3 (Fig. 30); spore wall layers 1 and 2 extend up to 70 μm below the spore base. *Pore* occluded by a septum, 1.2-2.7 μm wide, continuous with the innermost lamina of spore wall layer 3 (Fig. 30).

Collections examined. HOLOTYPE. POLAND. Szczecin, under pot-cultured *P. lanceolata*, 10 Jan. 1998, *Błaszkowski, J., 2202* (DPP); ISOTYPES: *Błaszkowski, J. 2203-2223* (DPP) and two slides at OSC.

Other material examined. ISRAEL. Near Tel-Aviv (32º4'N, 34º46'E), from the root zone of *C. drummondi*, 16 Dec. 1997 and 15 June 2000, *Błaszkowski, J., unnumbered collection* (DPP). SPAIN. Near Cape Salinas (36º19'N, 3º2'E), Majorca, from around roots of *A. arenaria*, 15 Aug. 2001, *Błaszkowski, J.*, unnumbered collection (DPP). TURKEY. Near Karabucak-Tuzla (36º43'N, 34º59'E), from among roots of *A. arenaria*, 7 June 2001, *Błaszkowski, J.*, unnumbered collection (DPP). ITALY. Near Calambrone (43º35'N, 10º18'E), from under *A. arenaria*, 11 Oct. 2002, *Błaszkowski, J.*, unnumbered collection (DPP).

Etymology. *Xanthium*, referring to the plant species with which this fungus was originally associated.

Distribution and habitat. Spores of *G. xanthium* were for the first time isolated from a trap culture established with a soil sample collected under *Xanthium* cf. *spinosum* L. colonizing maritime sand dunes adjacent to Veriko in northern Greece (22º35'E, 40º08'N). This fungus was not found in the field-sampled soil. The fungi occurring in the field soil from which *G. xanthium* inoculum originated included two unrecognized *Glomus* spp. and *S. persica*. The arbuscular mycorrhizal fungal species associated with *G. xanthium* in trap cultures were *G. clarum* Nicol. & N.C. Schenck, *G. gibbosum*, and an undescribed *Glomus* sp.

Subsequently, this fungus was revealed in 15 trap cultures with rhizosphere soils of other dune sites of the Mediterranean Sea. They were collected from under *C. drummondi* growing near Tel-Aviv, Israel, in 1997 (one sample) and 2000 (7 samples), from among roots of *A. arenaria* growing near Cape Salinas, Majorca, Spain, in 2001 (2 samples), from under *A. arenaria* growing near Karabucak-Tuzla, Turkey, in 2001 (4 samples), and under *A. arenaria* growing near Calambrone, Italy, in 2002 (one

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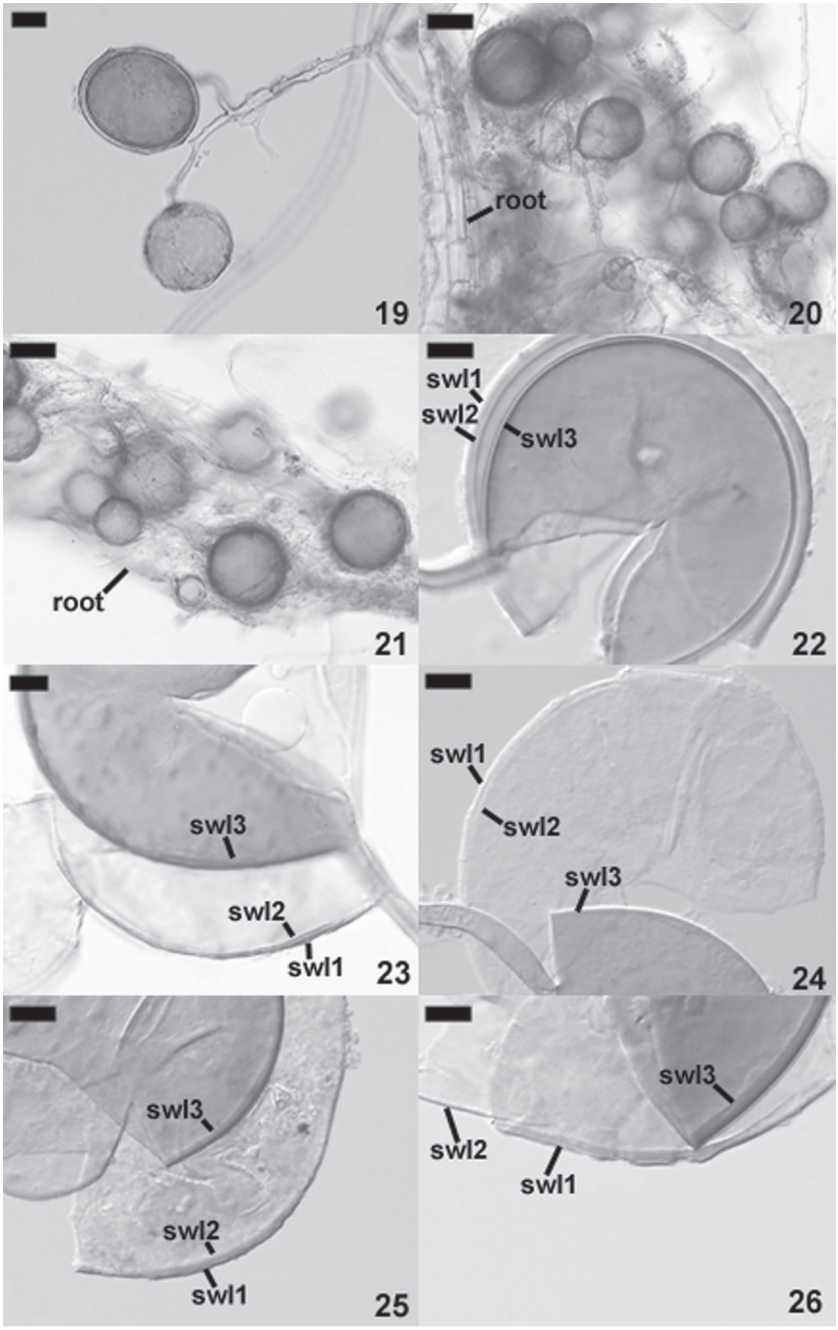
Figs. 19-34. Spores of *Glomus xanthium* and its mycorrhizae in roots of *Zea mays* stained in 0.1% trypan blue. **19.** Extraradical spores with halo formed by spore wall layers 1 and 2. **20.** Spores closely associated with root. 21. Intraradical spores. **22-26.** Adherent spore wall layers 1 (swl1) and 2 (swl2) separated from spore wall layer 3 (swl3). **27.** Spore wall layers (swl) 1-3 separated from each other. **28 and 29.** Highly deteriorated spore wall layer 1 (swl1) adherent to spore wall layer 2 (swl2) separated from spore wall layer 3 (swl3). **30.** Subtending hyphal wall layers (shwl) 1-3; curved septum (s) is visible. **31.** Arbuscules (arb). **32.** Arbuscule (arb), vesicle (ves), and coils of intraradical hyphae. **33.** Vesicles (ves). **34.** Straight (shyp) and coiled (chyp) intraradical hyphae. Figs. 19 and 20, intact spores in water. Fig. 21. Root with spores in PVLG. Figs. 22-24 and 27-30, spores crushed in PVLG. Figs. 25 and 26, spores crushed in PVLG+Melzer's reagent. Figs. 31-34, mycorrhizae in PVLG. Figs. 19-21 and 31-34, bright field microscopy; Figs. 22-30, differential interference contrast. Bars: Fig. 19=20 μm, Figs. 20 and 21=50 μm, Figs. 22-34=10 μm.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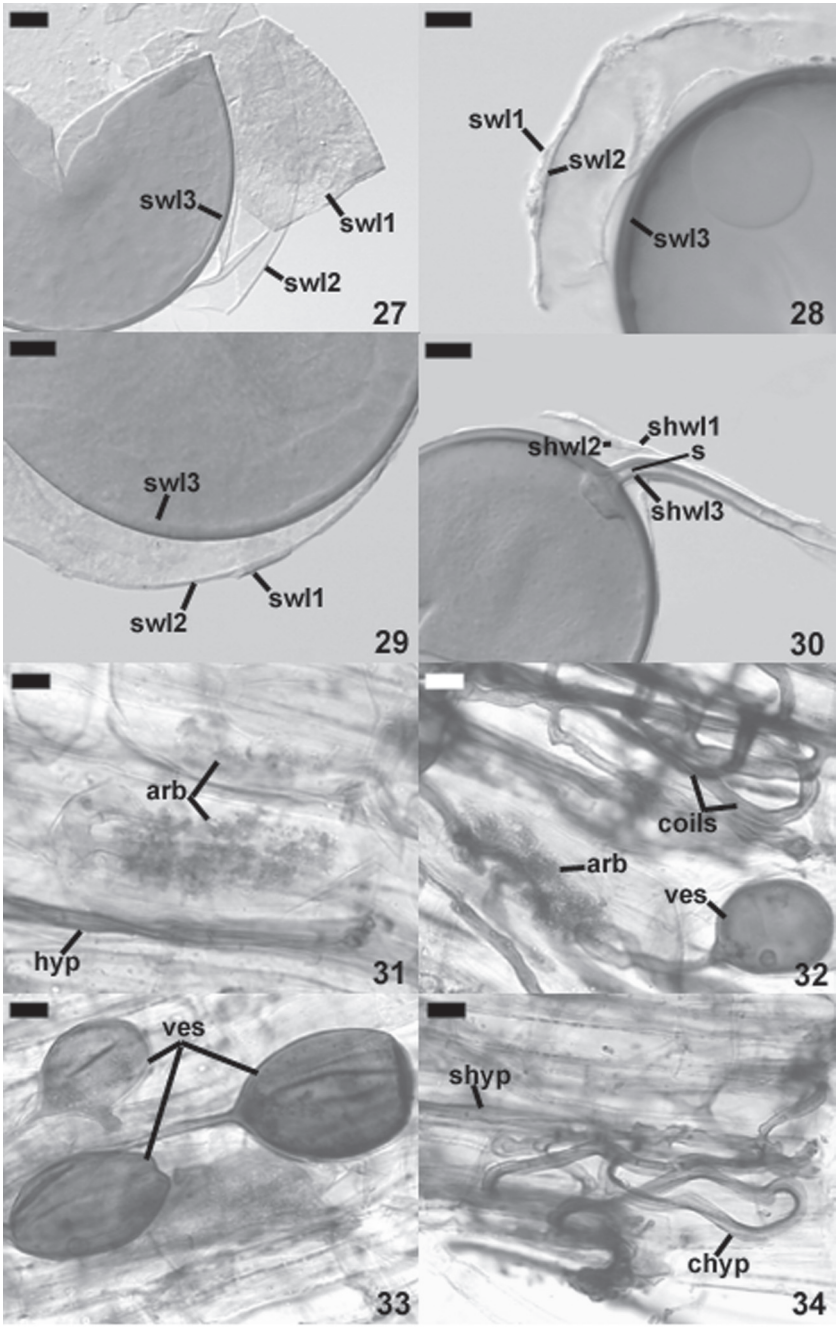
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sample). No study of the composition of arbuscular mycorrhizal fungal species in the field-collected soils was undertaken. The arbuscular mycorrhizal fungi co-occurring with *G. xanthium* in the trap cultures with Israeli soils were *Archaeospora trappei*, *G. constrictum*, *G. dominikii*, *G. claroideum*, *G. coronatum*, and *Scutellospora pellucida* (Nicol. & N.C. Schenck) C. Walker & F.E. Sanders. The Majorca's cultures contained *Ar. trappei*, *G. constrictum*, *G. corymbiforme*, *S. calospora*, those from Turkey *G. constrictum*, *G. coronatum*, *G. fasciculatum*, *G. aurantium*, and *S. calospora*, and those from Italy *Acaulospora bireticulata* F.M. Rothwell & Trappe, *G. aurantium*, *G. microcarpum* Tul. & C. Tul., and *S. persica*.

The chemical properties of the original soils were not determined.

Mycorrhizal associations. *Glomus xanthium* was associated in the field with vesicular-arbuscular mycorrhizae of *X.* cf. *spinosum* in Greece, *C. drummondi* in Israel, and *A. arenaria* in Spain and Turkey. In one-species cultures with *Z. mays* as the host plant, *G. xanthium* formed mycorrhizae with arbuscules, vesicles, as well as intra- and extraradical hyphae (Figs. 31-34). Arbuscules were numerous and generally evenly distributed along the root fragments examined (Fig. 31). Vesicles occurred numerously and were globose to subglobose, (20-)27(-40) μm diam, or ellipsoid, 17.5-40.0 x 22.5-120 μm (Figs. 32 and 33). Intraradical hyphae varied in thickness from (0.9-)5.9(-12.7) μm, grew parallel to the root axis, and sometimes formed Y- or H-shaped branches and coils, 12.5-27.5 x 17.5-55.0 μm (Figs. 31, 32 and 34). Extraradical hyphae were abundant, frequently associated with spores, and measured (3.2-)4.7(-7.4) μm wide. In 0.1% trypan blue, arbuscules stained violet white (16A2) to deep violet (16D8), vesicles violet white (16A2) to deep violet (16E8), intraradical hyphae violet white (16A2) to deep violet (16E8), coils violet white (16A2) to reddish violet (16B6), and extraradical hyphae deep violet (16D8-E8).

Phylogenetic position. Sequence data and phylogenetic analyses placed *Glomus xanthium* in *Glomus* Group A *sensu* Schüßler et al. (2001). Sequences of *G. xanthium* fell into a separate cluster (Fig. 17) or even formed a separate lineage (Fig. 18) within *Glomus* Group A, distinct from all well known species of this group (e.g. *G. mosseae*, *G. coronatum*, *G. caledonium* (Nicol. & Gerd.) Trappe & Gerd., *G. geosporum* (Nicol. & Gerd) C. Walker). However, *G. xanthium* clustered close to a preliminarily named *Glomus* sp. 'Bad Sachsa' (with no further correlation to morphological features) from a gypsum slope of the southern Harz mountains (Germany; Renker et al. 2003, Börstler et al. unpubl. data), displaying identities between 90 and 94% (Fig. 35).

Discussion. Two properties mainly distinguish *G. xanthium* from other *Glomus* species. First, spores of the new fungal species tend to form within or tightly adherent to roots (Figs. 20 and 21). Second, the spores are relatively small, with the outermost layer usually thicker than the innermost layer of the 3-layered spore wall (Figs. 19, 22-24, 27).

The pattern of spore wall differentiation in *G. xanthium* is similar to that of *Glomus* species investigated to date (Błaszkowski 1997; Błaszkowski & Tadych 1997; Morton 1996; Stürmer & Morton 1997), with discrete layers formed successively.

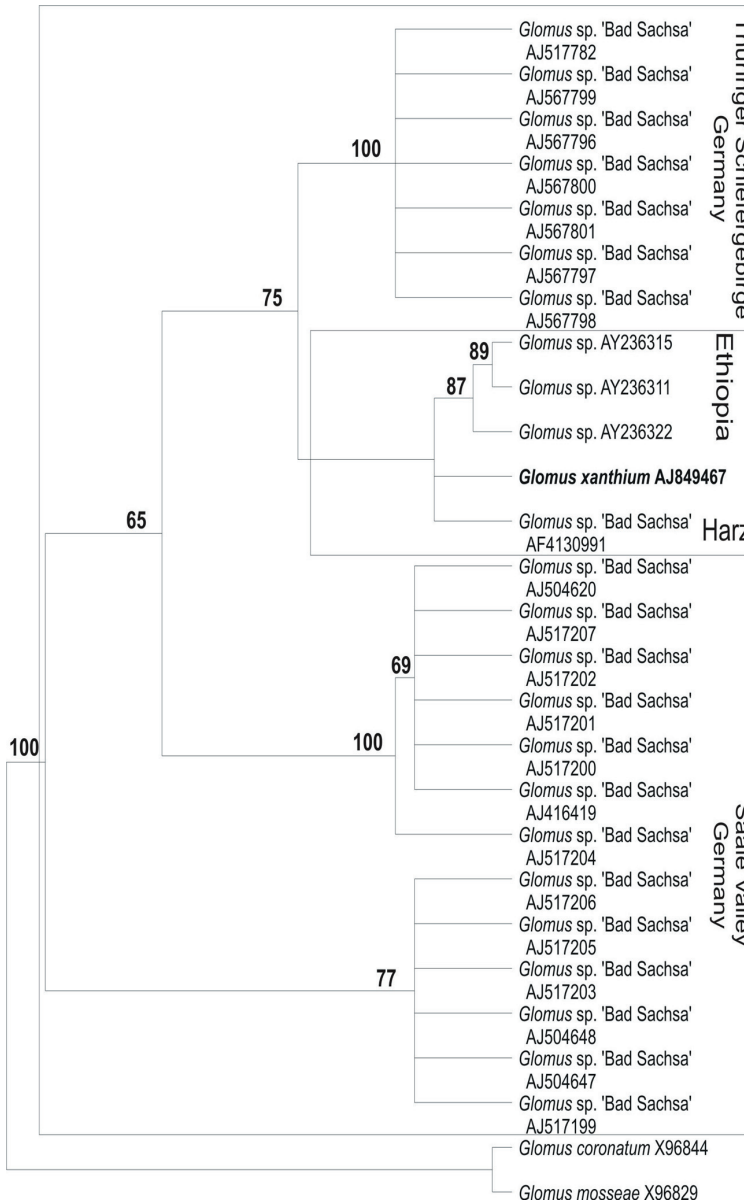

Fig. 35. Strict Consensus Tree of the Glomeromycota belonging to *Glomus* Group A (*sensu* Schüßler et al. 2001) based on 28 ITS sequences of the nrDNA. The phylogenetic position of the newly described *G. xanthium* is given in bold. Numbers above branches give bootstrap support derived from 1000 replicates. Sequences of *Glomus coronatum* and *G. mosseae* were used as outgroups.

When observed under a dissecting microscope, spores of *G. xanthium* most resemble small-spored isolates of *G. aggregatum* N.C. Schenck & Sm. emend. Koske and *G. intraradices* N.C. Schenck & S.M. Sm. These species produce yellow-colored spores that frequently occur in both aggregates tightly associated with roots and inside them (Błaszkowski 2003; Schenck & Smith 1982; Stürmer & Morton 1997).

Using a light microscope, examination of spores crushed in a mixture of PVLG and Melzer's reagent readily separates the three fungi. The spore wall of *G. xanthium* is composed of two, usually adherent rigid, semi-permanent and permanent layers, respectively, readily separating from a laminate innermost layer when crushed (Figs. 22-29). The spore wall of *G. aggregatum* and *G. intraradices* also consists of three layers, of which two outer ones usually detach from the innermost laminate layer in crushed spores (Błaszkowski 2003; Schenck & Smith 1982; Stürmer & Morton 1997). However, the two outer spore wall layers of the latter fungi are short-lived and usually are completely sloughed in mature spores (Błaszkowski 2003; Stürmer & Morton 1997). Additionally, the outermost wall layer of both *G. aggregatum* and *G. intraradices* stains red to purple in Melzer's reagent (Błaszkowski 2003; Stürmer & Morton 1997), whereas that of *G. xanthium* ramains non-reactive in this reagent. Finally, the unique character of *G. aggregatum* is the production of spores inside their parent spores by internal proliferation (Błaszkowski 2003; Koske 1985).

Although morphology placed *G. xanthium* close to *G. intraradices*, molecular data did not confirm this estimation. Unfortunately, there is lack of sequence data for *G. aggregatum*. Based on available molecular data, *G. xanthium* can be considered a member of *Glomus* Group A. While all the well known species of this group are distinct from *G. xanthium*, *Glomus* sp. 'Bad Sachsa'-sequences were found to be the closest relatives in the phylogentic analyses (Figs. 17 and 35). Firstly detected by Landwehr et al. (2002) at a gypsum slope in the southern Harz mountains (Germany), similar sequence types were found in further studies within Germany (Renker et al. 2003, Börstler et al. unpubl. data). Quite recently, Wubet et al. (2003) detected a *Glomus* sp. in Ethiopia colonizing roots of *Prunus africana*. ITS sequences of this fungus fall into the same sequence cluster like *G. xanthium* and the original *Glomus* sp. 'Bad Sachsa' sequence (Fig. 35).

Glomus aurantium and *G. xanthium* are probably adapted to warm soils of southern hemisphere. They have not been found in any of ca. 3000 soil samples collected in different dune and non-dune soils of northern Europe (Błaszkowski 2003). Koske (1987) found temperature to be the main abiotic factor influencing the structure of arbuscular fungi of the barrier dunes extending from New Jersey to Virginia. According to Pirozynski (1968), temperature is the major factor determining the distribution and occurrence of fungi in general.

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