Three new species of arbuscular mycorrhizal fungi of the genus *Diversispora* from maritime dunes of Poland

Janusz Błaszkowski[a], Piotr Niezgoda[a], Szymon Zubek[b], Edward Meller[c], Paweł Milczarski[d], Ryszard Malinowski[c] Monika Malicka[e], Sylwia Uszok[e], Bruno Tomio Goto[f], Wojciech Bierza[e], Leonardo Casieri[g] , and Franco Magurno[e]

[a]Department of Protection and Shaping of Environment, West Pomeranian University of Technology in Szczecin, Słowackiego 17, PL–71434 Szczecin, Poland; [b]Institute of Botany, Faculty of Biology, Jagiellonian University, Gronostajowa 3, 30-387 Kraków, Poland; [c]Laboratory of Soil Science and Environmental Chemistry, Department of Shaping of Environment, West Pomeranian University of Technology in Szczecin, Słowackiego 17, PL–71434 Szczecin, Poland; [d]Department of Genetics, Plant Breeding & Biotechnology, West Pomeranian University of Technology in Szczecin, Słowackiego 17, PL 71434 Szczecin, Poland; [e]Institute of Biology, Biotechnology and Environmental Protection, University of Silesia in Katowice, Jagiellońska 28, PL-40-032 Katowice, Poland; [f]Departamento de Botânica e Zoologia, Universidade Federal do Rio Grande do Norte, Campus Universitário, 59072–970, Natal, RN, Brazil; [g]Mycorrhizal Applications LLC at Bio-Research & Development Growth Park, 1005 N Warson Rd., STE 402, St. Louis, MO 63132, USA

ABSTRACT

Three new species of arbuscular mycorrhizal fungi of the genus *Diversispora* (phylum Glomeromycota) were described based on their morphology and molecular phylogeny. The phylogeny was inferred from the analyses of the partial 45S rDNA sequences (18S-ITS-28S) and the largest subunit of RNA polymerase II (*rpb1*) gene. These species were associated in the field with plants colonizing maritime sand dunes of the Baltic Sea in Poland and formed mycorrhiza in single-species cultures.

KEY WORDS: *Diversispora*; Glomeromycota; morphology; molecular phylogeny; new primers; nuc rDNA; *rpb1*

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) forming glomoid spores occur commonly in diverse terrestrial habitats, where they usually dominate in spore communities of this group of fungi (Polo-Marcial et al. 2021; Maia et al. 2020; Marinho et al. 2018). Glomoid spores arise blastically at tips of sporogenous hyphae as in *Glomus macrocarpum* (Morton and Redecker 2001), the type species of the genus *Glomus* and the phylum Glomeromycota (Clements and Shear 1931; Oehl et al 2011). Currently, glomoid spore-producing species of AMF are classified in 49 genera belonging to 16 families across four orders of the Glomeromycota (Wijayawardene et al. 2020; Błaszkowski et al. 2021a, b). Among them are species of the genus *Diversispora* in the family Diversisporaceae, order Diversisporales (Walker and Schüßler 2004; Schüßler and Walker 2010). According to Oehl et al. (2011), the main morphological characters distinguishing the glomoid spores of members of *Diversispora* reside between the spore subtending hypha and the spore wall at the spore base: the subtending hypha is colorless, even when the spore wall is pigmented, and cylindrical.

Species of the genus *Claroideoglomus* in the family Claroideoglomeraceae (order Glomerales) also produce glomoid spores with colorless subtending hyphae, which, however,

are conspicuously funnel- or bill-shaped at the spore base (Oehl et al. 2011). Moreover, Schüßler and Walker (2010) recognized *Claroideoglomus* as containing species that form spores with a flexible, thin, colorless innermost spore wall layer (originally called an inner wall). However, among the *Claroideoglomus* species listed by Schüßler and Walker (2010) is *C. etunicatum*, which lacks a flexible, colorless innermost spore wall layer (Becker and Gerdemann 1977; Błaszkowski 2012). Such a layer is also absent in the spore wall of *C. hanlinii* (Błaszkowski et al. 2015a) but present in *D. clara* (Estrada et al. 2011) and *D. sporocarpia* (Jobim et al. 2019). Moreover, the subtending hypha of some *Claroideoglomus* species is not always bill-shaped (pers. observ.) and colorless, cylindrical, or funnel-shaped subtending hyphae of pigmented spores are also produced by AMF species outside *Claroideoglomus* and *Diversispora*, e.g., *Orientoglomus emiratia*, originally described as *Dominikia emiratia* (Al-Yahya'ei et al. 2017). Finally, the colorless subtending hyphae of *D. clara* spores cannot suggest the generic affiliation of this species because the spores are colorless as well and the color change is invisible.

Consequently, the data about the morphological convergence of *Diversispora* and other genera of the Glomeromycota discussed above demonstrate that the morphological characters of glomoid spores are homoplastic and only the molecular identification can reliably recognize species.

Of the molecular markers tested so far, the highest taxonomic resolution resides in sequences covering the 18S-ITS-28S segment of the nuclear ribosomal DNA and partial sequences of the largest subunit of RNA polymerase II (*rpb1*), which allow to separate even very closely related species, including those hidden among so called complex species (Kohout et al. 2014; Krüger et al. 2012; Stockinger et al. 2014). Moreover, recent analyses indicated that phylogenies of members of the Glomeraceae reconstructed from concatenated sequences of the two unlinked loci (18S-ITS-28S+*rpb1*) were more robust than those obtained based on

these loci analyzed separately (Błaszkowski et al. 2021a, b), in agreement with the opinions of many researchers dealing with other fungal groups (Matheny 2005; Miadlikowska et al. 2006, 2014; Alves-Silva et al 2020; Salvador-Montoya et al. 2020). However, members of other families of the Glomeromycota have not been tested in this respect so far.

Three potentially new glomoid spore-producing AM species were trapped and maintained in pot cultures. Their morphological characters, particularly the colorless subtending hyphae of their pigmented spores suggested they may represent *Claroideoglomus* or *Diversispora*. Indeed, BLAST queries, using 18S-ITS-28S sequences, indicated that the three fungi were undescribed species of *Diversispora*. Therefore, the main aims of this study here were to characterize in detail the morphology of these fungi and to determine their phylogenetic positions among sequenced species of *Diversispora* based on 18S-ITS-28S and *rpb1* sequences.

MATERIALS AND METHODS

***Origin of biological material*.**—Spores of each of the three new species (hereafter referred to as *Diversispora* 1, *Diversispora* 2, and *Diversispora* 3) were originally extracted from trap pot cultures inoculated with field-collected rhizosphere soil and root fragments of *Ammophila arenaria* (L.) Link (*Diversispora* 1, *Diversispora* 3) and *Agrostis stolonifera* L. The spores were used to establish single-species pot cultures, from which originated spores we later analyzed morphologically and molecularly. The plant host of the trap and single-species cultures was *Plantago lanceolata* L. The field samples were collected as follows: under *A. arenaria* growing in dunes of the Baltic Sea near Świnoujście (53°55′03″N 14°17′39″E) in north-western Poland by J. Błaszkowski 14 Aug 2012 (*Diversispora* 1) and 19 Aug 2013 (*Diversispora* 3); under *A. stolonifera* that had colonized the 12 deflation pan of the Baltic Sea dunes located in Słowiński National Park (SNP) in northern Poland (54°38′–54°46′N 17°03′–17°33′E) by G. Chwat 2 Sep 2013 (*Diversispora* 2).

***Establishment and growth of trap and single-species cultures, extraction of spores, and staining of mycorrhizal structures*.—**Methods used to establish trap and single-species cultures, growing conditions, and methods of spore extraction and staining of mycorrhizal structures were as those described previously (Błaszkowski et al. 2012). Five to ten spores of uniform morphology of each AMF species were used to establish single-species cultures.

***Microscopy and nomenclature*.—**Morphological features of spores as well as phenotypic and histochemical characters of spore wall layers of the new species were characterized based on at least 50–100 spores of each species mounted in water, lactic acid, polyvinyl alcohol/lactic acid/glycerol (PVLG, Omar et al. 1979), and a mixture of PVLG and Melzer's reagent (1:1, v/v). The preparation of spores for study and photography were as described previously (Błaszkowski 2012; Błaszkowski et al. 2012). The types of spore wall layers were defined by Błaszkowski (2012) and Walker (1983). Color names were from Kornerup and Wanscher (1983). Nomenclature of fungi and the authors of fungal names are from the Index Fungorum website http://www.indexfungorum.org/AuthorsOfFungalNames.htm. The term "glomerospores" was used for spores produced by AMF as proposed by Goto and Maia (2006).

Voucher specimens of the proposed new species [spores permanently mounted in PVLG and a mixture of PVLG and Melzer's reagent (1:1, v/v) on slides] were deposited at Z+ZT (ETH Zurich, Switzerland; holotypes) and in the Laboratory of Plant Protection, Department of Shaping of Environment (LPPDSE), West Pomeranian University of Technology in Szczecin, Poland (isotypes).

Molecular phylogeny, DNA extraction, PCR, cloning, and DNA sequencing.—DNA of each species was extracted from eight single spores crushed in 5 µl of ultra clean water with a needle on sterile microscope slides under a dissecting microscope and incubated at 100 C for 10 min. To obtain SSU-ITS-LSU sequences, raw DNA was used as template for PCR with a

nested procedure following a protocol modified after Krüger et al. (2009). The reaction mix in the first PCR contained 10 µl of Phusion High-Fidelity DNA polymerase 2× Master Mix (Finnzymes, Espoo, Finland), 1 µl each of 10 µM SSUmAf and LSUmAr primers, 2 µl of DNA and 6 µl of ultra clean water (Water Molecular Biology Reagent, Sigma, Saint Louis, USA). In the second PCR, the template consisted of 5 µl of the product of the first PCR diluted 1:100 with ultra clean water, 10 µl of the master mix mentioned above, 1 µl each of 10 µM SSUmCf and LSUmBr primers, and 3 µl of water. Thermal cycling was done in the TPersonal 48-Biometra thermocycler (Biometra GmbH,Goettingen, Germany) with the following conditions for the first PCR: 5 min initial denaturation at 99 C, 40 cycles of 10 s denaturation at 99 C, 30 s annealing at 50 C, 60 s elongation at 72 C and 10 min at 72 C for final elongation. The conditions of the nested PCR differed in the annealing temperature (53 C) and the number of cycles (30). After gel visualization, the PCR products with the expected-size bands were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and then cloned with the Zero Blunt TOPO PCR Cloning Kit (Life Technologies, Carlsbad, USA) following the manufacturers' instructions. Eight positive (white) colonies per transformation were used for plasmid extraction with QIAGEN QIAprep Miniprep Kit (Qiagen, Hilden, Germany). Sequencing of the amplified SSU-ITS-LSU region was performed at LGC Genomics, Berlin, Germany (http://www.lgcgenomics.com/), using M13F and M13R primers.

Rpb1 sequences of *Diversispora* 1 and *Diversispora* 2 were obtained with a nested PCR performed under conditions recommended by and with primers designed by Stockinger et al. (2014). The first PCR was performed with primers DR160fmix and HS2680GPr, and the second with HS189GPf and RPB1-DR1210r. Similarly, a nested PCR was used to obtain the *rpb1* sequences of *Diversispora* 3, *D. aurantia*, *D. insculpta*, *D. peloponnesiaca*, and *D. spurca*, with the newly designed primers RPB1-4F2/ RPB1-5R1 and RPB1-4F3/ RPB1-5R2.

Primer specificity and sequences, and PCR conditions are described in SUPPLEMENTARY TABLE 1. DreamTaq DNA Polymerase (Thermo Fisher) was used for the amplifications in 20 µl final volume according to the manufacturer's specifications, adding $MgCl_2$ 3 mM and BSA 0.5 µg $µl^{-1}$ as final concentrations only in the PCR on raw DNA. The thermal cycling was as follows: 5 min initial denaturation (95 C), 40 cycles (30 cycles in the nested PCR) of 30 s denaturation (95 C), 30 s annealing, 1 min 30 s (1 min in the nested PCR) elongation at 72 C, and 5 min (72 C) final elongation. Cloning and sequencing of the PCR products obtained were performed identically to those used to obtain 18S-ITS-28S sequences. Both 18S-ITS-28S and *rpb1* sequences were deposited in GenBank (18S-ITS-28S: MT724382–MT724385, MT725497–MT725502, OL684642–OL684648; *rpb1*: MT733211–MT733213, OL690405-OL690414.

Sequence alignment and phylogenetic analyses.—Three main sequence alignments were prepared. The first consisted of 18S-ITS-28S sequences of the three new species, all other sequenced species of *Diversispora* (21 species), and five species of four genera other than *Diversispora* in the Diversisporaceae, to serve as outgroup. The second alignment contained *rpb1* sequences available for the species of the first alignment. The alignment covered the partial cds of the fourth and fifth exons of the *rpb1* gene, and the intron between them. The two sequence sets (18S-ITS-28S and *rpb1*) were aligned separately with MAFFT 7 using the E-INS-I strategy. Indels were coded only for the 18S-ITS-28S set as binary characters by means of FastGap 1.2 (Borchsenius 2009), with the possibility to code missing data to be recognized by the phylogenetic inference programs. The binary character set was added to the respective nucleotide alignment, as described in Błaszkowski et al. (2014). The *rpb1* set was not considered for indel coding because of the limited number of gaps. The third alignment, 18S-ITS-28S+*rpb1*, was resulting from the concatenation of the previous two. Nine environmental sequences (KP756537, KP756538, HF970197, HF970225, JF439146,

JF439144, JF439145, HG425938, JN180910) were retrieved by a BLAST analysis as likely related to the three new *Diversispora* species (identity >97.3%). To verify their placement, an additional 18S-ITS-28S alignment with the environmental sequences was produced. The alignments and tree files are available as SUPPLEMENTARY FILES.

The percentage sequence divergences of the three new species from sequences of their closest relatives were calculated separately using BioEdit (Hall 1999). All comparisons were performed between sequences of the same length, i.e., the sequence fragments longer than the shortest compared sequence were cut off.

The reconstruction of the phylogenetic positions of the three new *Diversispora* species was performed based on Bayesian inference (BI) and maximum likelihood (ML) phylogenetic analyses of the 18S-ITS-28S and 18S-ITS-28S+*rpb1* alignments, performed via CIPRES Science Gateway 3.1 (Miller et al. 2010). To improve the accuracy of phylogenetic reconstruction (Lanfear et al. 2012; Nagy et al. 2012), in both BI and ML analyses, the 18S-ITS-28S alignment was divided into six partitions: 18S, ITS1, 5.8S, ITS2, 28S, and the binary (indel) character set. In BI and ML analyses of the 18S-ITS-28S+*rpb1* alignment, the sequence set was divided into eleven partitions: six and five for the 18S-ITS-28S and *rpb1* parts, respectively. In the *rpb1* part, for each exon separate partitions were applied for the first two and for the third codon positions; a single partition was applied to the intron.

GTR+I+G was chosen as nucleotide substitution model for each nucleotide partition in both BI and ML analyses as suggested by Abadi et al. (2019). Substitution models selected by ModelTest-NG 0.1.5 (Darriba et al. 2020) were also tested in the ML analysis but the trees obtained a final loglikelihood value lower compared to those where GTR+I+G was used. For the indel partition in BI analysis, F81 model was chosen as suggested in the MrBayes manual.

Four Markov chains were run over one million generations in MrBayes 3.2 (Ronquist et al. 2012), sampling every 1000 generations, with a burn-in at 3000 sampled trees. The ML

phylogenetic tree inference, using a maximum likelihood/1000 rapid bootstrapping run, was computed with RAxML 8.2.12 (Stamatakis 2014).

We assumed that clades were supported when BI posterior probability and ML bootstrap support values were ≥ 0.95 and ≥ 70%, respectively. To evaluate possible conflicts between the genes, the topologies of the ML trees (collapsed at bootstrap values <70%) were compared. In addition, the trees were compared based on three measures: (i) the number of species clades supported with BI ≥0.95 and ML ≥70%, (ii) mean supports of nodes with BI ≥0.95 and ML ≥70%, and (iii) the amount of resolution of each tree. Mean supports of nodes were the sums of BI ≥0.95 and ML ≥70% supports divided by the number of nodes with BI ≥0.95 and ML ≥70% present in each tree. The amount of resolution is the number of significantly supported internal branches divided by the size of the tree (number of nodes–2) when rooted (Thorley and Wilkinson 2000). The phylogenetic trees were visualized and edited in MEGA6 (Tamura et al. 2013).

RESULTS

General data and phylogeny.—In this study, 101 sequences of the 18S-ITS-28S region or part thereof (the 28S *D. celata* AY639225 sequence only) and 41 sequences of the *rpb1* gene were analyzed. Of these, 30 were new (17 18S-ITS-28S and 13 *rpb1*, including two of *D. spurca*, the type species of *Diversispora*; Walker and Schüßler 2004). The 18S-ITS-28S and 28S sequences represented 21, and those of *rpb1* 18 species of *Diversispora*, including our three new species. These sequences were part of two alignments (18S-ITS-28S, 18S-ITS-28S+*rpb1*) that were analyzed using BI and ML algorithms. The ratios of variable sites to the total number of characters in the 18S-ITS-28S and 18S-ITS-28S+*rpb1* alignments were 496/1647 and 502/2340, respectively, and the number of parsimony informative sites in these alignments were 399/1647 and 365/2340, respectively.

Two phylogenetic trees were obtained, here named 18S-ITS-28S and 18S-ITS-28S+*rpb1* (SUPPLEMENTARY FIG. 1 and FIG. 1, respectively). The topologies of the trees were identical, but clade supports were higher in the 18S-ITS-28S+*rpb1* tree (FIG. 1, SUPPLEMENTARY FIG. 1). Almost all species clades had full BI support in both trees (FIG. 1, SUPPLEMENTARY TABLE 2). The mean ML supports of species clades in the two trees also were very high, with a slight predominance of the support value in the 18S-ITS-28S+*rpb1* tree. The BI and ML resolution values were similar in both trees.

In both trees, the three new species were fully or strongly supported in both BI and ML analyses and the relationships of these species were identical: *D. aestuarii* and *D. varaderana* formed a sister relationship in a larger clade with *D. insculpta,* and *D. densissima* was sister to *D. marina* (FIG. 1, SUPPLEMENTARY FIG. 1). Also, the relationships of the other species present in the trees were identical. The differences between 18S-ITS-28S sequences of *D. densissima* vs. *D. marina*, *D. aestuarii* vs. *D. varaderana*, and *D. aestuarii* vs. *D. insculpta* were 3.6%, 3.7%, and 5.0%, respectively. In the same comparisons, *rpb1* sequences differed by 1.1%, 1.9%, and 2.0%, respectively, and 18S-ITS-28S+*rpb1* sequences by 5.0%, 3.0%, and 7.2%, respectively.

TAXONOMY

Description of new species

Diversispora densissima Błaszk., B.T. Goto, Niezgoda & Magurno, sp. nov. FIG. 2A–H

MycoBank: MB836243

Typification: POLAND: Spores from a single-species culture established from spores extracted from a trap culture inoculated with rhizosphere soil of *Ammophila arenaria* from the Baltic Sea dunes (53º55′03″N 14º17′39″E), 14 Aug 2012, *J. Błaszkowski* (**holotype**: Slide with spores Z+ZT Myc 61119, **isotypes**: slides with spores no. 3711–3721, LPPDSE).

Diagnosis: Differs from *D. marina*, the closest phylogenetic relative (FIG. 1, Supplementary FIG. 1), in spore color and size, the number and phenotypic properties of spore wall layers (Fig. 2A–H), as well as in nucleotide composition of sequences of the 18S-ITS-28S nuc rDNA region and the *rpb1* gene (see the Discussion section for details).

Etymology: *densissima* (Latin), referring to the thick spore wall of this species.

Description: Glomerospores (= spores) formed singly in soil (FIG. 2A). Spores arising blastically at tips of sporogenous hyphae (FIG. 2A, F–H). *Spores* pale orange (5A3) to light brown (6D8); globose to subglobose; (53–)80(–108) µm diam, very rarely slightly ovoid; 52–75 × 60–97 µm; with one subtending hypha (FIG. 2A–H). *Spore wall* composed of three permanent layers (FIG. 2B–H). Layer 1, forming the spore surface, flexible to semi-flexible, smooth, pale yellow (3A3) to light brown (6D8), (1.0–)1.3(–1.8) µm thick (FIG. 2B–H). Layer 2 laminate, semi-flexible, pale orange (5A3) to light brown (6D8), (4.8–)8.3(–11.5) µm thick (FIG. 2B–H). Layer 3 uniform, flexible to semi-flexible, hyaline, (0.8–)1.0(–1.3) µm thick, usually tightly adherent to lower surface of layer 2, occasionally separating from this layer in vigorously crushed spores (FIG. 2B–H). Layers 1–3 do not stain in Melzer's reagent (FIG. 2H). *Subtending hypha* yellowish white (2A4) to greyish yellow (3B4); straight or recurved, cylindrical or slightly funnel-shaped, rarely slightly constricted at the spore base; (6.0–)7.9(–12.5) µm wide at the spore base (FIG. 2A, F–H); robust, not breaking in crushed spores. *Wall of subtending hypha* yellowish white (2A4) to greyish yellow (3B4); (1.5–)2.5(–3.7) µm thick at the spore base; consisting of two layers continuous with spore wall layers 1 and 2 (FIG. 2F–H). *Pore* (1.8–)2.2(–4.8) µm wide at the spore base, occluded by a straight or slightly curved septum continuous with a few innermost laminae of spore wall layer 2 and spore wall layer 3; septum 1.8–4.8 µm wide, 1.0–1.4 µm thick, positioned at or slightly above the spore base (FIG. 2H). *Germination* unknown.

Ecology and distribution: Associated in symbiosis with *Ammophila arenaria* in dunes of the Baltic Sea in north-western Poland, forming mycorrhiza with arbuscules, vesicles, and intraradical and extraradical hyphae in single-species cultures with *Plantago lanceolata* as the host (structures stained pale violet (16A3) to deep violet (16E8) in 0.1% Trypan blue). According to BLAST and phylogenetic analysis, environmental 18S-ITS-28S sequences with identity ≥ 97% and clustering inside the *D. densissima* clade (Supplementary FIG. 2) were obtained in China (JF439144–6 sequences), Czech Republic (HG425938), and Peruvian Andes (HF970197, HF970225).

Diversispora marina Błaszk., B.T. Goto, Niezgoda & Magurno, sp. nov. FIG. 3A–H

MycoBank: MB836242

Typification: POLAND. Spores from a single-species culture established from spores extracted from a trap culture inoculated with rhizosphere soil of *Agrostis stolonifera* from the 12 deflation pan of the Baltic Sea dunes (54º38′–54º46′N 17º03′–17º33′E), 2 Sep 2013, *G. Chwat* (**holotype**: Slide with spores Z+ZT Myc 61118, **isotypes**: slides with spores no. 3700–3710, LPPDSE).

Diagnosis: Differs from *D. densissima*, the closest phylogenetic relative (FIG. 1, Supplementary FIG. 1), in spore color and size, the number and phenotypic properties of spore wall layers (FIG. 3A–H), as well as in nucleotide composition of sequences of the 18S-ITS-28S nuc rDNA region and the *rpb1* gene (see the Discussion section for details).

Etymology: *marina* (Latin), referring to the coastal habitat, in which this species was originally found.

Description: Glomerospores (= spores) formed mainly singly in soil (FIG. 3A) and frequently inside roots (Fig. 3B). Spores arising blastically at tips of sporogenous hyphae (FIG. 3G, H). *Spores* pale yellow (3A3–4A3); globose to subglobose; (50–)66(–82) µm diam,

297 frequently ovoid; 38–65 × 52–77 µm; with one subtending hypha (FIG. 3A–H). *Spore wall*
298 composed of four layers (FIG. 3C–H). Layer 1, forming the spore surface, evanescent,
299 flexible, smooth in young spores, becoming roughened with age, usually completely sloughed
300 off in older spores, hyaline to yellowish white (4A2), (1.0–)1.2(–1.4) µm thick when intact
301 (FIG. 3C–H). Layer 2 permanent, uniform (without visible sublayers), semi-flexible, pale
302 yellow (3A3) to brownish yellow (5C5), (0.8–)1.1(–1.6) µm thick, occasionally separating
303 from upper surface of layer 3 (FIG. 3C–H). Layer 3 permanent, laminate, semi-flexible,
304 hyaline to pale yellow (4A3), (2.8–)4.0(–7.4) µm thick (FIG. 3C–H). Layer 4 permanent,
305 uniform, flexible, hyaline, 0.8–1.2 µm thick, usually tightly adherent to lower surface of layer
306 3, occasionally separating from this layer in vigorously crushed spores (FIG. 3C–H),
307 generally difficult to observe. Layers 1–4 do not stain in Melzer's reagent (FIG. 3D–F, H).
308 *Subtending hypha* hyaline to pale yellow (3A3–4A3) near the spore base, hyaline below the
309 pigmented portion; straight or recurved, cylindrical or slightly funnel-shaped, rarely slightly
310 constricted at the spore base; (4.8–)6.0(–9.6) µm wide at the spore base (FIG. 3G, H); not
311 breaking in crushed spores. *Wall of subtending hypha* hyaline to pale yellow (3A3–4A3); its
312 pigmented part extends up to 4.4 µm below the spore base, then it becomes hyaline; (1.2–
313)1.7(–2.2) µm thick at the spore base; consisting of four layers continuous with spore wall
314 layers 1–4; subtending hyphal wall layer (shwl) 1 usually highly deteriorated or completely
315 sloughed off in most mature spores; shwl 4 usually adherent to inner surface of a subtending
316 hyphal septum, even in vigorously crushed spores, and, therefore, difficult to see (FIG. 3G,
317 H). *Pore* (2.4–)4.0(–8.4) µm wide at the spore base, occluded by a straight or curved septum
318 continuous with a few innermost laminae of spore wall layer 3 and spore wall layer 4; septum
319 2.0–3.1 µm wide, 1.0–1.2 µm thick, positioned at or up to 4.4 µm below the spore base (FIG.
320 3H). *Germination* unknown.

Ecology and distribution: Associated in symbiosis with *Ammophila arenaria* in dunes of the Baltic Sea in north-western Poland, forming mycorrhiza with arbuscules, vesicles, and intraradical and extraradical hyphae in single-species cultures with *Plantago lanceolata* as the host (structures stained pale violet (16A3) to deep violet (16E8) in 0.1% Trypan blue). According to BLAST and phylogenetic analysis, environmental 18S-ITS-28S sequences with identity ≥ 98% and forming a supported cluster with *D. marina* sequences (Supplementary FIG. 2) were obtained from roots of *Picconia azorica* (Tutin) Knobl. in native forests of Azores (KP756537–8 sequences).

Diversispora aestuarii Błaszk., B.T. Goto, Niezgoda & Magurno, sp. nov. FIG. 4A–H

MycoBank: MB840934

Typification: POLAND: Spores from a single-species culture established from spores extracted from a trap culture inoculated with rhizosphere soil of *Ammophila arenaria* from the Baltic Sea dunes (53º55′03″N 14º17′39″E), 19 Aug 2013, *J. Błaszkowski* (**holotype**: Slide with spores Z+ZT Myc 66294, **isotypes**: slides with spores no. 3822–3838, LPPDSE).

Diagnosis: Differs from *D. varaderana*, the closest phylogenetic relative (FIG. 1, Supplementary FIG. 1), in the number of spore wall layers, the phenotypic properties of the spore wall layer forming the spore surface, the spore size and morphology of the spore subtending hypha, as well as in nucleotide composition of sequences of the 18S-ITS-28S nuc rDNA region and the *rpb1* gene (see the Discussion section for details).

Etymology: *aestuarii* (Latin), referring to the city of Świnoujście (= river mount), near which this species was originally found.

Description: Glomerospores (= spores) formed singly in soil (FIG. 4A). Spores arising blastically at tips of sporogenous hyphae (FIG. 4A, G, H). *Spores* pale yellow (3A3) to yellowish brown (5E8); globose to subglobose; (77–)109(–135) µm diam, rarely slightly

ovoid; 69–130 × 87–139 µm; with one subtending hypha (FIG. 4A–H). *Spore wall* composed of four layers (FIG. 4B–H). Layer 1, forming the spore surface, semi-permanent, flexible to semi-flexible, smooth, yellowish white (3A2) to greyish yellow (3B5), (1.2–)3.4(–7.0) µm thick, usually easily separating from layer 2 in crushed spores (FIG. 4B–H); this layer does not slough completely off in even old spores; sometimes, it is uneven in thickness and locally more or less sloughed off, thereby wavy when observed in a cross view (FIG. 4B, C). Layer 2 permanent, flexible to semi-flexible, smooth, hyaline to pale yellow (3A3), (0.8–)1.8(–2.6) µm thick, always tightly adherent to layer 3 (FIG. 4B–H). Layer 3 permanent, laminate, semi-flexible, smooth, pale yellow (3A3) to yellowish brown (5E8), (3.5–)5.8(–9.0) µm thick, composed of thin, <0.5 µm thick, tightly adherent sublayers, not separating from each other in even vigorously crushed spores (FIG. 4B–H). Layer 4 permanent, flexible, smooth, hyaline, 0.8–1.1 µm thick, usually separating from lower surface of the laminate layer 3 in even moderately crushed spores; probably beginning developing along inner surface of the laminate layer 3, at the spore base, forming the lumen connecting the subtending hypha with the spore interior. Layers 1–4 do not stain in Melzer's reagent (FIG. 4H). *Subtending hypha* hyaline to greyish yellow (3B5); straight or recurved, cylindrical or constricted, rarely slightly funnel-shaped at the spore base; (5.2–)8.0(–12.2) µm wide at the spore base (FIG. 4A, G, H), sometimes breaking at the spore base in crushed spores. *Wall of subtending hypha* hyaline to greyish yellow (3B5); (1.2–)2.7(–4.6) µm thick at the spore base; consisting of two or three layers continuous with spore wall layers 1 and 3 or 1–3 (FIG. 4G, H); subtending hyphal wall layer (shwl) 1 usually highly or completely sloughed off in mature spores; shwl2, if occurs, present only directly at the spore base; shwl3 hyaline, fragile, sometimes detaching from spores during crushing. *Pore* (1.6–)2.6(–6.0) µm wide at the spore base, occluded by a curved septum continuous with spore wall layer 4; septum 1.6–5.9 µm wide, 0.9–1.2 µm thick, positioned at or up to 5.2 µm below the spore base (FIG. 4G, H). *Germination* unknown.

Ecology and distribution: Associated in symbiosis with *Ammophila arenaria* in dunes of the Baltic Sea in north-western Poland, forming mycorrhiza with arbuscules, vesicles, and intraradical and extraradical hyphae in single-species cultures with *Plantago lanceolata* as the host (structures stained violet white (16A2) to deep violet (16D8) in 0.1% Trypan blue). Through a BLAST querying, it was possible to detect one environmental 28S sequence (JN180910) with identity >98% with the sequences of *D. aestuarii* and clustering inside the species clade (Supplementary FIG. 2). The environmental study was conducted in rangelands around the Missoula and Bitterroot Valleys of western Montana, USA.

DISCUSSION

The morphological and molecular phylogenetic analyses described above (*i*) confirmed our hypotheses that the three glomoid spore-producing morphotypes of AMF found in maritime sand dunes of Poland were new species of *Diversispora* (FIG. 1, SUPPLEMENTARY FIG. 1), (*ii*) showed that two of these new species, described here as *D. densissima* and *D. marina*, linked a sister relationship, and the closest relatives of the third new species, *D. aestuarii*, were *D. varaderana* and *D. insculpta*, and (*iii*) indicated that BI and ML analyses of two concatenated unlinked loci (18S-ITS-28S and *rpb1*) improved the robustness of the phylogenetic estimate for *Diversispora* compared to BI and ML analyses of the two individual gene regions, but the improvements were slight (SUPPLEMENTARY TABLE 2).

Diversispora densissima and *D. marina* differ clearly in many morphological characters. The spore wall of *D. densissima* consists of three layers (FIGS. 2C–H), lacking spore wall layer 2 present in the four-layered spore wall of *D. marina* (FIGS. 3B–H). Spore wall layer 1 of *D. densissima* is a permanent structure and was present intact in all examined spores (FIGS. 2B–H), whereas spore wall layer 1 of *D. marina* is short-lived and usually highly deteriorated in mature spores (FIGS. 3E–G) and completely sloughed off in older spores. In addition, the spore wall of *D. densissima* is ca. 1.3-fold thicker, the spores are

clearly darker, and up to 1.3-fold larger when globose (FIGS. 2A–H, 3A–H). Finally, the subtending hypha of *D. densissima* is ca. 1.3-fold wider, has a 1.3–1.6-fold thicker wall, but the pore of the subtending hypha is 1.3–1.8-fold narrower.

Many morphological characters also strongly separate *D. aestuarii* from *D. varaderana* and *D. insculpta*. The spore wall of *D. varaderana* is 1.3–2.1-fold thinner and consists of only two layers (Błaszkowski et al. 2015b), lacking the permanent spore wall layers 2 and 4 of *D. aestuarii* (FIG. 4B–H). In contrast to spore wall layer 1 of *D. aestuarii*, which is always present in even old specimens (FIG. 4B–H), spore wall layer 1 of *D. varaderana* is a short-lived structure that usually is strongly or completely sloughed off in mature spores. Finally, spores of *D. varaderana* are 1.3–1.5-fold smaller when globose, the subtending hypha is 1.2–1.4-fold narrower, may have a 1.2–1.8-fold thinner wall, and has a 1.2–2.4-fold narrower pore.

Spores of *D. insculpta* are ca. 1.6-fold smaller when globose, their spore wall is 1.3–2.4-fold thinner and consists of only two layers (Błaszkowski et al. 2004a; Błaszkowski 2012). Both spore wall layers are permanent and each of them is of equal thickness when observed in a cross view. In addition, the spore subtending hypha of *D. insculpta* is 1.2–1.6-fold narrower and has a wall 1.2–1.5-fold thinner at the spore base.

Results from our analyses indicated that *D. densissima*, *D. marina*, and *D. aestuarii* differ clearly from their closest relatives also at the molecular level. The calculated mean sequence divergences (see "*General data and phylogeny*") exceeded the widely accepted thresholds of conspecificity, i.e., 97% and ca. 99.0% for 18S-ITS-28S and *rpb1* sequences, respectively (Corazon-Guivin et al. 2019; Stockinger et al. 2014). Even though these thresholds are working well in most cases, caution should be used to overcome the risk of confusing species isolates as different species. Some species were proven to host an extremely high genetic variability, e.g., *Rhizoglomus irregulare* (Chen et al. 2018), that can exceed the

boundaries accepted for species delimitation. In the genus *Diversispora*, the intraspecific genetic dissimilarity of nrDNA sequences can reach almost 5% in *D. spurca*, while several neighboring species are sharing dissimilarity values lower than 3% (pers. observ.), e.g., *D. peloponnesiaca* vs. *D. clara*, which differ fundamentally in morphology (Estrada et al. 2011; Błaszkowski et al. 2019). For these reasons, when describing a new species, molecular data should always be supported by phylogenetic and morphological analyses.

When originally erected based on molecular phylogenetic analyses, the genus *Diversispora* contained only *D. spurca* (Walker and Schüßler 2004), originally described as *G. spurcum* (Pfeiffer et al. 1996). Later, Gamper et al. (2009) described *D. celata* and their molecular phylogenetic analyses suggested that *G. aurantium*, *G. eburneum*, and the fungus named *G. versiforme* BEG47 were also members of *Diversispora*. Schüßler et al. (2011) proved that *G. versiforme* BEG47 was phylogenetically conspecific with *G. epigaeum*, which along with *G. trimurales* also belong to *Diversispora*. In addition, these researchers concluded that *G. versiforme*, originally described as *Endogone versiformis* (Karsten 1884), was an autonomic taxon, which, despite the morphological similarity to *Diversispora* species, must be considered as a species of uncertain position in *Glomus* sensu lato because of the lack of molecular evidence.

The literature references (Balázs et al. 2015; Błaszkowski 1997; Błaszkowski et al. 2001, 2004a, b, 2015b, 2019; Estrada et al. 2011; Haug et al. 2021; Jobim et al. 2019; Oehl et al. 2011; Symanczik et al. 2014, 2018) demonstrated that *Diversispora* has a worldwide distribution and probably many species of this genus are waiting to be discovered and characterized. This strongly supports the conclusions of Gamper et al. (2009) and Schüßler et al. (2011), who found the presence of members of *Diversispora* in 21 countries of the world.

The wide distribution, as well as the ease of growing *Diversispora* species in culture (pers. observ.) prove the high ecological plasticity of this group of fungi. This property should

be used in choice of AMF to produce inocula intended for the use in practice, knowing the various helpful effects of AMF on plants and environments (Smith and Read 1997). The choice of functionally effective species for such inocula may be done only when tested AMF may be unambiguously identified. We believe that our work will facilitate further understanding of the morphological and molecular diversity of *Diversispora* fungi and will contribute to the expected shaping of their presence in various environments.

ACKNOWLEDGMENTS

Part of this work was supported by Polish National Centre of Science, grant no. 2020/37/N/NZ9/00509, as well as by Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq) that provided research grants to BT Goto (proc. 311945/2019-8). We also thank Universidade Federal do Rio Grande do Norte for covering the costs of B.T. Goto's stay as collaborative research in West Pomeranian University of Technology in Szczecin in the period from December 2019 to January 2020. The research was funded, in part, by the Institute of Botany at the Jagiellonian University, project no. N18/DBS/000002. We thank the associate editor and the anonymous reviewers of the manuscript for their useful comments.

ORCID

Janusz Błaszkowski http://orcid.org/0000-0003-3688-164X

Bruno Tomio Goto https://orcid.org/0000-0001-6157-4954

Wojciech Bierza https://orcid.org/0000-0002-1789-5512

Franco Magurno http://orcid.org/0000-0002-3117-8149

Monika Malicka https://orcid.org/0000-0002-1034-0995

Paweł Milczarski https://orcid.org/0000-0003-2622-8938

Szymon Zubek https://orcid.org/0000-0003-1338-9572

LITERATURE CITED

Abadi S, Azouri D, Pupko T, Mayrose I. 2019. Model selection may not be a mandatory step for phylogeny reconstruction. Nature Communications 10:934.

Al-Yahya'ei MN, Mullath SK, AlDhaheri LA, Kozłowska A, Błaszkowski J. 2017. *Dominikia emiratia* and *Rhizoglomus dunense*, two new species in the Glomeromycota. Botany 95:629–639.

Alves-Silva G, Drechler-Santos E R, Silveira R M B. 2020. Bambusicolous revisited: multilocus phylogeny reveals a clade of host-exclusive species. Mycologia 112:1–16.

Balázs TK, Błaszkowski J, Chwat G, Góralska A, Gaspar BK, Lukacs AF, Kovács GM. 2015. Spore-based study of arbuscular mycorrhizal fungi of semiarid sandy areas in Hungary, with *Diversispora jakucsiae* sp. nov. Mycological Progress 14:1021.

Becker WN, Gerdemann JW. 1977. *Glomus etunicatus* sp. nov. Mycotaxon 6:29–32.

Błaszkowski J. 1997. *Glomus gibbosum*, a new species from Poland. Mycologia 89:339–345.

Błaszkowski J. 2012. Glomeromycota. Kraków, Poland: W. Szafer Institute of Botany, Polish Academy of Sciences. 303 p.

Błaszkowski J, Adamska I, Czerniawska B. 2004a. *Glomus insculptum*, a new arbuscular mycorrhizal species from Poland. Mycotaxon 89:225–234.

494 Błaszkowski J., Blanke V., Renker C., Buscot F. 2004b. *Glomus aurantium* and *G. xanthium,*
495 new species in Glomeromycota. Mycotaxon 90:447–467.

496

497 Błaszkowski J, Chwat G, Góralska A. 2015a. *Acaulospora ignota* and *Claroideoglomus*
498 *hanlinii*, two new species of arbuscular mycorrhizal fungi (Glomeromycota) from Brazil and
499 Cuba. Mycological Progress 14:18.

500

501 Błaszkowski J, Chwat G, Góralska A, Ryszka P, Orfanoudakis M. 2014. *Septoglomus*
502 *jasnowskae* and *Septoglomus turnauae*, two new species of arbuscular mycorrhizal fungi
503 (Glomeromycota). Mycological Progress 13:999–1009.

504

505 Błaszkowski J, Furrazola E, Chwat G, Góralska A, Lukács AL, Kovács GM. 2015b. Three
506 new arbuscular mycorrhizal *Diversispora* species in Glomeromycota. Mycological Progress
507 14:105.

508

509 Błaszkowski J, Jobim K, Niezgoda P, Meller E, Malinowski M, Milczarski P, Zubek S,
510 Magurno F, Casieri L, Bierza W, Błaszkowski T, Crossay T, Goto BT. 2021a. New
511 glomeromycotan taxa, *Dominikia glomerocarpica* sp. nov. and *Epigeocarpum crypticum* gen.
512 nov. et sp. nov. from Brazil, and *Silvaspora* gen. nov. from New Caledonia. Frontiers in
513 Microbiology 12:655910.

514

515 Błaszkowski J, Kovács GM, Gáspár BK, Balázs TK, Buscot F, Ryszka P. 2012. The
516 arbuscular mycorrhizal *Paraglomus majewskii* sp. nov. represents a new distinct basal lineage
517 in Paraglomeraceae (Glomeromycota). Mycologia 104:148–156.

518

519 Błaszkowski J, Niezgoda P, Meller E, Milczarski P, Zubek S, Malicka M, Uszok S, Casieri L,
520 Goto BT, Magurno F. 2021b. New taxa in Glomeromycota: Polonosporaceae fam. nov.,
521 *Polonospora* gen. nov., and *P. polonica* comb. nov. Mycological Progress 20:941–951.
522
523 Błaszkowski J, Niezgoda P, de Paiva JN, da Silva KJG, Theodoro RC, Jobim K,
524 Orfanoudakis M, Goto BT. 2019. *Sieverdingia* gen. nov., *S. tortuosa* comb. nov., and
525 *Diversispora peloponnesiaca* sp. nov. in the Diversisporaceae (Glomeromycota). Mycological
526 Progress 18:1363–1382.
527
528 Błaszkowski J, Tadych M, Madej T. 2001. *Glomus arenarium*, a new species in Glomales
529 (Zygomycetes). Acta Societatis Botanicorum Poloniae 70:97–101.
530
531 Borchsenius F. 2009. FastGap 1.2. Department of Biosciences, Aarhus University, Denmark.
532
533 Chen ECH, Morin E, Beaudet D, Noel J, Yildirir G, Ndikumana S, Charron P, St-Onge C,
534 Giorgi J, Krüger M, Marton T, Ropars J, Grigoriev IV, Hainaut M, Henrissat B, Roux C,
535 Martin F, Corradi N. 2018. High intraspecific genome diversity in the model arbuscular
536 mycorrhizal symbiont *Rhizophagus irregularis*. New Phytol 220:1161–1171.
537
538 Clements FE, Shear CL. 1931. e genera of fungi. Hafner Publishing Co., New York, USA.
539
540 Corazon-Guivin MA, Cerna-Mendoza A, Guerrero-Abad JC, Vallejos-Tapullima A,
541 Carballar-Hernández S, da Silva GA, Oehl F. 2019. *Nanoglomus plukenetiae*, a new fungus
542 from Peru, and a key to small-spored Glomeraceae species, including three new genera in the
543 "*Dominikia* complex/clades". Mycological Progress 18:1395–1409.

Darriba D, Posada D, Kozlov AM, Stamatakis A, Morel B, Flouri T. 2020. ModelTest-NG: a new and scalable tool for the selection of DNA and protein evolutionary models. Molecular Biology and Evolution 37:291–294.

Estrada B, Palenzuela J, Barea J-M, Ruiz-Lozano JM, da Silva GA, Oehl F. 2011. *Diversispora clara* (*Glomeromycetes*) - a new species from saline dunes in the Natural Park Cabo de Gata (Spain). Mycotaxon 118:73–81.

Gamper HA, Walker C, Schüßler A. 2009. *Diversispora celata* sp. nov: molecular ecology and phylotaxonomy of an inconspicuous arbuscular mycorrhizal fungus. New Phytologist 182:495–506.

Goto BT, Maia LC. 2006. Glomerospores: a new denomination for the spore of Glomeromycota, a group molecularly distinct from the Zygomycota. Mycotaxon 96:129–132.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41:95–98.

Haug I, Setaro S, Suárez JP. 2021. Global AM fungi are dominating mycorrhizal communities in a tropical premontane dry forest in Laipuna, South Ecuador. Mycological Progress 20:837–845.

Jobim K, Błaszkowski J, Niezgoda P, Kozłowska A, Zubek S, Mleczko P, Chachuła P, Ishikawa NK, Goto BT. 2019. New sporocarpic taxa in the phylum Glomeromycota:

Sclerocarpum amazonicum gen. et sp. nov. in the family Glomeraceae (Glomerales) and *Diversispora sporocarpia* sp. nov. in the Diversisporaceae (Diversisporales). Mycological Progress 18:369–384.

Karsten PA. 1884. Fragmenta mycologica. Hedwigia 23:39–40.

Kohout P, Sudová R, Janoušková M, Čtvrtlíková M, Hejda M, Pánková H, Slavíková R, Stajerová K, Vosátka M, Sýkorová Z. 2014. Comparison of commonly used primer sets for evaluating arbuscular mycorrhizal fungal communities: Is there a universal solution? Soil Biology & Biochemistry 68:482–493.

Kornerup A, Wanscher JH. 1983. Methuen handbook of colour. 3rd ed. London: Eyre Methuen. 252 p.

Krüger M, Krüger C, Walker C, Stockinger H, Schüßler A. 2012. Phylogenetic reference data for systematics and phylotaxonomy of arbuscular mycorrhizal fungi from phylum to species-level. New Phytologist 193:970–984.

Krüger M, Stockinger H, Krüger C, Schüßler A. 2009. DNA-based level detection of Glomeromycota: one PCR primer set for all arbuscular mycorrhizal fungi. New Phytologist 183:212–223.

Lanfear R, Calcott B, Ho SYW, Guindon S. 2012. PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. Molecular Biology and Evolution 29:1695–1701.

Maia LC, Passos J H, Silva J A, Oehl F, Assis DMA. 2020. Species diversity of Glomeromycota in Brazilian biomes. Nova Hedwigia 72:181–205.

Marinho F, Silva IR, Oehl F, Maia LC. 2018. Checklist of arbuscular mycorrhizal fungi in tropical forests. Sydowia 70:107–127.

Matheny PB. 2005. Improving phylogenetic inference of mushrooms with RPB1 and RPB2 nucleotide sequences (*Inocybe*; Agaricales). Molecular Phylogenetics and Evolution 35:1–20.

Miadlikowska J, Kauff F, Hofstetter V, Fraker E, Grube M, Hafellner J, Reeb V, Hodkinson BP, Kukwa M, Lücking R, Hestmark G, Otalora MG, Rauhut A, Büdel B, Scheidegger C, Timdal E, Perlmutter GB, Ertz D, Diederich P, Lendemer JC, May P, Schoch CL, Arnold AE, Gueidan C, Tripp E, Yahr R, Robertson C, Lutzoni F. 2006. New insights into classification and evolution of the Lecanoromycetes (Pezizomycotina, Ascomycota) from phylogenetic analyses of three ribosomal RNA- and two protein-coding genes. Mycologia 98:1088–1103.

Miadlikowska J, Kauff F, Högnabba F, Oliver JC, Molnár K, Fraker E, Gaya E, Hafellner J, Hofstetter V, Gueidan C, Otálora MAG, Hodkinson B, Kukwa M, Lücking R, Björk C, Sipman HJM, Burgaz AR, Thell A, Passo A, Myllys L, Goward T, Fernández-Brimem S, Hestmark G, Lendemer J, Lumbsch HT, Schmull M, Schoch CL, Sérusiaux E, Maddison DR, Arnold AE, Lutzoni F, Stenroos S. 2014. A multigene phylogenetic synthesis for the class Lecanoromycetes (Ascomycota): 1307 fungi representing 1139 infrageneric taxa, 317 genera and 66 families. Molecular Phylogenetics and Evolution 79:132–168.

Miller MA, Pfeiffer W, Schwartz T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. 2010 Gateway Computing Environments Workshop (GCE), New Orleans, LA, USA. p. 1–8.

Morton JB, Redecker D. 2001. Two families of Glomales, Archaeosporaceae and Paraglomaceae, with two new genera *Archaeospora* and *Paraglomus*, based on concordant molecular and morphological characters. Mycologia 93:181–195.

Nagy LG, Kocsube S, Csana Z, Kovacs GM, Petkovits T, Lgyi CV, Papp T. 2012. Re-mind the gap! insertion – deletion data reveal neglected phylogenetic potential of the nuclear ribosomal internal transcribed spacer (ITS) of fungi. PLoS ONE 7:e49794.

Oehl F, da Silva GA, Goto BT, Sieverding E. 2011. Glomeromycota: three new genera and glomoid species reorganized. Mycotaxon 116:75–120.

Omar MB, Bollan L, Heather WA. 1979. A permanent mounting medium for fungi. Bulletin of the British Mycological Society 13:31–32.

Pfeiffer CM, Walker C, Bloss HE. 1996. *Glomus spurcum*: a new endomycorrhizal fungus from Arizona. Mycotaxon 59:373–382.

Polo-Marcial MH, Lara-Pérez LA, Goto BT, Margarito-Vista X, Andrade-Torres A. 2021. Glomeromycota in Mexico: a country with very high richness. Sydowia 74:33–63.

643 Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L,
644 Suchard MA, Huelsenbeck JP. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference
645 and model choice across a large model space. Systematic Biology 61:539–42.
646
647 Salvador-Montoya CA, Popoff O, Góes-Neto, AR, Drechsler-Santos ER. 2020. Global
648 phylogenetic and morphological reassessment of *Fomitiporella* s.l. (Hymenochaetales,
649 Basidiomycota): taxonomic delimitation of *Fomitiporella* s.s. and segregation of
650 Rajchenbergia, gen. nov. Plant Systematics and Evolution 306:34.
651
652 Schüßler A, Krüger M, Walker C. 2011. Revealing natural relationships among arbuscular
653 mycorrhizal fungi: culture line BEG47 represents *Diversispora epigaea*, not *Glomus*
654 *versiforme*. PLoS ONE 6(8), e23333.
655
656 Schüßler A, Walker C. 2010. The Glomeromycota. A species list with new families and new
657 genera. Edinburgh, UK: Royal Botanic Garden Edinburgh. 56 p.
658
659 Smith SE, Read DJ. 1997. Mycorhizal symbiosis. Academic Press. Harcourt Brace &
660 Company, Publishers. San Diego, London, New York, Boston, Sydney, Tokyo, Toronto.
661
662 Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of
663 large phylogenies. Bioinformatics 30:1312–1313.
664
665 Stockinger H, Peyret-Guzzon M, Koegel S, Bouffaud M-L, Redecker D. 2014. The Largest
666 Subunit of RNA Polymerase II as a New Marker Gene to Study Assemblages of Arbuscular
667 Mycorrhizal Fungi in the Field. PLoS ONE 9:e107783.

Symanczik S, Al-Yahya'ei MN, Kozłowska A, Ryszka P, Błaszkowski J. 2018. A new genus, *Desertispora*, and a new species, *Diversispora sabulosa*, in the family Diversisporaceae (order Diversisporales, subphylum Glomeromycotina). Mycological Progress 17:437–449.

Symanczik S, Błaszkowski J, Chwat G, Boller T, Wiemken A, Al-Yahya'ei MN. 2014. Three new species of arbuscular mycorrhizal fungi discovered at one location in a desert of Oman: *Diversispora omaniana*, *Septoglomus nakheelum* and *Rhizophagus arabicus*. Mycologia 106:243–259.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30:2725–2729.

Thorley JL, Wilkinson M. 2000. The RadCon Manual 1.1.2. Bristol University, UK.

Walker C. 1983. Taxonomic concepts in the Endogonaceae: spore wall characteristics in species descriptions. Mycotaxon 18:443–455.

Walker C, Schüßler A. 2004. Nomenclatural clarifications and new taxa in the Glomeromycota. Mycological Research 108:979–982.

Wijayawardene NN, Hyde KD, Al-Ani LKT et al. 2020. Outline of fungi and fungus-like taxa. Mycosphere 11:1060–1456.

List of figures

Figure 1. 50% majority-rule consensus tree from the Bayesian analysis of 18S-ITS-28S+*rpb1* sequences of *Diversispora aestuarii*, *D. densissima* and *D. marina*, 18 other species of *Diversispora*, and five species from four genera other than *Diversispora* in the Diversisporaceae to serve as outgroup. The Bayesian posterior probabilities ≥0.50 and ML bootstrap values ≥50% are shown near the branches, respectively. Bar indicates 0.05 expected change per site per branch.

Figure 2. *Diversispora densissima*. A. Intact spores. B–E. Spore wall layers (swl) 1–3. F–H. Subtending hyphal wall layers (shwl) 1 and 2 continuous with spore wall layers (swl) 1 and 2; swl 3 is also visible; note the robust subtending hypha; a septum (s) closing the pore connecting the subtending hyphal lumen with the spore interior is visible in H. A–G. Spores in PVLG. H. Spore in PVLG+Melzer's reagent. A–H. Differential interference microscopy. Scale bars: A = 20 μm, B–H = 10 μm.

Figure 3. *Diversispora marina*. A. Intact spores. B. Intraradical spores (is). C–F. Spore wall layers (swl) 1–4. G. Spore wall layers (swl) and subtending hypha (sh). H. Subtending hyphal wall layers (shwl) 1–4 continuous with spore wall layers (swl) 1–4. A–C, G. Spores in PVLG. D–F, H. Spores in PVLG+Melzer's reagent. A–H. Differential interference microscopy. Scale bars: A = 50 μm, B = 20 μm, C–H = 10 μm.

Figure 4. *Diversispora aestuarii*. A. Intact spores (sp) with one visible subtending hypha (sh). B–F. Spore wall layers (swl) 1–4; note that swl1 is partly more or less deteriorated (a semi-permanent layer), and swl2, 3, and 4 are permanent. G, H. Subtending hyphae with

718 subtending hyphal wall layers (shwl) 1–3 continuous with spore wall layers (swl) 1–3; shwl 2
719 and 3 are present only closely at the spore base in FIG. 4H; a curved septum (s) closing the
720 pore connecting the subtending hyphal lumen with the spore interior is visible in H. A, C, H.
721 Spores in PVLG. B, D–G. Spores in PVLG+Melzer's reagent. A–H. Differential interference
722 microscopy. Scale bars: A = 50 µm, B–H = 10 µm.
723
724 *Corresponding address: Janusz Błaszkowski; e-mail: janusz.blaszkowski@zut.edu.pl
725