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Glomus highlandensis and *G. mongioie*, Two New Arbuscular Mycorrhizal Fungi from Saltmarshes, Dunes and Mountains of Europe

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Abstract: Morphological and phylogenetic (45S nrDNA+*RPB1* gene) analyses of three glomoid spore-producing arbuscular mycorrhizal fungi revealed the presence of two new species belonging to the genus *Glomus* in the family Glomeraceae. In the field, *Glomus highlandensis* sp. nov. was found in a saltmarsh of the Scottish Highlands and in maritime sand dunes of the Baltic Sea in Poland, both saline environments, while *G. mongioie* sp. nov. originates from the Italian Alps. Phylogenetic placement analysis using environmental sequences indicated that *G. highlandensis* could have likely worldwide distribution while *G. mongioie* seems to be a rare species in the world. The molecular and phylogenetic analysis provided important insights about the presence of ribosomal variants in *Glomus*, with potential negative implication in phylogeny and species recognition.

Keywords: Glomeromycota; molecular phylogeny; ribosomal variants; taxonomy; two new *Glomus* species

1. Introduction

Arbuscular mycorrhizal fungi (AMF) are key components of the soil microbiome. They live in close relationship with the vast majority of plant species in forest or agricultural systems, benefiting them by increasing water and nutrient absorption and mitigating biotic and abiotic stress. They also enhance soil structure and stability by stimulating root expansion and developing a hyphal network that contributes to the formation of stable macroaggregates, thereby decreasing erosion [1,2]. Finally, AMF hyphae are able to recruit specific microbial populations with multiple plant growth promoting traits such as phosphate solubilization, nitrogen fixation, plant growth regulation and biocontrol activity, increasing the functional diversity and richness of rhizosphere microorganisms [3,4].

Because of the benefits they provide, AMF have high ecological and agronomic potential, and several species, especially from the Glomeraceae family, are frequently used in commercial inoculants [5]. However, the richness of species that make up this group is still poorly understood, with several species potentially highly effective in forming symbioses yet to be discovered, as evidenced by the disparity between the number of environmental sequences available and the number of species formally described. Currently, around 360 AMF species are recognized, distributed in 49 genera and 17 families within the phylum Glomeromycota [6–8].

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The description of *Glomus macrocarpum* and *G. microcarpum* by [9] marked the recognition of the first fungal genus within Glomeromycota, despite seven years later both species were transferred to the genus *Endogone* [10]. *Glomus* was reinstated by [11] with the revision of the family Endogonaceae, entailing the reestablishment of the original species (*G. macrocarpum* and *G. microcarpum*), the description of new *Glomus* species, and the transfer of other species from *Endogone* to *Glomus*. Subsequently, for at least four decades, all species with glomoid-type spores (spores produced terminally, sub-terminally or intercalary from subtending hyphae) were included in *Glomus*. Until 2010, *Glomus* encompassed nearly 50% of the described richness within Glomeromycota. However, phylogenetic analysis revealed that the genus was polyphyletic, and several *Glomus* species were reorganized into other taxonomic categories from genus to class level [7,12–16].

The phylogeny of *Glomus* has been constrained by the absence of molecular data from the type species, *G. macrocarpum*, lectotypified by [17]. [12] established a molecular epitype of *G. macrocarpum* and defined it as the only species in *Glomus sensu stricto*, while the other species were categorized as *Glomus sensu lato* due to their uncertain or unknown phylogenetic data. [15] described *G. tetrastratosum* Błaszk., Chwat & Góralska based on its phylogenetic proximity to *G. macrocarpum*, and other four new species (*G. bareae* Błaszk., Niezgoda, B.T. Goto & Kozłowska, *G. ibericum* A. Guillén, F.J. Serrano-Tamay, J.B. Peris & I. Arrillaga, *G. atlanticum* Błaszk., Niezgoda, B.T. Goto, Moreira & Magurno, and *G. chinense* F.X. Yu, B.T. Goto, H.Y. Feng & Yong Jun Liu) were recently described [18–21], enriching the Glomus *sensu stricto* clade. Morphologically, this clade is represented by glomoid species occurring in loose clusters or rarely in hypogeous or epigeous unorganized glomerocarps, forming a single spore wall with two to four layers.

During investigations of AMF assemblages associated with plants growing in a saltmarsh area, sandy dunes and mountains of Europe, we obtained single-spore cultures of three glomoid fungi, thereafter recognized as new members of the genus *Glomus*. The new species are here described under the name *G. highlandensis* and *G. mongioie* spp. nov., based on the morphology of the spores and the phylogenetic analysis of 45S nrDNA and the *RPB1* genes.

Moreover, the analyses performed showed the presence of two ribosomal variants in *G. highlandensis* and *G. tetrastratosum*, highly divergent and with potential negative implication in phylogeny and species recognition.

2. Materials and Methods

2.1. Sampling and Single Species Pot Cultures

The field inoculum containing *Glomus highlandensis* was collected in the saltmarsh habitat of Beauly Firth (57°30'13.9" N 4°19'06.6" W), Scotland, UK, which is a part of Moray Firth and together with Inverness Firth form the estuarine component of the Moray Basin system. The soils are mixosaline to saline, mainly mineral, and pH is circumneutral [22]. The climate of the area is temperate oceanic, with a mean annual temperature of 9.3°C. The mean annual rainfall is relatively low (624.4 mm) due to the "rain shadow" effect caused by surrounding mountains [22,23]. The plant communities consist of *Zostera angustifolia* Rchb., *Z. noltii* Hornem., *Carex recta* Boott as well as *Juncus gerardi* Loisel., *Plantago maritima* L., *Armeria maritima* (Mill.) Willd. and *Festuca rubra* L. [22,24]. The field samples were collected by F. Magurno on 29th October 2018.

The second occurrence of *G. highlandensis* was found associated with the roots of *Leymus arenarius* Hochst. growing on the sand dunes of the Baltic Sea near Jastarnia, Poland (54°42'23.2452" N 18°40'7.7988 E") on the Hel Peninsula. The coastal climate of the peninsula is shaped by oceanic and continental influences, with an average annual temperature of about 9°C and mean annual rainfall of 750 mm. The soils developed on the dunes consist of unconsolidated or partly consolidated material [25]. The material was collected on 9th July 2021 by K. Bierza from yellow dune communities, representing earlier succession stages of tall-grass perennial swards with the domination of *L. arenarius* and *Ammophila arenaria* (L.) Link.

The field inoculum containing *Glomus mongioie* was collected on the foothills of Monte Mongioie in the Ligurian Alps, Italy (44°09'34.7" N 7°46'29.2 E"), characterized by rich mesophilous grasslands

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and calcitic-dolomitic soil. The plant species composition included *Aquilegia atrata* W.D.J.Koch, *Gentiana ligustica* R.Vilm. & Chopinet, *Leontopodium nivale* subsp. *alpinum* (Cass.) Greuter, *Nigritella* sp., *Potentilla* sp., *Helictotrichon sedenense* (DC.) Holub, *Aster alpinus* L., *Pulsatilla alpina* (L.) Delarbre, *Linum alpinum* L., *Carex firma* Host, *Saxifraga caesia* L., *S. cernua* L., *Trifolium thalii* Vill., *Trollius europaeus* L., *Polygala alpestris* Rchb, and *Festucetum* sp. The site is characterized by low temperature climate with mean annual values of 3.0 to 5.9°C and mean annual precipitation of 1001-1500 mm [26]. The samples were collected by F. Magurno on 17th August 2019.

Field-collected mixtures of rhizosphere soils and root fragments collected from the three locations were used for inoculation of pot trap cultures with *Plantago lanceolata* L. as host plant. After approx. five months, spores and loose clusters were isolated from the substrate and used to establish pure species pot cultures in a mixture of sterile river sand and bentonite clay (9:1, v/v). The cultures were checked at regular intervals of three months to detect the presence of mycorrhizal structures (data not shown).

2.2. Morphological Analysis

Spore clusters and spores were mounted in water, polyvinyl alcohol-lactic acid-glycerol (PVLG), and a mixture of PVLG and Melzer's reagent (1:1, v/v). Morphological characteristics of spores and their sub-cellular structure were characterized based on at least 50–100 spores, examined and photographed using dissecting and compound microscopes. Color names were from [27]. The terminology of spore characters follows [21,28–30]. Types of spore wall layers are those defined by [31,32]. Nomenclature of fungi and the authors of fungal names are from the Index Fungorum database (www.indexfungorum.org). The terms "glomerospores" and "glomerocarps" were used for spores and fruit bodies (sporocarps) produced by AM fungi, respectively, as [33,34] proposed. Voucher specimens of the two new species were deposited in the ZT Myc (ETH Zu-rich, Switzerland; holotypes), and in the UFRN herbarium, Brazil (isotypes).

2.3. Molecular Analysis

For both species, genomic DNA was extracted from single clusters of spores with the DNeasy PowerSoil Pro kit (Qiagen), following the producer's instructions with the modifications as follows: the first step of vortexing was replaced by crushing the spores with a micropestle inside a vial containing 100 µl of CD1 solution; the kit's solutions volume was arranged proportionally till the first washing step; final elution was performed in 30 µl volume. Amplicons of 45S nrDNA partial genes (thereafter 45S) were obtained by nested PCR with the primer pairs SSUmAf–LSUmAr and SSUmCf–LSUmBr [35] using the Phusion Plus DNA Polymerase (Thermo Fisher Scientific) with universal annealing temperature of 60°C, according to producer's instructions. *RPB1* amplicons were obtained from *Glomus highlandensis* by PCR with the primers RPB1 3F alfa and RPB1 5R beta [36] targeting the region from exon 3 to 5, and from *G. mongioie* with the primers RPB1-4F1 in combination with RPB1-5R as in [30] targeting the region from exon 4 to 5. PCR amplicons were purified with GeneJET PCR Purification Kit (Thermo Fisher Scientific), cloned with CloneJET PCR Cloning Kit (Thermo Fisher Scientific) and sequenced at Genomed S.A. (Warsaw). Both 45S and RPB1 sequences were deposited in GenBank (accession numbers will be added after revision of the manuscript).

2.4. Phylogenetic Analyses

For the phylogenetic accommodation of the here presented new species, two datasets of 45S sequences and one dataset of *RPB1* partial gene sequences were prepared, representing all the sequenced members of the genus *Glomus*, upon verification by blastn analysis of the affiliation of the new species to genus. Sequences of the two described *Complexispora* species [36] were included to represent the outgroup in the phylogenetic analysis.

The 45S datasets contained 49 (ds1) and 41(ds2) sequences respectively, overlapping the partial 18S - ITS - partial 28S regions. The datasets differed by the presence of alternative gene variant sequences, to test their influence on the stability of species clades in the phylogenetic inference.

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Additionally, the ds1 included 4 sequences representing both variants previously obtained from an isolate of *Glomus tetrastratosum* from the AMF collection of the University of Silesia in Katowice.

The *RPB1* dataset contained 29 sequences, the longest spanning from exon 1 to 5, the shortest from exon 4 to 5.

The datasets were aligned separately with the online version of MAFFT 7 using the E-INS-i iterative refinement methods (http://mafft.cbrc.jp/alignment/server/). A concatenated alignment was prepared combining the 45S (ds2) and the RPB1 alignments using a custom Python script.

The two 45S and the concatenated alignments were used as input for the Bayesian inference (BI) and maximum likelihood (ML) phylogenetic inference, performed via CIPRES Science Gateway 3.1 [37]. The 45S alignments were divided into five partitions: 18S, ITS1, 5.8S, ITS2, 28S. Nine additional *RPB1* partitions, representing 5 exons and four introns, were employed in the concatenated alignment. In both BI and ML analyses GTR+I+G was used as nucleotide substitution model for each partition [38]. Four Markov chains were run over one million generations in MrBayes 3.2 [39], sampling every 1,000 generations, with a burn-in at 30% sampled trees. The ML phylogenetic tree inference was performed with RAxML-NG 1.0.1 [40], using a maximum likelihood/1000 bootstrapping run, and ML estimated proportion of invariable sites and base frequencies. For each alignment, the resulting phylogenetic trees were visualized, merged and edited in TreeGraph 2 [41]. Any discrepancy in the topology between BI and ML trees was recorded. Clades were considered supported with Bayesian posterior probabilities ≥ 0.95 and ML bootstrap values ≥ 70%.

The percentages of identity intraspecies and between the new species and the closest relatives was calculated in Mothur v.1.44.3 using the function dist.seqs (calc=eachgap, countends=F) using the aligned 45S and RPB1 datasets as input.

To detect other possible occurrences of the two species, blastn was used to retrieve uncultured Glomeromycota sequences from GenBank with percentage of identity > 96% with at least one of the 45S sequences of the species of interest. The environmental sequences were aligned with the db1 and their phylogenetic placement was established by RAxML-EPA [42] followed by the placement mass accumulation in GAPPA v0.8.0 [43] of each sequence upwards the 45S ML tree used as reference, with threshold > 0.9, similarly to [44].

3. Results

3.1. Phylogenetic Analyses

Overall, 16 sequences for the 45S locus and 7 sequences for the *RPB1* locus were obtained in the study. Among the 45S sequences, 11 were obtained for *Glomus highlandensis* (7 for the isolate "Highlands - Scotland", 4 for the isolate "Hel-Poland") and 5 for *G. mongioie* (Italian Alps). About the *RPB1* locus, 5 sequences were obtained for *G. highlandensis* (2 for the isolate "Highlands", 3 for the isolate "Hel") and 2 for *G. mongioie*.

The visual inspection of the two 45S alignments allowed to recognize the presence of two variants, S (=short) and L (=long) among the sequences of *G. highlandensis* (present in both isolates) and *G. tetrastratosum*, differing mostly in the ITS1 motif by an insertion of ca 40-50 bp (Figure S1).

Both the phylogenies generated from the concatenated alignment (Figure 1) and the two 45S alignments (Figures 2 and S2) showed identical topology. All parameters of the convergence diagnostic (potential scale reduction factor and standard deviation of split frequencies) indicated that the convergence was obtained in the BI analysis. The sequences of *G. highlandensis* and *G. mongioie* populated independent, significantly supported clades, both sister to *G. macrocarpum*.

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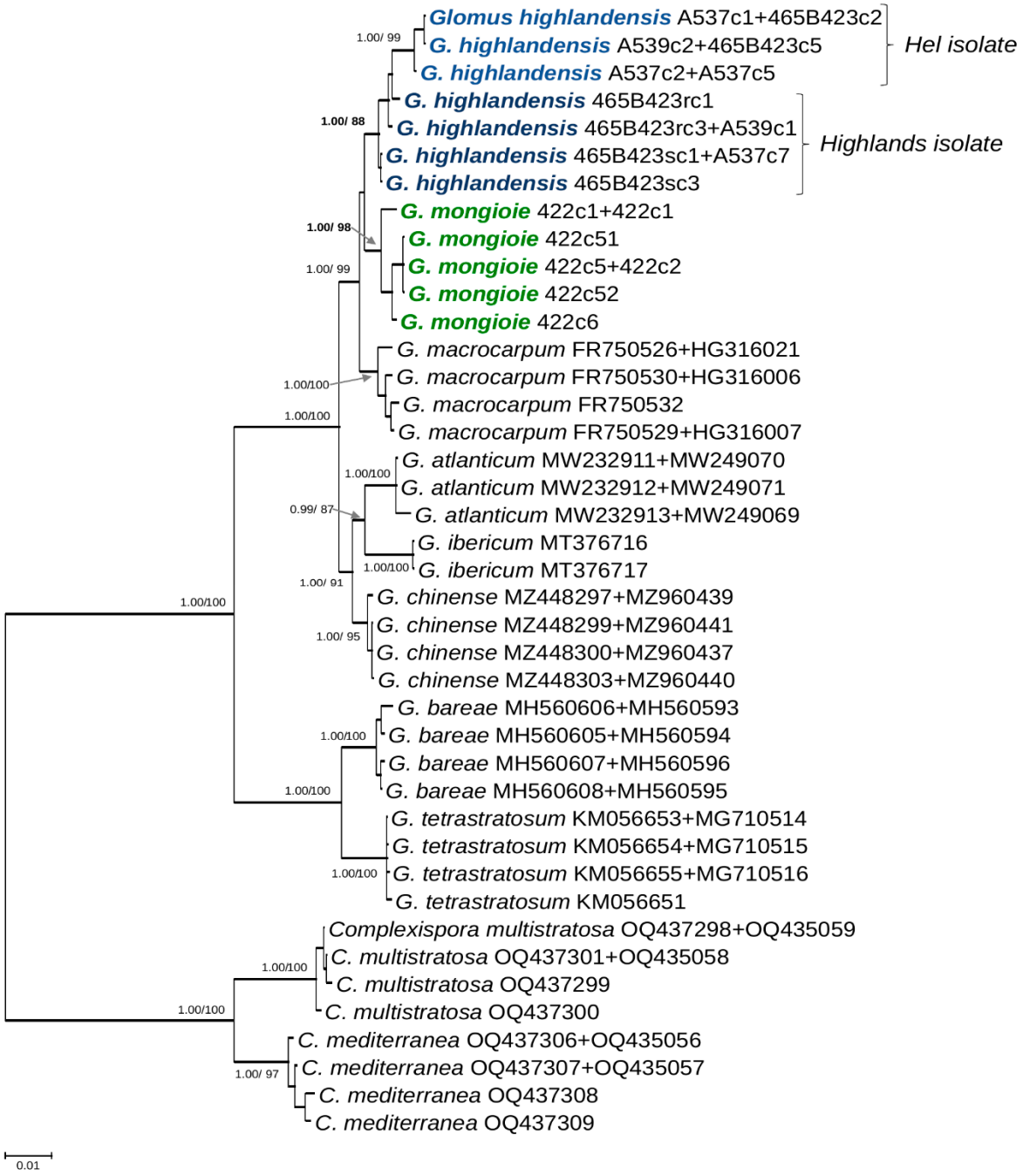

Figure 1. 50% majority-rule consensus tree from the Bayesian analysis of 45S sequences concatenated with *RPB1* sequences of *Glomus highlandensis*, *G. mongioie*, the 6 species of *Glomus sensu strictu* in possession of molecular data, and two *Complexispora* species (the closest taxa to *Glomus* in the family Glomeraceae) serving as outgroup. The two new species are in bold coloured font. Different shades of blue are used to distinguish the two isolates of *G. highlandensis*. Bayesian posterior probabilities ≥0.95 and ML bootstrap values ≥60% are shown near the branches, respectively. Bar indicates 0.1 expected change per site per branch.

The phylogenetic inference of the db1 (Figure 2) showed all 4 *G. highlandensis* sequences of the L variant clustering in a fully supported "long branch" clade inside the species clade that received full BI support (1.00) and moderate ML support (71%). In the consensus tree generated from the db2 alignment (without the L variant sequences) (Figure S2) and in the concatenated tree (Figure 1), the species clade ML support increased to 84% and 88%, respectively.

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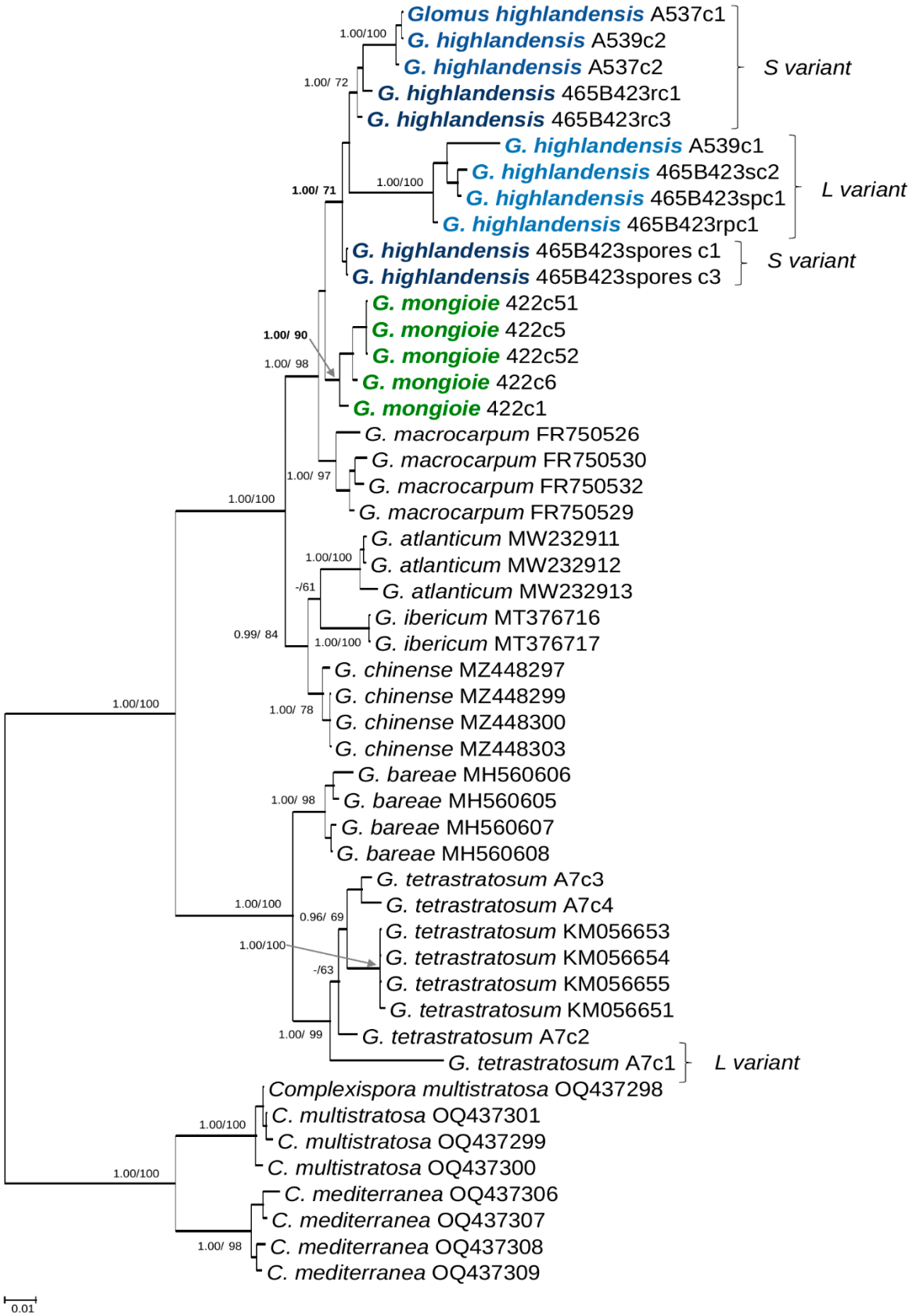

Figure 2. 50% majority-rule consensus tree from the Bayesian analysis of dataset ds1 (S- and L-variant sequences). The dataset consisted in 45S sequences of *Glomus highlandensis*, *G. mongioie*, six species of *Glomus sensu strictu* in possession of molecular data, and two *Complexispora* species (the closest taxa to *Glomus* in the family Glomeraceae) serving as outgroup. The two new species are in bold coloured font. Different shades of blue are used to distinguish the two isolates of *G. highlandensis*, and their L-

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variant sequences. Bayesian posterior probabilities ≥0.95 and ML bootstrap values ≥60% are shown near the branches, respectively. Bar indicates 0.1 expected change per site per branch.

The G. mongioie clade received in all analysis full BI support and ML support ranging from 88-90% in the 45S phylogenies (db2 and db1 alignments, respectively) to 98% in the 45S-*RPB1* phylogeny.

The L variant sequence of *G. tetrastratosum* clustered with the S variant sequences of the species in an almost fully supported clade (BI=1.00, ML=99%), while the S variant clade received only low ML support (63%).

Considering the two variants together, the 45S sequences of *G. highlandensis* displayed a high intraspecific variability, with a range of identity as 91.5-100%. The clade hosting the L variant shared 91.5-94.4% of identity with the sequences of the S variant. The S variant was considered as "conventional" since the visual inspection of the alignment showed a similar ITS1 organization with the sequences representing the other *Glomus* species (therefore used for species comparison). Considering the S variant, the percentage of identity between the Highlands and Hel isolates ranged between 96 and 97.9%. *G. highlandensis* differed from the neighbour species *G. macrocarpum* and *G. mongioie* by 95.9-97.6 and 94.5-98.3%, respectively. In *G. mongioie* the percentage of identity among 45S sequences was higher than 98.5%. The species differed from the neighbour species *G. macrocarpum* by 95.2 and 96.1%.

The percentage of identity of *RPB1* sequences was higher than 99.6% and 99.3% in *G. highlandensis* and *G. mongioie*, respectively. The two species differed by 99-99.2%, and from *G. macrocarpum* by 99.1-99.4 (*G. highlandensis*) and 98.4-98.6 (*G. mongioie*). In *G. tetrastratosum* the L variant shared ca 94% of identity with the S variant sequences.

Regarding possible environmental occurrences of the two species, 34 "Uncultured Glomus/Glomeromycota" sequences were retrieved from Genbank. RAxML-EPA and GAPPA analysis assigned six sequences to *G. highlandensis*, while no environmental occurrences were found for *G. mongioie* (Figure S3; see also *Ecology and distribution* in Taxonomy section).

3.2. Taxonomy

Glomus highlandensis B.T. Goto, Magurno, Uszok, M.B. Queiroz, **sp. nov.**

Figure 3A–G.

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

Etymology: English, *highlandensis*, referring to the Scottish Highlands, location where this species was originally found.

Typification: Scotland. Spores from a single-species culture established from a trap culture inoculated with a field-collected mixture of rhizospheric soil from a saltmarsh of the Beauly Firth, Scotland, UK (57°30'13.9" N 4°19'06.6" W), October 2018, F. Magurno. Holotype: slide with spores no. XXXX - will be added after reviews, collection name. Isotype: slides with spores no. XXXX - will be added after reviews, UFRN-Fungos.

Diagnosis: Differs from *G. macrocarpum*, the closest phylogenetic relative together with *G. mongioie* (Figure 1), in: (i) the spore wall and subtending hyphal wall structure, as well as (ii) nucleotide composition of sequences of the 45S nuc rDNA gene and the *RPB1* gene.

Description: Glomerospores formed in soil, in loose clusters with 2–18 spores or singly, arise blastically at tips of sporogenous hyphae directly continuous with extraradical mycorrhizal hyphae (Figure 3A–G). *Spores* pale yellow (4A3) to greyish yellow (4B3); globose to subglobose; (30–)62(–110) µm diam; rarely ovoid; 70–98 × 81–110 µm; with one subtending hypha (Figure 3A–F). *Spore wall* composed of four layers (Figure 3A,C–G).

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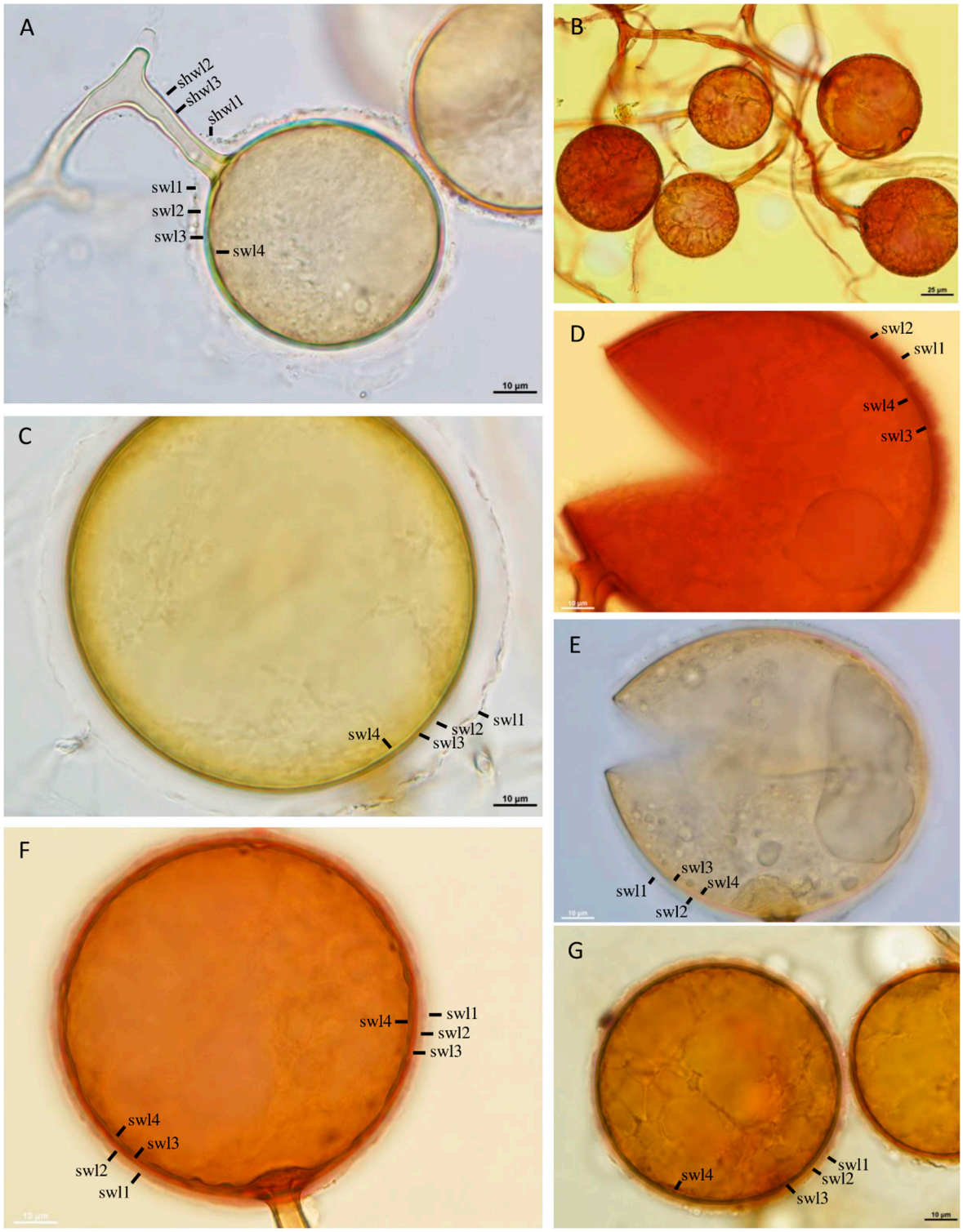

Figure 3. A–G. *Glomus highlandensis*. A–B. Cluster with sporogenous hyphae, spores, and a spore subtending hypha (sh). A, C–G. Spore wall layers (swl) 1–4. A, F. Subtending hyphal wall layers (shwl) 1–4 continuous with spore wall layers (swl) 1–4. A, C, E. Spores in PVLG. B, D, F, G. Spores in PVLG+Melzer's reagent. A–G. Differential interference microscopy.

Layer 1, forming the spore surface, evanescent, mucilaginous, short-lived, hyaline, (0.6–)0.7(–0.8) µm thick, often highly expanding in spores mounted in PVLG and separating from the upper surface of spore wall layer 2 by up to 15.0 µm, frequently entirely sloughed off in mature spores (Figure 3A,C). Layer 2 uniform, permanent, smooth, semi-flexible, hyaline, (0.5–)0.8(–1.3) µm thick, tightly adherent to the upper surface of layer 3, not separating from this layer in even vigorously

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crushed spores (Figure 3C–G). Layer 3 laminate, permanent, smooth, semi-flexible to semi-rigid, pale yellow (4A3) to greyish yellow (4B3), (0.8–)1.5(–3.0) µm thick, consisting of very thin, <0.5 µm thick, laminae, tightly adherent to and not separating from each other in even vigorously crushed spores (Figure 3C–F). Layer 4 permanent, flexible, hyaline, 0.5–1.0 µm thick, detected in mature spores and difficult to see in young spores. In Melzer's reagent, spore wall layers 1 and 3 stains reddish white (7A2) to deep red (8A3) (Figure 3B,D,F,G). *Subtending hypha* pale yellow (4A3) to greyish yellow (4B3); straight or recurved, cylindrical to slightly funnel-shaped at the spore base; (7.6–)9.8(–13.7) µm wide at the spore base (Figure 3A,B,D,F). *Wall of subtending hypha* pale yellow (4A3) to greyish yellow (4B3); (1.7–)2.7(–3.8) µm thick at the spore base; consisting of four layers continuous with spore wall layers 1–4; subtending hyphal wall layer 1 swelling in PVLG and usually highly deteriorated or, occasionally, entirely sloughed off in mature spores (Figure 3C). *Pore* (1.5–)4.5(–8.0) µm wide at the spore base, open, rarely closed by a curved septum connecting the inner surface of spore wall layer 4 (Figure 3A,F). Spore content of hyaline oily substance. *Germination* unknown.

Ecology and distribution: In the field, *G. highlandensis* probably lived in arbuscular mycorrhizal symbiosis with roots of salt-tolerant wetland plant species in Scotland, and with roots of *L. arenarius* in sand dunes of the Baltic sea, Poland, but no molecular analyses were performed to confirm this assumption. In single-species cultures with *P. lanceolata* as the host plant, *G. highlandensis* formed mycorrhiza with arbuscules, vesicles, as well as intra- and extraradical hyphae (data not shown). The RAxML-EPA and GAPPA affiliation analysis using environmental sequences indicated that *G. highlandensis* was putatively detected in prairie grasses of Canada (EU380107), in an ash sedimentation pond of Czech Republic (HG425911, HG425912) and in forest and groves areas in the USA (JQ029746, HQ895795, JX848925).

Glomus mongioie Magurno, B.T. Goto, Uszok, M.B. Queiroz, **sp. nov.**

Figure 4A–F.

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

Etymology: mongioie, referring to the Monte Mongioie, where this species was originally found.

Typification: Italy. Spores from a single-species culture established from a trap culture inoculated with a field-collected mixture of rhizospheric soil from Monte Mongioie, a mountain of the Ligurian Alps located in the southern Piedmont (44°09'34.7" N 7°46'29.2 E"), August 2019, F. Magurno. Holotype: slide with spores no. XXXX - will be added after reviews, collection name. Isotype: slides with spores no. XXXX - will be added after reviews, UFRN-Fungos.

Diagnosis: Differs from *G. highlandensis*, the closest phylogenetic relative together with *G. macrocarpum* (Figure 1), in: (i) the spore wall structure, (ii) morphometric features of the spore wall, the subtending hyphal wall and pore, and (iii) nucleotide composition of sequences of the 45S nuc rDNA region and the *RPB1* gene.

Description: Glomerospores formed in soil, in loose clusters with 2–21 spores or singly, arise blastically at tips of (i) sporogenous hyphae branched from a parent hypha continuous with an extraradical mycorrhizal hypha (spores in clusters) or (ii) sporogenous hyphae directly continuous with extraradical mycorrhizal hyphae (single spores), Figure 4A–F. *Spores* yellowish white (4A2) to greyish yellow (4B5); globose to subglobose; (45–)86(–110) µm diam; rarely ovoid; 37–73 × 45–83 µm; with one subtending hypha (Figure 4A–F). *Spore wall* composed of three layers (Figure 4A,C–F). Layer 1, forming the spore surface, uniform (not containing visible sublayers), mucilaginous, short-lived, flexible, hyaline, (0.8–)0.9(–1.1) µm thick, always highly swelling in spores mounted in PVLG and separating from the upper surface of spore wall layer 2 by up to 14.0 µm, frequently entirely sloughed off in mature spores (Figure 4C–E). Layer 2 uniform (without visible sublayers), permanent, semi-flexible, smooth, hyaline, (0.8–)1.1(–1.4) µm thick, tightly adherent to the upper surface of spore wall layer 3 (swl3), not separating from this layer in even vigorously crushed spores, occasionally difficult to detect when very thin, especially in young spores with a semi-hyaline or brightly coloured swl3 due to the lack of contrast.

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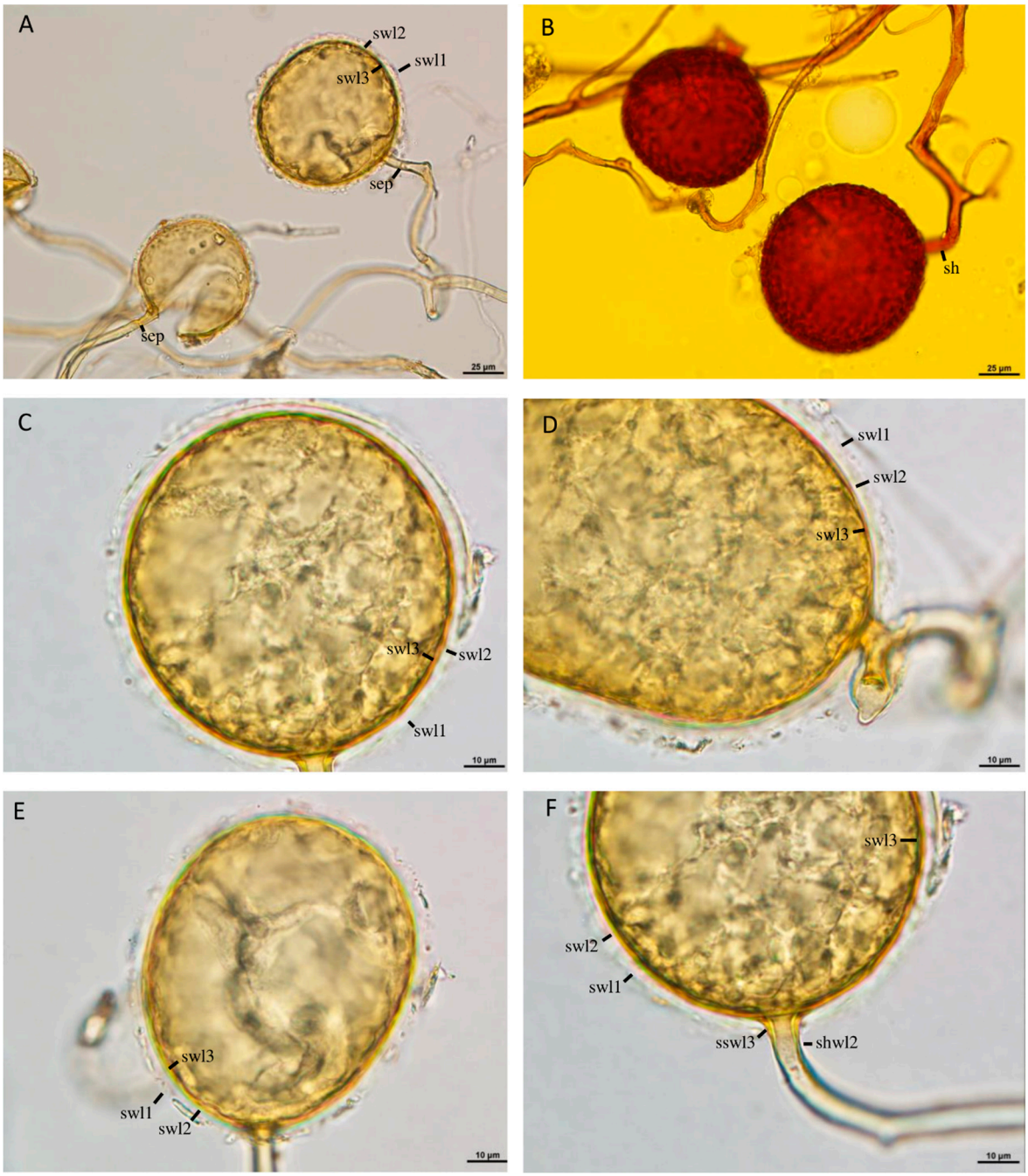

Figure 4. A–F. *Glomus mongioie*. A–B. Cluster with sporogenous hyphae, spores, and a spore subtending hypha (sh). A, C–F. Spore wall layers (swl) 1–3. A, F. Subtending hyphal wall layers (shwl) 1–3 continuous with spore wall layers (swl) 1–3. A, C–F. Spores in PVLG. B. Spores in PVLG+Melzer's reagent. A–F. Differential interference microscopy.

Layer 3 laminate, permanent, smooth, semi-flexible, yellowish white (4A2) to greyish yellow (4B5), (1.1–)1.3(–1.6) µm thick, consisting of very thin, <0.5 µm thick, laminae, tightly adherent to and not separating from each other in even vigorously crushed spores (Figure 4C–F). In Melzer's reagent, spore wall layer 1 and 3 usually stains reddish white (11A2) to brownish violet (11D8; Figure 4B). *Subtending hypha* yellowish white (4A2) to greyish yellow (4B5); straight or recurved, usually slightly funnel-shaped, rarely cylindrical or slightly constricted at the spore base; (5.3–)8.4(–12.4) µm wide at the spore base (Figure 4A–F); not braking in crushed spores. *Wall of subtending hypha* yellowish white (4A2) to greyish yellow (4B5); (1.3–)2.4(–3.2) µm thick at the spore base; consisting of three layers continuous with spore wall layers 1–3; subtending hyphal wall layer 1 swelling in PVLG and usually highly deteriorated or, occasionally, entirely sloughed off in mature spores (Figure 4D,F). Pore (2.5–

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)3.9(–6.4) µm wide at the spore base, usually open, very rarely closed by a curved septum, 0.6–1.0 µm thick, continuous with some innermost laminae of spore wall layer 3 (Figure 4A). Spore content of hyaline oily substance. *Germination* unknown.

Ecology and distribution: In the field, *G. mongioie* lived in arbuscular mycorrhizal symbiosis with roots of herbaceous mountain vegetation in the Ligurian Alps, Italy, but no molecular analyses were performed to confirm this assumption. In single-species cultures with *P. lanceolata* as the host plant, *G. mongioie* formed mycorrhiza with arbuscules, vesicles, as well as intra- and extraradical hyphae (data not shown). The RAxML-EPA and GAPPA affiliation analysis using environmental sequences showed no sequences potentially representing *G. mongioie*, suggesting that this is probably a rare species in the world.

4. Discussion

The morphological analysis supported by the phylogenetic inference using single and concatenated loci confirmed the three AMF isolates obtained from saltmarshes (Highlands, Scotland) and coastal sand dunes (Hel Peninsula, Poland), and from the Mountain Alps (Italy) represent two new species of the genus *Glomus*. The analysis accommodated the first two isolates, both from hypersaline environments, in *G. highlandensis*, while the third isolate from Alps represented *G. mongioie*. The phylogenetic analysis placed the two new species in a supported clade together with *G. macrocarpum*.

Finally, the molecular and phylogenetic analysis showed the presence of two ribosomal variants in *G. highlandensis* and *G. tetrastratosum*, highly divergent and negatively affecting the support of the species clade in *G. highlandensis*.

4.1. Comparison with Glomus Species

Morphologically, *G. highlandensis* differs substantially from *G. mongioie*. In *G. highlandensis*, the spore wall consists of four layers (Figure 3A,C–G), while the spore wall of *G. mongioie* contains three layers (Figure 4A,C–F), lacking the inner, hyaline, flexible swl4 of the former species. Both species differ fundamentally from *G. macrocarpum*, their closest species in the phylogenetic analysis. The most significant difference resides in the spore wall. *Glomus macrocarpum* contains only two layers [11,17], lacking the inner, hyaline, flexible swl4 of the *G. highlandensis* and the second permanent, uniform, hyaline layer of the *G. mongioie*. At the moment *G. macrocarpum* represents a species producing spores in unorganized glomerocarps without convincing evidence of their capacity to grow in single or trap culture. In contrast, *G. highlandensis* and *G. mongioie* produced hundreds of spores both in trap and single species culture. The only other glomoid spore-producing species without ornamentation and with a four-layered spore wall is *G. tetrastratosum* [45]. However, spores of *G. highlandensis* are clearly lighter (vs. brownish yellow in *G. tetrastratosum*), 2.2–2.4-fold smaller when globose.

The only species non-sequenced with similar morphology to that of *G. mongioie* is *G. kerguelense* Dalpé & Strullu. Both species produce yellow coloured glomoid-like spores with a three-layered spore wall and a subtending hypha [7,46]. The main differences between these species reside in the phenotypic and histochemical characters of the spore wall layers. Instead of the uniform, hyaline, thin (0.8–1.4 µm) spore wall layer 2 of *G. mongioie*, *G. kerguelense* has a laminate, hyaline to pale yellow, thicker (4.3–6.8 µm) layer. Spore wall layer 3 in both species is laminate and yellow coloured. But, in *G. kerguelense* it is clearly thicker (1.6–2.2 µm), and, most importantly, its inner surface in maturing spores is covered with granular processes, forming a granular/spongiform appearance that disappear with spore maturing (vs. always smooth in *G. mongioie*). None of the spore wall layers of *G. kerguelense* stains in Melzer's reagent like spore wall layer 1 of *G. mongioie*. *Glomus atlanticum*, *G. bareae* and *G. chinense*, all species of *Glomus sensu strictu*, also present three layers in the spore wall [18,20,21]. Similarly to *G. mongioie*, in *G. atlanticum* and *G. bareae* the Melzer's reaction is observed in the swl1, but these species differ significantly. The swl3 in *G. mongioie* is laminated and greyish yellow (vs. laminated and orange brown in *G. atlanticum* and flexible and hyaline in *G. bareae*) [20]. In *G. chinense*, the second, uniform, hyaline slw2 is missing as in *G. mongioie*. In addition, the Melzer's reaction in *G. chinense* is observed in swl1–2 (vs. in swl1 and swl3 in *G. mongioie*).

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4.2. Ribosomal Variants: A "Forgotten" Issue

The existence of ribosomal variants and their possible implication in the phylogenetic placement of AMF species have been known for a long time. Already 20 years ago, [47] was warning about the possibility of misleading phylogenetic information using ribosomal markers in environmental studies. Based on analysis of the 28S D2 region, [47] detected several variants in isolates of *Glomus claroideum* and *G. etunicatum* (currently *Entrophospora claroidea* and *E. etunicata*; [7]) with a level of divergence up to 19% bp differences among the sequences. [48] investigated the rRNA gene diversity amplifying the 5.8S-partial 28S region from single spores in nine populations of *Claroideoglomus* (=*Entrophospora*) species (*E. claroidea*, *E etunicata*, *E. lutea*). According to the study two groups of sequences, called S and L variants, were detected, clustering in two distinct supported clades as result of ancestral polymorphism. Each clade was populated by sequences from the three species investigated. The S (=short) variant differed from the L (=long) variant primarily by a 100 bp deletion in the ITS2 and by multiple nucleotide polymorphisms along the 28S region. Both variants were considered functional after verification of gene expression and estimation of the structural stability. Finally, recent comparative analysis of genomes from several isolates of *Rhizoglomus irregulare* confirmed the presence in this species of up to 11 divergent rRNA copies on each haplotype investigated, located on different chromosomes [49,50]. These late findings expanded our knowledge about the presence of divergent rRNA variants even outside the genus *Entrophospora*.

In the present study we had further confirmation, detecting two highly divergent variants among *G. highlandensis* and *G. tetrastratosum* sequences, probably as a consequence of ancestral polymorphism. Differently from what was observed by [48], the differences among the variants were observed mostly in the ITS1 locus, with an insertion of ca 40-60 bp. Furthermore, the variants were not forming independent clades trespassing the species boundaries, but they had a negative influence on the species clades' support of *G. highlandensis*. Further investigations, principally employing genome sequencing, are needed to understand the magnitude of this phenomenon in the Glomeromycota, how it could affect the phylogeny of closely related species, and how it could mislead taxonomists in recognizing "artificial" species.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: Bird's-Eye View of ITS1 alignment; Figure S2: 50% majority-rule consensus tree from the Bayesian analysis of dataset ds2 (S-variant sequences); Figure S3: Phylogenetic placement of environmental sequences potentially related to the two new *Glomus* species.

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Data Availability Statement: Datasets generated during and/or analyzed during the current study are available from the corresponding author upon request. Sequences obtained in the study are available in Genbank with the accession numbers: XXX will be added after review.

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