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ORIGINAL ARTICLE

Three new arbuscular mycorrhizal *Diversispora* species in Glomeromycota

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Abstract Morphological observations of spores and mycorrhizal structures of three arbuscular mycorrhizal fungi (Glomeromycota) prompted, and subsequent phylogenetic analyses of SSU–ITS–LSU nrDNA sequences confirmed, that they are undescribed species of the genus *Diversispora*. Morphologically, the first species, here named *D. varaderana*, is most distinguished by its relatively small (≤90 μm diam when globose) and yellow-coloured spores with a simple spore wall consisting of two layers, of which layer 1, forming the spore surface, is short-lived and usually completely sloughed in most spores. The distinctive features of the second species, *D. peridiata*, are the occasional formation of spores in clusters and peridium-like hyphae covering the clusters and single spores, and especially the permanent and relatively thick spore wall layer 1, which is the only coloured component of the two-layered spore wall of the yellow-coloured and relatively small spores (≤100 μm diam). The third species, *D. slowinskiensis*, is most characterized by its spore wall layer 1 that is the only coloured component of the three-layered spore wall and frequently is covered with blister-like swellings. All the three species were grown in single-species cultures established from spores extracted from trap cultures inoculated with rhizosphere soils of plants growing in maritime sand dunes: *D. varaderana* from those located near Varadero on the Hicacos Peninsula, Cuba, and the two others from those of the Słowiński National Park, northern Poland.

Keywords Diversisporaceae · *Diversispora varaderana* sp. nov · *D. peridiata* sp. nov · *D. slowinskiensis* sp. nov · Molecular phylogeny · Morphology

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Introduction

The genus *Diversispora* C. Walker & A. Schüssler of the family Diversisporaceae C. Walker & A. Schüssler and the order Diversisporales C. Walker & A. Schüssler belongs to the phylum Glomeromycota C. Walker & A. Schüssler that comprises arbuscular mycorrhizal fungi (AMF) (Schüßler et al. 2001). These fungi have a world-wide distribution and form symbiosis with ca. 70–90 % of vascular land plants (Smith and Read 2008; van der Heijden et al. 2015) and some plants whose roots are permanently flooded (Sudová et al. 2015).

The Diversisporales and Diversisporaceae were originally designated in the Glomeromycota based on phylogenetic analyses of sequences of the small subunit (SSU) rRNA gene of three former *Glomus* spp. (Schüßler et al. 2001), but validly published 3 years later (Walker and Schüßler 2004). At that time, the Diversisporaceae contained only the genus *Diversispora* with the type species *D. spurca* (C.M. Pfeiff., C. Walker & Bloss) C. Walker & A. Schüssler. Currently, the Diversisporaceae still comprises the genera *Corymbiglomus* Błaszk. & Chwat, *Redeckera* C. Walker & A. Schüssler, *Otospora* Oehl, Palenz. & N. Ferrol and *Tricispora* Oehl et al. (Redecker et al. 2013). Like *Diversispora* spp., species

of *Corymbiglomus* and *Redeckera* form glomoid spores, which develop blastically at the tip of a sporogenous hypha (Redecker et al. 2007; Schüßler and Walker 2010; Błaszkowski 1995, 2012). The genera *Otospora* and *Tricispora* are represented by single species, whose mode of spore formation differs substantially from those of *Diversispora* spp. *Otospora bareae* Palenz., N. Ferrol & Oehl produces acaulosporoid spores laterally from the neck of a sporiferous saccule (Palenzuela et al. 2008), and the entrophosporoid spores of *T. nevadensis* arise inside the neck of a sporiferous saccule (Oehl et al. 2011b).

Identification and classification of *Diversispora* spp. from their spore morphology is uncertain and difficult, even for experienced specialists. The spore wall structure and the phenotypic and histochemical traits of its components are similar or identical to those of species of other clades of the Glomeromycota. According to Oehl et al. (2011a), *Diversispora* spp. are most distinguished by characters of their subtending hyphae at the spore base, which are cylindrical and have a colourless or light-coloured wall abruptly passing into a coloured spore wall in coloured spores. However, the colour change is difficult to observe in, for example, *D. gibbosa* (Błaszk.) Błaszk. & Kovács, which produces very light-coloured spores or it is not visible at all in *D. clara* Oehl et al., whose spores are colourless, as those of many species of other genera of the Glomeromycota with cylindrical subtending hyphae (Estrada et al. 2011; Błaszkowski 2012).

Meanwhile, literature data suggest that the vast majority of AMF existing in the world remain unnamed and among them are many representatives of the genus *Diversispora* (Gamper et al. 2009; Schüßler et al. 2011). Thus, considering the difficulties of morphological identification of *Diversispora* spp. characterized above, certain recognition of *Diversispora* spp. and determination of the phylogenetic position of yet unnamed species among described relatives of the genus have to take into account their morphological and molecular traits, as Gamper et al. (2009) suggested, provided that the resolution of molecular data allows to separate even very closely related taxa.

Sequence data retrieved from the conserved SSU rRNA gene, used in the erection of the Glomeromycota (Schüßler et al. 2001) and many other studies of AMF (Walker et al. 2007; Krüger et al. 2012), frequently poorly recognized and resolved some taxa of AMF, especially those of the genus *Diversispora* (Gamper et al. 2009). Also, information coming from other regions of DNA, as the more variable internal transcribed spacer 1 (ITS1), the 5.8S rRNA gene, ITS2 rDNA (hereafter named ITS) and the large subunit (LSU) rRNA gene, the genes for mitochondrial LSU rRNA (Börstler et al. 2010; Sýkorová et al. 2012), β-tubulin (Msiska and Morton 2009) or H+−ATPase (Corradi et al. 2004; Sokolski et al. 2010), as well as the largest subunit of RNA polymerase II (RPB1; Stockinger et al. 2014) gene either occasionally were insufficient to know the identity of some AMF or sequences collected in public data bases regard only few named species, making certain identification of most closely related species impossible. For example, in the recently published set of sequences of the RPB1 gene the genus *Diversispora* is represented by one sequence of *D. epigaea* (B.A. Daniels & Trappe) C. Walker & A. Schüssler (Stockinger et al. 2014).

Krüger et al. (2012) proved and our numerous phylogenetic analyses (Błaszkowski et al. 2012, 2014, 2015a, b, c) also indicated that the sequences best resolving even morphologically and molecularly very closely related species are those spanning the SSU–ITS–LSU nrDNA region. In addition, the sequences concern a large proportion of described species of AMF, for example, 69 % of *Diversispora* spp. sensu Oehl et al. (2011a).

Our morphological observations of spores of three AMF extracted from single-species cultures prompted they are undescribed *Diversispora* spp. Subsequent phylogenetic analyses of SSU–ITS–LSU nrDNA sequences of the fungi confirmed the conclusion and revealed their closest molecular relatives. The fungi are described below as *D. varaderana* sp. nov., *D. peridiata* sp. nov., and *D. slowinskiensis* sp. nov.

Materials and methods

Establishment and growth of trap and single-species cultures, extraction of spores, and staining of mycorrhizal structures

Spores were first extracted from pot trap cultures that were established from the rhizosphere soils and roots of sampled plants mixed with autoclaved coarse grained sand and then grown in conditions described previously (Błaszkowski et al. 2012). The sampled plants are mentioned in the "*Mycorrhizal associations*" sections regarding each species of AMF (see below). Single-species cultures of each of the AMF species described below were also established and grown as given in Błaszkowski et al. (2012). The cultures were successfully established after inoculation of host plant roots with 10–20 spores per pot and their growing in conditions characterized in Błaszkowski et al. (2012). The host plant in both trap and single-species cultures was *Plantago lanceolata* L. Spores for morphological and molecular analyses and roots for studies of mycorrhizal structures were collected from 5-month-old cultures. Spores were extracted from trap and single-species cultures by the method described recently by Błaszkowski et al. (2015c). Roots were stained as Błaszkowski (2012) described.

Microscopy and nomenclature

Morphological features of spores and the phenotypic and histochemical characters of spore wall layers were determined

after examination of at least 100 spores 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 and mycorrhizal structures for study and photography were as those described previously (Błaszkowski 2012; Błaszkowski et al. 2012). Types of spore wall layers are those defined by Błaszkowski (2012), Stürmer and Morton (1997) and Walker (1983). Colour names are 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. Voucher specimens were mounted in PVLG and a mixture of PVLG and Melzer's reagent (1:1, *v/v*) on slides and deposited at the common mycological herbarium of the University and ETH of Zurich, Switzerland (Z+ZT; holotypes), the Department of Ecology, Protection and Shaping of Environment (DEPSE), West Pomeranian University of Technology in Szczecin, Szczecin, Poland, and in the herbarium at Oregon State University (OSC) in Corvallis, Oregon, USA (isotypes).

DNA extraction, polymerase chain reaction, cloning, and DNA sequencing

Crude DNA of each species was extracted from four single spores. The procedures with the spores prior to polymerase chain reactions (PCR), the conditions and primers used in the PCRs to obtain SSU–ITS–LSU nrDNA sequences, as well as cloning and sequencing were as those described in Błaszkowski et al. (2013). The sequences were deposited in GenBank (KT444708–KT444721).

Sequence alignment and phylogenetic analyses

Comparisons of sequences of our three AMF with sequences listed after BLAST queries showed that they all represent undescribed species of the Glomeromycota. Subsequent pilot analyses of the yet undescribed AMF and randomly selected published sequences of all named species with glomoid spores of known molecular phylogenies proved that all our AMF belong in the genus *Diversispora*. Then we established a set of sequences comprising 4–5 sequences each of the three new *Diversispora* spp. and 2–5 sequences each of all previously described *Diversispora* spp. of known phylogenies. *Corymbiglomus corymbiforme* Błaszk. & Chwat served as outgroup. All the sequences regarded the SSU–ITS–LSU nrDNA segment. The set of sequences was aligned with MAFFT v. 7 (Katoh and Standley 2013) using the auto option (http://mafft.cbrc.jp/alignment/server/). To improve phylogenetic resolution (Nagy et al. 2012) indels were coded by means of the simple indel coding algorithm (Simmons et al. 2001) as implemented in GapCoder (Young and Healy 2003) and this binary character set was added to the nucleotide alignment. Bayesian (BI) analyses were carried out with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003); SSU–ITS–LSU sequence data plus indel characters were divided into four partitions. GTR+G and two-parameter Markov (Mk2 Lewis) models were used for the nucleotide partitions and indels, respectively. Four Markov chains were run for 10,000,000 generations, sampling every 1,000 steps, with a burn in at 3,000 sampled trees. Convergence of the MCMC Bayesian phylogenetic inference was checked by AWTY online (Nylander et al. 2007).

Maximum likelihood (ML) phylogenetic analyses were carried out with the raxmlGUI (Silvestro and Michalak 2012) implementation of RAxML (Stamatakis 2014) with GTRGAMMA for DNA and default set for binary (indel) characters. Rapid bootstrap analysis with 1,000 replicates was used to test the support of the branches. The generated phylogenetic trees were visualized and edited in MEGA6 (Tamura et al. 2013).

Results

General data

Our phylogenetic analyses of SSU–ITS–LSU nrDNA sequences of three likely yet unnamed AMF confirmed our supposition and proved that they belong in the genus *Diversispora* and indicated their closest named relatives (Fig 1). The overall phylogenetic relations of the *Diversispora* species showed in our tree are in correspondence with previous phylogenetic analyses of the genus. Although species and some clades in the tree have strong support, relative position of some lineages and the intra-group topology of some clades could not be resolved.

Taxonomy

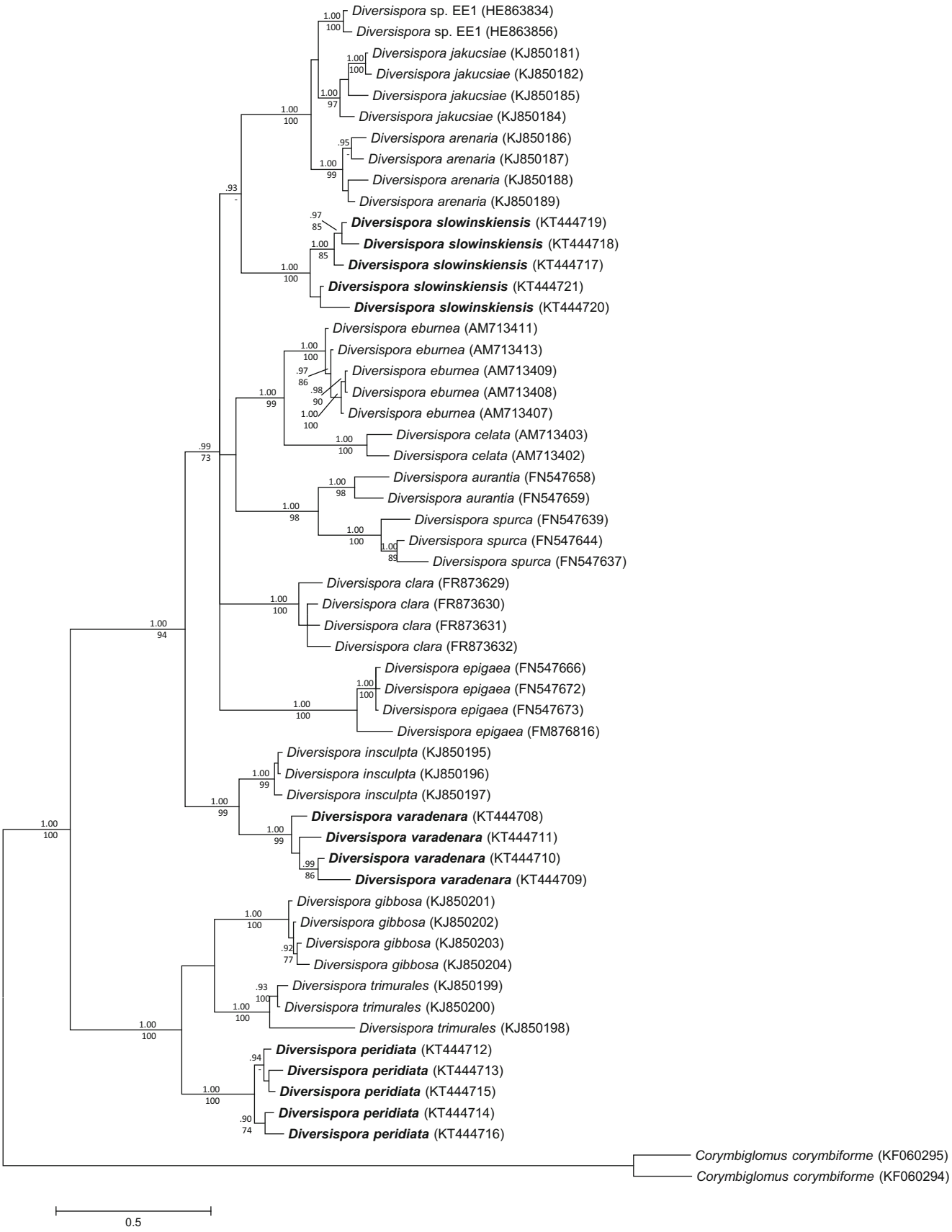
Diversispora varaderana Błaszk., Chwat, Kovács & Góralska, sp. nov. Figs. 2–8.

MycoBank No. MB 814172

Holotype: ZT Myc 55234 (Z+ZT), *isotypes*: 3460–3471 (DEPSE), and OSC 153622, OSC 153623 (OSC). Data on the origin of *D. varaderana* are given in Table 1.

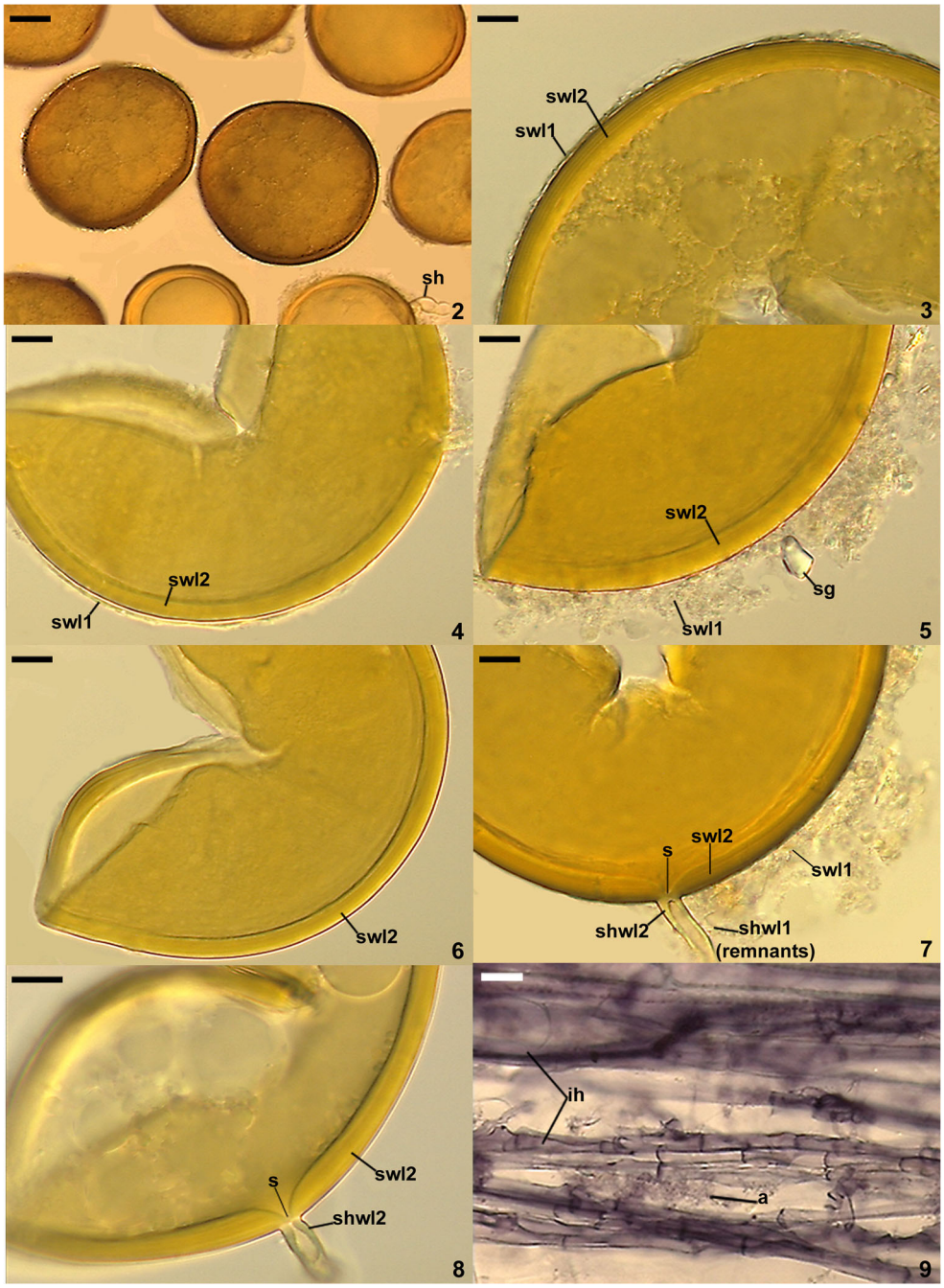
Etymology Latin, *varaderana*, referring to the city Varadero, Cuba, near where the species was found.

Sporocarps **unknown** Spores formed singly in soil (Fig. 1); develop blastically at the tip of sporogenous hyphae. *Spores* yellow (3A6) to dark yellow (4C8); globose to subglobose; (60–)75(−90) μm diam; rarely ovoid; 75–80×85–92 μm


Diversispora sp. EE1 (HE863834)
Diversispora sp. EE1 (HE863856)
Diversispora jakucsiae (KJ850181)
Diversispora jakucsiae (KJ850182)
Diversispora jakucsiae (KJ850185)
Diversispora jakucsiae (KJ850184)
Diversispora arenaria (KJ850186)
Diversispora arenaria (KJ850187)
Diversispora arenaria (KJ850188)
Diversispora arenaria (KJ850189)
Diversispora slowinskiensis (KT444719)
Diversispora slowinskiensis (KT444718)
Diversispora slowinskiensis (KT444717)
Diversispora slowinskiensis (KT444721)
Diversispora slowinskiensis (KT444720)
Diversispora eburnea (AM713411)
Diversispora eburnea (AM713413)
Diversispora eburnea (AM713409)
Diversispora eburnea (AM713408)
Diversispora eburnea (AM713407)
Diversispora celata (AM713403)
Diversispora celata (AM713402)
Diversispora aurantia (FN547658)
Diversispora aurantia (FN547659)
Diversispora spurca (FN547639)
Diversispora spurca (FN547644)
Diversispora spurca (FN547637)
Diversispora clara (FR873629)
Diversispora clara (FR873630)
Diversispora clara (FR873631)
Diversispora clara (FR873632)
Diversispora epigaea (FN547666)
Diversispora epigaea (FN547672)
Diversispora epigaea (FN547673)
Diversispora epigaea (FM876816)
Diversispora insculpta (KJ850195)
Diversispora insculpta (KJ850196)
Diversispora insculpta (KJ850197)
Diversispora varadenara (KT444708)
Diversispora varadenara (KT444711)
Diversispora varadenara (KT444710)
Diversispora varadenara (KT444709)
Diversispora gibbosa (KJ850201)
Diversispora gibbosa (KJ850202)
Diversispora gibbosa (KJ850203)
Diversispora gibbosa (KJ850204)
Diversispora trimurales (KJ850199)
Diversispora trimurales (KJ850200)
Diversispora trimurales (KJ850198)
Diversispora peridiata (KT444712)
Diversispora peridiata (KT444713)
Diversispora peridiata (KT444715)
Diversispora peridiata (KT444714)
Diversispora peridiata (KT444716)
Corymbiglomus corymbiforme (KF060295)
Corymbiglomus corymbiforme (KF060294)
0.5

◀ **Fig. 1** 50 % majority rule consensus phylogram inferred from a Bayesian analysis of SSU–ITS–LSU rDNA sequences of *Diversispora varaderana*, *D. peridiata*, and *D. slowinskiensis* among 11 known *Diversispora* spp. and an undescribed *Diversispora* sp. EE1. *Corymbiglomus corymbiforme* served as outgroup. Sequences of the three new *Diversispora* spp. are in boldface and are followed by GenBank accession numbers. The Bayesian posterior probabilities ≥0.50 and ML bootstrap values ≥50 % are shown above and below the branches, respectively. Bar indicates 0.5 expected change per site per branch

(Figs. 2–8). *Spore wall* consists of two layers (Figs 3–5, 7). Layer 1, forming the spore surface, evanescent, short-lived, hyaline to yellowish white (2A2), (1.0–)1.3(−1.5) μm thick, usually highly deteriorated or completely sloughed in most spores (Figs. 3–5, 7). Layer 2 laminate, smooth, yellow (3A6) to dark yellow (4C8), (3.3–)6.2(−7.5) μm thick (Figs. 3–8). None of layers 1 and 2 stains in Melzer's reagent (Figs 5–7). *Subtending hypha* hyaline to yellowish white (2A2); straight or recurved, cylindrical to funnel-shaped, sometimes slightly constricted at the spore base; (4.5–)6.4(−8.5) μm wide at the spore base (Figs 2, 7, 8). *Wall*



Figs. 2–8 *Diversispora varaderana* spores. 2. Intact spores with subtending hypha (sh). 3–5. Spore wall layers (swl) 1 and 2; in Fig. 5, swl1 is highly deteriorated and incorporated sand grain (sg). 6. Spore wall layer 2 (swl2); swl1 is completely sloughed. 7 and 8. Subtending hyphal wall layers (shwl) 1 and 2 continuous with spore wall layers (swl) 1 and 2 and septum (s) continuous with swl2. Fig. 9. Mycorrhizal structures of *D. varaderana* in roots of *Plantago lanceolata* stained in 0.1 % trypan blue: arbuscule (a) and intraradical hyphae (ih). Fig. 2. Spores in lactic acid. Figs. 3, 8. Spores in PVLG. Figs. 4–7. Spores in PVLG+ Melzer's reagent. Fig. 9. Mycorrhizal structures in PVLG. Figs. 2–9, differential interference microscopy. Bars: Fig. 2=20 μm, Figs. 3–9=10 μm

Table 1 The origin of three new *Diversispora* spp

Species	Host/substrate in the field	Locality	Date of collection of field sample/collected by	Date of collection from single-species culture/collected by
D. varaderana	*Phoenix dactylifera*/ maritime sand dune soil	Varadero (23°08′22″N, 81°17′10″W), Cuba	29 December 2009/J. Błaszkowski	12 March 2013/J. Błaszkowski
D. peridiata	*Ammophila arenaria*/ maritime sand dune soil	Słowiński National Park (54°45′N, 17°26′E), Poland	6 September 2011/G. Chwat	–"–
D.slovinskiensis	–"–	–"–	31 August 2011/G. Chwat	–"–

of subtending hypha hyaline to yellowish white (2A2); (1.3–)2.2(−2.6) μm thick at the spore base; continuous with spore wall layers 1 and 2; subtending hyphal wall layer 1 usually completely sloughed in most spores (Figs 7, 8). *Pore* (1.3–)1.9(−2.5) μm diam, occluded by a straight or slightly curved septum continuous with spore wall layer 2 (Figs 7, 8); septum positioned at or up to 1.8 μm below the spore base. *Germination* unknown.

Mycorrhizal associations In the field *D. varaderana* was likely associated with roots of *P. dactylifera* growing near Varadero on the Hicacos Peninsula, Cuba. However, the presence of the fungus inside the sampled roots was not examined using molecular methods.

The mycorrhiza of *D. varaderana* formed in single-species cultures with *P. lanceolata* as host plant consisted of arbuscules and intra- and extraradical hyphae (Fig. 9). The structures were uniformly distributed along the root fragments examined. Intraradical hyphae usually were straight, rarely slightly curved, and (2.8–)4.7(−5.8) μm wide. They frequently formed ellipsoidal coils, 13.3–26.5×39.0–45.2 μm. In 0.1 % trypan blue arbuscules stained very pale [violet white (16A2) to pale violet (16A3)], and the other structures pale violet (16A3) to lilac (16B4).

Phylogenetic position Phylogenetic analyses proved that the closest relative of *D. varaderana* is *D. insculpta* (Błaszk.) Oehl, G.A. Silva & Sieverd. and the clades of these sister groups were strongly supported (Fig. 1).

Distribution and habitat Spores of *D. varaderana* were found in only one trap culture inoculated with the rhizosphere soil and roots of *P. dactylifera* growing near Varadero on the Hicacos Peninsula. The presence of spores of the fungus in the mixture was not examined. *Diversispora varaderana* was not found in ca. 2500 other field-collected soils and ca. 2700 trap cultures (personally examined by J. Błaszkowski) that represented different cultivated and uncultivated sites of Africa, Asia, Europe, and USA.

However, BLAST queries indicated that *D. varaderana* sequences were similar in ≥97 % to nine SSU–ITS–LSU sequences (HG425941–45, HG425948, HG425951–53) obtained from roots of six plant species growing in Central Bohemia, Czech Republic and four SSU–ITS–LSU sequences (FR686946–49) coming from a spore cluster of a fungus named *Diversispora* sp. W5257 found in the United Kingdom. These similarities suggest *D. varaderana* is widely distributed in the world.

Notes Morphologically, *D. varaderana* is most distinguished by its relatively small and yellow-coloured spores with a simple spore wall consisting of two layers, of which layer 1 is short-lived and usually completely sloughed in most spores (Figs. 2–8).

The phylogenetically closest relative of *D. varaderana* is *D. insculpta* (Fig. 1), whose spores are similar in colour, size, and also have a spore wall with two layers (Błaszkowski et al. 2004; Błaszkowski 2012). However, the phenotypic features of the layers in the two species differ considerably. While spore wall layer 1 of *D. varaderana* is short-lived and usually completely sloughed in most spores extracted from ca. 5-month-old cultures (Figs. 3–8), that of *D. insculpta* is permanent and always present and intact in spores coming from cultures even older than 2 years (Błaszkowski, pers. observ.). Spore wall layer 2 in *D. varaderana* is smooth on its upper and lower surfaces (Figs. 3–8), and in *D. insculpta* the lower surface of this layer is ornamented with evenly distributed pits. In addition, at the spore base the subtending hypha of *D. insculpta* is 1.2–1.5-fold narrower and has a wall 1.4–1.7-fold thinner.

Another *Diversispora* sp. closely related morphologically to *D. varaderana* is *D. celata* C. Walker, Gamper & A. Schüssler, whose spores are similar in colour and spore wall comprises an evanescent outer layer 1 and a laminate inner layer 2 (Gamper et al. 2009; Błaszkowski 2012). In contrast to the very short-lived spore wall layer 1 of *D. varaderana* (Figs. 3–8) that of *D. celata* is much longer-lived and usually present in even the darkest (likely the oldest) spores. This is probably because the layer thickness is 1.3–1.7-fold thicker and, therefore, deteriorates slower. In addition, in *D. celata* the laminate spore wall layer 2 is clearly thinner (up to 1.7-fold), and its subtending hypha at the spore base is ca. 1.6-fold wider and has a wall up to 1.5-fold thicker than in *D. varaderana*.

Finally, *D. varaderana* and *D. celata* differ molecularly and the difference is large (Fig. 1).

Diversispora peridiata Błaszk., Chwat, Kovács & Góralska, sp. nov. Figs. 10–17.

MycoBank No. MB 814173

Holotype: ZT Myc 55235 (Z+ZT), *isotypes*: 3473–3495 (DEPSE) and OSC 153624, OSC 153625 (OSC). Data on the origin of *D. peridiata* are given in Table 1.

Etymology: Latin, *peridiata*, referring to the peridium-like hyphae occasionally formed by the species.

Sporocarps Unknown. Spores formed in soil, sometimes also inside roots. Hypogeous spores produced singly, more rarely in loose to compact clusters with 3–21 spores (Figs. 10–17); spores develop blastically at the tip of sporogenous hyphae. Single spores and spores in clusters occasionally covered with hyaline peridium-like hyphae, 2.8–8.5 μm wide (Figs. 11, 12). *Spores* yellowish white (4A2) to maize yellow (4A6); globose to subglobose; (35–)74(−100) μm diam; rarely ovoid; 70–80×90–100 μm (Figs. 10–17). *Spore wall* consists of two layers (Figs. 13–17). Layer 1, forming the spore surface, permanent, uniform (not divided into visible sublayers), pale yellow (3A3) to brownish yellow (5C8), (0.8–)1.5(−2.3) μm thick, always intact in all spores (Figs. 13–17). Layer 2 laminate, smooth, pliable, hyaline, (3.3–)5.1(−7.5) μm thick, frequently releasing single or groups of laminae in crushed spores (Figs. 13–17). Peridium-like hyphae and spore wall layers 1 and 2 do not stain in Melzer's reagent (Figs. 12, 14, 15, 17). *Subtending hypha* pale yellow (3A3) to brownish yellow (5C8) near the spore base, then hyaline; straight or recurved, cylindrical to funnel-shaped, sometimes slightly constricted at the spore base; (5.0–)6.1(−8.3) μm wide at the spore base (Figs. 16, 17). *Wall of subtending hypha* pale yellow (3A3) to brownish yellow (5C8) near the spore base, then hyaline; (1.4–)1.8(−2.8) μm thick at the spore base; continuous with either spore wall layer 1 only (when spore wall layer 2 started its development at the spore base and forms a septum at the level of its upper surface; Fig. 16) or with spore wall layers 1 and 2 (when spore wall layer 2 started developing below the spore base; Fig. 17). *Pore* (1.6–)2.7(−3.5) μm diam, occluded by a straight or slightly curved septum continuous with spore wall layer 2 (Figs. 16, 17); septum positioned at the spore base or up to 3.5 μm below the spore base. *Germination* unknown.

Mycorrhizal associations In the field *D. peridiata* was likely associated with roots of *A. arenaria* growing near a beach of the Baltic Sea and the deflation hollow no. 6 and in the deflation hollow no. 12 of SPN in northern Poland. However, no molecular studies were performed to check if roots *A. arenaria* harboured *D. peridiata*. Deflation hollows are depressions produced by wind erosion, during which dry sand is gradually blown away until moist sand is exposed (Piotrowska 1991). Soil of such hollows usually is wet, and its vegetation is frequently periodically under water.

In single-species cultures with *P. lanceolata* as host plant, the mycorrhiza of *D. peridiata* consisted of arbuscules and intra- and extraradical hyphae. No vesicles were found. Arbuscules and hyphae were widely dispersed along roots. The intraradical hyphae were (2.8–)5.5(−8.5) μm wide and sometimes formed circular to ellipsoidal coils, 24.8–66.0× 28.8–66.0 μm, when seen in plan view. The structures stained violet white (15A2) to light violet (17A5) in 0.1 % trypan blue.

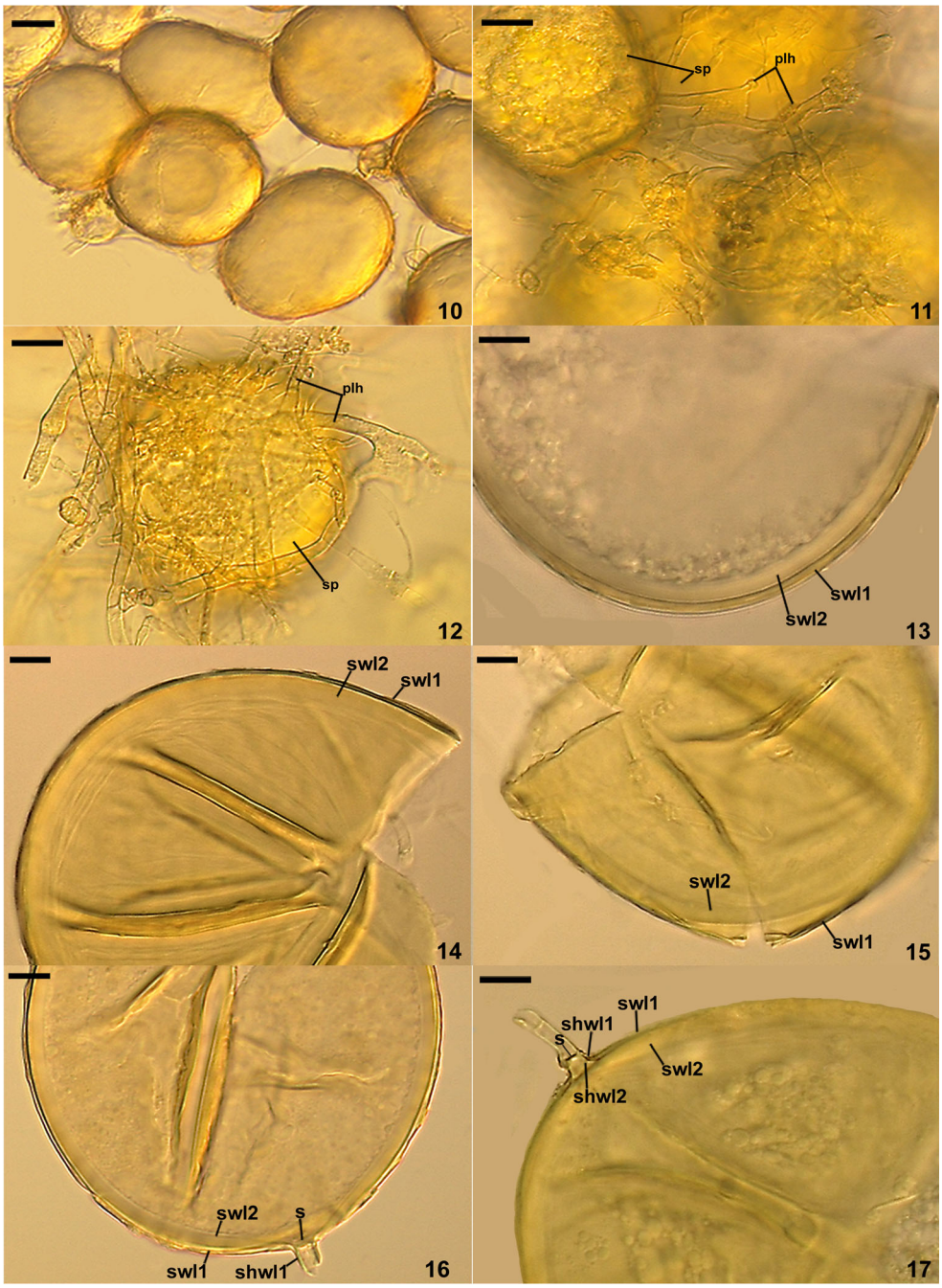
Phylogenetic position Phylogenetic analyses placed *D. peridiata* sequences in a strongly supported clade with *D. gibbosa* and *D. trimurales* (Koske & Halvorson) C. Walker & A. Schüssler (Fig. 1). The sequences of the three species unambiguously separated from each other, but the relative positions of the three species within the clade could not be resolved (Fig 1).

Distribution and habitat *Diversispora peridiata* was so far identified in three trap cultures inoculated with the rhizosphere soils and roots of *A. arenaria* colonizing maritime sand dunes belonging to SPN and was not revealed in ca. 5200 other soils from different sites and regions of the world (see "*Distribution and habitat*" regarding *D. varaderana* described above).

BLAST searches showed that, except for *D. gibbosa*, no other AMF was thus far recognized, whose sequences are similar in ≥97 % to SSU–ITS–LSU sequences of *D. peridiata*. Thus, *D. peridiata* may rarely occur in the world.

Notes Morphologically, *D. peridiata* is distinguished by the occasional formation of spores in clusters and the peridium-like hyphae covering the clusters and single spores (Figs. 10–12), and especially by the permanent and relatively thick spore wall layer 1, which is the only coloured component of the two-layered spore wall of the yellow-coloured and relatively small-spored species (Figs. 13–17).

Diversispora gibbosa, one of the two closest relatives of *D. peridiata* in molecular phylogenetic analyses (Fig. 1), sometimes also produces spores in clusters, but they usually contain fewer spores (2–8) and are completely enclosed by a hyphal mantle having a continuous wall, without openings (Błaszkowski 1997, 2012; vs. are partly covered with narrow peridium-like hyphae; Figs. 11, 12). Spores of *D. gibbosa* may also be lighter (hyaline vs. never are hyaline in *D. peridiata*), are 1.3–2.3-fold larger

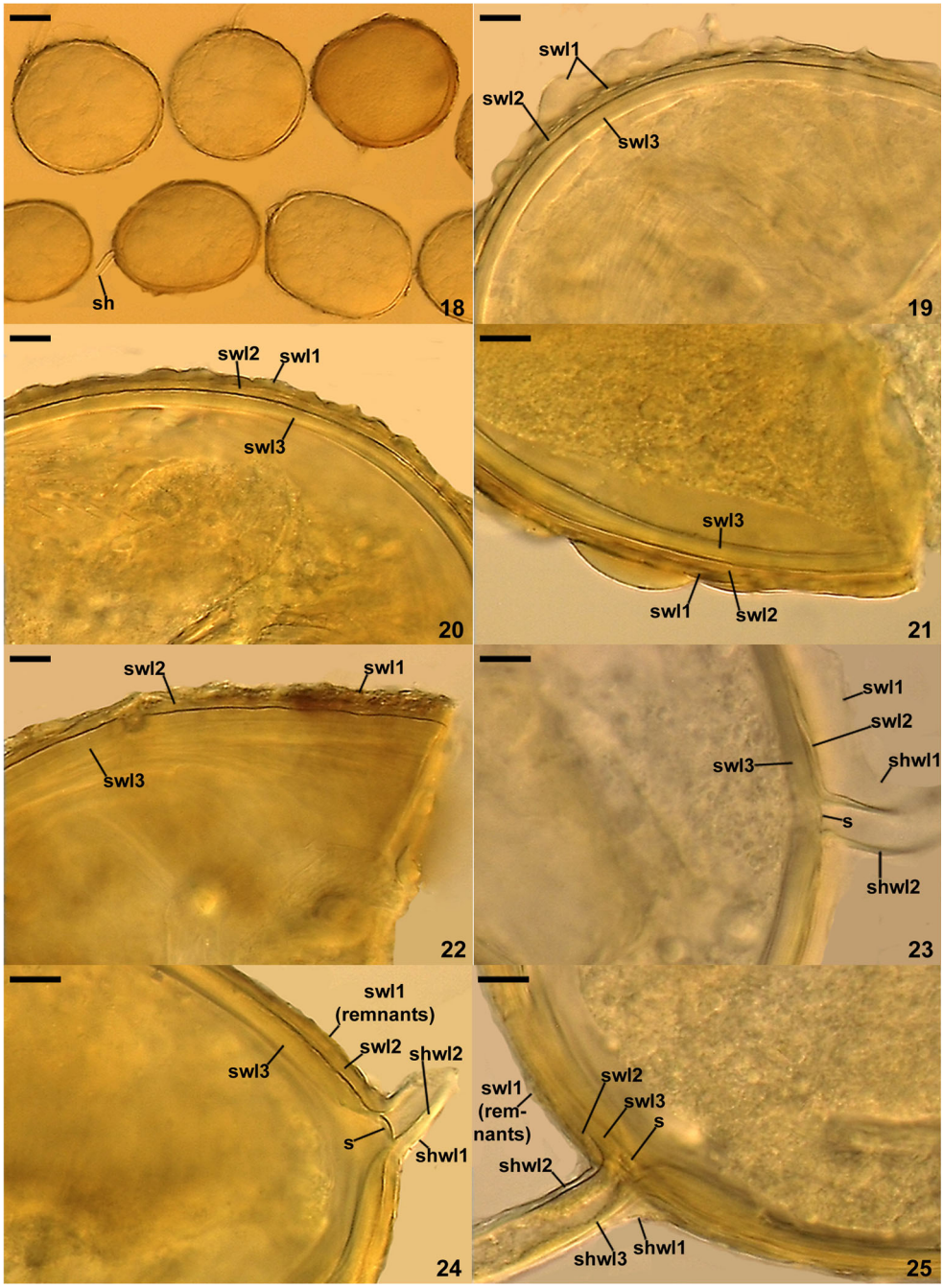


Figs. 10–17 *Diversispora peridiata* spores. 10. Cluster of intact spores without peridium-like hyphae. 11, 12. Cluster of intact spores (sp, Fig. 11) and single spore (Fig. 12) covered with peridium-like hyphae (plh). 13–15. Coloured layer 1 (swl1) and colourless layer 2 (swl2) of the spore wall. 16. Spore wall layers (swl) 1 and 2, one-layered subtending hyphal wall (shwl1) continuous with swl1 and septum (s) of the subtending hypha continuous with swl2 at the spore base. 17. Spore wall layers (swl) 1 and 2 continuous with subtending hyphal wall layers (shwl) 1 and 2 and septum (s) located below the spore base and continuous with swl2. Figs. 10, 11, 13, 16. Spores in PVLG. Figs. 12, 14, 15, 17. Spores in PVLG+Melzer's reagent. Figs. 10–17, differential interference microscopy. Bars: 10–12=20 μm, Figs. 13–17=10 μm

when globose and, most importantly, their spore wall comprises four layers, of which an evanescent, hyaline layer 1 and a flexible, hyaline layer 4 are lacking in the spore wall of *D. peridiata*.

The second species closely related in phylogenetic analyses to *D. peridiata* is *D. trimurales* (Fig. 1). However, morphologically the fungi also differ substantially. *Diversispora peridiata* occasionally forms spores in clusters (vs. only singly in *D. trimurales*) and peridium-like hyphae covering single spores and their clusters (Figs. 10–12; vs. no such hyphae) and the spores are maize yellow at most [4A6 vs. golden yellow (5B8)] and ca. 1.6–3.1-fold smaller when globose (Błaszkowski et al. 2003; Błaszkowski 2012). Most importantly, the spore wall of *D. peridiata* consists of two layers (Figs. 13–17) and does not possess layer 1 of the three-layered spore wall of *D. trimurales*, which, in addition, may be up to 1.9-fold thicker. Finally, in *D. peridiata* the subtending hypha and its pore at the spore base are 1.2-fold and up to 1.6-fold narrower, respectively.

Diversispora slowinskiensis Błaszk., Chwat, Góralska & Kovács, sp. nov. Figs. 18–25.



Figs. 18–25 *Diversispora slowinskiensis* spores. 18. Intact spores with subtending hyphae (sh). 19–22. Spore wall layers (swl) 1–3; note swollen swl1 of young spore in Fig. 19. 23, 24. Spore wall layers (swl) 1–3, subtending hyphal wall layers (shwl) 1 and 2 continuous with swl1 and 2 and septum (s) located at the spore base and continuous with swl3; note swollen swl1 of young spore in Fig. 23. 25. Spore wall layers (swl) 1–3 continuous with subtending hyphal wall layers (shwl) 1–3 and septum (s) continuous with swl3. Figure 18. Spores in lactic acid. Figs. 23, 25. Spores in PVLG. Figs. 19–22, 24. Spores in PVLG+Melzer's reagent. Figures 18–25, differential interference microscopy. Bars: Fig. 18=50 µm, Figs. 19–25=10 µm

MycoBank No. MB 814174

Holotype: ZT Myc 55236 (Z+ZT), *isotypes*: 3497–3516 (DEPSE) and OSC 153626, OSC 153627 (OSC). Data on the origin of *D. slowinskiensis* are given in Table 1.

Etymology. Latin, *slowinskiensis*, referring to SPN, Poland, in which the species was discovered.

Sporocarps unknown Spores formed singly in soil (Fig. 18); develop blastically at the tip of sporogenous hyphae. *Spores* pale yellow (2A3) to greyish yellow (3B5); globose to subglobose; (75–)138(−190) µm diam; rarely ovoid; 70–115×105–170 µm (Figs. 18–25). *Spore wall* consists of three layers (Figs. 19–25). Layer 1, forming the spore surface, evanescent, hyaline to yellowish brown (5E8), (1.0–)2.3(−5.3) µm thick, frequently with blister-like swellings, up to 7.0 µm high, on its upper surface, usually slowly deteriorating with age, rarely completely sloughed in older specimens (Figs. 18–25); in youth plastic, hyaline, with its upper part frequently swelling in PVLG (Figs. 19, 23), then darkening and becoming more compact (Figs. 20–22, 24). Layer 2 uniform (not divided into visible sublayers), smooth, permanent,

hyaline, (0.8–)2.3(−4.0) μm thick (Figs. 19–25). Layer 3 laminate, smooth, pliable, hyaline, (2.3–)5.1(−10.0) μm thick, consisting of very thin laminae, <0.5 μm thick, frequently stratifying into groups of laminate, rarely into single laminae in even slightly crushed spores (Figs. 19–25). None of layers 1–3 stains in Melzer's reagent (Figs. 19–22, 24). *Subtending hypha* usually hyaline, rarely yellowish brown (5E8) close at the spore base, when subtending hyphal wall layer 1 continuous with spore wall layer 1 remains intact or is slightly deteriorated; straight or recurved, cylindrical to funnel-shaped, sometimes slightly constricted at the spore base; (9.3–)12.3(−15.4) μm wide at the spore base (Figs. 18, 23–25). *Wall of subtending hypha* hyaline to yellowish brown (5E8); (2.6–)4.5(−7.0) μm thick at the spore base. The wall is continuous with spore wall layers 1 and 2, when spore wall layer 3 started developing at or slightly above the spore base (Figs. 23, 24), or spore wall layers 1–3, when spore wall layer 3 began arising below the spore base (Fig. 25). *Pore* (2.5–)3.7(−4.5) μm diam, occluded by a straight or slightly curved septum continuous with spore wall layer 3 (Figs. 23–25); septum positioned at the level of the center or the upper surface of spore wall layer 3 (Figs. 23–25). *Germination* unknown.

Mycorrhizal associations In the field *D. slowinskiensis* was likely associated with roots of *A. arenaria* and *Juncus articulatus* L. growing in the 12 deflation hollow of SPN. However, the existence of the fungus inside the roots of the plant species was not examined using molecular methods.

In single-species cultures with *P. lanceolata* as host plant, *D. slowinskiensis* formed mycorrhiza with arbuscules and intra- and extraradical hyphae. The structures were patchily distributed along root fragments. Intraradical hyphae were straight or slightly curved, (2.8–)6.3(−9.8) μm wide and rarely formed ellipsoidal coils, 21.3–41.5×65.0–87.3 μm, when observed in plain view. All of the structures stained violet white (17A2) to greyish violet (17C6) in 0.1 % trypan blue.

Phylogenetic position In the phylogenetic analyses, sequences of *D. slowinskiensis* formed a clade with the fully supported monophyletic group of *D. arenaria* (Błaszk., Tadych & Madej) Oehl, G.A. Silva & Sieverd., *D. jakucsiae* Błaszk., Balázs & Kovács, and an undescribed *Diversispora* sp. EE1 (Fig. 1).

Distribution and habitat Spores of *D. slowinskiensis* were extracted from 2 of 298 (0.7 %) trap cultures inoculated with field-collected mixtures of the rhizosphere soils and root fragments of *A. arenaria* and *J. articulatus* growing in the 12 deflation hollow of SPN.

Searches of BLAST did not indicate any sequence of similarity of ≥97 % to sequences of *D. slowinskiensis*. This and the lack of *D. slowinskiensis* spores in the large number of soils and sites so far examined by J. Błaszkowski (see above in "*Distribution and habitat*" regarding *D. varaderana*) suggest our new species has a very narrow distribution in the world.

Notes Two morphological structures distinguish *D. slowinskiensis* spores. The first is spore wall layer 1, which is the only coloured component of the three-layered spore wall, frequently is covered with blister-like swellings and in youth its upper part often swells in PVLG while its basal part always adherers to the upper surface of spore wall layer 2 (Figs. 19–25). The second is the pliable, laminate, hyaline spore wall layer 3, which consists of loosely associated laminae (Figs. 19–25). This layer either starts its development at the spore base, and then is not a component of the subtending hyphal wall, or arises far below the spore base forming subtending hyphal wall layer 3.

In addition, *D. slowinskiensis* is unique in molecular phylogeny: sequences of the species grouped in its own clade sister to a clade comprising *D. arenaria*, *D. jakucsiae* and an undescribed *Diversispora* sp. EE1 (Thiery et al. 2012) (Fig. 1).

None of the species mentioned above forms spores ornamented with blister-like swellings like those of *D. slowinskiensis* spores (Figs. 19–21). In addition, *D. arenaria* spores may be clearly darker, are 1.4–1.6-fold smaller when globose and at the spore base their subtending hypha is 1.9–2.5-fold narrower and has a 3.7–4.1-fold thinner wall (Błaszkowski et al. 2001; Błaszkowski 2012). Spore wall layer 1 in both species is evanescent, but in *D. arenaria* it is always hyaline [vs. hyaline to yellowish brown (5E8) in *D. slowinskiensis*; Figs. 19–25] and 2.0–3.5-fold thinner when intact. Spore wall layer 2 in *D. arenaria* also is much thinner (up to 2.7-fold) and sloughs with age (vs. it is permanent in *D. slowinskiensis*; Figs. 19–25), and layer 3 is coloured (vs. hyaline; Figs. 19–25).

Spores of *D. jakucsiae* also are much darker, ca. 1.3-fold smaller when globose and their subtending hypha may be up to 1.9-fold narrower at the spore base (Balazs et al. 2015). Most importantly, in *D. jacuksiae* spore wall layer 1 is permanent, smooth and does not give the spores their proper colour (vs. evanescent and the sole coloured component in the spore wall in *D. slowinskiensis*; Figs. 19–25), layer 2 is laminate and darkest (vs. uniform, hyaline; Figs. 19–25) and 3.6–5.3-fold thicker than that of *D. slowinskiensis*, and layer 3 is flexible to semi-flexible and up to 1.3 μm thick (vs. laminate, up to 10.0 μm thick; Figs. 19–25).

Diversispora Sp. EE1 was characterized by molecular phylogenetic methods with no information on morphology of its spores (Thiéry et al. 2012). From microphotographs of crushed spores of the fungus (kindly provided by Drs. Odile Thiéry and Maarja Öpik) we can see that our *D. slowinskiensis* and *Diversispora* sp. EE1 differ substantially: spores of the latter fungus are lighter and likely have a two-walled spore wall (vs. three-layered in *D. slowinskiensis*).

Based on morphology, without molecular evidence, Oehl et al. (2011a) transferred *G. pustulatum* Koske et al. to the genus *Diversispora* as a new combination, *D. pustulata* (Koske et al.) Oehl, G.A. Silva & Sieverd. The upper surface of layer 1 of its three-layered spore wall is covered with a pustulate ornamentation (Koske et al. 1986; Błaszkowski 1994, 2012) like that of *D. slowinskiensis* (Figs. 19, 21), but in *D. pustulata* this layer is permanent (vs. evanescent), layer 2 is laminate and coloured (vs. uniform, hyaline; Figs. 19–25), and layer 3 is flexible and <1 μm thick (vs. laminate, up to 10 μm thick; Figs. 19–25). Finally, *D. pustulata* spores may be much darker [up to deep orange (5A8)], are 1.4–1.7-fold smaller when globose and at the spore base their subtending hypha is 1.3–2.2-fold narrower and has a ca. 3.5-fold thinner wall.

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