A new genus, Complexispora, with two new species, C. multistratosa and C. mediterranea, and Epigeocarpum japonicum sp. nov.

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Abstract

Morphological analyses of three glomoid spore-producing fungi suggested that two of them are undescribed species of Glomeraceae or glomoid spore-producing members of *Entrophospora* in Entrophosporaceae (phylum Glomeromycota), and the third differed slightly from *Dominikia glomerocarpica* and *Epigeocarpum crypticum*, recently described in Glomeraceae. The first two fungi originated from the Mediterranean Sea sand dunes of the Peloponnese, Greece, and the third was originally found in a tree plantation in Yokohama City, Japan. Phylogenetic analyses of sequences of the 45S nuc rDNA region and the *RPB1* gene showed that the three fungi are undescribed species in Glomeraceae. The first two species represented a new genus, here described as *Complexispora* with *C. multistratosa* sp. nov. and *C. mediterranea* sp. nov. The third species enlarged the monospecific genus *Epigeocarpum*, as *E. japonicum*.

Introduction

The family Glomeraceae with two genera, *Glomus* and *Sclerocystis*, was originally erected in the order Endogonales, class Zygomycetes (Pirozynski and Dalpé 1989). Except for *Glomus* and *Sclerocystis* species, which have been known to form arbuscular mycorrhiza and fungi suspected to live in such symbiosis, the order contained taxa that differed fundamentally in the mode of reproduction and nutrition, as, for example, *Endogone* species (Gerdemann and Trappe 1974). Therefore, Morton and Benny (1990) transferred Glomeraceae to a new order, Glomerales in Zygomycetes, which included solely arbuscular mycorrhizal fungi (AMF). Based on phylogenetic analyses of sequences of the 18S nuc rDNA gene, Redecker et al. (2000) found that Glomerales is polyphyletic, and Schwarzott et al. (2001) distinguished in Glomeraceae *Glomus*-groups A and B. *Glomus*-group A was represented, among others, by *G. caledonium*, *G. mosseae* and *G. intraradices*, and *Glomus*-group B by *G. claroideum* and *G. lamellosum*. Following phylogenetic analyses of sequences of the same gene, Schüßler et al. (2001) accommodated Glomerales in a newly erected phylum, Glomeromycota. Schüßler and Walker (2010) sequenced *G. macrocarpum*, designated it as the type species of *Glomus* and Glomeromycota, and, consequently, retained in Glomeraceae only species of *Glomus*-group A sensu Schwarzott et al. (2001); *Glomus*-group B was raised to the rank of a monogeneric family, Claroideoglomeraceae with *Claroideoglomus*, in Glomerales.

Oehl et al. (2011) distinguished in Glomeraceae six subgroups depending on the shape of the spore subtending hypha, the thickness of its wall, the mode of pore closure, and the formation of spores singly, in clusters, or in un- or organized glomerocarps (= sporocarps). Consequently, the researchers distributed glomoid spore-producing species of Glomeraceae in four genera. The feature linking these subgroups was the similar pigmentation of the subtending hyphal wall at and below the spore base to that of the spore wall in coloured spores. In coloured glomoid spores of *Entrophospora* species (Entrophosporaceae, Entrophosporales; Błaszkowski et al. 2022b), which are morphologically the closest to members of Glomeraceae, the subtending hyphal wall, even at the spore base, is colourless or conspicuously lighter than the spore wall (Oehl et al. 2011; Błaszkowski et al. 2022b). However, the presence or absence of

pigment in the subtending hyphal wall and the other morphological features mentioned above, used singly and in combination, are generally not diagnostic for the phylogenetically recognized generic clades of Glomeraceae. Another reason that complicates the taxonomic diagnosis of several members of Glomeromycota, not only those producing glomoid spores, is their morphological crypticity and the difficulty of detecting morphological differences using traditional methods due to the simplicity of the construction of the spores, which are unicellular, and the high phenotypic variability of the spore components.

Consequently, the most reliable and useful way to characterize and identify members of Glomeromycota is to determine their molecular phylogeny. This, however, may also be difficult or impossible because a large number of species lack molecular characterization. For instance, although the Glomeraceae is the richest family in Glomeromycota, only ca. 57% of species of this family, distributed in 18 genera, have known molecular phylogeny. Interestingly, of the 18 genera, 13, including the genus *Epigeocarpum*, were recognized only in the last nine years (Błaszkowski et al. 2015; 2018a, b, 2021a; Corazon-Guivin et al. 2019a–c, Sieverding et al. 2014). So, it is expected that soon further new taxa at different ranks will be revealed among the not sequenced members of Glomeraceae. Moreover, such members of Glomeraceae can also be among the ca. 23% of glomoid spore-producing species, currently classified in other families of Glomeromycota, with uncertain or unknown phylogenies.

The aim of the studies described in this paper was to characterize the morphology and phylogeny of three fungi producing spores with features of species of Glomeraceae and glomoid spore-producing *Entrophospora* species, including a species of almost identical morphology to the recently newly described *Dominikia glomerocarpica* and *Epigeocarpum crypticum* (Błaszkowski et al. 2021a).

Materials And Methods

Origin of study material, establishment and growth of trap and single-species cultures, extraction of spores, and staining of mycorrhizal structures

The potentially new species were initially named Species 1–3. Spores of Species 1 and 2 were first grown in trap pot cultures. Then, the spores, as well as spores of Species 3, extracted from a field-collected glomerocarp (= sporocarp), were used to establish single-species pot cultures. Many attempts at establishing such cultures with Species 2 and 3 failed. Therefore, Species 1, 2, and 3 were characterized based on spores extracted from both types of cultures, only trap cultures, and only a glomerocarp, respectively. The trap cultures, in which Species 1 and 2 were found, had been originally inoculated with field-collected mixtures of rhizosphere soils and root fragments collected under five specimens of *Ammophila arenaria* (L.) Link ca. 100 m apart from each other. The plants colonized maritime sand dunes of the beach Voidokoilia (36°57'N 21°39'E) located on the Peloponnese peninsula, Greece. The soil samples were collected by Dr. Dimitris Arrianas on July 18, 2012, and J. Błaszkowski on September 8, 2015. Data about the climate and soil chemical properties of the sampled site are in Błaszkowski et al. (2019). The glomerocarp of Species 3 was collected on September 22 of 2020 from beneath the litter in a

plantation of *Cryptomeria japonica* (Thunb. Ex L.F.) D. Don in Yokohama City, Kanagawa Prefecture, Japan (35°30'N 139°30'E). The climate of the sampling site is warm and humid with an average annual temperature of 16.9 ºC and an annual rainfall of 1,937 mm recorded in 2019 at the nearest meteorological station Yokohama by the Japan Meteorological Agency.

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 and phenotypic and histochemical characters of spore wall layers of the new species presented here were characterized based on 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 terms "glomerospores" and "glomerocarps" were used for spores and sporocarps, respectively, produced by AMF as proposed by Goto and Maia (2006) and Jobim et al. (2019).

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 ZT Myc (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).

Dna Extraction, Pcr, Cloning, And Dna Sequencing

Genomic DNA of Species 1 and Species 2 was extracted from single or 2–5 spores of each fungus, and that of Species 3 was obtained from fragments of a glomerocarp, each containing ca. 10–30 spores. The method of processing the spores prior to PCR, conditions and primers used for PCR, as well as cloning and sequencing of PCR products to obtain 45S sequences of the three species were as those described by Błaszkowski et al. (2021b). Primers and PCR conditions to obtain *RPB1* amplicons are summarized in Table 1. Both 45S and *RPB1* sequences were deposited in GenBank (…will be added after reviews...).

Table 1
Characteristics of the sequence alignments analyzed.

Name of alignment	No. of sequences	No. of fungal species	No. of base pairs	No. of variable sites	No. of parsimony informative sites
45S	95	29	971	1868	891
RPB1	61	25	1712	706	608
45S + *RPB1*	95	29	3580	1677	1499

Phylogenetic Analyses

Preliminary BLAST analysis on the 45S sequences obtained suggested that Species 1–3 are undescribed taxa of Glomeraceae. To confirm the status and find position of these species within Glomeraceae, three alignments were produced (45S, *RPB1*, 45S + *RPB1*) separately using MAFFT 7 with the E-INS-i option. The 45S alignment contained 95 sequences of the 45S region or its part, which characterized Species 1–3 and 24 representative species of all recognized (17) genera of Glomeraceae, except for *Simiglomus*. For *Sclerocystis sinuosa*, to increase the phylogenetic signal, the 45S sequence was obtained by concatenation of the overlapping sequences AJ133706 (18S), AJ437106 (45S), and FJ461846 (28S) obtained from the same isolate MD126. The *RPB1* alignment consisted of 61 *RPB1* sequences of those species of the 45S alignment that have sequences of the two loci. This alignment represented 19 species in 16 genera of Glomeraceae and Species 1–3. The 45S + *RPB1* alignment was produced by manual concatenation of the 45S and *RPB1* alignments. In all alignments, the outgroup was represented by sequences of three same *Diversispora* species.

The percentage sequence divergences of Species 1–3 from sequences of their closest relatives (Fig. 1, Online Resources 2, 3) were calculated using BioEdit (Hall 1999). All comparisons were performed on sequences of the same length.

The phylogenetic position of Species 1–3 was reconstructed based on Bayesian inference (BI) and maximum likelihood (ML) phylogenetic analyses of the 45S and 45S + *RPB1* alignments, performed via CIPRES Science Gateway 3.1 (Miller et al. 2010). The 45S alignment was divided into five partitions: 18S, ITS1, 5.8S, ITS2, 28S. Five additional *RPB1* partitions, representing 3 exons and two introns, were added in the alignments with the concatenated genes, similarly as in Błaszkowski et al. (2021a). In addition, a tree with only *RPB1* sequences partitioned as in the analysis of the 45S + *RPB1* alignment was generated to reveal potential conflict in the topologies of the trees obtained from analyses of sequences of single loci.

GTR + I + G was chosen in both BI and ML analyses as nucleotide substitution model for each nucleotide partition (Abadi et al. 2019). Substitution models selected by ModelTest-NG 0.1.5 (Darriba et al. 2020) were also tested in ML analyses but the trees obtained final loglikelihood values lower than those where GTR + I + G was used.

Four Markov chains were run over one million generations in MrBayes 3.2 (Ronquist et al. 2012), sampling every 1,000 generations, with a burn-in at 3,000 sampled trees. The ML phylogenetic tree inference was performed with RAxML-NG 1.0.1 (Kozlov et al. 2019), using a maximum likelihood/1000 bootstrapping run, and ML estimated proportion of invariable sites and base frequencies. The alignments and tree files were deposited as online resources.

We assumed that clades were supported when the Bayesian posterior probabilities and the ML bootstrap values were ≥ 0.95 and ≥ 70%, respectively. The phylogenetic trees were visualized and edited in TreeGraph 2 (Stöver and Müller 2010). 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 four measures referred to the ingroup: (i) the number of species clades supported with BI ≥ 0.95 and ML ≥ 70%, (ii) mean BI and ML values for species clades when supported, (iii) mean supports of nodes with BI ≥ 0.95 and ML ≥ 70%, and (iv) the amount of resolution of each tree. The amount of resolution was calculated for the ingroup of the tree, dividing the number of significantly supported internal branches by the size of the ingroup (Thorley and Wilkinson 2000).

To detect possible other findings of the potential three new species, their 45S sequences were used as queries in BLASTn to retrieve nucleotide sequences from GenBank. The sequences were selected according to the percentage of identity > 96 with at least one of the queries. Sequences were clustered in Mothur 1.39 (Schloss et al. 2009) using a dissimilarity cutoff of 1%. The possible relatedness with any of the three new species was assessed repeating the BI and ML phylogenetic analyses of the 45S locus, including the representative environmental sequences in the 45S alignment.

Results

General data and phylogeny

Among the analyzed 45S and *RPB1* sequences, 18 and 6, respectively, were newly obtained in this study. Data about the numbers of variable and parsimony informative sites of the alignments are presented in Table 1.

The topologies of the trees generated in BI and ML analyses of the 45S and 45S + *RPB1* alignments were identical (Online Resource 1, Fig. 1). In all trees, Species 1 and 2 sequences clustered in two separate species subclades belonging to a clade at the rank of genus. This new generic clade formed a highly supported clade together with the *Glomus* and *Sclerocarpum* clades, occupying a sister position to the *Glomus* clade. In all analyses, Species 1 and 2, as well as the generic clade containing the two species obtained full or very high supports (BI = 1.0; ML = 97—100%). Instead, the node linking the new generic clade with the *Glomus* clade was not supported in any analysis.

All analyses of both alignments placed Species 3 in a species clade sister to the clade with *E. crypticum* sequences. Both the species clade and the generic *Epigeocarpum* clade were fully supported (Fig. 1,

Online Resource 1). All these analyses also showed that the sister of *Epigeocarpum* is the monospecific genus *Kamienskia* with *K. bistrata*.

The *RPB1* tree showed similar statuses and relationships of the analyzed species as the 45S and 45S + *RPB1* trees, when the species had *RPB1* sequences. Also, the supports of the new generic clade *Complexispora* and all species clades were significant.

In all trees, the considered measurements regarding the ingroup were identical or similar, except for the *RPB1* tree where the BI and ML resolutions were slightly lower than in the 45S and 45S + *RPB1* trees (Online Resource 4).

The divergences of Species 1 from Species 2, determined based on 45S and *RPB1* sequences, were 5.7–6.9% and 1.1%, respectively. The similarities of the 45S and *RPB1* sequences of Species 1 and 2 to those of *G. bareae* and *G. tetrastratosum*, which clustered in a neighbouring clade, were 76.6–84.8% and 91.7–92.1%, respectively. The 45S and *RPB1* sequences of Species 3 and *E. crypticum* differed by 8.9–9.3% and 4.1–4.6%, respectively.

Taxonomy

The results of the phylogenetic analyses and sequence comparisons described above proved that Species 1–3 are new taxa of Glomeraceae, of which Species 1 and 2 (below described as *Complexispora multistratosa* and *C. mediterranea*, respectively) represent a new genus (named *Complexispora*), and Species 3 extended the monospecific genus *Epigeocarpum* as *E. japonicum*.

Description Of A New Genus

Complexispora Błaszk., B.T. Goto, Niezgoda & Magurno, **gen. nov**.

MycoBank No. …….. (will be added after reviews)

Etymology

Latin, *Complexispora*, referring to the complex spore wall structure of species of this genus.

Type species: *Complexispora multistratosa* Błaszk., B.T. Goto, Niezgoda & Magurno

Diagnosis

Differs from glomoid spore-producing species of other genera of Glomeraceae in (i) having a swelling, colorless, laminate spore wall layer surrounding the main, much thicker, structural laminate spore wall layer and (ii) nucleotide composition of sequences of the 45S nuc rDNA region and the *RPB1* gene (see Discussion for details).

Genus description

Producing hypogeous glomoid spores singly and in clusters. Spores yellow to brown, usually globose to subglobose, 62–230 µm diam, with a spore wall composed of four to six layers, of which two consist of tightly adherent sublayers (laminae). Only spore wall layer 1 stains in Melzer's reagent. Subtending hypha cylindrical to funnel-shaped, concolorous with the spore wall, with a wall composed of layers continuous with all spore wall layers, except for the innermost layer. Subtending hyphal pore closed by a septum continuous with the innermost spore wall layer, occasionally also by a septum connecting the inner surfaces of the main structural laminate spore wall layer. Forming mycorrhiza with vesicles and arbuscules staining dark in 0.1% Trypan blue.

Description Of New Species

Complexispora multistratosa Błaszk., B.T. Goto, Niezgoda & Magurno, **sp. nov**.

Figures 2a–h, 3a–d

MycoBank ……. (will be added after reviews)

Typification: GREECE. PELOPONNESE: Spores from a single-species culture established from spores extracted from a trap culture inoculated with a field-collected mixture of rhizosphere soil and root fragments of *Ammophila arenaria* from a maritime sand dune site of the beach Voidokoilia (36°57'N 21°39'E), the Peloponnese peninsula, Greece, 8 September 2015, J. Błaszkowski (**holotype** slide with spores no. ZT Myc ……, **isotypes** slides with spores no. ….–….., LPPDSE). (missing data will be added after reviews)

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

Diagnosis

Differs from other glomoid spore-producing species with a six-layered spore wall in (i) the phenotypic and histochemical properties of the two outer spore wall layers surrounding the other spore wall layers, (ii) morphology of the spore pore closure, and (iii) nucleotide composition of sequences of the 45S nuc rDNA region and the *RPB1* gene (see Discussion for details).

Description

Glomerospores (= spores) glomoid, formed singly in the soil (Fig. 2a). Spores arise blastically at tips of sporogenous hyphae continuous with extraradical mycorrhizal hyphae. *Spores* pastel yellow (4B3) to brown (5E5); globose to subglobose; (130–)173(–230) µm diam, rarely ovoid; 140–180 × 150–210 µm; with one subtending hypha (Figs. 2a, 3c). *Spore wall* composed of six layers (Figs. 2a–h, 3a–d). Layer 1, forming the spore surface, evanescent, uniform (without visible sublayers), flexible, hyaline, ca. 0.8–1.3 µm thick when intact (Figs. 2a–h, 3a, d). Layer 2 evanescent, uniform, flexible, hyaline, ca. 1.0–1.5 µm

thick when intact and mounted in water, swelling up to a thickness of 90 µm and showing sublayers, < 0.5 µm thick, slightly separating from each other, or groups of sublayers, not separated from each other, when mounted in PVLG and PVLG + Melzer's reagent (Figs. 2a–h, 3a, d). Layers 1 and 2 are smooth in young spores but become roughened with age and are occasionally completely sloughed off in older spores. Layer 3 permanent, uniform, semi-flexible, smooth, hyaline, (0.8–)1.3(–2.0) µm thick, always tightly adherent to the upper surface of layer 3 (Figs. 2b–h, 3a–d). Layer 4 permanent, laminate, smooth, pastel yellow (4B3) to brown (5E5), (8.5–)12.4(–19.0) µm thick, consisting of thin, < 0.5 µm thick, laminae, tightly adherent to each other (Figs. 2b–h, 3a–d). Layer 5 permanent, uniform, semi-flexible, smooth, dull yellow (3B3) to grayish yellow (4B3), (1.0–)1.1(–1.5) µm thick, always tightly adherent to the lower surface of layer 4 (Figs. 2b–h, 3a–d). Layer 6 permanent, uniform, flexible to semi-flexible, smooth, hyaline, 0.8–1.0 µm thick, usually easily separating from the lower surface of layer 5 in even slightly crushed spores (Figs. 2b–h, 3a–d). Only layer 1 stains pale red (12A3) to ruby red (12D8) in Melzer's reagent (Figs. 2f–h, 3a, d). *Subtending hypha* pastel yellow (4B3) to brown (5E5); straight or recurved, cylindrical or slightly funnel-shaped, rarely slightly constricted at the spore base; (13.0–)23.6(–34.0) µm wide at the spore base (Figs. 2a, 3c, d); not braking in crushed spores. *Wall of subtending hypha* pastel yellow (4B3) to brown (5E5); (5.3–)9.3(–15.0) µm thick at the spore base; consisting of five layers continuous with spore wall layers 1–5; subtending hyphal wall layers 1 and 2 usually highly deteriorated or completely sloughed off in mature spores (Figs. 3c, d). *Pore* (2.5–)5.7(–22.3) µm wide at the spore base, always occluded by ingrowth of spore wall layer 6, reaching half the thickness of the laminate spore wall layer 4, and sometimes also by a straight or slightly curved septum connecting the inner surfaces of spore wall layer 5 near the half thickness of spore wall layer 4 or at the spore base (Figs. 3b–d). *Germination* unknown.

Ecology and distribution

In the field, associated with roots of *A. arenaria* in the Peloponnese peninsula, Greece. The geographic position, climate, and soil chemical properties of the sampled site are characterized in Błaszkowski et al. (2019). BLASTn and phylogenetic analyses involving environmental sequences indicated that *C. multistratosa* has not been detected so far. Forming mycorrhiza with arbuscules, vesicles, as well as intraradical and extraradical hyphae in single-species cultures with *P. lanceolata* as the host; fungal structures stained pale violet (16A3) to deep violet (16DE8) in 0.1% Trypan blue.

Complexispora mediterranea Błaszk., B.T. Goto, Niezgoda & Magurno, **sp. nov**.

Figures 4a–h

MycoBank No. MB …… (will be added after reviews)

Typification: GREECE. PELOPONNESE: Spores extracted from trap cultures inoculated with field-collected mixtures of rhizosphere soil and root fragments of *A. arenaria* from a maritime sand dune site of the beach Voidokoilia (36°57'N 21°39'E), the Peloponnese peninsula, Greece, 8 September 2015, J.

Błaszkowski (**holotype** slide with spores no. ZT Myc ……, **isotypes** slides with spores no. ….–….., LPPDSE). (missing data will be added after reviews)

Etymology

Latin, *mediterranea*, referring to the Mediterranean origin of this species.

Diagnosis

Differs from other glomoid spore-producing species with a four-layered spore wall in (i) the phenotypic properties of the outermost spore wall layer surrounding the other spore wall layers and (ii) nucleotide composition of sequences of the 45S nuc rDNA region and the *RPB1* gene (see Discussion for details).

Description

Glomerospores (= spores) glomoid, formed in clusters or singly in the soil (Fig. 4a). Clusters compact or loose, with two to more than hundred randomly distributed spores. Spores arise blastically at tips of sporogenous hyphae either branched from a parent hypha continuous with an extraradical mycorrhizal hypha (spores in clusters) or directly continuous with extraradical mycorrhizal hyphae (single spores) (Fig. 4a). *Spores* pale yellow (4A3) to olive (3E8); globose to subglobose; (62–)93(–121) µm diam; frequently ovoid; 40–92 × 54–108 µm; with one subtending hypha (Figs. 4a–h), occasionally with two. *Spore wall* composed of four layers (Figs. 4b–g). Layer 1, forming the spore surface, evanescent, uniform (without visible sublayers) in spores crushed in water, swelling up to a thickness of ca. 20 µm, and then uniform or, rarely, consisting of thin, < 0.5 µm thick, sublayers slightly separating from each other in spores mounted in PVLG, flexible to semi-flexible, smooth in young spores, becoming roughened with age, occasionally completely sloughed off in older spores, hyaline to pale yellow (4A3), (1.3–)2.4(–4.5) µm thick when intact (Figs. 4b–g). Layer 2 permanent, uniform, semi-flexible, smooth, hyaline, (0.8–)1.2(–2.0) µm thick, rarely separating from the upper surface of layer 2 (Figs. 4b–g). Layer 3 permanent, laminate, semi-flexible, pale yellow (4A3) to olive (3E8), (3.6–)8.8(–12.8) µm thick, consisting of thin, < 0.5 µm thick, laminae, tightly adherent to each other (Figs. 4b–g). Layer 4 permanent, uniform, flexible to semi-flexible, hyaline to yellowish white (2A2), (0.8–)1.0(–1.5) µm thick, usually separating from the lower surface of layer 3 in slightly crushed or even intact spores (Figs. 4b–g). Only layer 1 usually stains reddish white (9A2) to brownish violet (11D8) in Melzer's reagent, (Figs. 4e, f), occasionally is nonreactive or the staining reaction disappears with time (Figs. 4d, g). *Subtending hypha* pale yellow (4A3) to olive (3E8); straight or recurved, usually slightly funnel-shaped, rarely cylindrical or slightly constricted at the spore base; (8.3–)13.7(–20.8) µm wide at the spore base (Figs. 4g, h); not braking in crushed spores. *Wall of subtending hypha* pale yellow (4A3) to olive (3E8); (3.5–)6.0(–9.6) µm thick at the spore base; consisting of three layers continuous with spore wall layers 1–3; subtending hyphal wall layer 1 usually highly deteriorated or sloughed off in mature and older spores (Figs. 4g, h). *Pore* (1.3–)2.6(–6.0) µm wide at the spore base, closed by a curved septum formed by a protrusion of spore wall layer 4, located at the level of the lower surface of spore wall layer 3 or slightly below (Figs. 4g). Spore content of hyaline oily substance. *Germination* unknown.

Ecology and distribution

In the field, associated with roots of *A. arenaria* in the Peloponnese peninsula, Greece. The geographic position, climate, and soil chemical properties of the sampled site are characterized in Błaszkowski et al. (2019). Our sequence comparisons and phylogenetic analyses suggested that two 45S sequences (HE775333, HE775339) ascribed to uncultured Glomeraceae might represent *C. mediterranea*. The identity of these sequences to the *C. mediterranea* 45S sequences is 97–98%. The sequences were obtained from roots of *Briza media* L. growing in Malesov, North Bohemia, Czech Republic. Attempts to grow *C. mediterranea* in single-species cultures with *P. lanceolata* as host plant failed.

Epigeocarpum japonicum, Yamato, B.T. Goto, Niezgoda, Magurno & Błaszk., **sp. nov.**

Figures 5a–h

MycoBank No. MB …… (will be added after reviews)

Typification: JAPAN. YOKOHAMA: A glomerocarp found on September 22, 2020 from under the litter in a tree plantation of *Cryptomeria japonica* in Yokohama City, Kanagawa Prefecture, Japan (35°30'N 139°30'E), by Minoru Nakajima in Kanagawa Prefectural Museum of Natural History (**holotype** slide with spores no. ZT Myc ……., **isotypes** slides with spores no. …..–….., LPPDSE). (missing data will be added after reviews)

Etymology

Latin, *japonicum*, referring to Japan, origin of this species.

Diagnosis

Differs from *E. crypticum*, the sister relative, in (i) the reactivity of spore wall layers 2 and 3 in Melzer's reagent and (ii) nucleotide composition of sequences of the 45S nuc rDNA region and the *RPB1* gene (see Discussion for details).

Description

Glomerospores (= spores) formed in a compact epigeous glomerocarp (Fig. 5a). *Glomerocarp* mustard yellow (3B6); 5.5 × 5.0 mm (Fig. 5a). *Peridium* thin, yellowish white (3A2) to pale yellow (3A3), only partially covering spores' conglomerations. *Gleba* mustard yellow (3B6), with hyaline to yellowish white (3A2), straight or branched hyphae; (2.0–)8.1(–16.0) µm wide, with a wall (0.8–)1.7(4.2) µm thick; not staining or staining reddish white (9A2) in Melzer's reagent; glomerocarp hosting hundreds of spores (Figs. 5b–h). Spores arise blastically at tips of sporogenous hyphae. *Spores* pale grey (1B1) to pale yellow (4A3); globose to subglobose; (30–)38(–44) µm diam; rarely ovoid; 30–39 × 33–46 µm; with one subtending hypha (Figs. 5b–h). *Spore wall* composed of three permanent, smooth layers (Figs. 5c–h). Layer 1, forming the spore surface, uniform (not containing visible sublayers), semi-flexible, hyaline, (0.8–)1.0(–1.3) µm thick, tightly adherent to layer 2 even in vigorously crushed spores (Figs. 5c–h).

Layer 2 laminate, semi-flexible, pale grey (1B1) to pale yellow (4A3), (5.2–)6.5(–8.0) µm thick; consisting of very thin, < 0.5 µm thick, laminae, tightly adherent to each other, not separating even in vigorously crushed spores (Figs. 5c–h). Layer 3 flexible, hyaline, ca. 1.0 µm thick, frequently separating from the inner surface of layer 2 even in intact spores, except for its funnel-shaped part associated with the inner surfaces of the laminate layer 2 forming the channel that connect the spore interior with the lumen of the subtending hypha (Figs. 5c–h). In Melzer's reagent, spore wall layer 3 and often some innermost laminae of the laminate spore wall layer 2 stain pastel red (9A4) to brownish red (10C8); Figs. 5e, f, h). *Subtending hypha* pale grey (1B1) to pale yellow (4A3); straight or recurved, funnel-shaped; (5.2–)6.0(–7.8) µm wide at the spore base (Figs. 5c, g, h); not braking in crushed spores. *Wall of subtending hypha* pale grey (1B1) to pale yellow (4A3); (1.4–)2.6(–3.2) µm thick at the spore base; consisting of two layers continuous with spore wall layers 1 and 2 (Figs. 5g, h). *Pore* (0.6–)1.0(–1.4) µm wide and open at the spore base; the channel connecting the lumen of the subtending hypha with the interior of spores closed by a septum continuous with spore wall layer (swl) 3; the septum positioned slightly below the inner surface of up to ca. half the thickness of swl 2; the subtending hyphal lumen gradually narrowing in maturing spores due to thickening of subtending hyphal wall layer 2 (Figs. 5c, g, h). Spore content of hyaline oily substance. *Germination* unknown.

Ecology and distribution

The specimen of *E. japonicum* characterized in this study was found in a plantation of *C. japonica* in Yokochama City, Japan. The geographic position and climatic conditions of the sampling site are described in Materials and Methods/Origin of Study Material. Sequence comparison and BI and ML analyses showed 24 environmental sequences, clustered in four OTUs (Online Resource 3), whose phylogenetic affiliation suggested conspecificity to those of *E. japonicum*. All sequences originated from Japan. Most of them were obtained from five specimens (CE1408, CE1506, CE1509, CE1708, CE1709) collected from different locations and originally identified as *Glomus microcarpum*. Attempts to grow *E. japonicum* in single-species cultures with *P. lanceolata* as host plant failed.

Discussion

The morphological and phylogenetic analyses of three glomoid spore-producing fungi presented above confirmed our hypotheses that they are undescribed species of the family Glomeraceae. Moreover, the phylogenetic analyses showed that two of these species clustered in a new generic clade of this family (Fig. 1; Online Resources 1, 2). Consequently, the clade was described under the name *Complexispora* gen. nov., and the two new species were characterized as *C. multistratosa* and *C. mediterranea*. The third fungus enlarged the monospecific genus *Epigeocarpum* (Błaszkowski et al. 2021a) and was described as *E. japonicum* sp. nov.

The only morphological structure that appears to be diagnostic for *Complexispora* is the swelling, colorless, laminate spore wall layer surrounding the main, much thicker, structural laminate spore wall layer (Figs. 2b–h, 3a, 4b, c, e). A spore wall layer with a similar phenotype and location in the spore wall

was previously revealed only in *G. caesaris* (Figs. 4e–h; Oehl et al. 2002), which potentially belongs to this genus.

Morphologically, *C. multistratosa* most resembles *G. caesaris*. Both species produce single spores, which are similar in colour, size, the number of spore wall layers, as well as in the position and phenotypic features of spore wall layers 1 and 5 (Figs. 2a–h, 3a–h) (Oehl et al. 2002). Spore wall layer 2 in both species swells in PVLG (Figs. 2a–h, 3a, d). However, in *G. caesaris* this layer is a permanent structure (Figs. 3e–h), while in *C. multistratosa* it deteriorates with age and may be completely sloughed off in older specimens (Figs. 3b, c). The spore wall of both species contains layer 3, which tightly covers the laminate layer 4 (Figs. 2b–h, 3a–h), but spore wall layer 3 of *G. caesaris* may be coloured (Figs. 3e, f vs. is always hyaline in *C. multistratosa*; Figs. 2b–h, 3a–d) and over 2-fold thicker. Moreover, the spore wall of *G. caesaris* is ca. 1.7-fold thinner than that of *C. multistratosa* and the spore wall components staining in Melzer's reagent in these species are layers 2 and 1, respectively (Figs. 2f–h, 3a, d). Importantly, in *G. caesaris* the spore subtending pore is closed only by spore wall layer 5, and in *C. multistratosa* the pore is closed by spore wall layer 6, which is lacking in the *G. caesaris* spore wall, and occasionally also by a septum separating the subtending hyphal lumen from the spore interior (Figs. 3b–d). Finally, at the *G. caesaris* spore base the subtending hypha and its wall may be 1.4-fold and 1.7-fold narrower and thinner, respectively. Phylogenetically, the two species cannot be compared because no sequence of *G. caesaris* has been deposited in public databases.

The only other species characterized to form glomoid spores with a six-layered spore wall is *Funneliformis fragilistratum*, originally described as *G. fragilistratum* (Skou and Jakobsen 1989). Spores of this species are also similar in size, colour, and the phenotypes of their spore wall layers 1–4. Skou and Jakobsen (1989) described spore wall layers 5 and 6 as "hyaline membranous walls, each 1–2 µm thick", which correspond to flexible to semi-flexible layers in the terminology currently used in characterizing Glomeromycota spore morphology. The presence of two such layers would render *F. fragilistratum* a unique species among all glomoid spore-producing species of Glomeromycota. However, this finding appears to be an artifact of the condition of spores and the way they were crushed. Most importantly, phylogenetic analyses of 18S sequences placed this species in the *Funneliformis* clade (Krüger et al. 2012).

Among non-sequenced species of Glomeromycota forming glomoid spores, only *G. avelingiae* and *G. bagyarajii* were described to have a spore wall composed of four layers, of which none is ornamented, and the innermost layer is flexible to semi-flexible (Mehrotra 1997; Sinclair et al. 2000). Both species also produce spores in clusters and the spores are coloured similarly to those of *C. mediterranea*, but the largest globose *G. avelingiae* spores are up to 1.5-fold smaller and have a 2.4–5.6-fold thinner spore wall. Moreover, the subtending hypha of *G. avelingiae* may be up to 2.1-fold narrower at the spore base and its pore is closed by a septum continuous with spore wall layer 3 (vs. by a protrusion of spore wall layer 4; Fig. 4g). In *G. bagyarajii*, the main structural, thickest, laminate spore wall component is layer 2 (vs. layer 3 in *C. mediterranea*; Figs. 4b–g) and the spore wall is ca. 2.1-fold thinner. In addition, at the spore base the *G. bagyarajii* subtending hypha is 1.2–1.5-fold narrower, has a 1.9–3.5-fold thinner wall, and its pore

may be up to 1.7-fold narrower. Finally, none of the spore components of *G. avelingiae* and *G. bagyarajii* stains in Melzer's reagent, as does spore wall layer 1 of *C. mediterranea* (Figs. 4e, f).

The only phenotypic character separating *E. japonicum* from *E. crypticum* is the reactivity of their spore wall components in Melzer's reagent (Błaszkowski et al. 2021a). In *E. crypticum*, only the thickest, laminate middle spore wall layer 2 stains in this reagent, while in *E. japonicum* the staining reaction occurs in the thinnest, flexible innermost layer 3 and often in some innermost laminae of the laminate layer 2 (Figs. 5e, f, h). However, this histochemical property probably is of little phylogenetic significance as Kaonongbua et al. (2010) concluded with respect to layer 2 of the innermost spore wall 3 of *Acaulospora* species. Instead, the 45S and *RPB1* sequence divergences strongly confirmed the separateness of the two species (see "General data and phylogeny").

As we mentioned in "*Ecology and distribution*" regarding *E. japonicum*, this species has likely been previously identified in Japan as *Glomus microcarpum* because its 45S sequences were almost identical to the 45S sequences of our new species. However, we treat this species assignment as incorrect for the following reasons. First, the morphology of *E. japonicum* does not match the morphology of *G. microcarpum* originally described by the Tulasne brothers (Tulasne and Tulasne 1845) and that of the type material characterized by Berch and Fortin (1984) and Koske et al. (1986). Tulasne and Tulasne (1845) and Berch and Fortin (1984) described *G. microcarpum* with a one-layered spore wall. Koske et al. (1986) found that the *G. microcarpum* spore wall consists of two layers: a unit outer layer and a much thicker, laminate inner layer. Gerdemann and Trappe (1974), based on examination of spores originating from the field and cultures, found the presence of one laminate spore wall layer in specimens of this species coming from Idaho, Oregon, and Washington. Błaszkowski (2012) and Oehl et al. (2011) distinguished two layers in the *G. microcarpum* spore wall and spores identified as *G. microcarpum* with such a spore wall structure were found in Northeast Brazil (unpublished data). Thus, there is no consensus in the literature on the morphology of *G. microcarpum* and, regardless of whether the spore wall structure of the true *G. microcarpum* is one- or two-layered, *E. japonica* clearly differs in this respect. Second, *G. microcarpum* has not yet been sequenced, although it has been described as one of the first two species of this group of fungi and until 2010 regarded as the type species of *Glomus* (e.g., Gerdemann and Trappe 1974). Therefore, in order to lift the doubts about the morphology of and to know the phylogenetic placement of *G. microcarpum* in Glomeromycota, its molecular phylogeny should be determined. This should be reconstructed from analyses of an epitype of *G. microcarpum* designated from a specimen (s) collected from Paris or its vicinity, where this species was originally discovered (Tulasne and Tulasne 1845).

The present study and literature data prove that epigeous and hypogeous glomerocarpic species of Glomeromycota differ strongly phylogenetically. Currently, they are distributed in eight genera belonging to two orders (Błaszkowski et al. 2021a, b, 2022a; Jobim et al. 2019). However, the phylogenies of a number of glomerocarpic species remain unknown and certainly many species are undiscovered mainly due to much greater difficulty in finding them than other members of Glomeromycota (Jobim et al. 2019).

It is expected that these yet uncharacterized glomerocarpic fungi will represent new taxa at different ranks.

We excluded from phylogenetic analyses the monospecific *Simiglomus* with *Simiglomus hoi*. The genus was erected based on two sequences of the 18S gene (Oehl et al. 2011), which in our alignments is represented only by approximately 240 base pairs. In addition, the origination of these sequences is uncertain, as stated by Redecker et al. (2013). Further studies are needed to clarify the status of *Simiglomus* in Glomeraceae.

Declarations

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Author contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Janusz Błaszkowski, Bruno Tomio Goto, Leonardo Casieri, Franco Magurno, Monika Malicka, Edward Meller, Paweł Milczarski, Piotr Niezgoda, and Szymon Zubek. The first draft of the manuscript was written by Janusz Błaszkowski, and all authors commented on previous versions of the manuscript. Conceptualisation: Janusz Błaszkowski, Bruno Tomio Goto, Leonardo Casieri, Franco Magurno, Sylwia Uszok; methodology: Janusz Błaszkowski, Bruno Tomio Goto, Franco Magurno, Piotr Niezgoda; formal analysis and investigation: Janusz Błaszkowski, Bruno Tomio Goto, Franco Magurno, Monika Malicka, Edward Meller, Paweł Milczarski, Piotr Niezgoda, Sylwia Uszok, and Szymon Zubek; writing original draft preparation: Janusz

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Data availability Datasets generated during and/or analyzed during the current study are available from the corresponding author upon request.

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