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***Glomus badium*, a new sporocarpic mycorrhizal fungal species from European grasslands with higher soil pH**

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Summary

Glomus badium forms small sporocarps with about 180-280 µm in diameter. The sporocarps have no peridium and contain 5-30 spores. The spores are situated around and partly within a whitish to yellowish gleba of interwoven intrasporocarpic hyphae. Spores are reddish brown to dark brown to black, globose, subglobose to ovoid, 51-90 x 75-120 µm in diameter. Spores have three wall layers, in total (5.5-)7-14 µm thick. The innermost layer usually closes the pore at the spore base together with a bridging septum formed by the middle layer. The subtending hypha of each spore is usually very short (<1-2.5 µm) and, thus, the spore base is difficult to observe. The new species can easily be differentiated from other sporocarp-forming *Glomus* spp. by the structure and the size of the sporocarps and spores, the organisation of the spores in the sporocarps, the colour of the spore walls and in particular through the characteristics of the intrasporocarpic hyphae and the short subtending hypha at the spore base. A partial DNA sequence of the 18S ribosomal small subunit gene of spores of *G. badium* was determined. Phylogenetic analyses firmly placed the sequence into *Glomus* spp. of group A of the Glomeraceae, with no close matches among named sequences obtained from spores of other *Glomus* species. Several sequences from field collected roots infected with AMF showed a high similarity to *G. badium*. *Glomus badium* is a frequent member of the arbuscular mycorrhizal fungi community of grasslands, grass-intercropped vineyards or olive fields, or no-till arable lands in Germany, France, Switzerland and Italy. It has been found in grasslands up to the tree line in the Alps, but so far, only in soils with pH 6-8.

Introduction

Arbuscular mycorrhizal fungi (AMF) of the Glomeromycetes are important soil micro-organisms which are involved in the uptake and transport of nutrients to plant roots (BAREA et al., 2002). They play a role in the diversity and functioning of natural ecosystems (VAN DER HEIJDEN et al., 1998), and they are important for the functioning of low input agroecosystems (MÄDER et al., 2002). SIEVERDING (1991) showed that the composition of the AMF communities affects the benefit that a plant or a crop may obtain from the symbiosis. The composition of the AMF community is heavily influenced by the land use system and farming intensity (OEHL et al., 2003a; 2004; 2005). Hence, it is important to define and recognise the AMF species present in different ecosystems or agricultural land use systems. The identification of AMF species can either be done by observing the morphological characteristics of spores of these fungi or by using molecular biological tools, and hence by determining DNA gene sequences of these fungi (REDECKER, 2000).

Grasslands in Central and Southern Europe have a high diversity of AMF species (OEHL et al., 2003a; 2003b) and several new species have been reported already from such ecosystems (OEHL et al., 2003b; 2003c; OEHL and SIEVERDING, 2004). We have studied the ecology of AM fungi in different agro-ecosystems, especially in

extensively managed and semi-natural grasslands (OEHL et al., 2003a; 2005). In this context, soil samples were also taken from grass-intercropped vineyards, extensively managed vineyards with grass weeds, grass vegetation on the wayside of vineyards, and from grass vegetation under olive trees. One of the new AMF species found in the soil samples is presented here under the epithet *Glomus badium*. Spores of this new species were first found in extensively managed vineyards in the Rhine-Nahe valley (in the vine growing area 'Rheinessen' of the Bundesland Rheinland-Pfalz, Germany). We now consider this formerly unknown fungal species as a characteristic part of AMF communities at grass-grown agricultural sites in Central and Southern Europe, especially in soils of pH 6-8. In this paper we describe the morphological characteristics of the spores and sporocarps of the new species and we compare the DNA gene sequences of this species with those known from other *Glomus* species.

Materials and methods

Soil sampling

Undisturbed soil cores (sampling depth 0-10 cm) were taken from grasslands, grass-grown vineyards, extensively managed vineyards with grass weed cover, grass vegetation on the wayside of vineyards, and arable lands subjected to different agricultural management practices in the Upper Rhine valley between Mainz (Germany) and Basel (Switzerland) on several occasions between 1994 and 2003, and from grass cover under vineyards and olive plantations in Umbria, Italy in January 2002. Spores and sporocarps of arbuscular mycorrhizal fungi were separated from the soil samples by the wet sieving and decanting and successive sugar gradient centrifugation technique (SIEVERDING, 1991).

AMF bait cultures

Bait cultures of the native AMF populations and pot cultures of isolated species were also established using the techniques described in OEHL et al. (2003a). The new fungus only infrequently sporulated in the bait cultures (OEHL et al., 2003a; 2004; 2005; note that in these three studies *G. badium* was named '*Glomus* sp. BR4'). The establishment of single species cultures on *Plantago lanceolata* L. was not successful.

Morphological analyses

The morphological properties of sporocarps, spores and subcellular structures are based on observations of specimens mounted in polyvinyl alcohol-lactic acid-glycerol (PVLG; KOSKE and TESSIER, 1983), in a mixture of PVLG and Melzer's reagent (BRUNDRETT et al., 1994), a mixture of lactic acid to water at 1:1, Melzer's reagent, and in water. The terminology of the spore structure is as suggested by INVAM (International Culture Collection of Arbuscular and Vesicular-Arbuscular Endomycorrhizal Fungi, see homepage:

www.invam.caf.wvu.edu). Photographs in Fig. 1 were taken with a digital camera (Olympus model DP50-CU) on a dissecting microscope (Olympus SZX 12; Fig. 1 A) and on a compound microscope (Zeiss; Axioplan; Fig. 1 B-H), respectively. Air-dried sporocarps and specimens mounted in PVLG and the mixture of PVLG and Melzer's reagent were deposited at Z+ZT, FB and OSC herbaria.

Molecular analyses

DNA crude extracts were produced from sporocarps as described by REDECKER et al. (1997). The extracts were used as template for a two-step polymerase chain reaction (REDECKER et al., 2003) using the primers NS5/ITS4 and NS5/GLOM5.8R, respectively (REDECKER, 2000). PCR products were obtained from an isotype isolated from a grassland on a Mollic Leptosol in Axalp (Brienzer Sec, Berner Oberland, Switzerland). The PCR products were cloned into pGEM-T (Catalys, Wallisellen, Switzerland), reamplified from the clones, purified with QIAquick (Quiagen, Hilden, Germany) and sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) for labeling. Samples were run on an ABI 310 capillary sequencer (Applied Biosystems). The sequences were aligned in PAUP*4b10 (SWOFFORD, 2001) to a dataset comprising the 3' region of the 18S ribosomal small subunit of other fungi of the Glomeromycetes (BIDARTONDO et al., 2002) and analyzed phylogenetically. *Glomus etunicatum* and *Glomus claroideum* (*Glomus* spp. of group B; SCHWARZOTT et al., 2001) were used as outgroups. In order to allow comparisons to shorter sequences of fungi of the Glomeromycetes obtained from plant roots directly from field sites (so-called environmental sequences), only 370 bp of the small subunit were analyzed. Bootstrap analysis (FELSENSTEIN, 1985) was performed to estimate the robustness of the phylogeny. The ITS1 region was aligned to the respective part of an ITS dataset containing *Glomus* spp. of group A (SCHWARZOTT et al., 2001) sequences. As this region was too short and too variable for phylogenetic analyses, it was visually checked for similarity. Sequences were submitted to the EMBL database under the accession number AJ871990.

Latin diagnosis

Glomus badium sp. nov. Oehl, Redecker & Sieverd. (Fig. 1)

Sporocarpia rubro-brunnea vel atro-brunnea cum forma globosa vel subglobosa, 180-275 x 200-280(-350) µm, sine peridio, cum paucis sporis (5-30). *Sporae*, rubro-brunneae vel atro-brunneae ad badias ad nigrae, interdum irregulares, 51-90 x 75-120 µm. *Hyphae intrasporocarpicae densae*, sporas non cingentes. *Sporae cum tunica tribus*, (5.5-)7-14 µm crassis in totum. *Tunica prima hyalina*, *secunda brunnea*, *tertia flavo-brunnea*. *Tunica interior et septum tunicae secundae porum conjunctionis hyphae cum spora occludens*. *Hypha adhaerens spora non nisi 1-2.5 µm longa*, raro visibilis est. *Typus hic designatus # 4992 : Z+ZT.*

Description

Sporocarps formed in soils on a thick, 7-15 µm wide, hyaline to yellowish hypha or formed tightly adherent to the host roots, or in the roots itself. Sporocarps are compact, globose to subglobose, 180-275 x 200-280(-350) µm in diam, without peridium (Fig. 1 A and B), and contain 5-30 dark reddish-brown to black-brown spores in an irregular radial organisation (Fig. 1 C) around and within a dense whitish gleba (Fig. 1 D) that is visible between the spores of the sporocarp. Fragments of sporocarps with 3-10 spores are frequently found in field samples. Intrasporocarpic gleba consists of

interwoven, branching and anastomosing hyphae (Fig. 1 E) that never circum-grow the spores completely (Fig. 1 B). Intrasporocarpic hyphae, 5-13 µm in diam, with two hyphal wall layers, in total 1.2-2.5 µm thick. Outer intrasporocarpic hyphal wall layer hyaline, 0.4-0.7 µm thick, evanescent and usually hardly to detect, inner layer hyaline to yellow, 0.8-1.8 µm. Spores and gleba not staining in Melzer's reagent.

Spores only formed in sporocarps, arising blastically from a hyphal loop. Spores reddish-brown to dark reddish brown to brown-black, globose, subglobose to ovoid, or sometimes irregular, maize grain-shaped due to the dense packing of spores around and partly between the intrasporocarpic hyphae (Fig. 1 C), (33-)51-90 x (50-) 75-120 µm in diam.

Spore wall of three layers (sw1, sw2, and sw3) (Fig. 1 D, F, and G), in total (5.5-) 7-14 µm thick. Outer layer (sw1) hyaline, <1.5 µm thick, sloughing and, thus, usually absent on spores of mature sporocarps or in sporocarp fragments. Some intrasporocarpic hyphae are sometimes attached to the surface of sw1, on parts of the spore surface (Fig. 1 F). Second layer (sw2) reddish-brown to dark brown, laminated, (4-)6-13 µm thick. Inner wall layer (sw3) yellow to yellow brown, 0.5-2.0 µm thick, usually tightly adherent to sw2 and sometimes difficult to observe, in particular when <1.0 µm. At the spore base, sw2 is often thickened (Fig. 1 G), up to 18 µm. None of the wall layers stains in Melzer's reagent.

Subtending hypha of spores difficult to observe, regularly <1.0-2.5 µm short, maximum of 15-20 µm long (Fig. 1 D and F), light yellow to yellow-brown, 4-7 µm in diam at the spore base. The attachment is then widening abruptly and is then part of the intrasporocarpic hyphal mycelium (Fig. 1 G). The spore wall layers sw1 and sw2 continue with the subtending hypha but change colour at the spore base to hyaline to light yellow. Spores regularly have a single subtending hypha; two or three subtending hyphae attached to one spore were infrequently also found.

Pore of the subtending hypha is straight and about 1-1.5 µm wide. The pore is closed at the spore base by sw3 (Fig. 1 F and G.) and often additionally by a septum formed by the innermost lamina of spore wall layer sw2 (Fig. 1 G and H).

Forms mycorrhiza, as concluded from sporocarp formation in or tightly adherent to roots and from molecular analyses.

Etymology: Latin, *badium* referring to the red-brown to dark brown-black colour of mature spores in sporocarps.

Type: Isolated from soil samples taken from the rhizosphere of *Dactylis glomerata* L. on a wayside of a vineyard in St. Johann/Rheinhessen (Bundesland Rheinland-Pfalz, Germany). Holotype: Slide # 4992 Z+ZT, Isotypes: FB, OSC.

Distribution: Germany: *Glomus badium* has frequently been isolated from grasses of un-weeded vineyards and from grasslands at waysides of vineyards in the wine growing area of Rheinhessen, e.g. in communities of St. Johann and Langenlonsheim, near Mainz. The

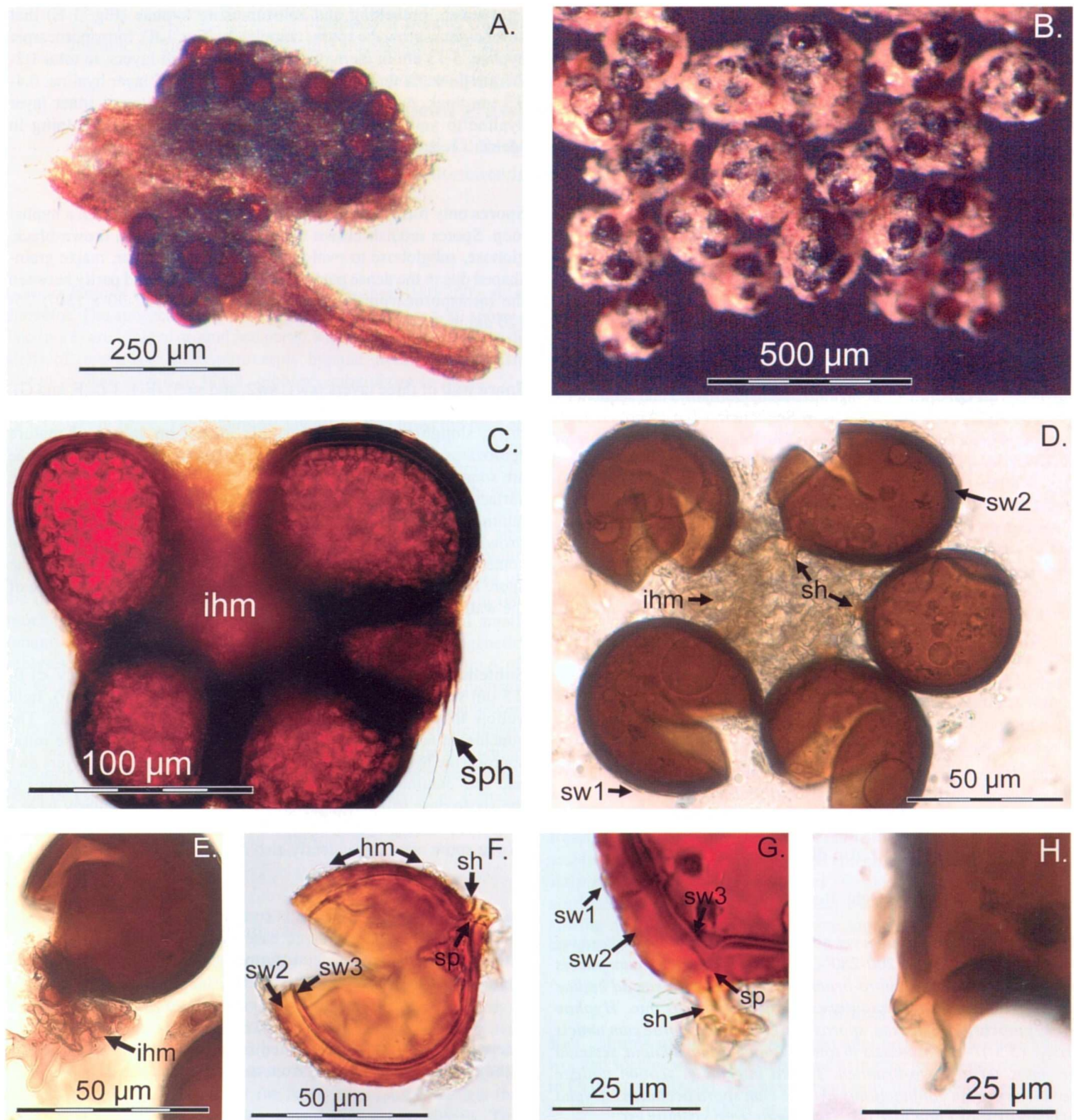


Fig. 1: *Glomus badium*. A. Spores formed in sporocarps, tightly adherent to host roots; without peridium. B. Sporocarps and sporocarp fragments isolated from field soil – whitish to cream gleba is visible between spores. C. Single sporocarp with five spores and with central intrasporocarpic hyphal mycelium (ihm) and the hyphal attachment of the sporocarp (sph). D. Sporocarp cracked; spores broken showing the short subtending hyphae (sh), intrasporocarpic hyphal mycelium (ihm), and spore wall layers 1 and 2 (sw1 and sw2). E. Intrasporocarpic hyphal mycelium (ihm). F. Broken mature spores with wall layers sw2 and sw3, the short subtending hypha (sh), and a septum (sp) closing the pore at the spore base. On parts of the surface of the spore, rests of gleba (hm) attached. G. Spore base with short subtending hypha (sh); the pore closed by inner wall layer sw3 and a bridging septum (sp) arising from wall layer sw2; sw 1 is thin. H. Bridging septum at the spore base. Specimen in Fig. 1 B in water; all other specimen (Fig. 1 A, C-H) mounted in PVLG.

soils there are Luvisols, Calcaric Regosols and Calcaric Phaeozems (LESSMANN et al., 1986) with pH 6-8. The Phaeozem soils are the typical soils for this region and are often called 'Rhine valley Chernozems', or 'Dunkelbraune Schwarzerden des Oberrheintales'

or 'Braune Steppenböden' in German language. *Glomus badium* was also isolated from Meso-Brometa and Arrhenathereta grasslands, on Calcaric Regosols and Chernozems near Vogtsburg im Kaiserstuhl. France: Isolated from a grassland on a Rendzic Leptosol near Leymen,

Departement Haut Rhin, **Switzerland**: Isolated from grasslands on a Rendzic Leptosol near Nenzlingen and on a Haplic Luvisol near Therwil, Kanton Basel Landschaft; a no-till arable land on a Rendzic Leptosol near Reigoldswil, Kanton Basel Landschaft; from several grasslands on calcareous bedrocks (with topsoil pH 6-8) at mountainous altitudes of 1000-1500 meter above sea level near Sent, Engiadina bassa, Chantun Grischun/Graubünden; near Prato, Cantone Ticino, and near Axalp, Kanton Bern; from grasslands up to the tree line in the Swiss Alps near Ovronnaz, Canton Valais, and close to Haldenstein (Chur), Kanton Graubünden. **Italy**: From grasslands grown under grapes and olive trees on Calcaric Cambisols and Rendzic Leptosols near Assisi, Umbria.

baccata (Fig. 2; WUBET et al., 2003). Sequences from root samples of different plant species from a species-rich calcareous grassland near Leymen (France, close to Basel, Switzerland) also fell within that group and were equally similar (ZUZANA SYKOROVA, unpublished results). From that grassland field site, numerous spores of *G. badium* were isolated previously (OEHL et al., 2003a), suggesting that the fungus identified within the roots was in fact *G. badium*. In the short sequence obtained from the ITS1 region, the *G. badium* sequence obtained from spores from Axalp was almost identical to the sequences obtained from roots (mentioned here above), only differing by an insertion of two base pairs. This similarity is remarkable in a variable region that is impossible to completely align across the *Glomus* spp. of group A according to SCHWARZOTT et al. (2001).

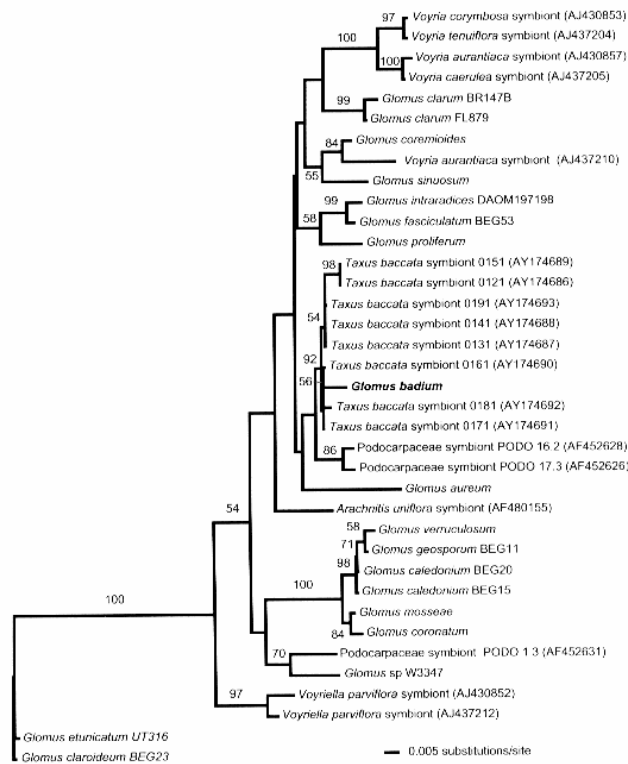


Fig. 2: Phylogenetic tree of *Glomus* spp. of group A according to SCHWARZOTT et al. (2001) obtained by neighbor-joining of partial 18s ribosomal small subunit sequences. Numbers above the branches denote bootstrap values from 1000 replicates (FELSENSTEIN, 1985). Environmental sequences obtained from uncultured root symbionts (see Materials and methods) are labeled with host plant and sequence accession numbers in brackets. Sequences with AMF species names originate from spores. In addition, isolate numbers are provided where multiple sequences from a single morphospecies are available.

Molecular analyses: A sequence of 617 bp length was obtained, comprising approximately 564 bp of the 18S small ribosomal subunit, and 53 bp of the ITS1 region. Phylogenetic analyses firmly placed the sequence into *Glomus* spp. of group A (SCHWARZOTT et al., 2001), with no close matches among named sequences obtained from spores. However, several environmental sequences from field collected roots showed a high similarity to *G. badium*: e.g. 99.5% over 375 bp with sequence AY174690 from the public databases. Together, they form a monophyletic clade which is supported well in bootstrap analyses (92% in Fig. 2). The related sequences originate from roots of *Taxus*

Discussion

A characteristic feature of the new species is the exclusive formation of spores in a compact, rather small sporocarp without a peridium containing 5-30 dark red to black spores irregularly arranged around and within a whitish to yellowish gleba. The extremely short subtending hypha of the spores is also typical. By these characteristics the new species can be differentiated from other sporocarpic species in the genus *Glomus* of the Glomeraceae family (following the classification of MORTON and BENNY, 1990). We could not establish pure pot cultures of *G. badium*. Hence, the direct confirmation could not be found that this new species forms vesicular arbuscular mycorrhiza. However, the morphological characteristics of the sporocarps and spores, and the formation of sporocarps attached to or in roots all are typical features for AMF species of the genus *Glomus*, which was confirmed by the molecular analyses.

Several other AMF species form dark red to black spores in sporocarps. These are *G. ambisporum* and *G. heterosporum* G.S. Sm. & N.C. Schenck (SMITH and SCHENCK, 1985), *G. atrouva* and *G. pellucidum* McGee & Pattinson (MCGEE and TRAPPE, 2002), *G. boreale* (Thaxter) Trappe & Gerd. (GERDEMANN and TRAPPE, 1974), *G. cuneatum* McGee & Cooper (MCGEE and TRAPPE, 2002), *G. botryoides* F.M. Rothwell & Victor (ROTHWELL and VICTOR, 1984), *G. invermaium* I.R. Hall (HALL, 1977), *G. flavisporum* (M. Lange & Lund) Trappe & Gerd. (GERDEMANN and TRAPPE, 1974), *G. melanosporum* Gerd. & Trappe (GERDEMANN and TRAPPE, 1974), *G. mertonii* Bentiv. & Hetrick (BENTIVENGA and HETRICK, 1991), *G. rubiforme* (Gerd. & Trappe) R.C. Almeida & N.C. Schenck (ALMEIDA and SCHENCK, 1990) and *G. tenebrosum* (Thaxter) Berch (BERCH and FORTIN, 1983).

The sporocarps and spores of the following species are significantly larger in size than those of *G. badium*, and thus, are easily distinguished from the new species: These are *G. ambisporum*, *G. atrouva*, *G. heterosporum*, *G. boreale*, *G. flavisporum*, *G. botryoides*, *G. melanosporum*, and *G. tenebrosum*.

Several sporocarpic AMF species are known with spores similar in size and colour to those of *G. badium*: *G. invermaium*, *G. cuneatum*, *G. pellucidum* and *G. rubiforme*. Sporocarps of *G. invermaium* are not arranged around intrasporocarpic hyphal mycelia, their sporocarps are loose and degrade rapidly in soils. This is the reason why usually only single spores or small spore clusters of *G. invermaium* are found in field samples. *Glomus cuneatum* has similar coloured spores to *G. badium* but spores are generally elongate, the sporocarps are bigger and have a peridium. Spores of *G. pellucidum* are lighter yellow brown in colour and they have a relatively long and clearly distinguishable subtending hypha which differentiates this species

from *G. badium*. *Glomus rubiforme* has a significantly higher number of spores in the sporocarp, the spores are densely organized around a central plexus of hyphae and, though some gleba is present inside the sporocarps of *G. rubiforme*, there is no gleba between the spores of *G. rubiforme*. The spore wall of *G. rubiforme* is also generally thinner and only bi-layered.

Spores and sporocarps of *G. mortonii* are lighter in colour, and the spores have a much larger size range in the sporocarp than those of *G. badium*. Additionally, each of the *G. mortonii* spores within the sporocarp is completely surrounded by a hyphal mantle that is about 10-20 µm thick and composed of sinuous hyphae.

Spores and sporocarps of *G. fuegianum* (Spegazzini) Trappe & Gerd. (GERDEMANN and TRAPPE, 1974) have a similar size to those of *G. badium*. Both the species are similar in wall structure – through our own observations we can confirm the findings of MCGEE and TRAPPE (2002) that the spores of *G. fuegianum* have three wall layers. However, the sporocarps of *G. fuegianum* have a peridium and spores of *G. fuegianum* are pale yellow to yellowish brown in colour. Also, the thick-walled subtending hypha of spores of *G. fuegianum* continues over about 20-40 µm distance from the spore base, a feature not found with *G. badium*.

Glomus badium is the second new small-spored sporocarpic species we describe from European grasslands. The first was *Glomus aureum* Oehl & Sieverd.¹ (OEHL et al., 2003b) which is frequent in European Arrhenathereta and Mesobrometa vegetations and occurs over a broad range of different soil types. In contrast, *G. badium* is found as a characteristic part of the AMF community either of grasslands, grass-grown vineyards or olive fields, or no-till arable lands, in soils with pH 6-8. More pronounced than for *G. aureum*, *Glomus badium* is usually completely absent at regularly tilled soils (OEHL et al., 2003a; 2004; 2005: Note that in those recent publications *G. badium* was named *Glomus* sp. BR4.). The DNA sequence data from *G. badium* allowed to infer that this or a closely related AMF species was in fact found in two different field sites at the border line between Switzerland and France with soil pH 8.0 (OEHL et al., 2003a) and in Southwest Germany at a soil pH of 6.0 (WUBET et al., 2003). The DNA sequence of *G. badium* is the first one that can be assigned to a described species in a clade of otherwise so-called 'environmental sequences' obtained from AM infected roots only, where the AMF species responsible for the infection are not yet known.

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¹ The phylogeny of a paratype isolate of *G. aureum* is presented in Fig. 2; the corresponding sequences were submitted to the EMBL database under the accession number AJ871991

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