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# Badania możliwości wykorzystania arbuskularnych grzybów mykoryzowych (gromada Glomeromycota) w ochronie ekosystemów wydmowych Peloponezu i arbuskularne grzyby mykoryzowe innych stanowisk

# Research on the possibility of using arbuscular mycorrhizal fungi (AMF; phylum Glomeromycota) in the protection of dune ecosystems of the Peloponnese and AMF of other sites

*Praca doktorska*

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*Składam serdeczne podziękowania*
*Panu prof. dr. hab. Januszowi Błaszkowskiemu*
*za wszechstronną pomoc udzieloną mi*
*w trakcie przygotowywania tej pracy.*

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## 1. SUMMARY

Arbuscular mycorrhizal fungi (AMF) of the phylum Glomeromycota form a symbiosis with 70–90% of vascular terrestrial plants. This symbiosis is of great interest to biologists and ecologists because AMF, among others, (*i*) regulate the carbon, nitrogen and phosphorus cycle, (*ii*) affect soil structure, productivity and diversity of plants and plant communities, and (*iii*) increase plant tolerance to heavy metals, soil salinity, water stresses, as well as pathogenic fungi and nematodes.

Of the described AMF species, *ca*. 21% were originally defined on the basis of spores originating from coastal dunes, and 46% of the others were associated with plants of such soils, indicating that dune soils (*i*) are particularly favorable for these fungi, (*ii*) the symbiosis of AMF with plants of these exceptionally difficult sites brings enormous benefits to the plants, AMF and soils of these sites, and (*iii*) coastal dune vegetation most certainly hosts numerous uncharacterized taxa of Glomeromycota. Therefore, attempts to further recognize these fungi are continued in the world in order to use them in the protection of plants and soils of dune sites, mainly by increasing the occurrence of the most functionally effective species. Among the indicators of such potential effectiveness, the frequency and abundance of sporulation of AGM present in a given site are considered very reliable.

One area where AMF was not explored prior to the research discussed in this PhD dissertation was the Mediterranean dunes of the Peloponnese Peninsula in Greece. In addition, the Plant Protection Laboratory grows pot cultures with AMF from numerous dune sites located in various regions of the world. Therefore, the aims of my own research were: (1) to characterize the morphological and molecular properties of AMF associated with dominant plant species of three coastal dune sites of the Peloponnese Peninsula, (2) to finally determine the systematic positions of *Acaulospora polonica*, *Entrophospora infrequens*, *Glomus diaphanum*, *G. drummondii*, *G. pansihalos*, *G. tortuosum*, and *G. walkeri* in Glomeromycota based on phylogenetic analyzes of sequences of the largest RNA polymerase II subunit (*RPB1*) gene and those covering the SSU-ITS-LSU nrDNA region plus *RPB1*, (3) to verify and supplement published diagnostic descriptions and systematic positions within Glomeromycota of AMF species based on: (*i*) phenotypic and histochemical features of spore components determined at different stages of their ontogenetic development, (*ii*) features of mycorrhizal structures, (*iii*) comparative studies of spore morphology and mycorrhizal structures, and (*iv*) phylogenetic analyzes of SSU-ITS-LSU nrDNA and *RPB1* sequences, using species collected during the implementation of the doctorate and live specimens from pot cultures established during earlier research by J. Błaszkowski, the dissertation supervisor, and (4) to collect and analyze morphological and molecular data for diagnostic characteristics of new AMF species discovered in cultures with AMF from Peloponnese and those grown in the collection of the dissertation supervisor.

The occurrence of AMF in the dunes of the Peloponnese was investigated based on spores extracted from 240 trap cultures. Of these, 104 were inoculated with a mixture of rhizosphere soil and root fragments of *Ammophila arenaria*, 45 represented *Eryngium maritimum*, 31 – *Euphorbia paralias*, 36 – *Medicago littoralis*, and 24 – *Medicago marina*. AMF spores were found in 238 cultures. The spores represented 38 species of 20 genera, 13 families, six orders of Glomeromycota. In addition, 18 morphotypes were identified that are likely undescribed taxa and require further study. The groups of species occurring very often (present in ≥60% of cultures) and common (30–60%) included only *Septoglomus constrictum* (80.42%) and *Diversispora epigaea* (53.33%), respectively. The species most abundantly sporulating were: *Acaulospora kentinensis*, *Archaeospora trappei*, *Diversispora aurantia*, *D. epigaea*, *Dominikia aurea*, *Glomus macrocarpum*, *Innospora majewskii*, and *Rhizoglomus irregulare*.

Thus, the data presented above suggest that for the protection of plants and dunes of the Peloponnese it should be used an inoculum containing either only *Septoglomus constrictum*, or *S. constrictum* together with one or more of the most frequently occurring species in the area, i.e., *Acaulospora kentinensis*, *Archaeospora trappei*, *Diversispora aurantia*, *D. epigaea*, *Dominikia aurea*, *Glomus macrocarpum*, *Innospora majewskii*, and *Rhizoglomus irregulare*.

Based on analyzes of spores extracted from cultures grown in the Laboratory of Plant Protection, a new order, a new family, six new genera, 15 new species were described, and 13 new nomenclature combinations in Glomeromycota were created.

## 2. STRESZCZENIE

Arbuskularne grzyby mykoryzowe (AGM) z gromady Glomeromycota tworzą symbiozę z 70–90% naczyniowych roślin lądowych. Symbioza ta jest obiektem dużego zainteresowania biologów i ekologów, ponieważ AGM m.in. (i) regulują obieg węgla, azotu i fosforu, (ii) wpływają na strukturę gleby, produktywność i różnorodność roślin oraz zbiorowisk roślinnych i (iii) zwiększają tolerancję roślin na metale ciężkie, zasolenie gleby, stresy wodne oraz patogeniczne grzyby i nicienie.

Z opisanych gatunków AGM, ca. 21% zostało oryginalnie zdefiniowanych na podstawie zarodników pochodzących z wydm nadmorskich, a 46% innych wiązało się z roślinami takich gleb, co świadczy, że gleby wydmowe (i) szczególnie sprzyjają tym grzybom, (ii) symbioza AGM z roślinami tych wyjątkowo uciążliwych stanowisk przynosi olbrzymie korzyści dla roślin, AGM i gleb tych stanowisk oraz (iii) roślinność wydm nadmorskich z dużą pewnością utrzymuje liczne nie scharakteryzowane taksony gromady Glomeromycota. Dlatego w świecie kontynuowane są próby dalszego poznania tych grzybów w celu ich użycia w ochronie roślin i gleb stanowisk wydmowych, głównie przez zwiększenie występowania gatunków funkcjonalnie najbardziej efektywnych. Wśród wskaźników takiej potencjalnej efektywności za bardzo wiarygodne uznaje się częstość występowania i obfitość zarodnikowania AGM obecnych w określonym stanowisku.

Jednym z obszarów, w którym nie poznano AGM przed podjęciem badań omówionych w niniejszej dysertacji doktorskiej, były wydmy śródziemnomorskie Półwyspu Peloponez w Grecji. Ponadto w Pracowni Ochrony Roślin hodowane są kultury wazonowe z AGM pochodzącymi z licznych stanowisk wydmowych położonych w różnych regionach świata. Dlatego celami badań własnych były: (1) scharakteryzowanie własności morfologicznych i molekularnych AGM związanych z dominującymi gatunkami roślin trzech nadmorskich stanowisk wydmowych Półwyspu Peloponeskiego, (2) ostateczne określenie stanowisk systematycznych Acaulospora polonica, Entrophospora infrequens, Glomus diaphanum, G. drummondii, G. pansihalos, G. tortuosum i G. walkeri w Glomeromycota na podstawie analiz filogenetycznych sekwencji genu największej podjednostki polimerazy II RNA (RPB1) i sekwencji obejmujących region SSU-ITS-LSU nrDNA plus RPB1, (3) zweryfikowanie i uzupełnienie opublikowanych opisów diagnostycznych i stanowisk systematycznych wewnątrz Glomeromycota gatunków AGM na podstawie: (i) cech fenotypowych i histochemicznych składowych zarodników określonych w różnych stadiach ich rozwoju ontogenetyczengo, (ii) cech struktur mykoryzowych, (iii) badań porównawczych morfologii zarodników i struktur mykoryzowych oraz (iv) analiz filogenetycznych sekwencji SSU-ITS-LSU nrDNA i RPB1, wykorzystując gatunki zebrane podczas realizacji doktoratu i żywe okazy z kultur wazonowych utworzonych w czasie wcześniejszych badań J. Błaszkowskiego, promotora rozprawy oraz (4) zebranie i przeanalizowanie danych morfologicznych i molekularnych do charakterystyk diagnostycznych nowych gatunków AGM odkrytych podczas badań prowadzonych w czasie przygotowywania rozprawy doktorskiej i znajdujących się w zbiorach promotora rozprawy.

Występowanie AGM w wydmach Peloponezu zbadano na podstawie zarodników tych grzybów wyekstrahowanych z 240 kultur pułapkowych. Spośród nich 104 zostały zainokulowane mieszaniną gleby ryzosferowej i fragmentów korzeni *Ammophila arenaria*, 45 reprezentowało *Eryngium maritimum*, 31 – *Euphorbia paralias*, 36 – *Medicago littoralis* i 24 – *Medicago marina*. Zarodniki AGM znaleziono w 238 kulturach. Zarodniki te reprezentowały 38 gatunków z 20 rodzajów, 13 rodzin, sześciu rzędów gromady Glomeromycota. Ponadto zidentyfikowano 18 morfotypów, które prawdopodobnie są nieopisanymi taksonami i wymagają dalszych badań. W grupach gatunków występujących bardzo często (obecne w ≥60% kultur) i często (30–60%) znalazły się tylko odpowiednio *Septoglomus constrictum* (80,42%) i *Diversispora epigaea* (53,33%). Gatunkami najobficiej zarodnikującymi były *Acaulospora kentinensis*, *Archaeospora trappei*, *Diversispora aurantia*, *D. epigaea*, *Dominikia aurea*, *Glomus macrocarpum*, *Innospora majewskii*, *Rhizoglomus irregulare*, *Septoglomus africanum* i *S. constrictum*.

A więc dane przedstawione wyżej sugerują, że do ochrony roślin i wydm Peloponezu należy wykorzystać inokulum zawierające wyłącznie *Septoglomus constrictum* lub *S. constrictum* wraz z jednym do kilku spośród następujących gatunków: *Acaulospora kentinensis*, *Archaeospora trappei*, *Diversispora aurantia*, *D. epigaea*, *Dominikia aurea*, *Glomus macrocarpum*, *Innospora majewskii* i *Rhizoglomus irregulare*.

Na podstawie analiz zarodników wyekstrahowanych z kultur hodowanych w Pracowni Ochrony Roślin opisano nowy rząd, nową rodzinę, sześć nowych rodzajów, 15 nowych gatunków i utworzono 13 nowych kombinacji nomenklaturowych w Glomeromycota.

## 3. WSTĘP

### Hipotezy i cele naukowe rozprawy

### Hipotezy:

1. Rośliny wydm nadmorskich Półwyspu Peloponeskiego utrzymują obfite zbiorowiska arbuskularnych grzybów mykoryzowych (AGM), wśród których są gatunki nieopisane, gatunki dotychczas znajdywane w świecie rzadko i gatunki o niekompletnie zdefiniowanych cechach morfologicznych oraz molekularnych.
2. Stanowiska systematyczne siedmiu gatunków AGM, oryginalnie opisanych w gromadzie Glomeromycota jako *Acaulospora polonica* Błaszk., *Entrophospora infrequens* (I.R. Hall) R.N. Ames & R.W. Schneid., *Glomus diaphanum* J.B. Morton & C. Walker, *Glomus drummondii* Błaszk. & Renker, *Glomus pansihalos* S.M. Berch & Koske, *Glomus tortuosum* N.C. Schenck & G.S. Sm. i *Glomus walkeri* Błaszk. & Renker, są niepewne.
3. Wiele AGM, które wyhodowano w jednogatunkowych kulturach, są nieopisanymi gatunkami z Glomeromycota. Wśród nich są gatunki pochodzące z Peloponezu.

### Cele rozprawy:

1. Scharakteryzowanie własności morfologicznych i molekularnych AGM związanych z dominującymi gatunkami roślin trzech nadmorskich stanowisk wydmowych Półwyspu Peloponeskiego, Grecja.
2. Ostateczne określenie stanowisk systematycznych *Acaulospora polonica*, *Entrophospora infrequens*, *Glomus diaphanum*, *G. drummondii*, *G. pansihalos*, *G. tortuosum* i *G. walkeri* w Glomeromycota na podstawie analiz filogenetycznych sekwencji genu największej podjednostki polimerazy II RNA (*RPB1*) i sekwencji obejmujących region SSU-ITS-LSU nrDNA plus *RPB1*.
3. Zweryfikowanie i uzupełnienie opublikowanych opisów diagnostycznych i stanowisk systematycznych wewnątrz Glomeromycota gatunków AGM na podstawie: (*i*) cech fenotypowych i histochemicznych składowych zarodników określonych w różnych stadiach ich rozwoju ontogenetyczengo, (*ii*) cech struktur mykoryzowych, (*iii*) badań porównawczych morfologii zarodniów i struktur mykoryzowych oraz (*iv*) analiz filogenetycznych sekwencji SSU-ITS-LSU nrDNA i *RPB1*, wykorzystując gatunki zebrane podczas realizacji doktoratu i żywe okazy z kultur wazonowych utworzonych w czasie wcześniejszych badań J. Błaszkowskiego, promotora rozprawy.
4. Zebranie i przeanalizowanie danych morfologicznych i molekularnych do charakterystyk diagnostycznych nowych gatunków AGM odkrytych podczas badań prowadzonych w czasie przygotowywania rozprawy doktorskiej i znajdujących się w zbiorach promotora rozprawy.

**Uzasadnienie celu 1.** Arbuskularne grzyby mykoryzowe (AGM) z gromady Glomeromycota tworzą symbiozę z 70–90% naczyniowych roślin lądowych (Smith, Read 2008). Symbioza ta jest obiektem dużego zainteresowania biologów i ekologów, ponieważ AGM m.in. (*i*) regulują obieg węgla, azotu i fosforu, (*ii*) wpływają na strukturę gleby, produktywność i różnorodność roślin oraz zbiorowisk roślinnych i (*iii*) zwiększają tolerancję roślin na metale ciężkie, zasolenie gleby, stresy wodne oraz patogeniczne grzyby i nicienie (van der Heijden et al. 2015). Z drugiej strony AGM pobierają z roślin do 20% wytworzonych produktów fotosyntezy, aby żyć.

Obecnie gromada Glomeromycota obejmuje jedną klasę, sześć rzędów, 17 rodzin, 50 rodzajów i dużą grupę taksonów o nie poznanej lub niepewnej filogenezie molekularnej (Redecker et al. 2013; Błaszkowski et al. 2015a, 2023; Marinho et al. 2014; Oehl et al. 2014).

Mimo, że AGM występują powszechnie i ich związek z roślinami utworzył się *ca.* 460 milionów lat temu, liczba poznanych gatunków tych grzybów (obecnie *ca*. 370) w porównaniu do liczby gatunków znacznie młodszych grup grzybów (700-66000; Ohsowski et al. 2014) jest bardzo mała. Jednak środowiskowe badania molekularne sugerują, że liczba ta może wahać się od 348 do ponad 1600.

Z opisanych gatunków AGM, *ca.* 21% zostało oryginalnie zdefiniowanych na podstawie zarodników pochodzących z wydm nadmorskich, a 46% innych wiązało się z roślinami takich gleb, co świadczy, że gleby wydmowe szczególnie sprzyjają tym grzybom i że ich symbioza z roślinami tych wyjątkowo uciążliwych stanowisk przynosi olbrzymie korzyści dla roślin, AGM i gleb tych stanowisk (Błaszkowski 2012).

Półwysep Peloponeski leży w południowej Grecji i zajmuje powierzchnię *ca.* 21549,6 km$^2$. W literaturze nie ma żadnych danych o AGM tego półwyspu. W latach 2014–2016 J. Błaszkowski wraz P. Niezgodą, utworzyli 240 wazonowych kultur pułapkowych z mieszaninami gleby ryzosferowej i korzeni ośmiu gatunków roślin kolonizujących trzy obszary wydmowe Peloponezu. Pierwszym były wydmy Romanos położone w południowo-wschodniej linii brzegowej Peloponezu. Stanowisko to należy do sieci NATURA 2000 i jest chronione. Wzdłuż wydm czołowych tego stanowiska roślinami najczęściej występującymi są *Ammophila arenaria*, *Anthemis peregrina*, *Eryngium maritimum*, *Euphorbia paralias*, *Medicago marina*, *Otanthus maritimus* i *Pancratium maritimum*. Drugim obszarem badawczym były wydmy Poseidi w Prowincji Chalkidiki. Nie są one chronione i są bardzo atrakcyjnym regionem turystycznym, który z tego powodu jest pod silną presją szkodliwego oddziaływania ludzi. Roślinność wydmowa głównie składa się z *A. arenaria*, *Elymus farctus* i *Calystegia soldanella*. Plaża Paraporti, trzecie stanowisko badawcze, jest położone na północno-wschodniej linii brzegowej półwyspu i jest rzadko odwiedzane przez turystów z powodu bardzo silnych wiatrów. Nie jest ono objęte ochroną. Wzdłuż plaży rośnie niemal wyłącznie *O. maritimus*, a *A. arenaria* nie występuje w ogóle. W zależności od badanego stanowiska, próbkowanymi roślinami były *A. arenaria*, *E. farctus*, *Er. maritimum*, *Eu. paralias*, *M. marina*, *Medicago littoralis*, *O. maritimus* i *P. maritimum*.

Kultury wspomniane wyżej utworzono w celu (*i*) ujawnienia AGM, które nie tworzyły zarodników w czasie zbioru prób gleby i korzeni, (*ii*) otrzymania dużej liczby żywych zarodników potrzebnych do badań morfologicznych i molekularnych oraz (*iii*) założenia kultur jednogatunkowych z wyselekcjonowanych morfotypów.

Pilotażowe badania prób polowych wspomnianych wyżej wykazały, że zawierają one obfite i różnorodne zbiorowiska AGM, wśród których są gatunki nieopisane. Dlatego autor rozprawy i Promotor spodziewali się wzbogacić znacząco wiedzę na temat biologii, rozmieszczenia i różnorodności AGM oraz uznali, że w przyszłości wiedza ta będzie wykorzystana w ochronie badanych roślin i stanowisk.

**Uzasadnienie celu 2.** Trafne zidentyfikowanie i klasyfikowanie AGM jest niezbędne dla opisania i zrozumienia filogenetycznej oraz funkcjonalnej różnorodności, która wpływa na produktywność zbiorowisk roślin i AGM. W wielu badaniach poznanie tożsamości AGM jest ważne, gdyż ich wpływ na rośliny może różnić się istotnie (Błaszkowski et al. 2023).

Identyfikowanie AGM jest trudne, ponieważ morfologia zarodników tych grzybów jest mało zróżnicowana (zarodniki są jednokomórkowe) i cechy fenotypowe oraz histochemiczne składowych zarodników zwykle zmieniają się w czasie lub zanikają. Z tych powodów wiele

gatunków AGM zostało błędnie umiejscowionych wewnątrz Glomeromycota. Niektóre z nich zostały przeniesione z ich oryginalnych rodzajów do innych rodzajów, zgodnie z poznaną filogenezą molekularną. Jednak filogeneza molekularna niektórych taksonów jest wątpliwa. Ponadto filogeneza wielu gatunków pozostaje nieznana i transfer niektórych innych gatunków do nowych rodzajów, dokonany na podstawie analiz sekwencji DNA, nie został zaakceptowany lub został przyjęty z sugestiami uzupełnienia danych i przeprowadzenia dodatkowych analiz w celu lepszego potwierdzenia słuszności tego transferu. Wśród tych taksonów są m.in. gatunki *Glomus drummondii* i *G. walkeri*, opisane przez Błaszkowskiego et al. (2006).

Schüßler i Walker (2010) przenieśli *Glomus drummondii* i *G. walkeri* do rodzaju *Claroideoglomus* (rodzina Claroideoglomeraceae). Następnie Oehl et al. (2011) utworzyli nowy rodzaj *Albahypha* z *A. drummondii* comb. nov. i *A. walkeri* comb. nov. Redecker et al. (2013) nie zaakceptowali rodzaju *Albahypha*. Rozpoznawcze analizy filogenetyczne sekwencji SSU-ITS-LSU nrDNA *A. drummondii* i *A. walkeri* przeprowadzone przez Promotora rozprawy również sugerowały, że *Albahypha* nie jest pewnym taksonem (Błaszkowski, obserw. własne). Niejasności dotyczące pozycji taksonomicznej pozostałych gatunków wymienionych w punkcie 2. „Hipotezy" i „Cele rozprawy" zostaną omówione w rodziale „Wyniki i dyskusja" niniejszej rozprawy. A więc pewne określenie przynależności taksonomicznej *Acaulospora polonica, Entrophospora infrequens, Glomus diaphanum, G. drummondii, G. pansihalos, G. tortuosum* i *G. walkeri* wymagało otrzymania jednoznacznych informacji o ich filogenezie molekularnej, które Autor rozprawy spodziewał się otrzymać po zsekwencjonowaniu genu *RPB1* tych grzybów i analizach filogenetycznych ich powiązanych sekwencji SSU-ITS-LSU i *RPB1,* których zalety scharakteryzowano niżej.

We wcześniejszych badaniach filogenetycznych głównie wykorzystywano sekwencje z pojedynczych, często bardzo zmiennych regionów jądrowego RNA, na podstawie których rozdzielanie gatunków było trudne lub niemożliwe (Krüger et al. 2012). Filogenezy zrekonstruowane z analiz takich zwykle krótkich sekwencji również były niewystarczająco miarodajne i uznawano, że nie powinny być wykorzystywane do tworzenia taksonów wyższych niż gatunek, szczególnie przy braku unikatowych cech morfologicznych. Z tego powodu, na przykład, próby utworzenia nowego rodzaju dla *Paraglomus majewskii* Błaszk. et Kovács zostały odrzucone przez recenzentów. Jednak późniejsze analizy długich sekwencji tego grzyba dowiodły, że filogeneza *P. majewskii* jest unikatowa; obecnie gatunek ten reprezentuje oddzielny rodzaj, *Innospora* Błaszk. et al. jako *I. majewskii* (Błaszk. et Kovács) Błaszk. et al. (Błaszkowski et al. 2017).

Obecnie uznaje się, że badanie wielu regionów (genów) DNA znacząco poprawia wiarygodność i rozdzielczość zrekonstruowanych filogenez. Dlatego Krüger et al. (2012) skonstruowali startery specyficzne dla AGM, które amplifikują region SSU-ITS-LSU nrDNA o długości *ca.* 1600 par zasad. Sekwencje tego odcinka DNA rozdzielały nawet bardzo blisko spokrewnione gatunki, ale nie wszystkie, mimo że różniły się morfologicznie. Również grupowanie, na przykład wspomnianych wyżej *Entrophospora infrequens*, *Glomus drummondii* i *G. walkeri* w drzewach filogenetycznych zmieniało się w zależności od liczby sekwencji tych gatunków i liczby oraz długości sekwencji innych porównywanych AGM. Innym przykładem demonstrującym słabość sekwencji SSU-ITS-LSU jest 98% podobieństwo molekularne nieopisanego grzyba *Glomus* 317, pochodzącego z wydm Peloponezu, i *Diversispora clara*, które pod względem morfologii podziela tylko tworzenie kulistych zarodników, a takie zarodniki produkuje 98% wszystkich AGM. Słabości te mogły wyniknąć z (*i*) dużej zmienności składowych cząstkowych sekwencji SSU-ITS-LSU, zwłaszcza segmentu ITS, która w jednym zarodniku i u jednego gatunku Glomeromycota może

dochodzić do 20% i jest wśród najwyższych poziomów zmienności notowanych w królestwie grzybów (Stockinger et al. 2014) i (*ii*) zbyt małej ilości informacji pochodzącej z krótkich sekwencji.

Słabości danych sekwencyjny omówione wyżej prawdopodobnie nie dotyczą genu *RPB1*, gdyż wszystkie dowody wskazują, że jest on monomorficzny. Zakres odchyleń sekwencji tego genu np. u *Rhizophagus irregularis* nie przekraczał 0,6% (Stockinger et al. 2014). Niestety gen ten był dotychczas sekwencjonowany bardzo rzadko i do roku 2014 sekwencje *RPB1* charakteryzowały tylko 30 gatunków (z 270 opisanych) z 15 rodzajów (29) AGM, z których np. rodzaj *Diversispora* był reprezentowany tylko przez *D. epigaea*, tj. 7,7% wszystkich poznanych gatunków rodzaju *Diversispora* sensu Oehl et al. (2011). Nieznane były sekwencje *RPB1* wszystkich gatunków wymienionych wyżej, jak również gatunków z rodzajów *Dominikia* i *Kamienskia* oraz wielu grzybów, które uznano za jeszcze nieopisane; większość z nich była hodowana w kulturach jednogatunkowych utworzonych przez J. Błaszkowskiego. Fakt ten uczynił rzadko spotykaną okazję do dodania do wiedzy o AGM olbrzymiej ilości nowych danych.

**Uzasadnienie celu 4.** Opublikowane opisy co najmniej 20% gatunków AGM z pewnością są niekompletne lub błędne. Definicje cech fenotypowych i histochemicznych struktur zarodnika są dwuznaczne, co powoduje, że porównywanie i klasyfikowanie wielu AGM jest trudne oraz jest główną przyczyną częstego braku korelacji między filogenezami odtworzonymi z badań morfologicznych i analiz molekularnych. Odsetek gatunków z rozpoznaną filogenezą molekularną wynosi *ca.* 61%, ale filogenezy *ca.* 38% gatunków są mniej wiarygodne, gdyż zostały zrekonstruowane z pojedynczych i zwykle krótkich odcinków DNA, których słabości omówiono wyżej. Filogenezy odczytane z analiz sekwencji regionu SSU-ITS-LSU nrDNA i genu *RPB1* pozostają nieznane u *ca.* odpowiednio 62% i 89% opisanych gatunków. Zdecydowana większość zdefiniowanych gatunków AGM nie została scharakteryzowana na podstawie sekwencji obejmujących SSU-ITS-LSU plus *RPB1*, chociaż szeroko uznaje się, że trafność i siła rozdzielcza filogenez wielogenowych jest znacznie większa.

Podczas ostatnich czterech lat w Pracowni Ochrony Roślin otrzymano *ca.* 400 sekwencji SSU-ITS-LSU nrDNA, które charakteryzowały 55 opisanych (bez danych molekularnych) i nieopisanych gatunków AGM pochodzących z żywej kolekcji z taksonami reprezentującymi różne regiony Afryki, Azji, Brazylii, Europy, Omanu i USA. DNA wszystkich tych AGM jest przechowywane w celu otrzymania sekwencji genu *RPB1*. Ponadto wiele innych AGM utrzymywanych w tej kolekcji, w tym przypuszczalnie jeszcze nienazwane lub niekompletnie zdefiniowane grzyby i te z wydm Peloponezu, oczekują na badania.

Przynajmniej częściowe oczyszczenie dostępnych danych z niedoskonałości omówionych wyżej spowoduje, że w przyszłości rozpoznawanie opisanych gatunków i definiowanie jeszcze nienazwanych taksonów będzie znacznie łatwiejsze, jak również umożliwi śledzenie ich występowania w świecie i zachowań w różnych okresach rozwoju roślin oraz zbiorowisk roślinnych (Sýkorová et al. 2012). Dane z takich obserwacji z kolei mogą być z powodzeniem wykorzystane np. w ochronie pojedynczych gatunków roślin lub zbiorowisk roślinnych i stanowisk zagrożonych oddziaływaniem różnych szkodliwych czynników abiotycznych i biotycznych, np. erozji.

## 4. JEDNOTEMATYCZNY CYKL PUBLIKACJI

| Lp. | Tytuł publikacji | Pkt.* | IF** |
|---|---|---|---|
| 1. | Błaszkowski J., Kozłowska A., **Niezgoda P.**, Goto B.T., Dalpé Y. 2018. A new genus, *Oehlia* with *Oehlia diaphana* comb. nov. and an emended description of *Rhizoglomus vesiculiferum* comb. nov. in the Glomeromycotina. Nova Hedwigia 107 (3-4), 501-518. doi: 10.1127/nova_hedwigia/2018/0488 | 40 | 1,254 |
| 2. | Błaszkowski J., **Niezgoda P.**, Goto B. T., Kozłowska A. 2018. *Halonatospora* gen. nov. with *H. pansihalos* comb. nov. and *Glomus bareae* sp. nov. (Glomeromycota; Glomeraceae). Botany 96: 737–748. dx.doi.org/10.1139/cjb-2018-0107 | 70 | 1,568 |
| 3. | Jobim K., Błaszkowski J., **Niezgoda P.**, Kozłowska A., Zubek Sz., Mleczko P., Chachuła P., Ishikawa N. K., Goto B. T. 2019. New sporocarpic taxa in the phylum Glomeromycota: *Sclerocarpum amazonicum* gen. et sp. nov. in the family Glomeraceae (Glomerales) and *Diversispora sporocarpia* sp. nov. in Diversisporaceae (Diversisporales). Mycological Progress 18, 369–384, doi: https://doi.org/10.1007/s11557-018-01462-2 | 70 | 2,713 |
| 4. | Błaszkowski J., **Niezgoda P.**, Piątek M., Magurno F., Malicka M., Zubek Sz., Mleczko P., Yorou N. S., Jobim K., Vista X. M., Lima J. L. R., Goto B. T. 2019. *Rhizoglomus dalpeae*, *R. maiae*, and *R. silesianum*, new species. Mycologia 6, 965–980, doi: 10.1080/00275514.2019.1654637 | 100 | 3,325 |
| 5. | Błaszkowski J., **Niezgoda P.**, de Paiva J. N., da Silva K. J. G., Theodoro R. C., Jobim K., Orfanoudakis M., Goto B. T. 2019. *Sieverdingia* gen. nov., *S. tortuosa* comb. nov., and *Diversispora peloponnesiaca* sp. nov. in the Diversisporaceae (Glomeromycota). Mycological Progress 18, 1363–1382, doi: https://doi.org/10.1007/s11557-019-01534-x | 70 | 2,713 |
| 6. | Błaszkowski J., Jobim K., **Niezgoda P.**, Meller E., Malinowski M., Milczarski P., Zubek Sz., Magurno F., Casieri L., Bierza W., Błaszkowski T., Crossay T., Goto B. T. 2021. New glomeromycotan taxa, *Dominikia glomerocarpica* sp. nov. and *Epigeocarpum crypticum* gen. nov. et sp. nov. from Brazil, and *Silvaspora* gen. nov. from New Caledonia. Frontiers in Microbiology 12, 655910. doi: 10.3389/fmicb.2021.655910 | 100 | 6,064 |
| 7. | Błaszkowski J., **Niezgoda P.**, Meller E., Milczarski P., Zubek Sz., Malicka M., Uszok S., Casieri L., Goto B. T., Magurno F. 2021. New taxa in Glomeromycota: Polonosporaceae fam. nov., *Polonospora* gen. nov., and *P. polonica* comb. nov. Mycological Progress 20, 941–951, doi: 10.1007/s11557-021-01726-4 | 70 | 2,713 |
| 8. | Błaszkowski J., **Niezgoda P.**, Zubek S., Meller E., Milczarski P., Malinowski R., Malicka M., Uszok S., Goto B. T., Bierza W., Casieri L., Magurno F. 2022. Three new species of arbuscular mycorrhizal fungi of the genus *Diversispora* from maritime dunes of Poland. Mycologia 114, 453–466, | 100 | 3,325 |

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|---|---|---|---|
| | doi:10.1080/00275514.2022.2030081 | | |
| 9. | Błaszkowski J., Sánchez-García M., **Niezgoda P.**, Zubek Sz., Fernández F., Vila A., Al-Yahya'ei M. N., Symanczik S., Milczarski P., Malinowski R., Cabello M., Goto B. T., Casieri L., Malicka M., Bierza W., Magurno F. 2022. A new order, Entrophosporales, and three new *Entrophospora* species in Glomeromycota. Frontiers in Microbiology 13, 962856, doi:10.3389/fmicb.2022.962856 | 100 | 6,064 |
| | **Suma** | **720** | **29,739** |

* Liczba punktów według listy MNiSW w roku opublikowania pracy
** Impact Factor według bazy Journal Citation Reports w roku opublikownia pracy

## 5. MATERIAŁ I METODY

**5.1.** *Hodowla kultur pułapkowych zainokulowanych mieszaninami gleb ryzosferowych i korzeni roślin wydm Peloponezu oraz kultur jednogatunkowych z morfotypami wyekstrahowanymi z kultur pułapkowych, które przypuszczalnie były nieopisanymi AMF lub gatunkami wcześniej rzadko notowanymi w świecie.* Założono 240 kultur pułapkowych, które zostały zainokulowane glebami ryzosferowymi i fragmentami korzeni ośmiu gatunków roślin. Rośliny te zasiedlały trzy obszary wydm peloponeskich scharakteryzowane wyżej; próby gleby i korzeni pobrano w latach 2014–2016. Inokulum każdej kultury pułapkowej była polowo zebrana mieszanina gleby ryzosferowej i korzeni (50 g) zmieszana (1:2, v/v) ze sterylnym gruboziarnistym piaskiem, w celu zapewnienia odpowiedniego napowietrzenia. Inokulum to umieszczano na powierzchni *ca*. 300 ml sterylnego piasku gruboziarnistego wprowadzonego do 0,5-litrowych plastikowych doniczek (wysokość 14 cm, szerokość 10 cm), po czym powierzchnię tak utworzonej kultury obsiewano *ca*. 20 ziarnami *Plantago lanceolata*, która pełniła rolę rośliny gospodarza. Kultury te uprawiano w szklarni i laboratorium do czasu rozpoczęcia ekstrakcji zarodników AGM. W szklarni, w okresie jesiennozimowym, kultury utrzymywano w temperaturze 17–21$^{o}$C przy 16-godzinnym naświetlaniu, o natężeniu 180 $\mu Em^{-1}s^{-1}$, lampami sodowymi SON-T AGRO (Philips LightingPoland S.A.), umieszczonymi 1 m nad górną powierzchnią wazonów. W okresie wiosennoletnim nie wykorzystywano ogrzewania i naświetlania. Nawadnianie roślin stosowano 2–3 razy w tygodniu wodą wodociągową. Gdy pojawiły się objawy niedoboru składników pokarmowych, kultury nawożono preparatem Florovit. W laboratorium kultury naświetlano lampami AQUAEL LEDDY TUBE RETRO FIT 18W.

Kultury jednogatunkowe zakładano i uprawiano w styropianowych pojemnikach (wysokość 10 cm, szerokość 8 cm). Ich podłożem hodowlanym był zautoklawowany piasek gruboziarnisty (ziarna śred. 1,0–10,0 mm - 80,50%; śr. 0,1–1,0 mm - 17,28%; śr.<0,1 mm - 2,22%) zmieszany (5:1, v/v) z klinoptylolitem (Zeocem, Bystré, Słowacja) o ziarnach 2,5–5 mm śr. Klinoptylolit jest krystalicznym uwodnionym glinokrzemianem metali alkalicznych i metali ziem alkalicznych o dużej zdolności jonowymiennej oraz odwracalnych właściwościach hydratacyjnych i odwadniających. pH mieszaniny piasku i klinoptylolitu wynosiło *ca*. 7,3. Pojemniki umieszczano w przezroczystych plastikowych torebkach o szerokości 15 cm i wysokości 22 cm, zgodnie z sugestią Walkera i Vestberga (1994) w celu ochrony przed kontaminacją. Kultury hodowano w warunkach identycznych do tych dotyczących kultur pułapkowych. Kolekcjonowanie zarodników z obu rodzjów kultur rozpoczynano nie wcześniej niż po pięciu miesiącach hodowli.

W celu ujawnienia i scharakteryzowania struktur mykoryzowych, z kultur jednogatunkowych, odcinano fragmenty korzeni *P. lanceolata* z głębokości *ca*. 1–5 cm poniżej górnego poziomu podłoża, po czym barwiono je 0,1% błękitem trypanu (Błaszkowski 2012). We wcześniejszych badaniach porównawczych stwierdzono, że *P. lanceolata* jest jedynym gatunkiem rośliny bardzo dobrze rosnącym w jałowym podłożu, jakim jest piasek z wydm zmieszany z piaskiem wielkoziarnistym. Ponieważ zarodnikowanie AMF jest ściśle skorelowane z wigorem ich rośliny żywicielskiej (Baszkowski, pers. obserw.; Walker, Vestberg 1994), gatunek ten okazał się idealny do hodowania AMF pochodzących z wydm nadmorskich.

**5.2.** *Ekstrakcja zarodników i identyfikacja AMF.* Zarodniki ekstrahowano metodą opisaną przez Błaszkowskiego et al. (2015b), z 50 g powietrznie wysuszonego podłoża kultur pułapkowych i jednogatunkowych pobranego ze środka każdej doniczki, z głębokość 0–10 cm. Zarodniki z kultur jednogatunkowych wyodrębniano w celu zbadania ich morfologii i ontogenezy.

Identyfikowanie i charakteryzowanie AGM prowadzono głównie na podstawie zarodników pochodzących z kultur jednogatunkowych, rzadko, gdy nie stwierdzono zarodnikowania w tych kulturach, do tego celu wykorzystywano zarodniki z kultur pułapkowych.

Cechy morfologiczne zarodników określano na podstawie badań co najmniej 100 zarodników umieszczonych w wodzie, kwasie mlekowym, alkoholu poliwinylowym/kwasie mlekowym/glicerolu (PVLG; Omar et al. 1979) oraz mieszaninie PVLG i odczynnika Melzera (1:1, v/v) na szkiełkach mikroskopowych. W celu scharakteryzowania składowych zarodników, kruszono je przez naciskanie górnej powierzchni szkiełka nakrywkowego końcem igły preparacyjnej, obserwując stopień skruszenia pod mikroskopem stereoskopowym. Tak przygotowane preparaty inkubowano w cieplarce, w temperaturze 65$^{o}$C przez kilka minut do 1 h w celu spłaszczenia struktur zarodników i oczyszczenia ich wnętrza z kropel oleju. Następnie zarodniki obserwowano pod mikroskopem Olympus BX 50 wyposażonym w kontrast interferencyjny Nomarkiego. Badano sposób powstawania zarodników, wielkość i kolor zarodników, ich strukturę wewnątrzkomórkową oraz właściwości fenotypowe i histochemiczne składowych zarodników. Wielkość nienaruszonych zarodników i innych struktur mierzono mikrometrem okularowym. Kolor zarodników określano pod mikroskopem stereoskopowym, gdy były zanurzone w wodzie.

Kolory i ich nazwy określano na podstawie atlasu Kornerupa i Wanschera (1983). Mikrofotografie otrzymywano używając kamery wideo Sony 3CDD sprzężonej z mikroskopem. Okazy zarodników, utrwalone w PVLG i mieszaninie PVLG oraz odczynnika Melzera (1:1, v/v) na szkiełkach mikroskopowych, zdeponowano w Pracowni Ochrony Roślin Katedry Kształtowania Środowiska Zachodniopomorskiego Uniwersytetu Technologicznego w Szczecinie (rzadkie gatunki dla świata, nowe dla Polski, izotypy nowych gatunków), w zielniku Oregon State University w Corvallis, Oregon, USA, i herbarium Z+ZT w Zurychu, Szwajcaria (holotypy nowych gatunków).

AMF identyfikowano na podstawie oryginalnych i uzupełnionych opisów gatunków (Błaszkowski 2012), kolekcji zdeponownej w Pracowni Ochrony Roślin ZUT (ok. 5000 stałych okazów i ok. 1000 żywych kultur doniczkowych), analiz filogenetycznych sekwencji ich nrDNA i genu *RPB1* (patrz niżej) oraz okazów wypożyczonych z zielników innych instytucji.

**5.3.** *Określenie właściwości molekularnych i pozycji filogenetycznej AMF na podstawie analiz sekwencji regionu SSU-ITS-LSU nrDNA i genu RPB1 oraz sekwencji SSU ITS-LSU*

*plus RPB1*. W analizach filogenetycznych wykorzystano genomowe DNA, które pochodziło z zarodników AMF zebranych podczas wcześniejszych badań J. Błaszkowskiego i było przechowywane w temperaturze -20$^0$C, oraz DNA świeżo wyekstrahowane z zarodników, które zostały wyhodowane w kulturach z glebami Peloponezu i innych stanowisk.

DNA ekstrahowano ze skruszonych pojedynczych zarodników, po czym amplifikowano je za pomocą zestawu DNeasy Plant Mini Kit (Qiagen, Niemcy), zgodnie z protokołem producenta. Amplifikację segmentu nrDNA SSU-ITS-LSU przeprowadzono w reakcji łańcuchowej polimerazy (PCR), używając starterów Krüger et al. (2009). Fragment genu *RPB1* amplifikowano przy użyciu starterów Stockingera et al. (2014) i starterów zaprojektowanych przez dr. Franco Magurno z Uniwersytetu Śląskiego, ponieważ wiele starterów Stockingera et al. (2014) było nieskutecznych. Warunki PCR były takie, jak opisane przez Kohout et al. (2014), Stockingera et al. (2014) i Błaszkowskiego et al. (2017) lub zoptymalizowane w odpowiednim czasie. Otrzymane produkty, po wizualizacji na żelach agarozowych, były oczyszczane, klonowane do wektora i przekształcane w kompetentne komórki *Escherichia coli*. Pozytywne klony sekwencjonowano na obu niciach DNA za pomocą uniwersalnych starterów (reverse, forward) w LGC Genomics Company, Berlin. Reprezentatywne sekwencje zdeponowano w GenBank.

W celu sprawdzenia przynależność otrzymanych sekwencji do grzybów gromady Glomeromycota, najpierw porównywano je z sekwencjami pochodzącymi z analiz własnych i sekwencjami zdeponowanymi w GenBanku za pomocą narzędzia Basic Local Alignment Search Tool (BLAST; Altschul et al. 1997). Przyrównania zestawów sekwencyjnych przeprowadzono używając programu MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server/). Analizy filogenetyczne przeprowadzono metodami Bayesianowską (BI) w programie MrBayes 3.1.2 (Ronquist, Huelsenbeck 2003) i maksymalnej wiarygodności (ML) w programie RAxML (Silvestro, Michalak 2012; Stamatakis 2014). Najlepiej dopasowany do analiz model substytucji nukleotydów wybrano za pomocą jModelTest (Posada 2008), biorąc pod uwagę wybór Akaike Information Criterion. W analizie BI łańcuch Markova ustawiono na 10000000 generacji, próbkowano co 1000 powtórzeń i spalano pierwsze 3000 generacji. Wsparcie gałęzi w analizach ML oszacowano metodą samopróbkowania (ang. bootstrap) na podstawie 1000 powtórzeń. Drzewa wizualizowano i edytowano używając Tree Explorer programu MEGA 6.0 (Tamura et al. 2013) oraz edytora tekstu.

**5.4.** *Inne badania*. Analizy chemiczne gleby dotyczyły odczynu, zawartości azotu azotanowego, fosforu, potasu i węgla organicznego. Własności te określano używając standardowych metod.

## 6. OMÓWIENIE WYNIKÓW BADAŃ PRZEDSTAWIONYCH W PUBLIKACJACH STANOWIĄCYCH OSIĄGNIĘCIE NAUKOWE

### 6.1. Występowanie AGM w wydmach Peloponezu

Spośród 240 zbadanych kultur pułapkowych, z których ekstrahowano zarodniki AGM, 104 zostały zainokulowane mieszaniną gleby ryzosferowej i fragmentów korzeni *Ammophila arenaria*, 45 reprezentowało *Eryngium maritimum*, 31 – *Euphorbia paralias*, 36 – *Medicago littoralis* i 24 – *Medicago marina*. Zarodniki AGM znaleziono w 238 kulturach. Reprezentowały one 38 gatunków z 20 rodzajów, 13 rodzin, sześciu rzędów gromady Glomeromycota, w tym cztery gatunki z rodzaju *Septoglomus*, po trzy gatunki z rodzajów *Acaulospora*, *Diversispora*, *Funneliformis*, *Glomus* i *Rhizoglomus*, po dwa gatunki z rodzajów *Cetraspora*, *Entrophospora*, *Racocetra* i *Scutellospora* oraz po jednym gatunku z

rodzajów *Ambispora*, *Archaeospora*, *Corymbiglomus*, *Dominikia*, *Gigaspora*, *Innospora*, *Pacispora*, *Paraglomus*, *Pervetustus*, *Polonospora* i *Sacculospora*. Ponadto zidentyfikowano 18 morfotypów, które oznaczono numerami, ponieważ prawdopodobnie są nieopisanymi taksonami i wymagają dalszych badań.

W grupach gatunków występujących bardzo często (obecne w ≥60% kultur) i często (30–60%) znalazły się tylko odpowiednio *Septoglomus constrictum* (80,42%) i *Diversispora epigaea* (53,33%; Tab. 1). Wśród gatunków rzadkich (10–30%) były *Archaeospora trappei* (15,42%), *Dominikia aurea* (16,67%), *Innospora majewskii* (27,5%), *Rhizoglomus irregulare* (13,33%) i *Septoglomus africanum* (12,50%). Pozostałe gatunki występowały bardzo rzadko (0,1–10%).

Według przyjętego 4-stopniowego zakresu obfitości zarodnikowania w 100 g suchego podłoża wzrostowego kultur, w których gatunek występował w więcej niż 10 kulturach (poziom ten utworzono w celu zachowania wiarygodności informacji), gdzie 1 = <10 zarodników, 2 = 11–50, 3 = 51–100 i 4 = >100, gatunkami najobficiej zarodnikującymi były *Acaulospora kentinensis* (śr. 2,36), *Archaeospora trappei* (1,59), *Diversispora aurantia* (1,91), *D. epigaea* (1,8), *Dominikia aurea* (2,70), *Glomus macrocarpum* (1,56), *Innospora majewskii* (1,52), *Rhizoglomus irregulare* (1,50), *Septoglomus africanum* (1,53) i *S. constrictum* (2,22).

Ponieważ własności chemiczne w wybranych analizowanych próbach glebowych były podobne i typowe dla wydm nadmorskich, nie badano ich wpływu na występownie AGM.

### 6.2. Utworzenie nowego rzędu i nowych kombinacji nomenklaturowych

### P10: A new order, Entrophosporales, and three new *Entrophospora* species in Glomeromycota

Jednym z przedstawicieli arbuskularnych grzybów mykoryzowych (AGM), obecnie zaklasyfikowanych do gromady Glomeromycota, który ukazuje trudności identyfikowania i klasyfikowania tej grupy grzybów jest gatunek oryginalnie opisany jako *Glomus infrequens* w rodzinie Endogonaceae (Hall 1977). Gatunek ten został scharakteryzowany i zaklasyfikowany do rodzaju *Glomus* na podstawie zarodników wyekstrahowanych z prób polowych, których urzeźbienie było unikatowe, ale zarodniki te były pozbawione struktur świadczących o sposobie ich powstawania. Ames i Schneider (1979) wyhodowali w kulturze zarodniki identycznie urzeźbione, które powstawały inaczej niż zarodniki gatunków z rodzaju *Glomus*. W konsekwencji badacze ci utworzyli nowy rodzaj w Endogonaceae, *Entrophospora*, z gatunkiem typowym *E. infrequens*. Rodzina Endogonaceae sensu Gerdemann i Trappe (1974) zawierała AGM, jak również grzyby ektomykoryzowe i saprotroficzne. Dlatego Morton i Benny (1990) przenieśli *Entrophospora* do nowo utworzonej rodziny Acaulosporaceae w nowych rzędzie Glomales (później poprawionym ortograficznie na Glomerales; Schüßler et al. 2001), który skupiał wyłącznie grzyby tworzące mykoryzę arbuskularną. Sieverding i Oehl (2006) umieścili *E. infrequens* w nowej rodzinie, Entrophosporaceae. Schüßler i Walker (2010) wprowadzili do Glomerales nową rodzinę, Claroideoglomeraceae, z nowym rodzajem *Claroideoglomus*. Gatunkiem typowym Claroideoglomeraceae, zawierającym jeden rodzaj, *Claroideoglomus*, zostało *C. claroideum* (Schüßler, Walker 2010), oryginalnie opisane jako *Glomus claroides* (Schenck, Smith 1982). Schüßler i Walker (2010) uznali Entrophosporaceae i *Entrophospora* za taksony o niepewnej pozycji systematycznej w rzędzie Diversisporales, nowo utworzonym w Glomeromycota.

Oehl et al. (2011b) zsynonimizowali Claroideoglomeraceae z Entrophosporaceae na podstawie analiz filogenetycznych sekwencji genów 18S i 28S *E. infrequens*, trzech gatunków z *Claroideoglomus* i innych reprezentantów Glomerales oraz innych rodzin

Glomeromycota. Rodzaj *Entrophospora* pozostał w Entrophosporaceae, a *Entrophospora* z *E. infrequens* została rodzajowym typem tej rodziny. Te same analizy również przekonały Oehla et al. (2011b) do przeniesienia *Clarodeoglomus drummondii* i *C. walkeri,* oryginalnie opisanych jako *Glomus drummondii* i *G. walkeri* (Błaszkowski et al. 2006), do nowo utworzonego rodzaju, *Albahypha,* z *A. drummondii* comb. nov. i *A. walkeri* comb. nov.

Redecker et al. (2013) odrzucili zmiany omówione wyżej i przywrócili Claroideoglomeraceae. Stwierdzili oni, że sekwencje 18S i 28S *E. infrequens* zagnieżdżają się wśród sekwencji *Claroideoglomus* i niezgodności między cechami morfologicznymi i molekularnymi tych taksonów nie można wytłumaczyć na podstawie dostępnych danych.

Główny argument za odrzuceniem *Albahypha* pochodził z analiz przeprowadzonych przez Krüger et al. (2012), które wykazały grupowanie sekwencji *A. drummondii* i *A. walkeri* wśród sekwencji *Claroideoglomus*. Ponadto Redecker et al. (2013) podkreślili, że rodzajowy klad *Albahypha* w drzewie 28S Oehla et al. (2011b) nie otrzymał wystarczającego wsparcia i przez to uczynił *Claroideoglomus* taksonem parafiletycznym.

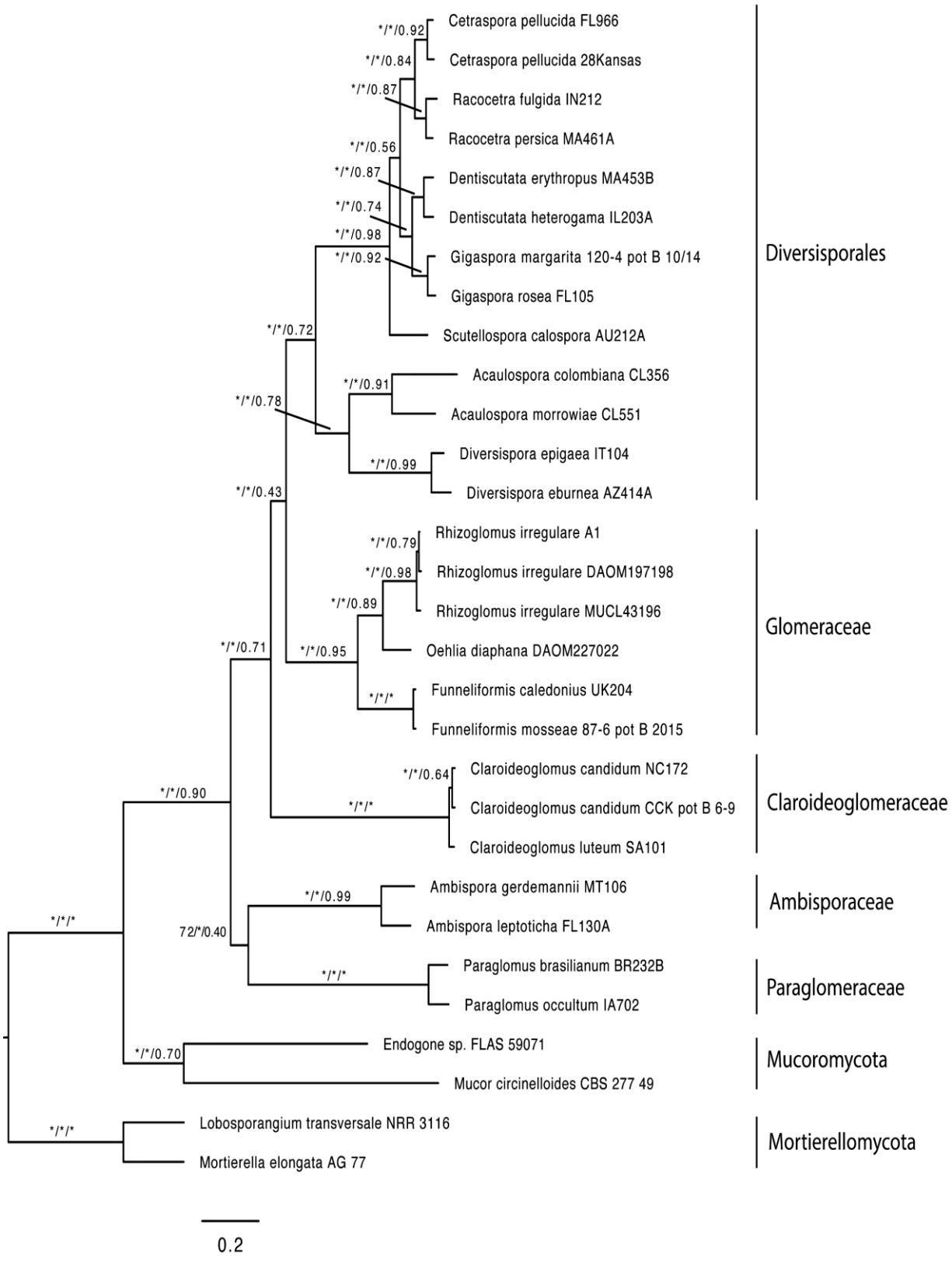
W klasyfikacji Schüßlera i Walkera (2010) rodzina Claroideoglomeraceae była siostrzanym taksonem Glomeraceae w rzędzie Glomerales. Niedawno opublikowane dwie filogenezy zasugerowały inne pokrewieństwa Claroideoglomeraceae wewnątrz Glomeromycota sensu Redecker et al. (2013). Beaudet et al. (2018), używając danych transkryptomicznych w analizach dziewięciu taksonów z siedmiu rodzin, ujawnili *Claroideoglomus* jako siostrę rodzajów *Ambispora* i *Paraglomus*, przynależących do odpowiednio rzędów Archaeosporales i Paraglomerales. Montoliu-Nerin et al. (2021), wykorzystując sekwencjonowanie pojedynczych jąder w celu otrzymania nowych genomów 15 gatunków reprezentujących siedem rodzin, wykazali, że Claroideoglomeraceae jest autonomiczną, dobrze wspartą grupą, siostrzaną względem kladu tworzonego przez Glomeraceae i rząd Diversisporales. Jednak żadna z przedstawionych hipotez nie korelowała jednoznacznie z morfologią uwzględnionych taksonów. Należy podkreślić, że obecnie powszechnie uznaje się priorytet danych molekularnych w identyfikowaniu i klasyfikowaniu grzybów, w tym AGM (Błaszkowski et al. 2023).

Rozbieżność poglądów o Claroideoglomeraceae/Entrophosporaceae, jak również o filogenezie *E. infrequens* i *C./A. drummondii* oraz *C./A. walkeri* prawdopodobnie wynikła z następujących przyczyn. Po pierwsze, jak zasugerowali Montoliu-Nerin et al. (2021), wnioski Beaudet et al. (2018) były stronnicze z powodu niewielkiej liczby analizowanych taksonów. Po drugie, Błaszkowski et al. (2022) uznali, że niejasne filogenezy *E. infrequens*, *C./A. drummondii* i *C./A. walkeri* wynikły z zbyt małego próbkowania sekwencyjnego tych taksonów i użycia sekwencji o zbyt małej rozdzielczości taksonomicznej. Dlatego, Błaszkowski et al. (2022) założyli, że (*i*) genomowe analizy filogenetyczne rzucą nowe światło na filogenetyczne umiejscowienie rodziny Claroideoglomeraceae/Entrophosporaceae i (*ii*) zwiększone próbkowanie oraz użycie sekwencji region SSU-ITS-LSU powiązanego z sekwencjami genu *RPB1* wyjaśni filogenezę *E. infrequens*, *C./A drummondii* oraz *C./A. walkeri*. Sekwencje każdego z tych lokusów na ogół rozdzielały nawet bardzo blisko spokrewnione gatunki (Krüger et al. 2012; Kohout et al. 2014; Stockinger et al. 2014). Jednak filogenezy zrekonstruowane z powiązanych sekwencji tych dwóch lokusów (SSU-ITS-LSU+*RPB1*) były bardziej solidne i ujawniały pokrewieństwa nie wykazane, gdy sekwencje tych lokusów były analizowane oddzielnie (Błaszkowski et al. 2019, 2021a, 2015a, b; Yu et al. 2022).

Posiadając kultury jednogatunkowe *Entrophospora infrequens* i czterech potencjalnie nowych gatunków AGM, wstępnie nazwanych „*Claroideoglomus* 1–4", tworzących glomoidalne zarodniki o morfologii podobnej do morfologii zarodników *Albahypha*/*Claroideoglomus* lub

*Diversispora*, celami omawianych badań były: (*i*) określenie, która z opublikowanych hipotez o statusie i pokrewieństwie Claroideoglomeraceae/Entrophosporaceae jest wiarygodna, (*ii*) wyjaśnienie umiejscowienia *E. infrequens* w Glomeromycota, używając genu *RPB1*, jako dodatkowego wskaźnika filogenetycznego, (*iii*) szczegółowe scharakteryzowanie morfologii *Claroideoglomus* 1–4 i określenie ich pozycji filogenetycznej wśród zsekwencjonowanych gatunków *Albahypha*/*Claroideoglomus* na podstawie sekwencji SSU-ITS-LSU i *RPB1* oraz (*iv*) rozstrzygnięcie konfliktu o statusie taksonomicznym *Albahypha*, używając wcześniej dostępnych i nowo wygenerowanych danych morfologicznych i molekularnych (SSU-ITS-LSU, *RPB1*).

Wszystkie analizy filogenetyczne przeprowadzone oddzielnie i łącznie z danymi genomowymi otrzymanymi przez Beaudet et al. (2018) i Montoliu-Nerin et al. (2021) wykazały polifiletyczność rzędu Glomerales, z rodziną Claroideoglomeraceae siostrzaną względem rzędu Diversisporales i rodziny Glomeraceae (Ryc.1).




**Ryc. 1.** Filogeneza najlepszego maksymalnego prawdopodobieństwa wywnioskowana za pomocą IQ-TREE z połączonego dopasowania 1260 ortologów z jedną kopią, które były wspólne dla co najmniej 50% taksonów. Ta sama topologia została odzyskana w ASTRAL III. Wartości w pobliżu gałęzi odpowiadają wsparciu bootstrap (BS) z analizy ML, lokalnemu prawdopodobieństwu a posteriori (LPP) i wsparciu kwartetowemu z analizy ASTRAL (BS/LPP/wsparcie kwartetowe). Gwiazdki oznaczają maksymalne wsparcie (100 lub 1,0). Jako grupy zewnętrzne użyto Mucoromycota i Mortierellomycota.

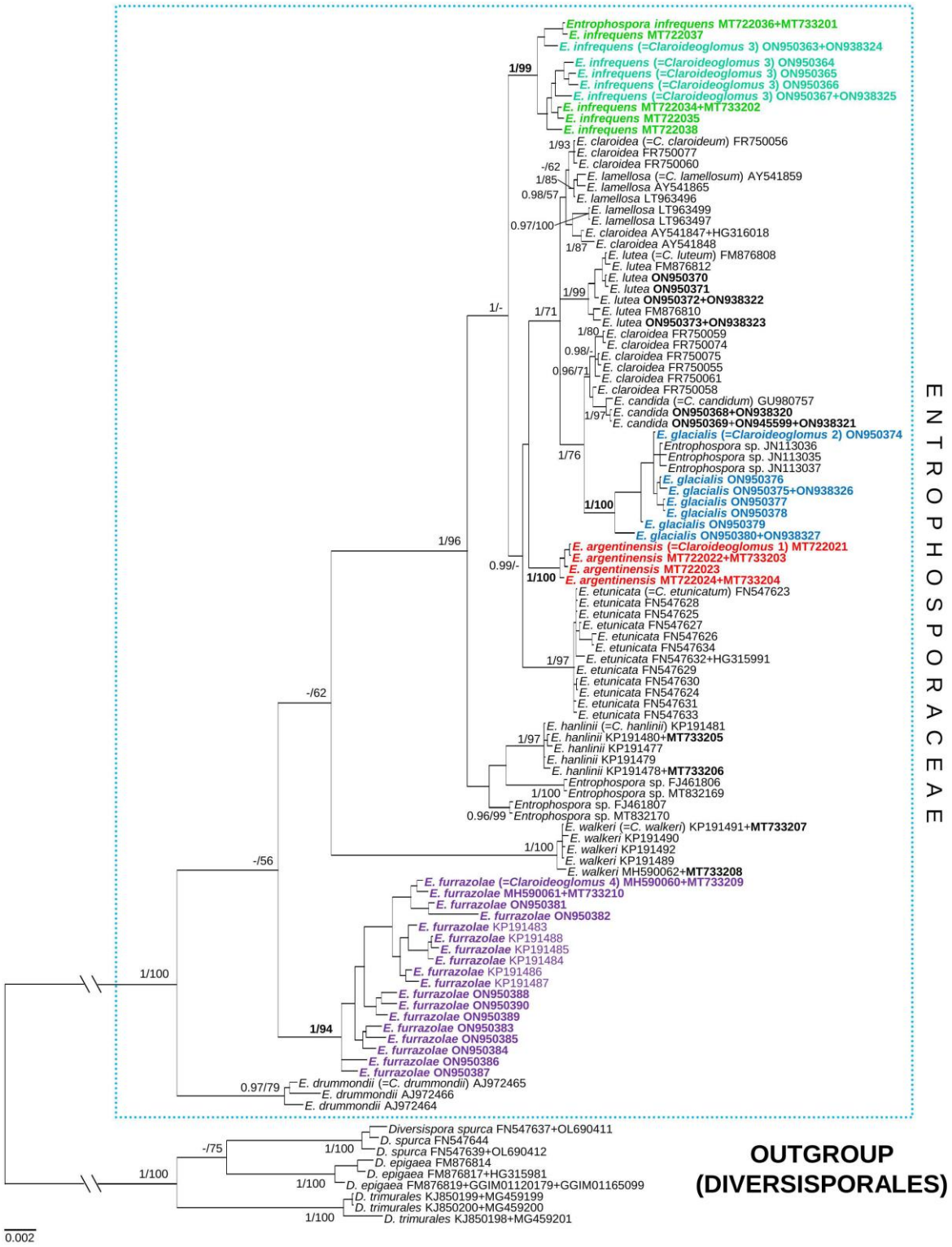
We wszystkich drzewach (SSU-ITS-LSU, *RPB1*, SSU-ITS-LSU+*RPB1*), *Claroideoglomus* 3 utworzyło klad z *Entrophospora infrequens*, którego sekwencje SSU-ITS-LSU i *RPB1* otrzymano z kolekcji własnej (Ryc. 2). W drzewach SSU-ITS-LSU+*RPB1* i SSU-ITS-LSU, *Claroideoglomus* 2 również zgrupowało się z *E. infrequens*, którego sekwencje 28S otrzymał Oehl et al. (2011b); nie ma dostępnych sekwencji *RPB1* tego grzyba.

We wszystkich drzewach *Claroideoglomus* 1 i *Claroideoglomus* 3, gdy statystycznie wsparte, zajęły autonomiczne pozycje (Błaszkowski et al. 2022). *Claroideoglomus* 2 utworzyło klad z *C. candidum* i sześcioma z 11 analizowanych sekwencji *C. claroideum* w drzewach SSU-ITS-LSU (bez wsparcia), SSU-ITS-LSU+*RPB1* i SSU-ITS-LSU+*RPB1*_G (z *Glomus* spp. w grupie zewnętrznej, wcześniej uznawanych za gatunki najbliżej spokrewnione; w pozostałych drzewach grupą zewnętrzną były sekwencje *Diversispora* spp., zgodnie z wynikami analiz genomowych, *vide* wyżej). *Claroideoglomus* 4 zostało umieszczone albo w silnie wspartym kladzie autonomicznym (w drzewach SSU-ITS-LSU+*RPB1* i *RPB1*) lub kladzie siostrzanym względem *C. drummondii*, który otrzymał tylko wsparcie BI.

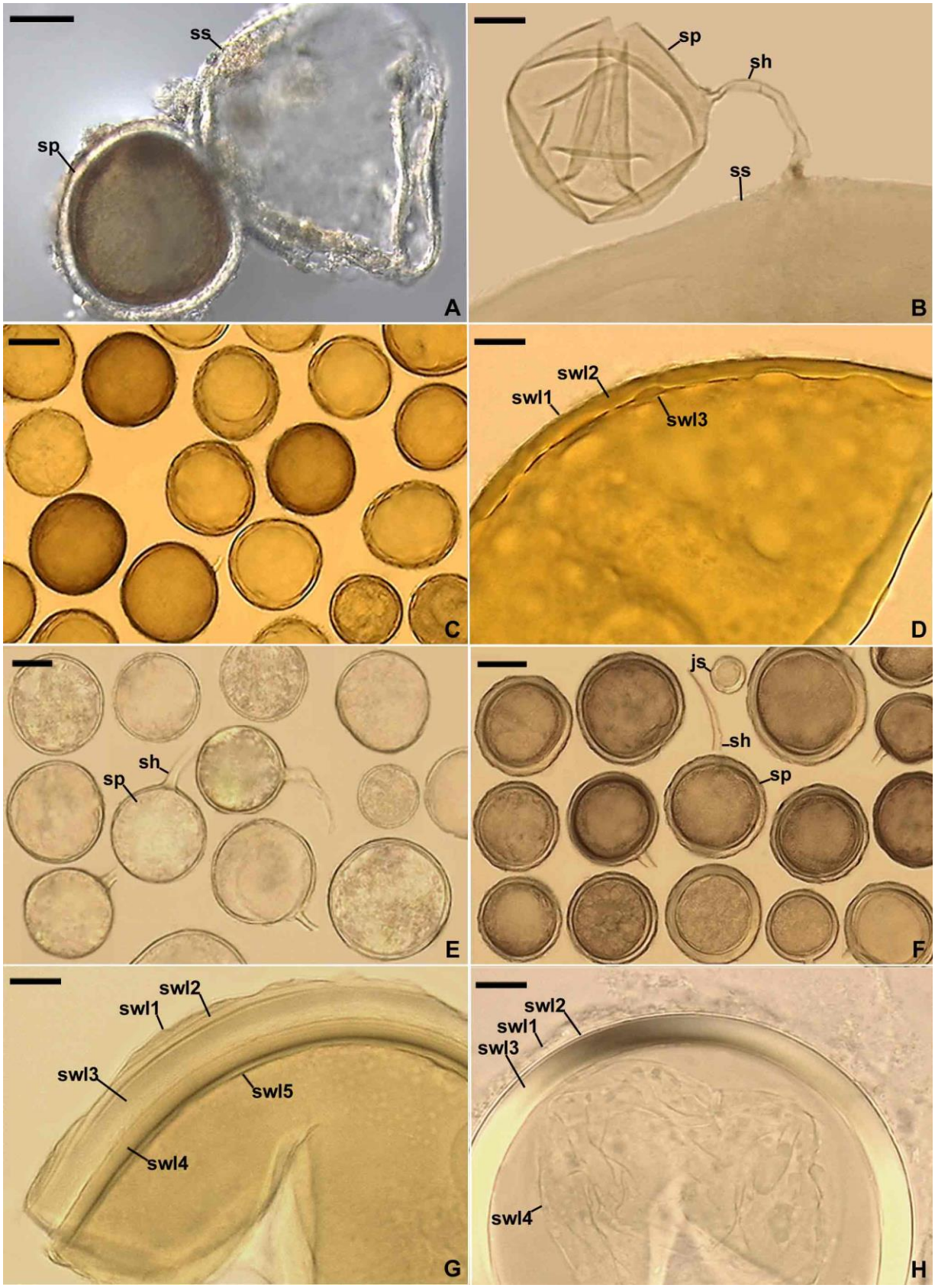
Wyniki analiz filogenomowych i filogenetycznych opisane wyżej skłoniły do (*i*) zaproponowania nowego rzędu, Entrophosporales, dla Entrophosporaceae w miejsce Claroideoglomeraceae, (*ii*) uzupełnienia definicji Glomerales, Entrophosporaceae, *Entrophospora* i *E. infrequens*, (*iii*) wyznaczenia epitypu *E. infrequens* o morfologii i filogenezie określonych z analiz entrofosproidalnej morfy *E. infrequens* i glomoidalnej morfy wstępnie nazwanej *Claroideoglomus* 3, (*iv*) wprowadzenia ośmiu nowych kombinacji nomenklaturowych i (v) opisania *Claroideoglomus* 1, 2 oraz 4 jako odpowiednio *Entrophospora argentinensis* sp. nov., *E. glacialis* sp. nov. i *E. furrazolae* sp. nov.

W wyniku wprowadzonych zmian rząd Glomerales pozostał z jedną rodziną, Glomeraceae, a rodzaj *Albahypha* został uznany za nieważny takson. Przeprowadzone badania istotnie wzbogaciły wiedzę o Glomeromycota przez ukazanie dymorfizmu i kryptycznej natury gatunku nazwanego *Entrophospora infrequens*, wcześniej znanego tylko z jego entrofosporoidalnej morfy, z wyróżniającą i jednolitą morfologią. Zachowanie dymorficzne AGM wcześniej obserwowano wyłącznie u gatunków z rodzajów *Ambispora* i *Archaeospora*, które tworzyły zarodniki akaulosporoidalne i glomoidalne (Goto et al. 2008; Palenzuela et al. 2011; Bills, Morton 2015; Oehl et al. 2019).

Podobnie jak analizy filogenetyczne, porównania morfologiczne również wykazały, że pokrewieństwa między trzema nowo opisanymi gatunkami *Entrophospora* tworzącymi glomoidalne zarodniki i glomoidalną morfą epitypu *E. infrequens* względem wszystkich wcześniej zsekwencjonowanych gatunków *Entrophospora* były nijakie i bardzo słabe. Żaden z poznanych gatunków *Entrophospora* nie tworzy zarodników (*i*) z laminowaną warstwą ściany zarodnika, która jest nierówna w grubości w jej różnych regionach, jak u *E. argentinensis* (Ryc. 3C, D), (*ii*) tak mocno przypominających zarodniki gatunków z rodzaju *Dominikia* i rodzajów spokrewnionych pod względem ich częstego tworzenia w luźnych skupieniach, wyglądu, wymiaru, zabarwienia, struktury ściany zarodnika, jak również pod względem cech fenotypowych i histochemicznych składowych tej ściany (Błaszkowski et al. 2021b), jak glomoidalne zarodniki *E. infrequens* (Ryc. 3E), (*iii*) ze ścianą zarodnika składającą się z pięciu trwałych warstw, jak u *E. glacialis* (Ryc. 3F, G) i (*iv*) ze ścianą zarodnika złożoną z trzech trwałych warstw, z których dwie barwią się w odczynniku Melzera, z wyjątkiem *E. furrazolae* (Ryc. 3H).




**Ryc. 2.** Filogram konsensusu 50% reguły większości z analizy bayesowskiej sekwencji SSU-ITS-LSU połączonych z sekwencjami *RPB1* Claroideoglomus 1, 2, 4 (nowo opisanych odpowiednio jako *Entrophospora argentinensis*, *E. glacialis* i *E. furrazolae*), *Claroideoglomus* 3 (glomoidalny morf epitypu *E. infrequens*), ośmiu innych gatunków *Claroideoglomus* sensu C. Walker i A. Schüßler oraz trzech *Diversispora* spp. w grupie zewnętrznej. Dawne nazwy gatunków zawarte w Entrophosporales podano w nawiasach. Nowe gatunki i numery sekwencji uzyskanych w badaniach wlasnych są pogrubione. Bayesowskie prawdopodobieństwo a posteriori ≥0,90 i wartości wsparcia ML ≥50% są podane w pobliżu gałęzi.




**Ryc. 3. A, B., E.** *Entrophospora infrequens*, **C, D.** *E. argentinensis*, **F, G.** *E. glacialis*, **H.** *E. furrazolae* (sh, trzonek, sp, zarodnik, ss, pęcherz zarodnikotwórczy, swl, warstwa ściany zarodnika).

### 6.3. Utworzenie nowej rodziny i nowej kombinacji nomenklaturowej

**P8: New taxa in Glomeromycota: Polonosporaceae fam. nov., *Polonospora* gen. nov., and *P. polonica* comb. nov.**

Jednym z gatunków rodzaju *Acaulospora* (rodzina Acaulosporaceae, rząd Diversisporales) jest *A. polonica*, która została umieszczona w tym rodzaju z powodu tworzenia akaulosporoidalnych zarodników, tj. bocznie, bezpośrednio na ramieniu pęcherza zarodnikotwórczego (Gerdemann, Trappe 1974; Błaszkowski 1988, 2012), podobnie jak u *A. laevis*, gatunku typowego dla *Acaulospora*. Jednak szereg cech morfologicznych *A. polonica* nie pasowało do cech większości *Acaulospora* spp. Wszystkie typowe *Acaulospora* spp. mają zarodniki z zabarwioną ścianą 1. zarodnika, tworzącą powierzchnię zarodnika, która u wielu gatunków jest urzeźbiona, i dwie bezbarwne ściany wewnętrzne (ściany 2. i 3. zarodnika). Wewnątrzkomórkowa struktura zarodników *A. polonica* również składa się z trzech ścian zarodnika, ale ściana 1. pozostaje bezbarwna przez cały cykl życiowy i jest gładka (Błaszkowski 1988, 2012). Zasadnicze różnice między *A. polonica* i typowymi gatunkami *Acaulospora* tkwią w składzie, jak również w własnościach fenotypowych i histochemicznych ściany 3. zarodnika. U *A. polonica* warstwa 1. ściany 3. zarodnika jest gruba, skórzasta i gładka (vs. cienka i urzeźbiona ziarnistymi naroślami u innych *Acaulospora* spp.), a warstwa 2. tej ściany jest cienka, błoniasta, nie pogrubia się w PVLG i nie barwi się w odczynniku Melzera (vs. plastyczna, silnie pęcznieje w PVLG, barwi się w odczynniku Melzera).

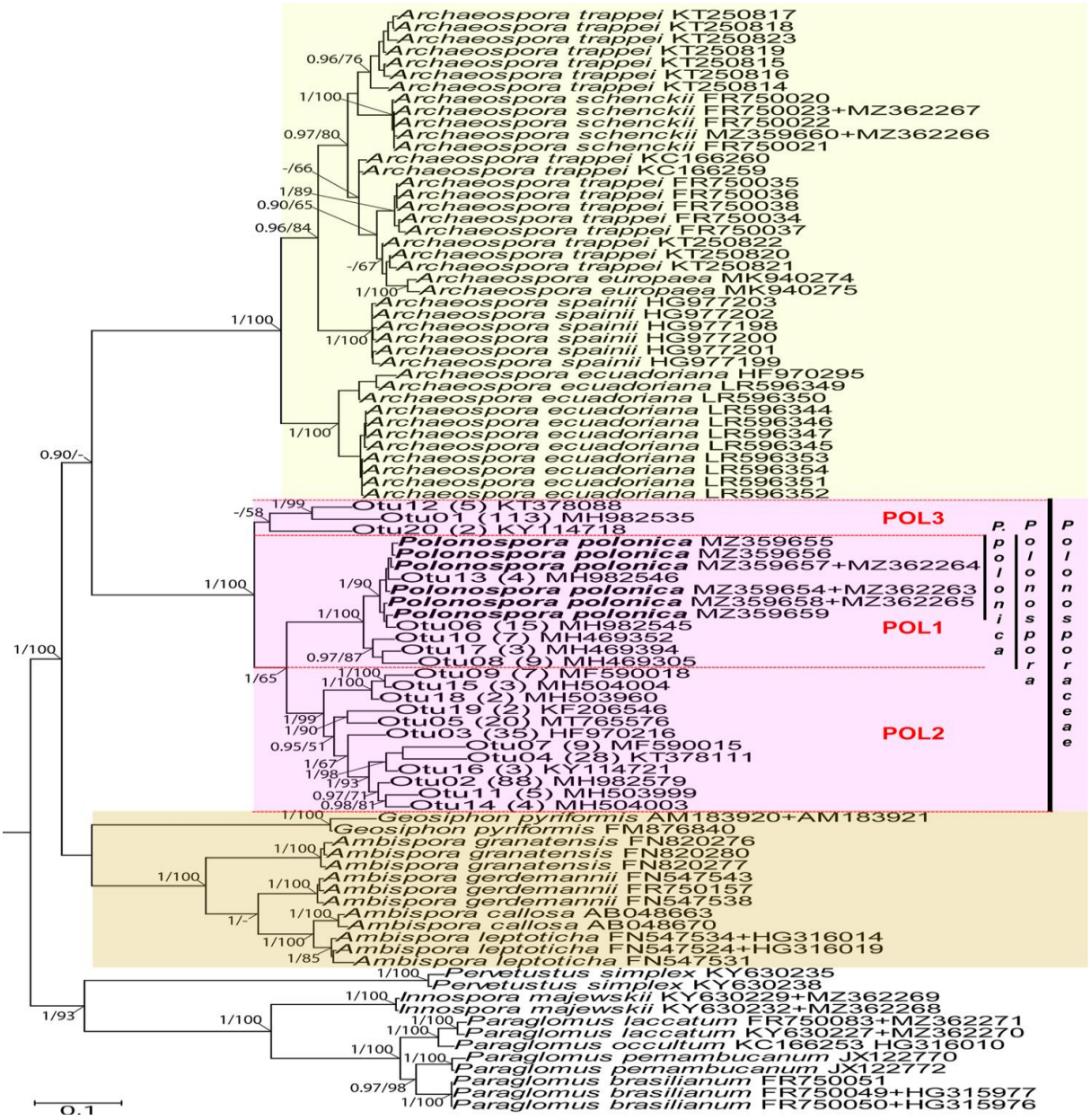
Spośród przedstawicieli Glomeromycota tworzących akaulosporoidalne zarodniki, cechy ściany 3. zarodnika *A. polonica* najbardziej przypominały cechy najbardziej wewnętrznej ściany 2. zarodnika wszystkich gatunków z rodzajów *Ambispora* i *Archaeospora*, przynależących do rodzin odpowiednio Ambisporaceae i Archaeosporaceae, które reprezentują starożytne taksony Glomeromycota (Morton, Redecker 2001) i filogenetycznie są silnie odchylone od rodzajowego kladu *Acaulospora* (Oehl et al. 2011b).

Mimo posiadania unikatowych cech morfologicznych scharakteryzowanych wyżej, umiejscowienie w Glomeromycota gatunków obecnie zaklasyfikowanych do *Ambispora* i *Archaeospora*, z których większość oryginalnie opisano jako *Acaulospora* spp., było możliwe tylko na podstawie analiz filogenetycznych ich molekularnych sekwencji (Morton, Redecker 2001; Oehl et al. 2011a). Morfologiczne odchylenia *A. polonica* od typowych *Acaulospora* spp., jak również morfologiczne podobieństwa i różnice względem *Ambispora* i *Archaeospora* spp. również sugerowały, że *A. polonica* powinna reprezentować nowy klad, umiejscowiony wśród bazowych kladów Glomeromycota. Jednak określenie pozycji tego kladu na podstawie tylko morfologii zarodników *A. polonica* nie było możliwe.

*Acaulospora polonica,* po jej opisaniu, była bardzo rzadko znajdywana fizycznie w próbach polowych i nie została wyhodowana w kulturach. Badania kultur pułapkowych założonych przez J. Błaszkowskiego ujawniły obfite zarodnikowanie tego gatunku. To zainicjowało badania, których celami były: (*i*) wyhodowanie *A. polonica* w kulturach jednogatunkowych, (*ii*) sprawdzenie poprawności opisów cech morfologicznych i histochemicznych zarodników tego gatunku sporządzonych przez Błaszkowskiego (1988, 2012), (*iii*) scharakteryzowanie struktur mykoryzowych *A. polonica,* (*iv*) określenie potencjalnej dymorficzności tego grzyba, jak również (*v*) przeprowadzenie stosownych analiz filogenetycznych w celu potwierdzenia hipotezy, że *A. polonica* przynależy do nieopisanego kladu położonego wśród bazowych kladów gromady Glomeromycota i określenie pozycji tego kladu wewnątrz tej gromady.

Wyniki analiz filogenetycznych i porównań sekwencji potwierdziły hipotezę, że gatunek oryginalnie opisany jako *A. polonica* (Błaszkowski 1988) nie przynależy do *Acaulospora,* ale

reprezentuje nowy rodzaj w nowej rodzinie rzędu Archaeosporales (Ryc. 4). Dlatego taksony te wprowadzono do Glomeromycota pod nazwami odpowiednio *Polonospora* i Polonosporaceae. Co więcej, analizy te wykazały, że (*i*) Polonosporaceae składa się z trzech grup w randze rodzaju (POL1–3): *Polonospora* z gatunkiem typowym *P. polonica* comb. nov. i AGM poznanych tylko ze środowiskowych badań molekularnych oraz (*ii*) te jeszcze nieopisane taksony mają ogólnoświatowe rozmieszczenie; ich sekwencje pochodziły m.in. z Australii, Chin, Japonii, Norwegii, Południowej i Północnej Ameryki, Portugalii oraz Republiki Czeskiej.




**Ryc. 4.** Filogram konsensusu 50% reguły większości z analizy bayesowskiej sekwencji 18S-ITS-28S+*RPB1* *Polonospora polonica*, 10 gatunków rzędu Archaeosporales, 20 reprezentatywnych sekwencji OTU z badań środowiskowych (z liczbą każdej OTU w nawiasach) i 6 gatunków Paraglomerales służących jako grupa zewnętrzna. Trzy odrębne klady (POL1-3) w Polonosporaceae są oddzielone czerwonymi liniami. Bayesowskie prawdopodobieństwo a posteriori ≥ 0,90 i wartości ładowania początkowego ML ≥ 50% podano w pobliżu gałęzi.

Liczne próby wyhodowania *Polonospora polonica* w kulturach jednogatunkowych nie powiodły się. Dlatego cechy struktur mykoryzowych tego gatunku i jego potencjalna

zdolność do tworzenia zarodników innych niż akaulosporoidalne pozostają nieznane. Analizy zarodników wyekstrahowanych z kultur pułapkowych wykazały, że opisy *A. polonica* sporządzone przez Błaszkowskiego (1988, 2012) są poprawne.

### 6.4. Utworzenie nowych rodzajów i kombinacji nomenklaturowych

### P1: A new genus, *Oehlia* with *Oehlia diaphana* comb. nov. and an emended description of *Rhizoglomus vesiculiferum* comb. nov. in the Glomeromycotina

Największą grupą w Glomeromycota są gatunki tworzące glomoidalne zarodniki, jak *Glomus macrocarpum*, typ rodzaju *Glomus*, zwłaszcza gatunki oryginalnie opisane jako *Glomus* spp. Jednak filogeneza wielu *Glomus* spp. jest nieznana lub wątpliwa (Oehl et al. 2011a, b; Schüßler, Walker 2010). Ponadto przypuszczano, że sekwencje przypisane do wielu pojedynczych gatunków mogą reprezentować różne gatunki i *vice versa*, grzyby o różnych nazwach mogą być tożsame (Błaszkowski, obserw. własne; Stockinger et al. 2014). Jednym z takich gatunków było *Glomus diaphanum*, oryginalnie opisane przez Mortona i Walkera (1984) z Zachodniej Wirginii.

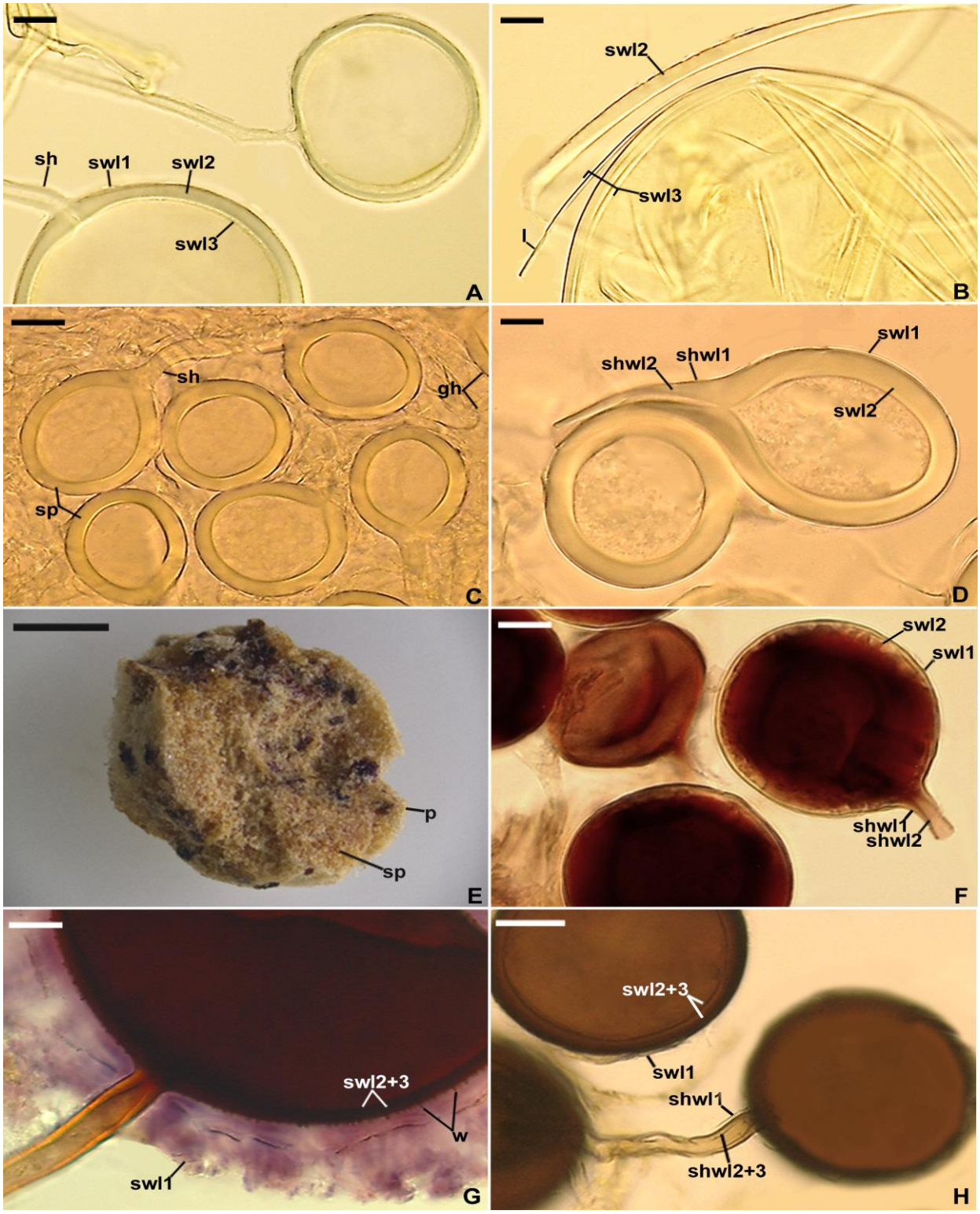
Pierwsze dane o molekularnej filogenezie *G. diaphanum* opublikowali Stockinger et al. (2009). W przedstawionym drzewie filogenetycznym siedem sekwencji SSU-ITS-LSU otrzymanych z zarodników pochodzących od dr. J.B. Mortona zgrupowało się w oddzielnym, w pełni wspartym kladzie w randze rodzaju. Klad ten był położony poniżej rodzajowego kladu z *Sclerocystis sinuosa* i kladu z sekwencjami *Glomus* spp., które później Schüßler i Walker (2010) przenieśli do nowo utworzonego rodzaju *Rhizoglomus*. Identyczną filogenezę *G. diaphanum* względem *S. sinuosa* i *Glomus*/*Rhizoglomus* spp. odtworzyli Msiska i Morton (2009) po analizach genu *β-tubuliny*. Stockinger et al. (2014) zrekonstruowali filogenezę *G. diaphanum* (nazwanego *G.* cf. *diaphanum*) na podstawie analiz sekwencji genu *RPB1*, która była identyczna względem filogenezy wcześniej wygenerowanej z sekwncji SSU-ITS-LSU (Stockinger et al. 2009).

Morfologicznie *G. diaphanum* różniło się zasadniczo pod względem wielu cech od *S. sinuosa* i zawierało cechy nieobecne u jakiegokolwiek innego gatunku tworzącego zarodniki glomoidalne (Błaszkowski 2012). Badania własne dwóch kanadyjskich AGM nazwanych DAOM745424 i DAOM227022 wykazały, że cechy morfologiczne i histochemiczne zarodników tych grzybów dokładnie pasowały do cech zarodników *G. diaphanum* zdefiniowanych przez Mortona i Walkera (1984), Mortona (http://invam.wvu.edu/) oraz Błaszkowskiego (2012). Ponadto analizy filogenetyczne sekwencji SSU-ITS-LSU i *RPB1* otrzymane w badaniach własnych i sekwencji tych lokusów pobranych z publicznych baz danych potwierdziły, że te dwa grzyby są tożsame względem odpowiednio *G. diaphanum* sensu Morton i Walker (1984) oraz *G.* cf. *diaphanum* sensu Stockinger et al. (2014), jak również ukazały dużą molekularną odległość między *G. diaphanum*, *S. sinuosa* i *Rhizoglomus* spp. A więc analizy te ponownie podpowiedziały, że *G. diaphanum* nie może przynależeć ani do rodzaju *Glomus*, ani do rodzaju *Rhizoglomus*, ale powinno reprezentować nowy rodzaj.

Dlatego celami dalszych badań były: (*i*) zdefiniowane nowego rodzaju, używając zgromadzonych danych morfologicznych i molekularnych i (*ii*) zilustrowanie cech morfologicznych oraz histochemicznych zarodników jego gatunku typowego na podstawie okazów *G. diaphanum* otrzymanych od dr. J.B. Mortona i grzybów DAOM745424 oraz DAOM227022.

Badania morfologiczne wykazały, że najbardziej wewnętrzna warstwa 3. ściany zarodnika *G. diaphanum* jest zlaminowana (Ryc. 5A, B) i przez to unikatowa w porównaniu do najbardziej wewnętrznej warstwy ściany zarodnika wszystkich innych poznanych gatunków Glomeromycota, którą tworzą glomoidalne zarodniki. Ta unikatowa morfologia również była

zgodna się z unikatową filogenezą (Ryc. 6), którą zrekonstruowano na podstawie analiz sekwencji SSU-ITS-LSU i *RPB1*. Analizy te podniosły ten gatunek do rangi rodzaju, opisanego pod nazwą *Oehlia*, z gatunkiem typowym *O. diaphana* comb. nov.




**Ryc. 5. A, B.** *Oehlia diaphana*, **B, C.** *Sclerocarpum amazonicum*, **E, F.** *Epigeocarpum crypticum*, **G.** *Halonatospora pansihalos*, **H.** *Silvaspora neocaledonica* (p, peridium, sp, zarodnik, sh, trzonek, shwl, warstwa ściany trzonka, swl, warstwa ściany zarodnika).




**Ryc. 6.** Filogram konsensusu reguły 50% z analizy bayesowskiej sekwencji 18S-ITS-28S+*RPB1 D. glomerocarpica*, *E. crypticum* i 34 innych gatunków AMF, w tym *C. claroideum*, *C. drummondii*, *C. walkerii* i *E. infrequens* w grupie zewnętrznej. Bayesowskie prawdopodobieństwa a posteriori ≥0,90 i wartości wsparcia ML ≥50% podano pobliżu gałęzi.

**P3: New sporocarpic taxa in the phylum Glomeromycota: *Sclerocarpum amazonicum* gen. et sp. nov. in the family Glomeraceae (Glomerales) and *Diversispora sporocarpia* sp. nov. in Diversisporaceae (Diversisporales)**

Spośród około 360 gatunków Glomeromycota tylko 57 zostało opisanych, że tworzą zarodniki glomoidalne w epigeicznych (naziemnych) lub podziemnych sporokarpach (owocnikach). Wszystkie gatunki sporokarpiczne z losowo rozmieszczonymi zarodnikami pierwotnie opisano w rodzaju *Glomus*. Późniejsze analizy filogenetyczne dały podstawę do przeniesienia niektórych z nich, np. *G. fulvum* i *G. megalocarpum*, do nowego rodzaju, *Redeckera*, w rodzinie Diversisporaceae (Walker, Schüßler 2004). Jednak molekularna filogeneza pozostała nieznana dla wielu innych sporokarpicznych gatunków, które Schüßler i Walker (2010) umieścili w ich oryginalnych rodzajach, jako gatunki o niepewnej pozycji.

Grzyby tworzące glomoidalne zarodniki w sporokarpach stanowią niewielki odsetek gatunków Glomeromycota i są jednymi z najmniej poznanych grzybów tej gromady pod względem ekofizjologii, statusu troficznego i molekularnej filogenezy, głównie z powodu trudności ich znajdywania i hodowania w kulturach jednogatunkowych. Zdaniem Gerdemanna i Trappego (1974) efektywność kolekcjonowania tej grupy grzybów nawet wynika z nabycia instynktu podziemnego.

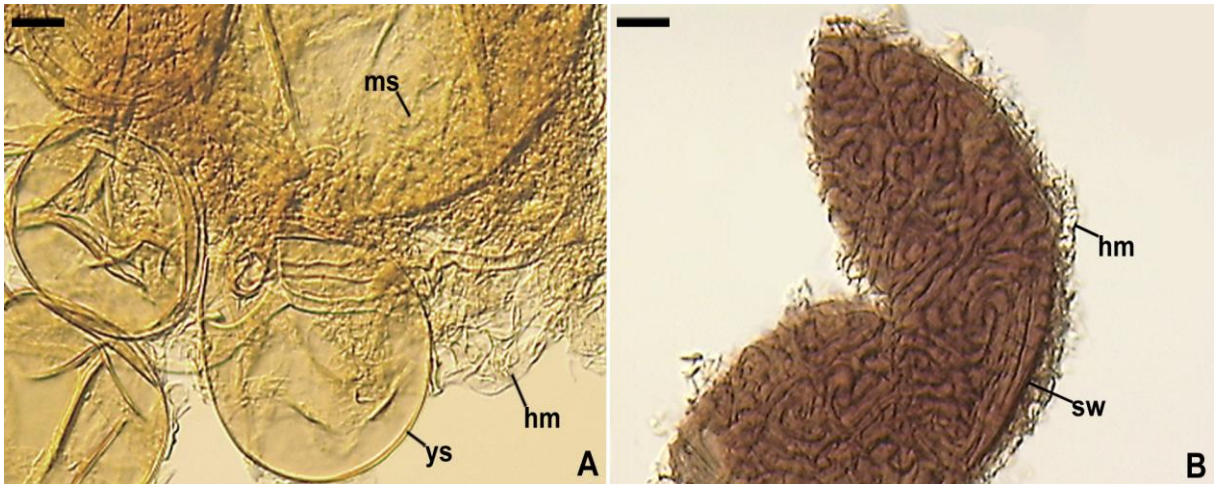
W badaniach własnych wykorzystano sporokarpy znalezione w Brazylii. Wstępne analizy morfologiczne i filogenetyczne z użyciem tylko sekwencji SSU-ITS-LSU podpowiedziały, że jest to nieopisany gatunek i powinien zostać szczegółowo scharakteryzowany w celu potwierdzenia tej hipotezy.

Późniejsze, rozszerzone analizy jednoznacznie potwierdziły konieczność utworzenia nowego, jednogatunkowego rodzaju, nazwanego *Sclerocarpum* z *S. amazonicum*, w rodzinie Glomeraceae. Po pierwsze, analizy filogenetyczne umiejscowiły *S. amazonicum* w kladzie równoważnym względem innych kładów w randze rodzaju (Ryc. 6). Po drugie, odchylenia sekwencji SSU-ITS-LSU *S. amazonicum* od sekwencji SSU-ITS-LSU *Glomus bareae, G. macrocarpum* i *G. tetrastratosum*, które zgrupowały się w siostrzanym kladzie, i odchylenia sekwencji *RPB1 S. amazonicum* od sekwencji *RPB1* tych samych *Glomus* spp. wynosiły odpowiednio 22.1–23.6% i 14.4–15.0%. A więc odchylenia te były znacznie większe niż odchylenia sekwencji tych samych lokusów innych filogenetycznie scharakteryzowanych rodzajowych taksonów Glomeraceae (Błaszkowski et al. 2018a, b).

Również cechy morfologiczne *S. amazonicum*, takie jak bardzo małe, hialinowe, grubościenne zarodniki i trzonek zarodnika, którego warstwy ściany powstają znacznie poniżej podstawy zarodnika oraz są ciągłe z warstwami ściany zarodnika, wyraźnie różnią się od cech morfologicznych innych reprezentantów Glomeromycota tworzących sporokarpy z losowo rozmieszczonymi zarodnikami (Ryc. 5C, D).

**P5: *Sieverdingia* gen. nov., *S. tortuosa* comb. nov., and *Diversispora peloponnesiaca* sp. nov. in the Diversisporaceae (Glomeromycota)**

Grzyb oryginalnie opisany jako *Glomus tortuosum* jest jednym z niewielu gatunków Glomeromycota, którego zarodniki powstają pojedynczo lub są losowo rozmieszczone w skupieniach i mają ścianę zarodnika zbudowaną z tylko jednej zabarwionej warstwy (Ryc. 7; Schenck, Smith 1982). Na podstawie analiz filogenetycznych sekwencji genu 28S (= LSU) Błaszkowski i Chwat (2013) przenieśli *G. tortuosum*, wraz z *G. globiferum*, do rodzaju *Corymbiglomus* w rodzinie Diversisporaceae (rząd Diversisporales) i przemianowali te gatunki na *C. tortuosum* comb. nov. i *C. globiferum* com. nov. Rodzaj *Corymbiglomus*, z gatunkiem typowym *C. corymbiforme*, został utworzony w następstwie analiz filogenetycznych sekwencji 28S *G. corymbiforme* (Błaszkowski 2012). W drzewie



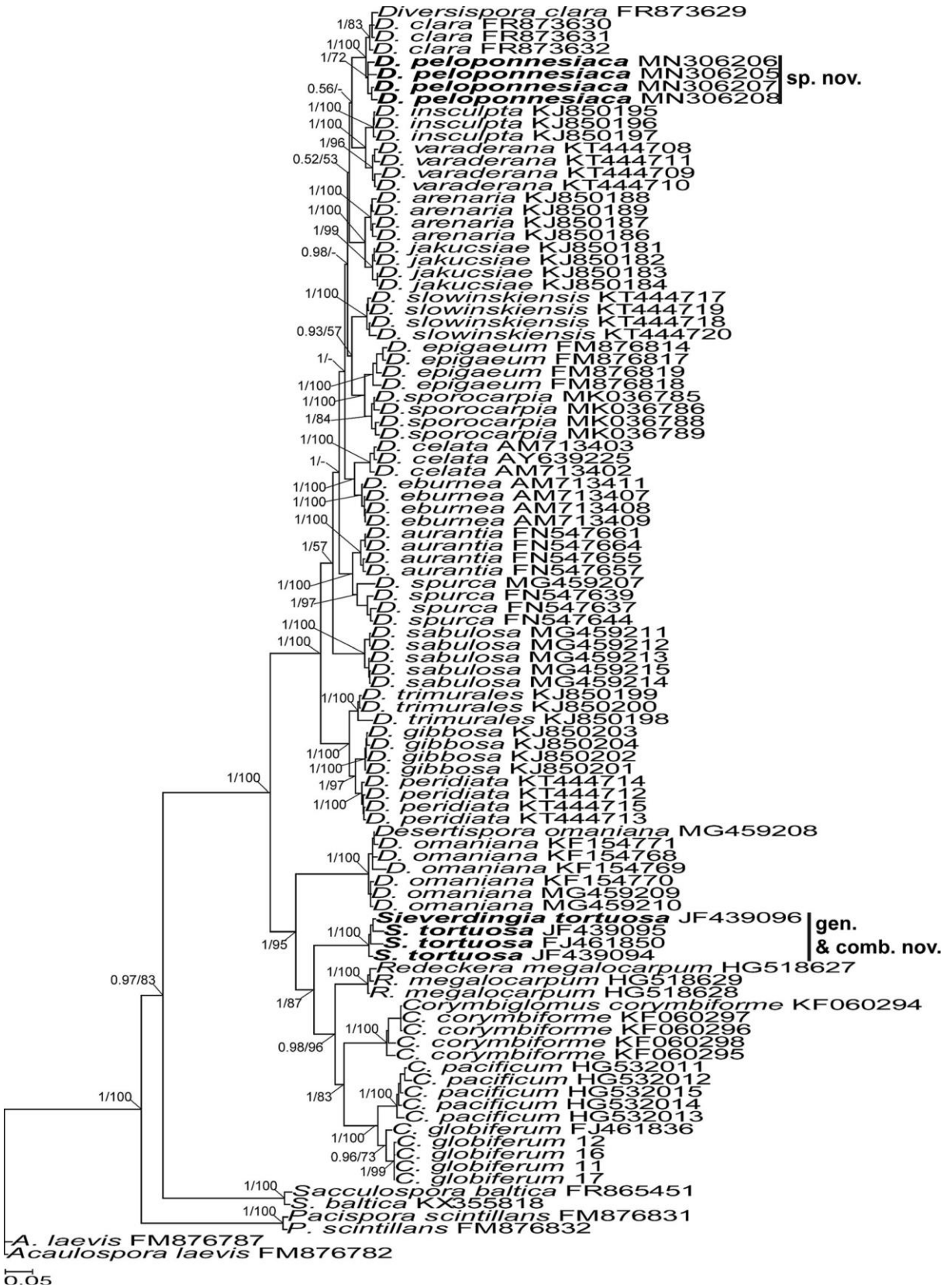

**Ryc. 7.** *Sieverdingia tortuosa* (hm, płaszcz grzybniowy, sw, ściana zarodnika, ys, młody nieopłaszczony zarodnik).

filogenetycznym Błaszkowskiego i Chwata (2013) każdy z tych gatunków był reprezentowany przez jedną sekwencję 28S, które zgrupowały się w siostrzanym kladzie względem kladu reprezentującego rodzaj *Diversispora*. Co ciekawe, ten trzygatunkowy klad składał się z dwóch kładów podrzędnych, jednego z sekwencjami *C. corymbiforme* i *C. globiferum*, i drugiego z sekwencjami *C. tortuosum*. Analizy te nie uwzględniły sekwencji *Redeckera* spp., które również mają glomoidalne zarodniki, ponieważ ekologia i wszystkie inne cechy morfologiczne *Redeckera* spp. oraz *C. tortuosum* różniły się zasadniczo (Redecker et al. 2007; Schenck, Smith 1982; Oehl et al. 2011a).

Rozpoznawcze analizy filogenetyczne dostępnych sekwencji genu 18S, regionu ITS i genu 28S, które reprezentowały wszystkie rodzaje rodziny Diversisporaceae tworzące glomoidalne zarodniki, zasugerowały, że *C. tortuosum* nie jest członkiem *Corymbiglomus*, ale reprezentuje nieopisany rodzaj w Diversisporaceae, co również postulowali Medina et al. (2014) w odniesieniu do nowo opisanego gatunku, *C. pacificum*, którego zarodniki nie były otoczone płaszczem grzybniowym, jak zarodniki *C. corymbiforme*, *C. globiferum* i *C. tortuosum*. Ponadto badacze ci stwierdzili, że wewnątrzkomórkowa struktura zarodników *C. pacificum* składa się z dwóch ścian i zasugerowali, że zarodniki *C. corymbiforme* oraz *C. globiferum* również są dwuścienne.

*Corymbiglomus corymbiforme* zostało oryginalnie scharakteryzowane (pod nazwą *Glomus corymbiforme*), jako tworzące zarodniki z pojedynczą ścianą zarodnika (Błaszkowski 1995). Oryginalny opis *C. globiferum* (jako *G. globiferum*) również świadczy, że jego zarodniki mają jedną ścianę zarodnika (Koske, Walker 1986). Ponadto obserwacje własne mikrofotografii zarodników *C. pacificum* opublikowane przez Medina et al. (2014) sugerowały, że ściana 2. zarodników *C. pacificum* sensu Medina et al. (2014) nie jest oddzielną strukturą i nie powstaje identycznie jak np. ściana 2. zarodników gatunków z rodzaju *Pacispora* (Błaszkowski 2012).

Dlatego celami podjętych badań były (*i*) sprawdzenie pozycji filogenetycznej czterech opisanych gatunków *Corymbiglomus* wśród taksonów rodziny Diversisporaceae z glomoidalnymi zarodnikami, (*ii*) ostateczne zdefiniowanie struktury śródkomórkowej zarodników tych gatunków i (*iii*) uzupełnienie opisu diagnostycznego rodzaju *Corymbiglomus*, gdyby to było konieczne.




**Ryc. 8.** Filogram 50% konsensusu reguły większości z analizy bayesowskiej sekwencji 18S-ITS-28S *Diversispora peloponnesiaca*, *Sieverdingia tortuosa* i 24 innych gatunków AMF, w tym *Acaulospora laevis*, *Pacispora scintillans* i *Sacculospora baltica* w grupie zewnętrznej. Bayesowskie prawdopodobieństwo a posteriori ≥0,50 i wartości wsparcia ML ≥50% podano w pobliżu gałęzi.

Przeprowadzone analizy filogenetyczne w pełni potwierdziły przypuszczenie, że *Corymbiglomus tortuosum* powinno być przeniesione do nowego rodzaju, który w konsekwencji opisano jako *Sieverdingia* w rodzinie Diversisporaceae (Ryc. 8).

W konsekwencji przeniesienie to i skorygowanie opisu diagnostycznego rodzaju *Corymbiglomus* wyeliminowało niezgodności między cechami morfologicznymi zarodników *C. corymbiforme, C. globiferum, C. pacificum* i *S. tortuosa* (Schenck, Smith 1982; Koske, Walker 1986; Błaszkowski 2012; Medina et al. 2014; http://fungi.invam.wvu.edu/the-fungi/classification/diversisporaceae/diversispora/tortuosum.html; http://fungi.invam.wvu.edu/thefungi/classification/diversisporaceae/diversispora/globiferum.

html).

Chociaż rodzaj *Sieverdingia* opisano na podstawie analiz filogenetycznych sekwencji 18S-ITS-28S i wyłącznie genu 28S tylko jednego gatunku, *S. tortuosa* (Ryc. 8), zagnieżdżenie stosunkowo krótkiej sekwencji FJ461850 genu 28S otrzymanej z *S. tortuosa* hodowanej w International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi, USA między trzema sekwencjami 18S-ITS-28S, o znacznie większej informatywności filogenetycznej, pochodzącymi z zarodników *S. tortuosa* znalezionych w Chinach dowiodły, że te cztery sekwencje charakteryzują jeden gatunek i silnie wsparły słuszność utworzenia rodzaju *Sieverdingia*.

Przeprowadzone analizy morfologiczne również potwierdziły hipotezę, że rodzaj *Corymbiglomus* powinien skupiać tylko gatunki z zarodnikami mającymi jedną ścianę zarodnika, w której najbardziej wewnętrznym komponentem jest stosunkowo gruba, zlaminowana warstwa, często oddzielająca się od pozostałych warstw, ale zwykle przylegająca do wewnętrznej powierzchni ściany trzonka u skruszonych zarodników. Taki wymóg spełniały tylko *C. corymbiforme, C. globiferum* i *C. pacificum*. Mimo, że Medina et al. (2014) opisali *C. pacificum* z dwuścienną strukturą wewnątrzkomórkową i uznali, że taką strukturę mają również zarodniki *C. corymbiforme* i *C. globiferum*, badania własne nie potwierdziły tych stwierdzeń. Wykazały one, że najbardziej wewnętrzny komponent ściany zarodnika wszystkich tych gatunków jest związany ze ścianą trzonka, z której prawdopodobnie powstaje, podobnie jak u *Claroideoglomus claroideum*. Badania ontogenezy *C. claroideum* wykazały, że związek najbardziej wewnętrznej warstwy ściany zarodnika ze ścianą trzonka zarodnika świadczy o wspólnym powstawaniu tych struktur i dlatego ta najbardziej wewnętrzna warstwa ściany zarodnika reprezentuje jedną strukturę, ścianę zarodnika (Stürmer, Morton 1997).

Dostępne dane własne i literaturowe, jak również porównania sekwencji molekularnych *C. corymbiforme, C. globiferum, C. pacificum* i *S. tortuosum* świadczą, że gatunki te prawdopodobnie występują raczej rzadko w świecie.

**P6: New glomeromycotan taxa, *Dominikia glomerocarpica* sp. nov. and *Epigeocarpum crypticum* gen. nov. et sp. nov. from Brazil, and *Silvaspora* gen. nov. from New Caledonia**

Spośród okazów AGM pochodzących z Północno Wschodniej Brazylii zbadano również dwa inne grzyby tworzące sporokarpy z losowo rozmieszczonymi zarodnikami glomoidalnymi. Po pierwszych, ogólnych obserwacjach grzyby te wydawały się być identyczne pod względem morfologii, ale różniły się od opisanych gatunków Glomeromycota (Ryc. 5E, F, 10A, B). Następne badania cech fenotypowych ujawniły bardzo małe różnice i zasugerowały, że sporokarpy te reprezentują dwa kryptyczne (o podobnej lub identycznej morfologii, ale wyraźnie odchylonej filogenezie molekularnej), nieopisane gatunki. Wykorzystując rozpoznawcze, niekompletne dane molekularne, przeszukiwania bazy BLAST potwierdziły potencjalną nowość tych grzybów i wykazały,

że są one prawdopodobnie spokrewnione z przedstawicielami rodzajów *Dominikia* i *Kamienskia*.

Crossay et al. (2018) opisali nowy gatunek w rodzinie Glomeraceae, *Rhizophagus neocaledonicus* (później przemianowany na *Rhizoglomus neocaledonicum*; Turrini et al. 2018), na podstawie analiz ML sekwencji 18S-ITS-28S, mimo, że klad *R. neocaledonicum* otrzymał tylko 49% wsparcie i odchylenie genetyczne tego gatunku od sześciu innych *Rhizoglomus* spp. było w rzędzie wartości rozdzielających klady Glomeromycota w randze rodzaju. Nieco później opublikowane wyniki innych analiz sekwencji 18S-ITS-28S i wszystkich zsekwencjonowanych *Rhizoglomus* spp. oraz reprezentantów pozostałych rodzajów Glomeraceae umieściły *R. neocaledonicum* między nowo opisanym rodzajem *Halonatospora* z *H. pansihalos* (Ryc. 5G, 6) i *S. sinuosa*, z których ostatni był położony bazowo względem pozostałych *Rhizoglomus* spp. (Błaszkowski et al. 2018b, 2019). A więc *R. neocaledonicum* powinno reprezentować nowy, autonomiczny klad.

Uwzględniając informacje opisane wyżej, celami dalszych badań były (*i*) udokumentowanie nowości dwóch brazylijskich grzybów, (*ii*) określenie ich pozycji filogenetycznej wśród rodzajowych krewniaków o poznanej filogenezie, (*iii*) scharakteryzowanie ich morfologii i (*iv*) przetestowanie hipotezy, że *R. neocaledonicum* jest członkiem nieopisanego rodzaju w Glomeromycota, wykorzystując wcześniej dostępne i nowo zebrane dane morfologiczne oraz molekularne po otrzymaniu zarodników tego grzyba od dr. T. Crossay z Nowej Kaledonii.

Jak spodziewano się po wstępnych badaniach morfologicznych i molekularnych, rozszerzone analizy filogenetyczne przyrównań z sekwencjami 18S-ITS-28S i *RPB1* reprezentantów wszystkich rodzajów rodziny Glomeraceae jednoznacznie i silnie dowiodły, że te dwa sporokarpiczne grzyby z Brazylii były nowymi gatunkami i mimo, że były one morfologicznie prawie identyczne (Ryc. 5E, F, 10A, B), filogenetycznie były silnie odchylonymi taksonami Glomeraceae. Pierwszy, nieformalnie nazwany „Gatunek 1" został umiejscowiony w rodzaju *Dominikia*, a drugi, „Gatunek 2", w nowym kladzie rodzajowym, sąsiadującym z rodzajem *Kamienskia* (Ryc. 6).

Analizy sekwencji obu lokusów, rozpatrywanych oddzielnie i łącznie, również potwierdziły wskazania wstępne, które sugerowały autonomiczność *R. neocaledonicum* (Ryc. 6).

W konsekwencji „Gatunek 1" i „Gatunek 2" opisano jako odpowiednio *Dominikia glomerocarpica* sp. nov. i *Epigeocarpum crypticum* gen. nov. et sp. nov., a *R. neocaledonicum* przeniesiono do nowego rodzaju, *Silvaspora* gen. nov., z *S. neocaledonica* comb. nov. (Ryc. 5E, F, H, 6, 10A, B).

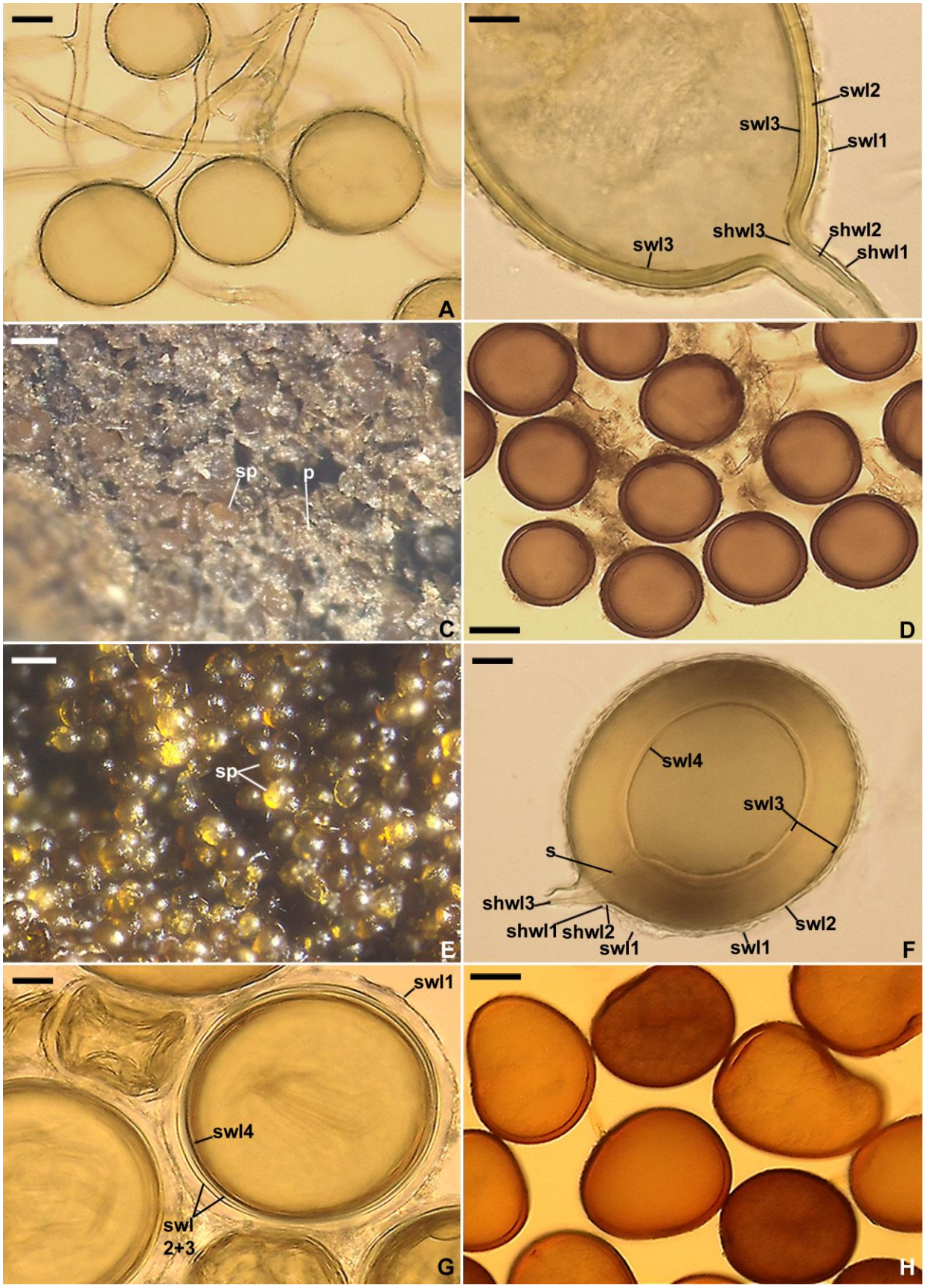
**P8: New taxa in Glomeromycota: Polonosporaceae fam. nov., *Polonospora* gen. nov., and *P. polonica* comb. nov.**

Dane o rodzaju *Polonospora* przedstawiono wyżej.

### 6.5. Opisanie nowych gatunków

**P2: *Halonatospora* gen. nov. with *H. pansihalos* comb. nov. and *Glomus bareae* sp. nov. (Glomeromycota; Glomeraceae)**

Analizy morfologiczne i filogenetyczne grzyba z zarodnikami glomoidalnymi, które wyhodowano w kulturach pułapkowych i jednogatunkowych, wykazały, że jest on nieopisanym gatunkiem w rodzaju *Glomus*, którego najbliższym krewniakiem filogenetycznym było *G. tetrastratosum* (Ryc. 6, 9A, B).




**Ryc. 9. A, B.** *Glomus bareae*, **C.** *Diversispora sporocarpia*, **D.** *Rhizoglomus dalpeae*, **E, F.** *R. maiae*, G. *R. silesianum*, **H.** *D. peloponesiaca* (p, peridium, sp, zarodnik, shwl, warstwa ściany trzonka, swl, warstwa ściany zarodnika).

Klad z tym nowym grzybem otrzymał pełne wsparcia BI (= 1.0) i ML (= 100%) w obu drzewach z sekwencjami SSU-ITS-LSU i *RPB1*. Gatunek ten opisano pod nazwą *G. bareae*, który różnił się wyraźnie od *G. tetrastratosum* pod względem zarówno morfologii (wymiar i kolor zarodników, struktura ściany zarodnika, cechy fenotypowe jej składowych, własności trzonka zarodnika), jak i składu nukleotydowego sekwencji SSU-ITS-LSU oraz *RPB1*. Sekwencje SSU-ITS-LSU i *RPB1 G. bareae* oraz *G. tetrastratosum* różniły się o odpowiednio 3,7–4,1% i 1,4–1,6%.

Dotychczas *G. bareae* znaleziono tylko w wydmie nadmorskiej Świnoujścia, której glebę ryzosferową *Ammophila arenaria* użyto do zainokulowania kultury pułapkowej wspomnianej wyżej.

**P3: New sporocarpic taxa in the phylum Glomeromycota: *Sclerocarpum amazonicum* gen. et sp. nov. in the family Glomeraceae (Glomerales) and *Diversispora sporocarpia* sp. nov. in Diversisporaceae (Diversisporales)**

Uzasadnienie opisania *Sclerocarpum amazonicum* sp. nov. przedstawiono w charakterystyce publikacji o utworzeniu nowego rodzaju, *Sclerocarpum*, *vide* wyżej P3, Ryc. 5B, C.

*Sclerocarpum amazonicum* dotychczas fizycznie poznano tylko z jego oryginalnego stanowiska zbioru, rezerwatu leśnego Adolfo Ducke położonego w centrum lasu amazońskiego. Brak sekwencji otrzymanych z molekularnych badań środowiskowych zdeponowanych w publicznych bazach danych również świadczyło, że *S. amazonicum* występuje rzadko w świecie.

*Diversispora sporocarpia* sp. nov. opisano na podstawie analiz morfologicznych i filogenetycznych sporokarpu (Ryc. 9C) znalezionego w lesie sosnowo świerkowym z domieszką leszczyny, położonym w Pienińskim Parku Narodowym. Próby wyhodowania tego grzyba w kulturach nie powiodły się. Analizy filogenetyczne umiejscowiły ten gatunek w sąsiedztwie *D. epigaea* z pełnymi lub silnymi wsparciami BI i ML (Ryc. 8). Różnice między sekwencjami SSU-ITS-LSU i *RPB1 D. sporocarpia* i *D. epigaea* były duże oraz wynosiły odpowiednio 3,7–9,9% i 1,5%. Morfologicznie *D. sporocarpia* różniła się również wyraźnie względem *D. epigaea* i główne odrębności dotyczą liczby oraz własności fenotypowych składowych ich ściany zarodnika (Jobim et al. 2019).

Poza stanowiskiem zbioru sporokarpu *D. sporocarpia* podanym wyżej, porównania sekwencji środowiskowych zdeponowanych w publicznych bazach danych zasugerowały, że gatunek ten wcześniej zidentyfikowano tylko w Republice Czeskiej.

**P4: *Rhizoglomus dalpeae*, *R. maiae*, and *R. silesianum*, new species**

*Rhizoglomus dalpeae* sp. nov. i *R. silesianum* sp. nov. zdefiniowano po analizach morfologicznych zarodników (Ryc. 9D–G) oraz filogenetycznych sekwencji SSU-ITS-LSU i *RPB1* otrzymanych z tych zarodników, które wyekstrahowano z kultur jednogatunkowych, a *R. maiae* sp. nov. po podobnych badaniach zarodników i sekwencji otrzymanych z epigeicznego sporokarpu.

Analizy BI i ML użytych sekwencji umieściły te grzyby w trzech w pełni lub silnie wspartych kladach gatunkowych. Najbliższymi krewniakami *R. dalpeae* były *R. clarum* i *R. dunense*, *R. maiae* – *R. arabicum* i *R. custos*, a *R. silesianum* – *R. natalense* (Ryc. 6). Różnice między sekwencjami SSU-ITS-LSU (*i*) *R. dalpeae* i *R. clarum* oraz *R. dunense* wynosiły odpowiednio 9,2–11,1% i 10,0–11,5%, (*ii*) *R. maiae* i *R. arabicum* oraz *R. custos* – odpowiednio 7,0–9,1% i 12,4–12,8% i (*iii*) *R. silesianum* oraz *R. natalense* – 1,6–2,8%.

Pod względem morfologii, różnice między (*i*) *R. dalpeae* i *R. clarum* oraz *R. dunense* tkwiły w własnościach fenotypowych i histochemicznych warstw 1–3 ściany zarodnika, (*ii*) *R. maiae* i *R. arabicum* oraz *R. custos* dotyczyły liczby warstw ściany zarodnika, własności fenotypowych warstwy 4. tej ściany i cech histochemicznych warstw 1., 3. oraz 4. ściany zarodnika i (*iii*) *R. silesianum* oraz *R. natalense* występowały w fenotypach warstw 3. i 4. ściany zarodnika oraz własnościach histochemicznych warstwy 1. tej ściany.

*Rhizoglomus dalpeae* pochodziło z Beninu, Zachodnia Afryka, gdzie było związane z korzeniami trzech gatunków traw rosnących na inselbergu (odosobnione skaliste wzgórze) położonym na obszarze, gdzie temperatura osiągała 40–60ºC. *Rhizoglomus maiae* oryginalnie znaleziono w tropikalnym rezerwacie leśnym w stanie Rio Grande do Norte, Brazylia, z średnimi rocznymi opadami wynoszącymi 1700 mm. *Rhizoglomus silesianum* w polu współżyło z korzeniami *Daucus carota*, które rosło na hałdzie Kostuchna, niedaleko Katowic, gdzie średni roczny opad i średnia roczna temperatura wynosiły odpowiednio *ca*. 580 mm oraz 7,6ºC. Przeszukiwania publicznie dostępnych zbiorów sekwencji, używając narzędzia BLAST, wykazały, że żaden z tych trzech nowych gatunków nie został wcześniej zidentyfikowany w molekularnych badaniach środowiskowych.

**P5: *Sieverdingia* gen. nov., *S. tortuosa* comb. nov., and *Diversispora peloponnesiaca* sp. nov. in the Diversisporaceae (Glomeromycota)**

*Diversispora peloponnesiaca* sp. nov. została scharakteryzowana z glomoidalnych zarodników wyekstrahowanych z kultur jednogatunkowych, wcześniej wyhodowanych w kulturze pułapkowej; morfologia tych zarodników podpowiadała, że reprezentują one nieopisany gatunek (Ryc. 9H).

Analizy BI i ML sekwencji SSU-ITS-LSU potwierdziły tę podpowiedź i ujawniły, że ten nowy gatunek jest siostrą *Diversispora clara* (Ryc. 8). Odchylenie sekwencji, tego gatunku, opisanego pod nazwą *D. peloponnesiaca*, od *D. clara* było duże i wahało się od 4,3% do 5,2%.

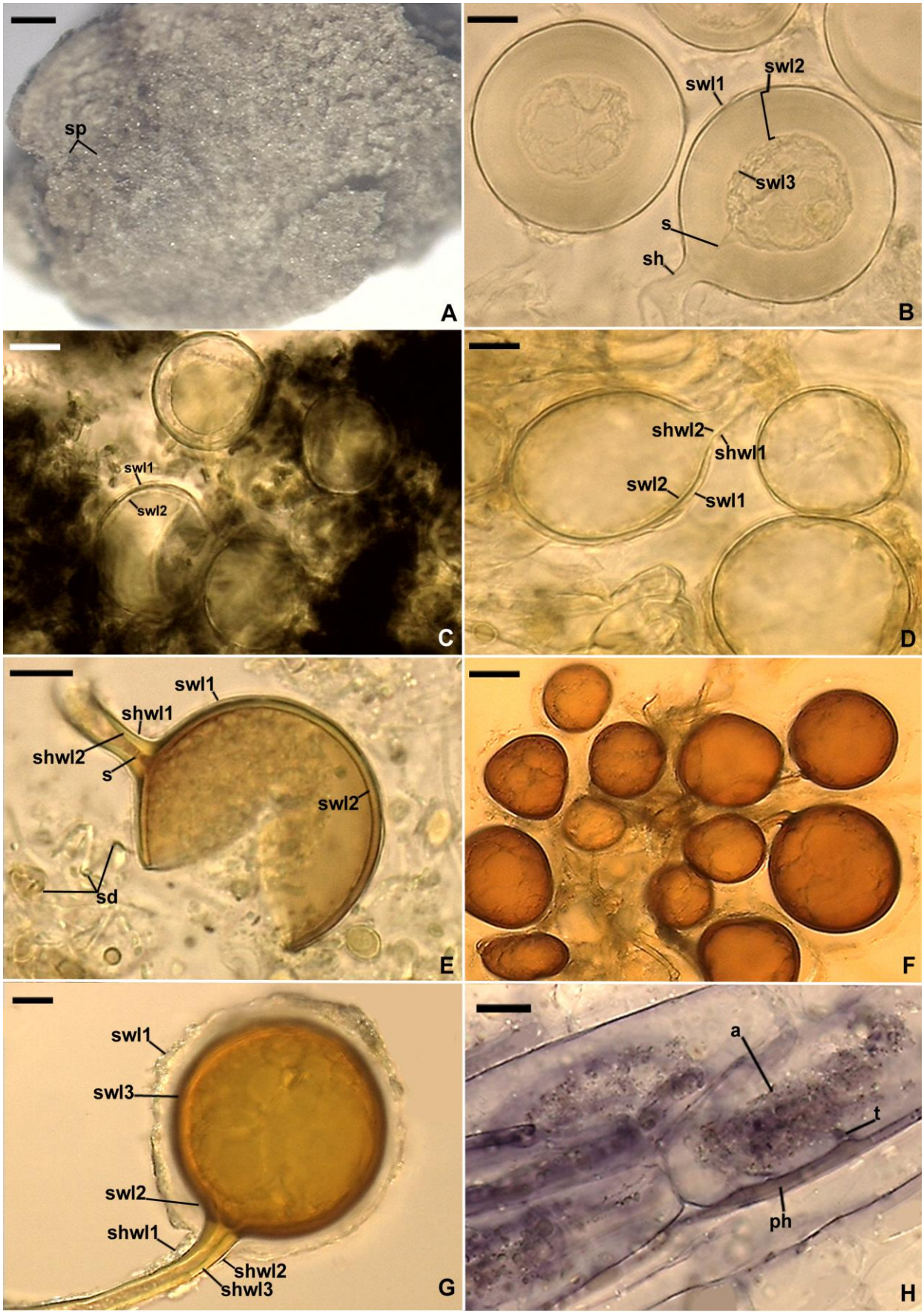
Morfologicznie te dwa gatunki różniły się zasadniczo, gdyż, przede wszystkim, zarodniki *D. peloponnesiaca* były zabarwione (Ryc. 9H), podczas gdy *D. clara* tworzy wyłącznie bezbarwne zarodniki.

Kultura pułapkowa wspomniana wyżej, w której pierwotnie znaleziono *D. peloponnesiaca*, została zainokulowana glebą ryzosferową *Ammophila arenaria*, która kolonizowała wydmy nadmorskie Peloponezu, Grecja. Nazwę tego półwyspu wykorzystano do utworzenia epitetu tego gatunku.

**P6: New glomeromycotan taxa, *Dominikia glomerocarpica* sp. nov. and *Epigeocarpum crypticum* gen. nov. et sp. nov. from Brazil, and *Silvaspora* gen. nov. from New Caledonia**

Po rozpoznawczych badaniach zarodników sporokarpicznego grzyba, wstępnie nazwanego „Gatunek 1", szczegółowe analizy morfologiczne i filogenetyczne potwierdziły przypuszczenie, że grzyb ten jest nieopisanym gatunkiem. Analizy BI i ML sekwencji SSU-ITS-LSU i *RPB1* wykazały jego w pełni lub silnie wsparte siostrzane pokrewieństwo z *Dominikia aurea* w rodzinie Glomeraceae (Ryc. 6). Odległości molekularne między tymi grzybami, wyliczone na podstawie porównań sekwencji 18S-ITS-28S i *RPB1*, wynosiły odpowiednio 8,5–10,2% i 4,3–4,8%. W konsekwencji grzyb ten został opisany jako *Dominikia glomerocarpica*.

Ekologicznymi i morfologicznymi cechami diagnostycznymi silnie odróżniającymi *D. glomerocarpica* od *D. aurea* były (*i*) siedlisko przebywania oraz cechy struktury zawierającej skupienia zarodników, (*ii*) kolor zarodników, skład ściany zarodnika i własności fenotypowe




**Ryc. 10. A, B.** *Dominikia glomerocarpica*, **C-E.** *D. bonfanteae*, **F-H.** *Glomus atlanticum* (sp, zarodnik, sh, trzonek, shwl, warstwa ściany trzonka, swl, warstwa ściany zarodnika, a, arbuskula, pg, strzępka).

oraz histochemiczne warstw tej ściany oraz (*iii*) cechy przegrody oddzielającej kanał trzonka zarodnika od wnętrza zarodnika (Ryc. 10A, B).

Sporokarp *D. glomerocarpica* pochodził z chronionego obszaru, Parque das Trilhas, który jest częścią Lasu Atlantyckiego i przynależy do gminy Guaramiranga w Brazylii. Przeszukiwania repozytoriów z sekwencjami, używając BLASTu, nie wykazały stwierdzenia tego gatunku w innych regionach świata.

Uzasadnienie opisania *Epigeocarpum crypticum* gen. nov. et sp. nov. przedstawiono w charakterystyce publikacji o utworzeniu nowego rodzaju, *Epigeocarpum, vide* wyżej P6.

**P7: *Dominikia bonfanteae* and *Glomus atlanticum*, two new species in the Glomeraceae (phylum Glomeromycota) with molecular phylogenies reconstructed from two unlinked loci**

Analizy dwóch grzybów, które w kulturach jednogatunkowych tworzyły glomoidalne zarodniki w skupieniach, dowiodły przypuszczenia, że były to nowe gatunki, które opisano jako *Dominikia bonfanteae* i *Glomus atlanticum* w rodzinie Glomeraceae. W drzewie filogenetycznym z sekwencjami SSU-ITS-LSU+*RPB1* klad z *D. bonfanteae*, w pełni wsparty w analizach BI and ML, został umiejscowiony w siostrzanej pozycji względem *D. difficilevidera* (Ryc. 6; Błaszkowski et al. 2021), a siostrzanym kładem *G. atlanticum*, również w pełni wspartym w obu analizach, było *G. macrocarpum* (Błaszkowski et al. 2021).

W porównaniu do *D. difficilevidera*, *D. bonfanteae* tworzyła zarodniki w skupieniach (vs. tylko pojedynczo u *D. difficilevidera*), zarodniki te miały dwuwarstwową ścianę zarodnika z warstwą 1. czasami barwiącą się w odczynniku Melzera (vs. trzywarstwowa, z żadną nie reagującą w tym odczynniku) i ich otwór trzonka zwykle był otwarty (vs. zamknięty przegrodą; Ryc. 10C–E).

Zarodniki *G. atlanticum* były wyraźnie ciemniej zabarwione niż zarodniki *G. macrocarpum* (szarawo-pomarańczowe do żółtawo-brązowych vs. jasnożółte do żółtych) i ich ściana zarodnika była bardziej złożona (trzywarstwowa vs. dwuwarstwowa; Ryc. 10F, G).

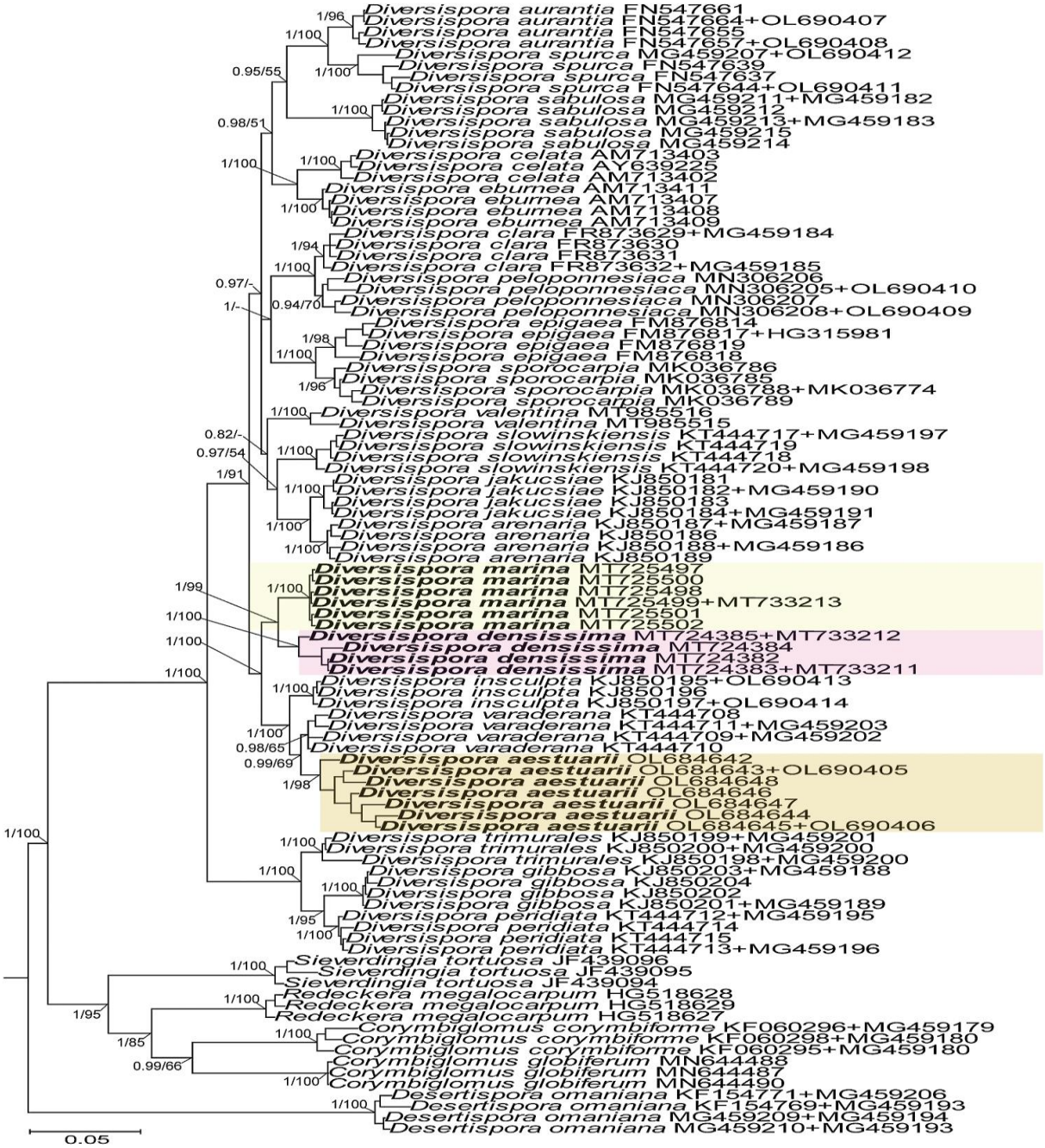
Zarodnikowanie w kulturach pułapkowych świadczyło, że w warunkach naturalnych *D. bonfanteae* współżyła z *Poa trivialis*, która rosła w zbiorowisku roślinnym przy brzegu stawu Kokotek II położonym w Lubieńcu na Górnym Śląsku, a *G. atlanticum* było związane z *Carpobrotus edulis* (Aizoaceae), które kolonizowało wydmy nadmorskie parku wodnego położonego w pobliżu miasta Porto w Portugalii.

Odpowiedzi na pytania zadane bazie BLAST, wykorzystując sekwencje SSU-ITS-LSU, wskazały, że *D. bonfanteae* była wcześniej identyfikowana w molekularnych badaniach środowiskowych przeprowadzonych w Republice Czeskiej, Niemczech, Chinach i Japonii, a *G. atlanticum* nie było notowane w żadnym innym stanowisku świata.

**P9: Three new species of arbuscular mycorrhizal fungi of the genus *Diversispora* from maritime dunes of Poland**

Glomoidalne zarodniki trzech AGM, nieformalnie nazwane „Gatunek 1", „Gatunek 2", „Gatunek 3", które wyhodowano w kulturach jednogatunkowych, pod względem morfologii przypominały zarodniki gatunków z rodzajów *Claroideoglomus* (obecnie *Entrophospora*; Błaszkowski et al. 2022) i *Diversispora*. Analizy porównawcze, wykorzystując narzędzie BLAST, i filogenetyczne ich sekwencji SSU-ITS-LSU i SSU-ITS-LSU+*RPB1* wykazały, że te trzy grzyby przynależą do *Diversispora* i są nieopisanymi gatunkami (Ryc. 11).

Topologie drzew z sekwencji SSU-ITS-LSU i SSU-ITS-LSU+*RPB1* były identyczne i w drzewach tych „Gatunek 1" został siostrą kladu z *D. varaderana*; identyczne pokrewieństwo utworzyły klady z „Gatunkiem 2" i „Gatunkiem 3" (Ryc. 11). Każdy z gatunków tych nowych kładów, które nazwano odpowiednio *D. aestuarii* sp. nov., *D. densissima* sp. nov. i *D. marina* sp. nov., otrzymał pełne lub silne wsparcie w analizach BI i ML, z nieznaczną przewagą wartości wsparć w drzewie SSU-ITS-LSU+*RPB1*. Różnice między sekwencjami SSU-ITS-LSU *D. aestuarii* vs. *D. varaderana* i *D. densissima* vs. *D. marina* wynosiły odpowiednio 3,7% oraz 3,6%.




**Ryc. 11.** Filogram 50% konsensusu reguły większości z analizy bayesowskiej sekwencji 18S-ITS-28S+*rpb1* *Diversispora aestuarii*, *D. densissima* i *D. marina*, 18 innych gatunków *Diversispora* i pięciu gatunków z czterech rodzajów innych niż *Diversispora* w Diversisporaceae jako grupa zewnętrzna. Bayesowskie prawdopodobieństwo a posteriori ≥ 0,50 i wartości ładowania początkowego ML ≥ 50% są podane w pobliżu gałęzi.

Morfologicznie *D. aestuarii* różniła się od *D. varaderana* pod względem liczby warstw w ścianie zarodnika (4-warstwowa vs. 2-warstwowa), własności warstwy 1. ściany zarodnika, tworzącej powierzchnię zarodnika (trwała vs. zwykle złuszczona u dojrzałych zarodników) i wymiaru oraz innych cech trzonka zarodnika (wyraźnie węższy, z cieńszą ścianą i mniejszym otworem u *D. varaderana*), a *D. densissima* od *D. marina* pod względem koloru i wymiaru zarodników (wyraźnie ciemniejsze i większe u *D. densissima*) oraz liczby i własności fenotypowych składowych ściany zarodnika (3-warstwowa vs. 4-warstwowa; trwała warstwa 1. ściany zarodnika vs. rozkładająca się z wiekiem; Ryc. 12).

Jak wynikało z badań kultur pułapkowych, w warunkach naturalnych *D. aestuarii* i *D. marina* żyły w symbiozie z korzeniami *Ammophila arenaria*, która kolonizowała wydmy nadmorskie w pobliżu Świnoujścia, a *D. marina* była związana z *Agrostis stolonifera*, która rosła w 12. zagłębieniu deflacyjnym Słowińskiego Parku Narodowego.

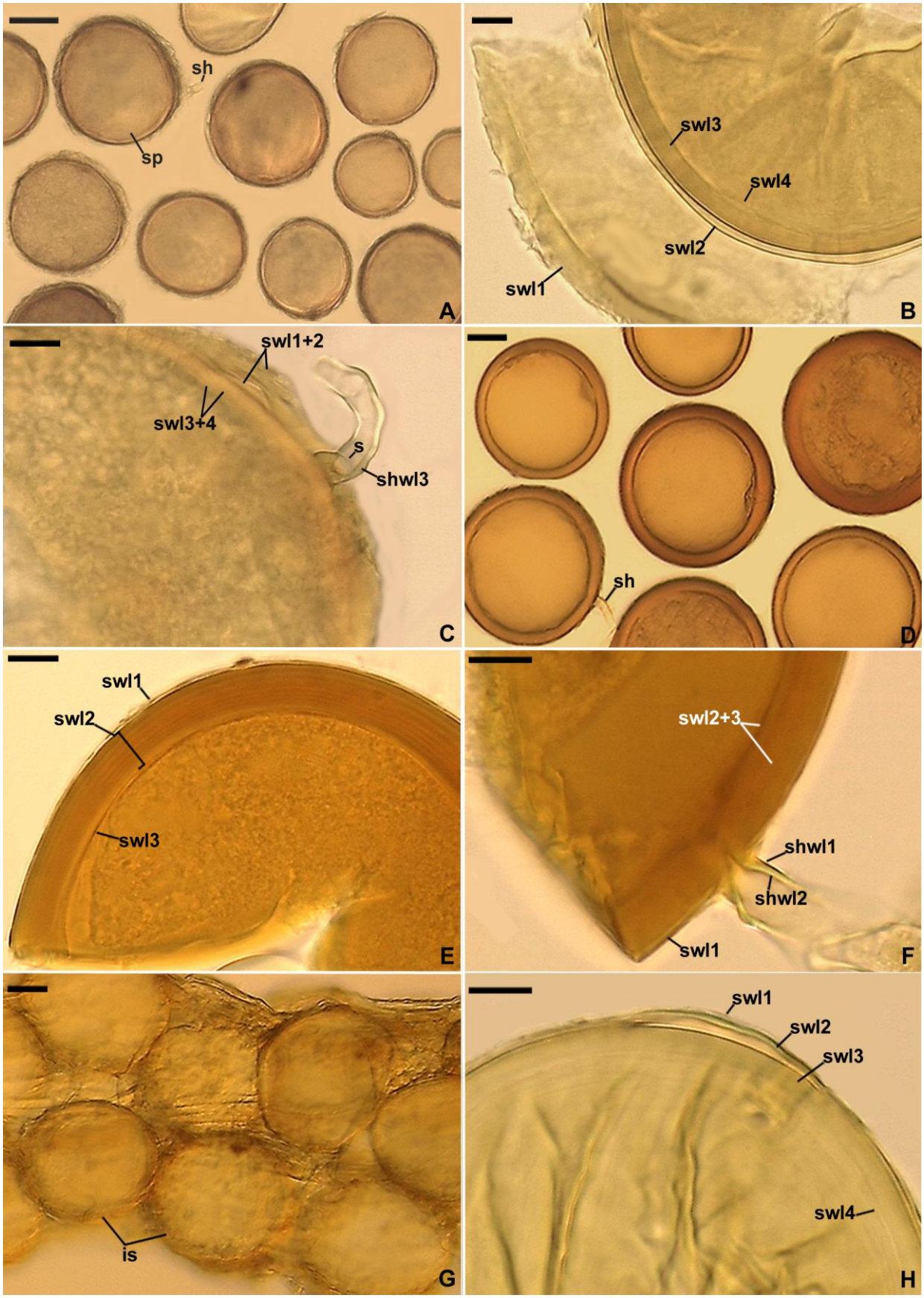
Według porównań sekwencji środowiskowych wykazanych przez BLAST i analiz filogenetycznych tych sekwencji z sekwencjami SSU-ITS-LSU trzech omawianych nowych gatunków, (*i*) *D. densissima* była wcześniej notowana w Chinach, Republice Czeskiej i peruwiańskich Andach, (*ii*) *D. marina* – w rodzimych lasach Azorów, a (*iii*) *D. aestuarii* – w pastwiskach wokół dolin Missoula i Bitterroot zachodniej Montany, USA.

**P10: A new order, Entrophosporales, and three new *Entrophospora* species in Glomeromycota**

Uzasadnienie opisania *Entrophospora argentinensis* sp. nov., *E. glacialis* sp. nov. i *E. furrazolae* sp. nov. przedstawiono w charakterystyce publikacji o utworzeniu nowego rzędu, *Entrophosporales*, *vide* wyżej P10.

Badania kultur pułapkowych wykazały, że w warunkach naturalnych prawdopodobnymi (nie badano obecności tych grzybów w korzeniach) roślinami gospodarzami (*i*) *E. argentinensis* były *Deschampsia flexuosa* i *Poa rigidifolia*, które rosły w stepie położonym w północnym regionie o nazwie Tierra del Fuego, Argentyna, (*ii*) *E. glacialis* – *Festuca vivipara*, *Poa alpina*, *Salix herbacea*, *S. polaris* i *Silene acaulis*, które zasiedlały przedgórze lodowca Isfallglaciarän położonego w dolinie Tarfala w północnej Szwecji i (*iii*) *E. furrazolae* – *Prosopis cineraria*, która rosła w sąsiedztwie plantacji palmy daktylowej (*Phoenix dactylifera*) w stacji doświadczalnej Ministerstwa Rolnictwa, Rybołówstwa i Zasobów Wodnych w Omanie, oraz *Poa trivialis*, która występowała w zbiorowisku roślinnym przy brzegu stawu Kokotek II położonym w Lubieńcu na Górnym Śląsku, gdzie również znaleziono *Dominikia bonfanteae* (*vide* wyżej).

Używając sekwencji SSU-ITS-LSU tych nowych gatunków, przeszukiwania baz danych, wykorzystując narzędzie BLAST, i analizy filogenetyczne wykazanych sekwencji, których podobieństwo sugerowało tożsamość z tymi gatunkami, ujawniły, że (*i*) *E. argentinensis* była notowana w Szwajcarii i USA, (*ii*) *E. glacialis* – w Brazylii, Chinach, Holandii, Japonii, Norwegii, Polsce, Szwajcarii i USA a (iii) *E. furrazolae* – w Chinach, Hiszpanii, Republice Czeskiej i Szwajcarii.




**Ryc. 12. A–C.** *Diversispora aestuarii*, **D–F.** *D. densissima*, **G, H.** *D. marina* (is, zarodnik wewnątrzkorzeniowy, sp, zarodnik, sh, trzonek, shwl, warstwa ściany trzonka, swl, warstwa ściany zarodnika).

## 7. MOŻLIWOŚCI WYKORZYSTANIA ARBUSKULARNYCH GRZYBÓW MYKORYZOWYCH W OCHRONIE ROŚLIN I WYDM PELOPONEZU

Badania występowania i różnorodności zbiorowisk AGM obecnych w wydmach Peloponezu, przeprowadzone na podstawie dużej liczby (240) prób gleby ryzosferowej i korzeni, wyraźnie ujawniły gatunki występujące najczęściej i dominujące w zbiorowiskach zarodników wytworzonych w wazonowych kulturach pułapkowych. Ponadto badania własne ukazały gatunki, które łatwo adaptują się do warunków znacznie różniących się od warunków polowych, a więc takich, które występują w czasie formulacji potencjalnego inokulum, następnie wykorzystywanego do produkcji szczepionek mykoryzowych.

Według Vogelsang et al. (2004) ekotypy gatunków AGM najczęściej obecne w danym stanowisku lub obszarze najlepiej nadają się do tworzenia szczepionek mykoryzowych, gdyż są one zaadoptowane do warunków tych obszarów i przez to zwykle najkorzystniej wpływają na ich rośliny-gospodarzy oraz najbliższe otoczenie (Vogelsang et al. 2004). Jednak biorąc pod uwagę dane literaturowe świadczące, że różne gatunki, a nawet ich szczepy mogą różnić się pod względem (*i*) zwiększania pobierania substancji odżywczych, zwłaszcza tych niezbędnych, przez rośliny wydmowe i przez to wspomagania ich wzrostu oraz kondycji (Koske, Polson 1984) (*ii*) łagodzenia skutów destrukcyjnego wpływu przemieszczającego się piasku, (*iii*) tworzenia glomaliny (Wright, Upadhyaya 1999), kleistej substancji wiążącej ziarna piasku w stabilne agregaty i chroniącej wydmy przed erozją (Koske 1975) i (*iv*) zdolności chronienia roślin wydmowych przed oddziaływaniem patogenicznych grzybów i nicieni (Greipsson, El-Mayas 2002) oraz toksycznych metali ciężkich, soli i innych substancji, szczepionka zalecana do stosowania w wydmach Peloponezu powinna zawierać albo wyłącznie *Septoglomus constrictum,* lub *S. constrictum* wraz z jednym do kilku spośród najczęściej stwierdzanych gatunków AGM na tym obszarze, tj. *Acaulospora kentinensis*, *Archaeospora trappei*, *Diversispora aurantia*, *D. epigaea*, *Dominikia aurea*, *Glomus macrocarpum*, *Innospora majewskii* i *Rhizoglomus irregulare*. Jednak ostateczna decyzja o składzie takiej szczepionki powinna jeszcze uwzględnić wyniki testów efektywności działania tych gatunków, gdy obecne samodzielnie w inokulum i w różnych kombinacjach. Dane literaturowe i informacje nieopublikowane jednoznacznie podkreślają potrzebę kontynowania badań nad szczepionkami mykoryzowymi i ich produkowania z powodów m.in. rosnących kosztów ochrony roślin i konieczność ochrony środowiska naturalnego (Kubiak 2005).

Jedną z metod zalecanych do stosowania szczepionek mykoryzowych z AGM, wyprodukowanych według zasad omówionych wyżej, jest najpierw użycie szczepionki do zainokulowania sadzonek roślin wydmowych hodowanych na rozsadnikach po czym przesadzanie zmykoryzownych sadzonek wraz z ich symbiontami do wybranych stanowisk. Korzyść tej metody wynika przede wszystkim z pewności utworzenia efektywnego związku mykoryzowego z AGM u roślin introdukowanych do nowego stanowiska. W konsekwencji tak zmykoryzowane rośliny będą miały przewagę nad gatunkami rodzimymi tego stanowiska. Metodę tę stosowano z powodzeniem z różnymi gatunkami roślin i AGM, które rosły w warunkach polowych (Bothe et al. 2010; Smith, Read 2008). Zaroślinnianie wydm sadzonkami *Ammophila arenaria* jest powszechnie stosowanym zabiegiem w Polsce.

## 8. WNIOSKI

Badania występowania i różnorodności zbiorowisk zarodników arbuskularnych grzybów mykoryzowych (AGM) w wydmach śródziemnomorskich Peloponezu, które przeprowadzono na podstawie 240 kultur pułapkowych zainokulowanych mieszaninami gleby ryzosferowej i fragmentów korzeni pięciu gatunków roślin rosnących w tych wydmach, pozwalają na sformułowanie następujących wniosków:

1. *Ammophila arenaria*, *Eryngium maritimum*, *Euphorbia paralias*, *Medicago littoralis* i *Medicago marina* wydm Peloponezu powszechnie współżyły z różnorodnymi zbiorowiskami AGM (zarodnikowały w 99,2% kultur), wśród których było 38 gatunków z 20 rodzajów 13 rodzin gromady Glomeromycota, w tym 17 gatunków nowych, z siedmiu nowo opisanych rodzajów i 18 morfotypów, które prawdopodobnie są nieopisanymi taksonami i wymagają dalszych badań.
2. Gatunkami AGM występującymi bardzo często (obecne w ≥60% kultur) i często (30–60%) w wydmach Peloponezu były *Septoglomus constrictum* (80,42%) i *Diversispora epigaea* (53,33%).
3. Gatunkiem AGM wydm Peloponezu wyraźniej najobficiej zarodnikującym w kulturach pułapkowych było *Septoglomus constrictum*. Innymi gatunkami tworzącymi liczne zarodniki były *Acaulospora kentinensis*, *Archaeospora trappei*, *Diversispora aurantia*, *D. epigaea*, *Dominikia aurea*, *Glomus macrocarpum*, *Innospora majewskii*, *Rhizoglomus irregulare* i *Septoglomus africanum*.
4. Do ochrony roślin i wydm Peloponezu należy wykorzystać inokulum zawierające wyłącznie *Septoglomus constrictum* lub *S. constrictum* wraz z jednym do kilku spośród następujących gatunków: *Acaulospora kentinensis*, *Archaeospora trappei*, *Diversispora aurantia*, *D. epigaea*, *Dominikia aurea*, *Glomus macrocarpum*, *Innospora majewskii* i *Rhizoglomus irregulare*.
5. Dotychczasowy stan poznania występowania, rozmieszczenia, znaczenia funkcjonalnego i statusu taksonomicznego AGM z pewnością jest słabo poznany. Na przykład na podstawie analiz zarodników wyekstrahowanych z kultur hodowanych w Pracowni Ochrony Roślin opisano nowy rząd, nową rodzinę, sześć nowych rodzajów, 15 nowych gatunków i utworzono 13 nowych kombinacji nomenklaturowych w Glomeromycota.
6. Dostępne systemy klasyfikowania przedstawicieli Glomeromycota wymagają dalszych weryfikacji, używając m.in. wysokiej jakości okazów do badań morfologicznych i filogenetycznych, z których ostatnie powinny być oparte, jako najbardziej wiarygodne, na danych molekularnych pochodzących z lokusów o najwyższej rozdzielczości taksonomicznej.

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**Tabela 1.** Występowanie i obfitość zarodnikowania AGM w wydmach Peloponezu.

| **Gatunek** | **Częstotliwość występowania (%)*** | **Obfitość zarodnikowania**** |
|---|---|---|
| *Acaulospora kentinensis* | 4,58 | 2,36 |
| *Acaulospora mellea* | 1,25 | 1 |
| *Acaulospora paulinae* | 1,25 | 1 |
| *Ambispora gerdemannii* | 0,42 | 1 |
| *Archaeospora trappei* | 15,42 | 1,59 |
| *Cetraspora armeniaca* | 1,67 | 1 |
| *Cetraspora pellucida* | 0,42 | 1 |
| *Corymbiglomus corymbiforme* | 0,42 | 1 |
| *Diversispora aurantia* | 4,58 | 1,91 |
| *Diversispora epigaea* | 53,33 | 1,8 |
| *Diversispora spurca* | 0,83 | 1 |
| *Dominikia aurea* | 16,67 | 2,7 |
| *Entrophospora infrequens* | 0,42 | 1 |
| *Entrophospora walker* | 0,83 | 2 |
| *Funneliformis coronatus* | 3,54 | 1,53 |
| *Funneliformis mosseae* | 1,67 | 1 |
| *Gigaspora margarita* | 0,42 | 1 |
| *Glomus* 410 | 0,42 | 2 |
| *Glomus* 413 | 0,42 | 4 |
| *Glomus* 415 | 0,42 | 3 |
| *Glomus* 424 | 0,42 | 4 |
| *Glomus* 433 | 2,08 | 3 |
| *Glomus* 435 | 0,42 | 3 |
| *Glomus* 436 | 0,42 | 2 |
| *Glomus* 442 | 0,42 | 2 |
| *Glomus* 446 | 0,83 | 2 |
| *Glomus* 447 | 0,42 | 2 |
| *Glomus* 448 | 0,42 | 2 |
| *Glomus* 449 | 0,42 | 3 |
| *Glomus* 452 | 0,42 | 4 |
| *Glomus* 457 | 0,83 | 2 |
| *Glomus macrocarpum* | 7,50 | 1,56 |
| *Glomus pachycaule* | 0,42 | 2 |
| *Glomus rubiforme* | 2,08 | 1,6 |
| *Innospora majewskii* | 27,50 | 1,52 |
| *Pacispora scintillans* | 5,42 | 1,23 |
| *Paraglomus laccatum* | 0,42 | 1 |

**Tabela 1, cd.**

| | | |
|---|---|---|
| *Pervetustus simplex* | 2,08 | 2,25 |
| *Polonospora polonica* | 2,92 | 1,71 |
| *Racocetra* 178 | 0,42 | 1 |
| *Racocetra* 179 | 0,42 | 1 |
| *Racocetra fulgida* | 0,83 | 1 |
| *Racocetra persica* | 0,42 | 2 |
| *Rhizoglomus fasciculatum* | 2,50 | 2,5 |
| *Rhizoglomus intraradices* | 0,42 | 2 |
| *Rhizoglomus irregulare* | 13,33 | 1,5 |
| *Sacculospora baltica* | 1,25 | 1 |
| *Scutellospora* 431 | 5,00 | 2,92 |
| *Scutellospora* 437 | 0,42 | 2 |
| *Scutellospora calospora* | 4,58 | 1,36 |
| *Scutellospora dipurpurescens* | 3,75 | 2,44 |
| *Septoglomus africanum* | 12,50 | 1,53 |
| *Septoglomus altomontanum* | 0,42 | 3 |
| *Septoglomus constrictum* | 80,42 | 2,22 |
| *Septoglomus xanthium* | 3,33 | 1,88 |

* procent kultur, w których znaleziono zarodniki.

** liczba zarodników w 100 g suchego podłoża wzrostowego, gdzie 1 = <10 zarodników, 2 = 11–50, 3 = 51–100 i 4 = >100.

KOPIE ARTUKUŁÓW STANOWIĄCYCH JEDNOTEMATYCZNY CYKL PUBLIKACJI
I OŚWIADCZENIA WSPÓŁAUTORÓW

**Artykuł nr 1**

| | |
|---|---|
| **Tytuł:** | **A new genus, Oehlia with Oehlia diaphana comb. nov. and an emended description of Rhizoglomus vesiculiferum comb. nov. in the Glomeromycotina** |
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| **Streszczenie:** | Morphological comparisons of spores and phylogenetic analyses of sequences of the SSU–ITS–LSU nrDNA region and the RPB1 gene indicated that an arbuscular mycorrhizal fungus (AMF) originally described as Glomus diaphanum and two Canadian AMF, DAOM745424 and DAOM227022, are conspecific and represent a separate clade at the rank of genus in the subphylum Glomeromycotina. The genus was here erected under the name Oehlia and morphological and molecular descriptions of its type species, O. diaphana comb. nov., were presented. The analyses also proved that the strain originally identified as G. diaphanum MUCL 43196 (DAOM229456) in fact is Rhizoglomus irregulare. In addition, the morphology and molecular phylogeny of an AMF originally named Endogone vesiculifera were revised based on spore clusters grown in culture. The revision indicated that the morphological description of the species is incomplete. It also confirmed that the fungus is a member of the genus Rhizoglomus and for the first time showed that the closest morphological and molecular relative of Rh. vesiculiferum comb. nov. is Rh. irregulare. |
| **Słowa kluczowe:** | arbuscular mycorrhizal fungi, molecular phylogeny, morphology |
| **DOI** | https://doi.org/10.1127/nova_hedwigia/2018/0488 |

**Artykuł nr 2**

| | |
|---|---|
| **Tytuł:** | ***Halonatospora* gen. nov.with *H. pansihalos* comb. nov.and *Glomus bareae* sp. nov. (Glomeromycota;Glomeraceae)** |
| **Autorzy:** | Janusz Błaszkowski,[1] PiotrNiezgoda[1], BrunoT.Goto[2], and Anna Kozłowska[1] |
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| **Streszczenie:** | We established single-species pot cultures of the former *Glomus pansihalos*, a member of the Glomeraceae, and obtained sequences of the SSU–ITS–LSU nrDNA segment and the RPB1 gene of the species. Phylogenetic analyses of the sequences indicated that *G. pansihalos* represents a separate clade at the rank of genus in the Glomeraceae. Consequently, the new genus was named *Halonatospora*, and *G. pansihalos* was renamed ***Halonatospora pansihalos*** comb. nov. We also grew an AMF that produced clusters with glomoid spores in single-species cultures and obtained SSU–ITS–LSU and RPB1 sequences of the fungus. Studies of pot cultures, morphological and histochemical characters of the spores, as well as phylogenetic analyses of the sequences proved that it is an undescribed species of the genus *Glomus* sensu stricto, which is associated with roots of *Ammophila arenaria* colonizing maritime sand dunes located in north-western Poland. |
| **Słowa kluczowe**: | arbuscular mycorrhizal fungi, morphology, molecular phylogeny, RPB1, SSU–ITS–LSU nrDNA |
| **DOI** | https://doi.org/10.1139/cjb-2018-0107 |

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DGfM

ORIGINAL ARTICLE



# New sporocarpic taxa in the phylum Glomeromycota: *Sclerocarpum amazonicum* gen. et sp. nov. in the family Glomeraceae (Glomerales) and *Diversispora sporocarpia* sp. nov. in the Diversisporaceae (Diversisporales)

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## Abstract

Of the nearly 300 species of the phylum Glomeromycota comprising arbuscular mycorrhizal fungi (AMF), only 24 were originally described to form glomoid spores in unorganized sporocarps with a peridium and a gleba, in which the spores are distributed randomly. However, the natural (molecular) phylogeny of most of these species remains unknown. We found unorganized sporocarps of two fungi-producing glomoid spores: one in the Amazonian forest in Brazil (tropical forest) and the second in a forest of Poland (temperate forest). The unique spore morphology of the two fungi suggested that they are undescribed species. Subsequent phylogenetic analyses of sequences of the small subunit–internal transcribed spacer–large subunit nrDNA region and the RPB1 gene confirmed this assumption and placed the Brazilian fungus in a separate clade at the rank of genus, very strongly divergent from its sister clade representing the genus *Glomus* sensu stricto in the family Glomeraceae (order Glomerales). The Polish fungus was accommodated in a sister clade to a clade grouping sequences of *Diversispora epigaea*, a fungus that also occasionally produces spores in sporocarps, belonging in the Diversisporaceae (Diversisporales). Consequently, the Brazilian fungus was here described as the new genus and new species *Sclerocarpum* gen. nov. and *S. amazonicum* sp. nov., respectively. The Polish fungus was described as *D. sporocarpia* sp. nov. In addition, the supposed reasons for the low representation of sporocarpic species in the Glomeromycota were discussed and the known distribution of sporocarp-producing Glomeromycota was outlined.

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**Keywords** Arbuscular mycorrhizal fungi · Glomerocarps · Morphology · Molecular phylogeny · RPB1 · SSU-ITS-LSU nrDNA

## Introduction

Of the nearly 300 species of the phylum Glomeromycota C. Walker & A. Schüßler (Schüßler et al. 2001; Tedersoo et al. 2018; Turrini et al. 2018) comprising arbuscular mycorrhizal fungi (AMF), only 57 were originally described to form spores at the tip of sporogenous hyphae in epigeous or hypogeous sporocarps, i.e., fruit bodies, with or without a peridium and with a gleba. The peridium is a network of hyphae that partially or completely covers the gleba, which is composed of spores embedded in interwoven hyphae (Gerdemann and Trappe 1974; Morton 1988). Sporocarps that have highly ordered spores, usually distributed side-by-side in a single layer around a central hyphal plexus, are called organized sporocarps and within the Glomeromycota are obligatorily produced mainly by species of the genus *Sclerocystis* Berk. & Broome (Morton 1988). Sporocarps with spores randomly distributed in the gleba are called "unorganized sporocarps." However, the formation of unorganized sporocarps is not always a stable character because some species that form such sporocarps, as for example *Glomus macrocarpum* Tul. & C. Tul., also produced spores in more- or less-compact naked clusters (without a glebal hyphae and a peridium) and/or single naked spores (Berch and Fortin 1983). According to Morton (1988), the formation of spores in naked clusters and singly by sporocarpic species probably is associated with undetermined conditions functioning in different geographical locations.

All current Glomeromycota species that produced unorganized sporocarps with glomoid spores, i.e., similar to those of *G. macrocarpum*, were originally described in the genus *Glomus* Tul. & C. Tul. (Gerdemann and Trappe 1974; Redecker et al. 2007), which currently belongs to the family Glomeraceae Piroz. & Dalpé in the order Glomerales J.B. Morton & Benny (Morton and Benny 1990; Tedersoo et al. 2018). However, molecular phylogenetic analyses placed, for example, *G. fulvum* (Berk. & Broome) Trappe & Gerd. and *G. megalocarpum* D. Redecker in a separate clade at the rank of genus next to *Diversispora spurca* (C.M. Pfeiff., C. Walker & Bloss) C. Walker & A. Schüßler (formerly *G. spurcum* C.M. Pfeiff., C. Walker & Bloss), which represents the family Diversisporaceae C. Walker & A. Schüßler in the order Diversisporales C. Walker & A. Schüßler (Walker and Schüßler 2004). Consequently, Schüßler and Walker (2010) erected a new genus, *Redeckera* C. Walker & A. Schüßler, with three species, including *R. fulvum* (Berk. & Broome) C. Walker & A. Schüßler (formerly *G. fulvum*) and *R. megalocarpum* (D. Redecker) C. Walker & A. Schüßler (formerly *G. megalocarpum*). Unfortunately, the molecular phylogenetic position remained unknown for many other sporocarpic species within the Glomeromycota, which Schüßler and Walker (2010) retained in their original genera, as species of uncertain position.

Despite the lack of molecular evidence, Oehl et al. (2011) transferred some of the sporocarpic *Glomus* spp. of *Glomus* sensu lato to other genera of the Glomeromycota. For example, *G. canadense* (Thaxt.) Trappe & Gerd. and *G. versiforme* (P. Karst.) S.M. Berch were accommodated in the genera *Redeckera* and *Diversispora* C. Walker & A. Schüßler (Diversisporaceae), respectively, because the morphology of the subtending hyphal wall and the spore wall at the spore base in these species clearly differed from the type species of the genus *Glomus*, *G. macrocarpum*, (Schüßler and Walker 2010). These transfers were probably correct, but many glomoid spore-producing species of the Glomeromycota do not have a synapomorphy, i.e., a unique morphological character defining a monophyletic group, or have synapomorphies that are invisible under a compound microscope (Błaszkowski et al. 2018a). Therefore, the taxonomic placement of such species within the Glomeromycota is impossible without molecular phylogenetic analyses of sequences of loci with high species resolution. Such well-tested combination of loci include the partial small subunit (SSU), i.e., 18S, rRNA gene; the nuclear internal transcribed spacer 1 (ITS1), 5.8S rRNA gene, and internal transcribed spacer 2 (ITS2) of the rDNA (together called ITS); and the partial large subunit (LSU), i.e., 28S, rRNA gene (together referred to as SSU-ITS-LSU rDNA), as well as the largest subunit of RNA polymerase II (RPB1) gene, which have been recommended as the basis for AM fungal DNA barcoding (Krüger et al. 2009; Stockinger et al. 2014; de Souza et al. 2018).

The fact that fungi-producing glomoid spores in unorganized sporocarps with a peridium constitute a small proportion of the Glomeromycota species and that they are some of the least-known groups of fungi in the phylum with respect to ecophysiology, trophic status, and molecular phylogeny, is mainly the result of the difficulty in collecting these species and growing them in single-species cultures. The collection of sporocarps usually requires the use of specific methods that are much more time-consuming and taxing than the wet-sieving and decanting method (Gerdemann and Nicolson 1963), commonly used to extract hypogeous AMF spores produced singly and in clusters. Because many sporocarpic species are found at or slightly below the soil surface and on plant residues, and their sporocarps are often large enough to be seen with the naked eye, the most frequently used method for collecting these fungi in the field is raking and searching the upper soil layer and buried plant fragments. Such onerous work has certainly discouraged to continue exploration of sporocarpic Glomeromycota. The effective collection of this

group of fungi is also depended on the development of a so-called hypogeous instinct (Gerdemann and Trappe 1974).

Of the described species producing glomoid spores in unorganized sporocarps with a peridium, probably only *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler (formerly *G. mosseae* T.H. Nicolson & Gerd.), *Glomus arborense* McGee, *G. macrocarpum*, *G. pallidum* I.R. Hall, *G. tenerum* P.A. Tandy, and *G. warcupii* McGee were grown in single-species cultures and proven to belong in AMF (Gerdemann and Trappe 1974; Hall 1977; McGee 1986; Schüßler and Walker 2010; http://fungi.invam.wvu.edu/). Attempts at growing other sporocarpic species in culture, for example *R. megalocarpum*, failed, and, therefore, their mycorrhizal status remains unknown (McGee and Trappe 2002; Redecker et al. 2007). For this reason, it is believed that some of the sporocarpic Glomeromycota may be facultative AMF or even saprotrophs (Goto et al. 2016), which requires further testing.

The failure in obtaining these fungi in culture has significantly limited or prevented the understanding of their morphology, molecular properties, functional diversity, and distribution. Field-collected sporocarps are often incomplete, with partially or completely decomposed external and internal structures, and their spores are often parasitized or empty, and, when DNA is present, it is often degraded. This makes obtaining sequence data from these species for phylogenetic analyses, environmental ecological surveys, and online databases relying on rDNA markers difficult or impossible (pers. observ.).

We found many fungi-producing glomoid spores in unorganized sporocarps and successfully obtained sequence data from them. Detailed morphological and molecular phylogenetic analyses revealed that one of the fungi, collected in Brazil, is an undescribed species of a new genus in the family Glomeraceae, and another fungus, found in Poland, is a new species of the genus *Diversispora* in the Diversisporaceae. Both fungi are described here.

## Materials and methods

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

Sporocarps of the Brazilian fungus were collected by K. Jobim at two sites of the Adolpho Ducke Forest Reserve (ADFR) in November 2017. The area of the ADFR is ca. 100 km$^2$ and it is located northeast of the city of Manaus (02° 55′ S, 59° 59′ W; Fig. 5). The vegetation of the terra firme forests of the ADFR, which is in the neotropical Campinarana ecoregion, is mainly composed of plant species in the families Arecaceae Bercht. & J.Presl, Burseraceae Kunth, Fabaceae Lindl., Lecythidaceae A. Rich., Malpiguiaceae Juss., and Polygonaceae Juss. (Ribero et al. 1999). Its soils are typically acidic and very poor in nutrients, and are predominantly clayey in the higher sites, becoming sandy as the elevation decreases, as characteristic of podzolic and hydromorphic soils (Chauvel 1982). According to Koppen's classifications (Peel et al. 2007), the region has a tropical humid climate (type Afi), with an average annual temperature of 26 °C and an annual average rainfall between 1500 and 2500 mm (Alencar et al. 1979; Ribeiro and Adis 1984). Plant species present at the collection sites included, among others, *Ecclinusa guianensis* Eyma, *Eschweilera atropetiolata* S.A. Mori, *Es. coriaceae* (DC.) S. A. Mori, *Es. pseudodecolorans* S.A. Mori, *Es. truncata* A.C.Sm., *Es. wachenheimii* (Benoist) Sandwith, *Oenocarpus bacaba* Mart., *Pouteria anomala* (Pires) T.D. Penn., *Protium hebetatum* D. C. Daly, *Pr. paniculatum* Engl. and *Scleronema micranthum* Ducke.

A sporocarp of the Polish fungus was found by Piotr Chachuła in the Pieniny National Park located in southern Poland, on October 10, 2017. The habitat at the collection site (49° 24′ 56.6′ ′N, 20° 19′ 56.5″ E; 552 m above sea level) was a pine and spruce forest with hazel that had typical soil classified as appropriate typical rendzina (Pancer-Koteja et al. 2004; Zaleski et al. 2016). The dominant herbaceous plant species was *Maianthemum bifolium* (L.) F. W. Schmidt. Average annual rainfall and temperature in the region where the Pieniny National Park is located are 700–900 mm and 6–8 °C, respectively.

Attempts at establishing and growing single-species cultures were performed using sporocarp fragments, (each containing ca. 20–50 spores) as described by Błaszkowski et al. (2012), using *Plantago lanceolata* L. as the host plant. Because all attempts at establishing single-species cultures failed, morphological and molecular analyses were performed on spores that were extracted from field-collected sporocarps, using a preparation needle under a stereomicroscope.

### Microscopy and nomenclature

At least 50–100 spores of each species were 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*) and examined to determine the morphological features and the phenotypic and histochemical characters of their spore wall layers. The preparation of spores for study and photography were as those described previously (Błaszkowski 2012; Błaszkowski et al. 2012). Types of spore wall layers were as defined by Błaszkowski (2012), and Walker (1983). Color 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),

except for those referring to the genus represented by a species originally described as *Glomus intraradices* N.C. Schenck & G.S. Sm. We accepted the arguments presented by Sieverding et al. (2014) and used the generic name *Rhizoglomus* Sieverd., G.A. Silva & Oehl instead of *Rhizophagus* P.A. Dang. for the clade with *Rh. intraradices* (N.C. Schenck & G.S. Sm.) Sieverd., G.A. Silva & Oehl.

Goto and Maia (2006) coined the term "glomerospores" to refer to the spores produced by members of the Glomeromycota because of their unique ontogeny and morphology compared to spores of other phyla of the Kingdom Fungi. In addition, the term unambiguously indicates the taxonomic affiliation of these spores, as do, for example, the ascospores (sexual spores) and ascocarps (fruiting bodies) of the phylum Ascomycota. Consequently, we propose to use the term "glomerocarps" (singular = glomercarp) for conglomerations of glomerospores, previously referred to as sporocarps. We use the terms "glomerocarp(s)" and "glomerospore(s)" only at the beginning of some sections of the paper. In other parts of the text, to avoid unnecessarily increasing their usage, we use the terms "sporocarps," "sporocarpic," and "spore(s)".

Voucher specimens of the new species consisting of dried fragments of glomerocarps in vials and slides with spores mounted in PVLG and a mixture of PVLG and Melzer's reagent (1:1, *v*/*v*) were deposited at the Herbarium of the Federal University of Rio Grande do Norte (UFRN Herbarium; holotype of *Sclerocarpum amazonicum*), Herbarium of the Instituto Nacional de Pesquisas da Amazônia (INPA Herbarium), Herbarium of the Mycology Department of the Federal University of Pernambuco (URM; isotypes of *S. amazonicum*), ETH in Zurich, Switzerland (Z+ZT; holotype of *Diversispora sporocarpia*), and the Department of Ecology, Protection and Shaping of Environment (DEPSE), at West Pomeranian University of Technology, Szczecin (isotypes of both fungi).

## Molecular phylogeny, DNA extraction, polymerase chain reaction, cloning, and DNA sequencing

Crude DNA was extracted from eight single spores of each fungus. Details of the treatment of the spores prior to polymerase chain reactions (PCRs), the conditions, and primers used in the PCRs to obtain SSU-ITS-LSU sequences were as those described in Błaszkowski et al. (2015a, 2015b), Krüger et al. (2009), and Symanczik et al. (2014).

In order to obtain RPB1 sequences of the two fungi, nested PCRs were performed in conditions recommended by and with primers designed by Stockinger et al. (2014). The first PCR with DNA of the Brazilian fungus was performed with the primers HS375mix and RPB1-DR1210r, and the second with DR160fmix and RPB1-DR1210r. The first PCR with DNA of the Polish fungus was performed with the primers DR160fmix and HS2680GPr, and the second with HS189GPf and RPB1-DR1210r. For both fungi, in the second nested PCR, 0.5 μl of product from the first amplification was used as template. Cloning and sequencing of PCR products to obtain both types of sequences were performed as described by Błaszkowski et al. (2015a). The sequences were deposited in GenBank (MK036773–MK036789).

## Sequence alignment and phylogenetic analyses

Preliminary phylogenetic analyses of sequences of the SSU-ITS-LSU nrDNA region indicated that the Brazilian fungus belonged in the family Glomeraceae, but it was strongly divergent from other representatives of the family. The Polish fungus was closely related to, but distinct from *Diversispora epigaea* (B.A. Daniels & Trappe) C. Walker & A. Schüßler of the family Diversisporaceae. Consequently, two sequence sets each were assembled, separately for each fungus. One set comprised SSU-ITS-LSU sequences and the second consisted of sequences of the RPB1 gene. Identity values of sequences of the Brazilian fungus only and of the Polish fungus only, as well as the magnitude of sequence divergence of the two fungi from their closest relatives were calculated in BioEdit (Hall 1999). All comparisons were performed on sequences of the same length.

The SSU-ITS-LSU alignment for the Brazilian fungus included sequences of species representing all recognized genera of the Glomeraceae (Redecker et al. 2013; Błaszkowski et al. 2018a, 2018b). The outgroup taxon was *Claroideoglomus claroideum* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler of the family Entrophosporaceae Oehl & Sieverd. emend. Oehl, Sieverd. Palez. & G.A. Silva. The set did not contain any sequence of the monospecific genera *Simiglomus* Sieverd., G.A. Silva & Oehl and *Viscospora* Sieverd., G.A. Silva & Oehl, both erected by Oehl et al. (2011), because (i) their taxonomic status is uncertain; (ii) there is no SSU-ITS-LSU sequence of *S. hoi* (S.M. Berch & Trappe) G.A. Silva, Oehl & Sieverd. (formerly *Glomus hoi* S.M. Berch & Trappe) available in public databases; and (iii) both *S. hoi* and *V. viscosa* (T.H. Nicolson) Sieverd., Oehl & G.A. Silva (originally described as *G. viscosum* T.H. Nicolson) differ fundamentally in morphology from the Brazilian fungus (Berch and Trappe 1985; Walker et al. 1995; Redecker et al. 2013; https://invam.wvu.edu/home). The alignment included 112 sequences that represented 36 species of known natural phylogeny, including *G. bareae* Błaszk. et al., *Halonatospora pansihalos* (S.M. Berch & Koske) Błaszk. et al. (Błaszkowski et al. 2018b), *C. claroideum*, and the undescribed Brazilian fungus (Fig. 1). Excluding the Brazilian fungus and *C. claroideum*, previous molecular phylogenetic analyses placed the other species in eight genera of the Glomeraceae (Redecker et al. 2013; Błaszkowski et al. 2018a, 2018b). Except for *G.* cf. *aggregatum* N.C. Schenck & G.S. Sm. and *Sclerocystis*

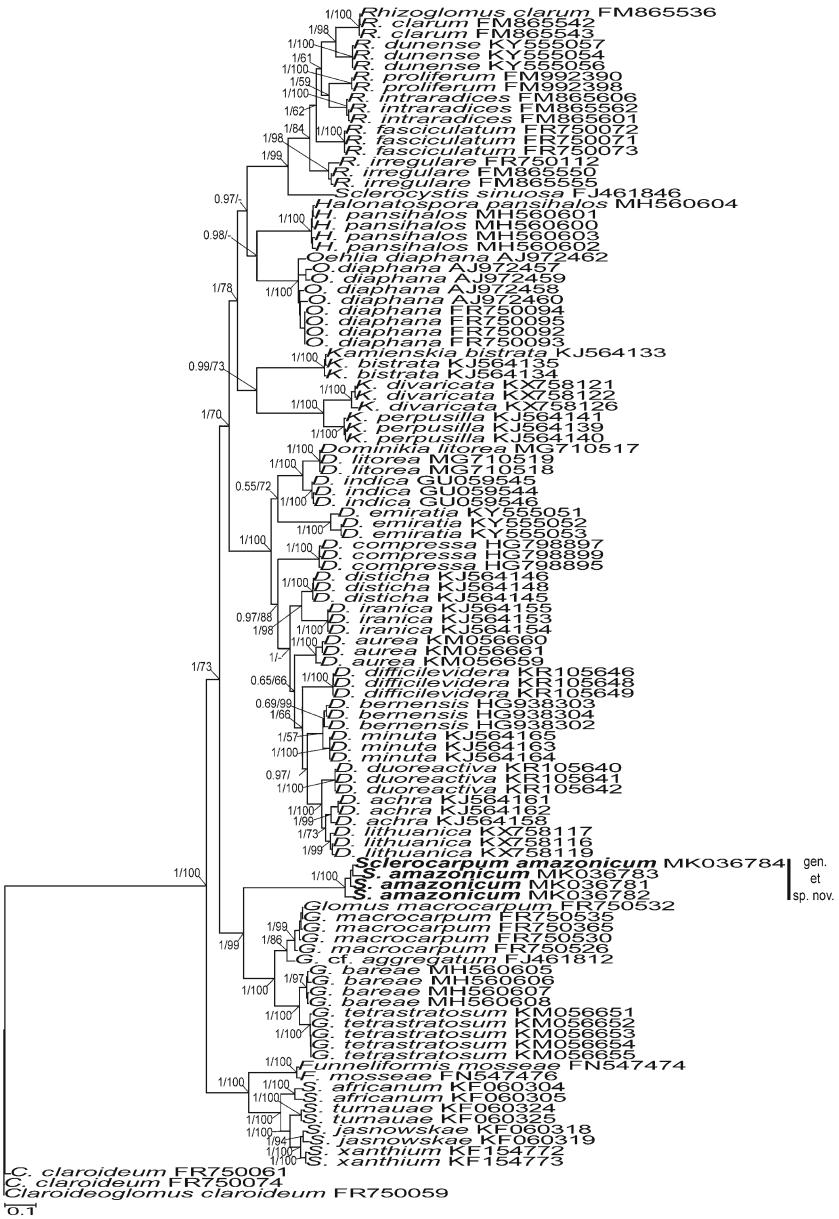



**Fig. 1** A 50% majority rule consensus phylogram produced from a Bayesian Inference analysis of SSU-ITS-LSU nrDNA sequences of *Sclerocarpum amazonicum* and 33 other species of AMF in the Glomeraceae, with *Claroideoglomus claroideum* (Entrophosporaceae) as outgroup. Bayesian Inference posterior probability values ≥ 0.50 and ML bootstrap values ≥ 50% are shown near the branches, respectively. Bar indicates 0.1 expected changes per site per branch

*sinuosa* Gerd. & B.K. Bakshi, which were both represented by single LSU sequences, the other species were each characterized by 2–9 SSU-ITS-LSU sequences covering the whole nrDNA segment amplified by the primers of Krüger et al. (2009).

The RPB1 alignment for the Brazilian fungus consisted of RPB1 sequences from species in all genera represented in the SSU-ITS-LSU set, including the genus *Claroideoglomus* as outgroup (Figs. 1, 2). The alignment comprised 85 sequences, which characterized 32 species of the Glomeraceae, the undescribed Brazilian fungus, and two *Claroideoglomus* spp. (Fig. 2). In contrast to the SSU-ITS-LSU alignment, the RPB1 alignment did not contain several species for which RPB1 sequences were unavailable, including *Dominikia bernensis* Oehl et al., *Do. compressa* (Sieverd. et al.) Oehl et al., *D. minuta* (Błaszk., Tadych & Madej) Błaszk., Chwat & Kovács, *G.* cf. *aggregatum*, *Rhizoglomus fasciculatum* (Thaxt.) Sieverd., G.A. Silva & Oehl, and *Septoglomus jasnowskae* Błaszk., Chwat & Ryszka. Except for *Sc. sinuosa*, *Se. deserticola* (Trappe, Bloss & J.A. Menge) G.A. Silva, Oehl & Sieverd., *Se. viscosum* (T.H. Nicolson) C. Walker et al., *Rh. intraradices*, and the Brazilian fungus, which were each represented by a single RPB1 sequence, each AMF species was characterized by 2–5 sequences.

The SSU-ITS-LSU alignment for the Polish fungus, which was putatively placed in the family Diversisporaceae based on preliminary analyses, consisted of 86 sequences of 19 species (including the Polish species) in four genera of the Diversisporaceae (Fig. 2). Because the preliminary analyses placed the Polish fungus in the genus *Diversispora* and

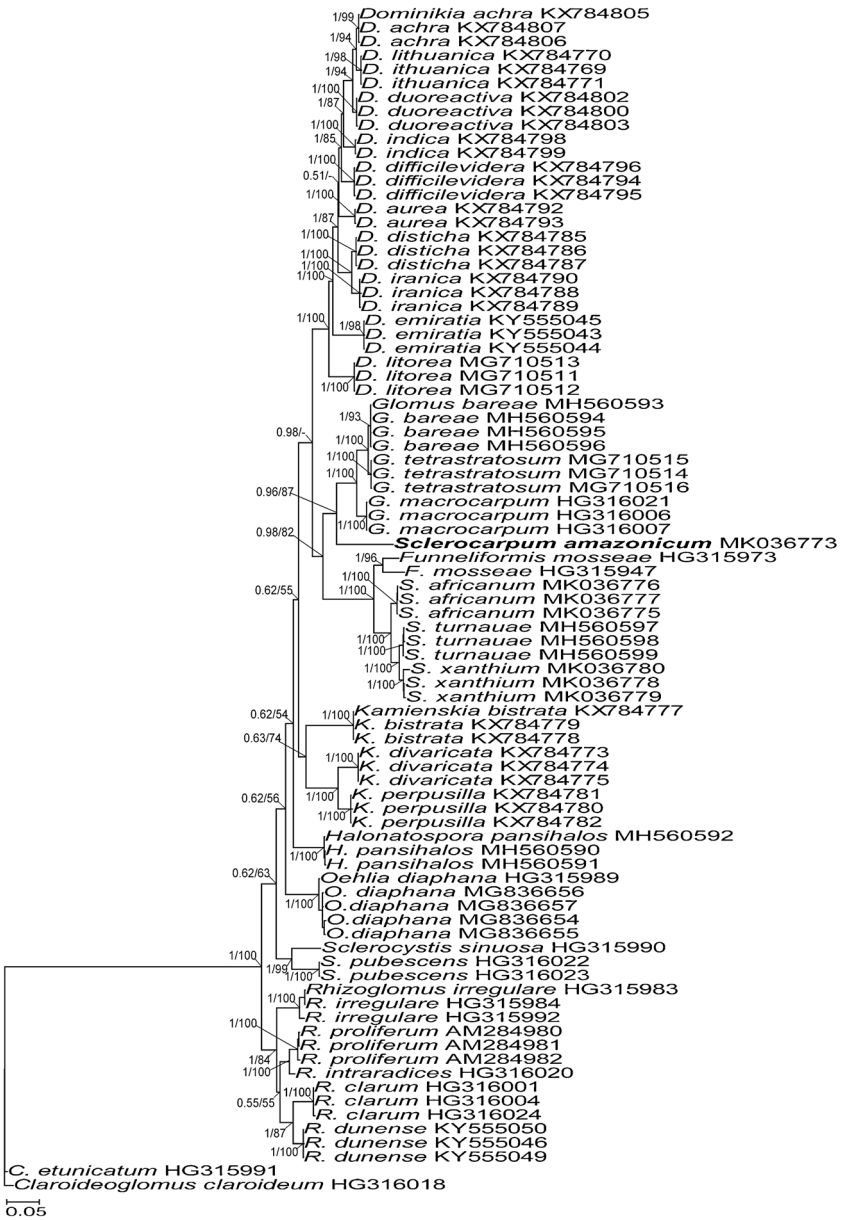



**Fig. 2** A 50% majority rule consensus phylogram produced from a Bayesian Inference analysis of RPB1 sequences of *Sclerocarpum amazonicum* and 30 other species of AMF in the Glomeraceae, with two *Claroideoglomus* spp. (Entrophosporaceae) as outgroup. Bayesian Inference posterior probability values ≥ 0.50 and ML bootstrap values ≥ 50% are shown near the branches, respectively. Bar indicates 0.05 expected changes per site per branch

indicated that the fungus is most closely related to *D. epigaea*, the SSU-ITS-LSU alignment included all available sequences of *D. epigaea* (24), five sequences of the Polish fungus, and 2–3 sequences each of the 14 other *Diversispora* spp. of known natural phylogeny (Symanczik et al. 2018). The outgroup was two sequences of *Pacispora scintillans* (S.L. Rose & Trappe) Sieverd. & Oehl ex C. Walker, Vestberg & A. Schüßler of the family Pacisporaceae C. Walker et al. Except for the AM418552 SSU-ITS sequence of *Redeckera megalocarpa* (D. Redecker) C. Walker & A. Schüßler, the other sequences of the analyzed fungi comprised the entire SSU-ITS-LSU nrDNA region.

The RPB1 alignment for the Polish fungus included 33 sequences of 14 species, including the Polish species, representing four genera of the Diversisporaceae, as well as *Sacculospora baltica* (Błaszk., Madej & Tadych) Oehl et al. of the family Sacculosporaceae Oehl et al., which served as an outgroup (Fig. 4). Except for *D. epigaea* and the Polish species, which each had a single RPB1 sequence available in public databases, all other species were represented by two to four RPB1 sequences.

All four sequence alignments were separately aligned with MAFFT v. 7 using the auto option (http://mafft.cbrc.jp/alignment/server/). For the SSU-ITS-LSU alignment, indels were coded by 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, as described and justified in Błaszkowski et al. (2014). The RPB1 set comprised only sequences of the RPB1 gene. Bayesian inference (BI) phylogenetic analyses

of the SSU-ITS-LSU and RPB1 alignments were conducted with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). In both analyses, the GTR nucleotide substitution model was used, as selected by jModelTest (Posada 2008), using the Akaike information criterion. Four Markov chains were run for 5,000,000 generations, sampling every 100 steps, with a burn-in at 7500 sampled trees. Maximum likelihood (ML) phylogenetic analyses of the alignments were carried out with the raxmlGUI (Silvestro and Michalak 2012) implementation of RAxML (Stamatakis 2014) using the GTRGAMMA algorithm. Then, 1000-replicate rapid bootstrap analysis was performed to determine the support of branches. In both BI and ML analyses of the SSU-ITS-LSU sequences, the analyzed set (the nucleotide alignment plus the binary (indel) character set) was divided into four partitions, knowing that analyses of partitioned data generally increase the accuracy of phylogenetic reconstruction (Lanfear et al. 2012; Nagy et al. 2012). Generated phylogenetic trees were visualized and edited in MEGA6 (Tamura et al. 2013).

## Results

### General data and phylogeny

The molecular phylogeny for each of the two sporocarpic fungi discussed here was reconstructed on the basis of analyses of two sequence sets, one with sequences of the SSU-ITS-LSU nrDNA region and the second with sequences of the RPB1 gene (Figs. 1–4). The SSU-ITS-LSU alignment of the Glomeraceae for the Brazilian fungus comprised 2348 characters, of which 972 and 892 were variable and parsimony-informative, respectively, as analyzes of the alignment with MEGA6 (Tamura et al. 2013) indicated. The identity values of the four SSU-ITS-LSU sequences of the new fungus ranged from 96.0 to 98.5%. The RPB1 alignment for the Brazilian fungus comprised 2899 characters, of which 1377 and 1288 were variable and parsimony-informative, respectively.

The second SSU-ITS-LSU alignment of the Diversisporaceae for the Polish fungus comprised 1959 characters, of which 710 and 627 were variable and parsimony-informative, respectively. The identity values of the five SSU-ITS-LSU sequences of the fungus ranged from 97.2 to 98.9%. The RPB1 alignment for the Polish fungus comprised 811 characters, of which 223 and 212 were variable and parsimony-informative, respectively.

Bayesian and ML analyses of both the SSU-ITS-LSU and RPB1 alignments placed the Brazilian fungus in a separate clade at the rank of genus that was sister to a clade representing the genus *Glomus* sensu stricto (Figs. 1, 2). The new generic clade had full support in all analyses (BI = 1, ML = 100%). The common node of the new generic clade and *Glomus* and the crown node of *Glomus* itself were also fully or strongly supported.

The identity values of SSU-ITS-LSU sequences of the Brazilian fungus with respect to those of its closest relatives, i.e., *G. macrocarpum*, *G.* cf. *aggregatum*, *G. bareae*, and *G. tetrastratosum* (Fig. 1), were 77.1–77.9%, 81.8–82.3%, 76.4–77.4%, and 76.8–77.6%, respectively. The identity values of the RPB1 sequence of the same fungus versus those of *G. macrocarpum*, *G. bareae*, and *G. tetrastratosum* were 86.6%, 85.0%, and 85.2–85.3%, respectively. The molecular identity between the Brazilian fungus and *G.* cf. *aggregatum* was not calculated because there is no RPB1 sequence for *G.* cf. *aggregatum* in public databases.

Bayesian and ML analyses of both the SSU-ITS-LSU and RPB1 alignments for the Polish fungus placed it as a new species, most closely related to *D. epigaea* (Figs. 3, 4), thus confirming results of the preliminary analyses mentioned above. In all analyses of both alignments, the clade comprising sequences of the Polish fungus and the common node for that clade and *D. epigaea* had full or strong support. The identity values of SSU-ITS-LSU and RPB1 sequences of the undescribed fungus compared with SSU-ITS-LSU and RPB1 sequences of *D. epigaea* were 90.1–96.3% and 98.5%, respectively.

Considering the unambiguously convincing results of the molecular phylogenetic analyses and the comparisons of sequence identities discussed above, we conclude that the Brazilian fungus is a new species representing a new genus in the family Glomeraceae of the Glomeromycota, and the Polish fungus is a new species in the Diversisporaceae. The fungi are described below as *Sclerocarpum amazonicum* gen. et sp. nov. and *D. sporocarpia* sp. nov.

### Taxonomy

#### Erection of a new genus

***Sclerocarpum*** B.T. Goto, Błaszk., Niezgoda, Kozłowska & Jobim, gen. nov.

**MycoBank No. MB** 828316.

***Etymology*** Latin, *Sclerocarpum*, *Sclero* (= hard) and *carpum* (= fruitbody), referring to the very hard glomerocarps (= sporocarps) formed by the type species of the new genus.

*Type species:* ***Sclerocarpum amazonicum*** Jobim, Błaszk., Niezgoda, Kozłowska & B.T. Goto.

***Diagnosis*** Differs from other genera in the Glomeraceae in producing pale-colored, scleroid glomerocarps with hyaline, glomoid spores that have a relatively thick, compared to the small spores, structural laminate spore wall layer, and in having the specific sequences of the nrDNA SSU gene: GGTCTTTGGTTGGTGAGAAG, regions of ITS1: AATGAAATTACGATCATTTA, ITS2: AAAAGATC GATTTTGTCGCCTTTC, AGCTCATCTTTTGAACCTTT

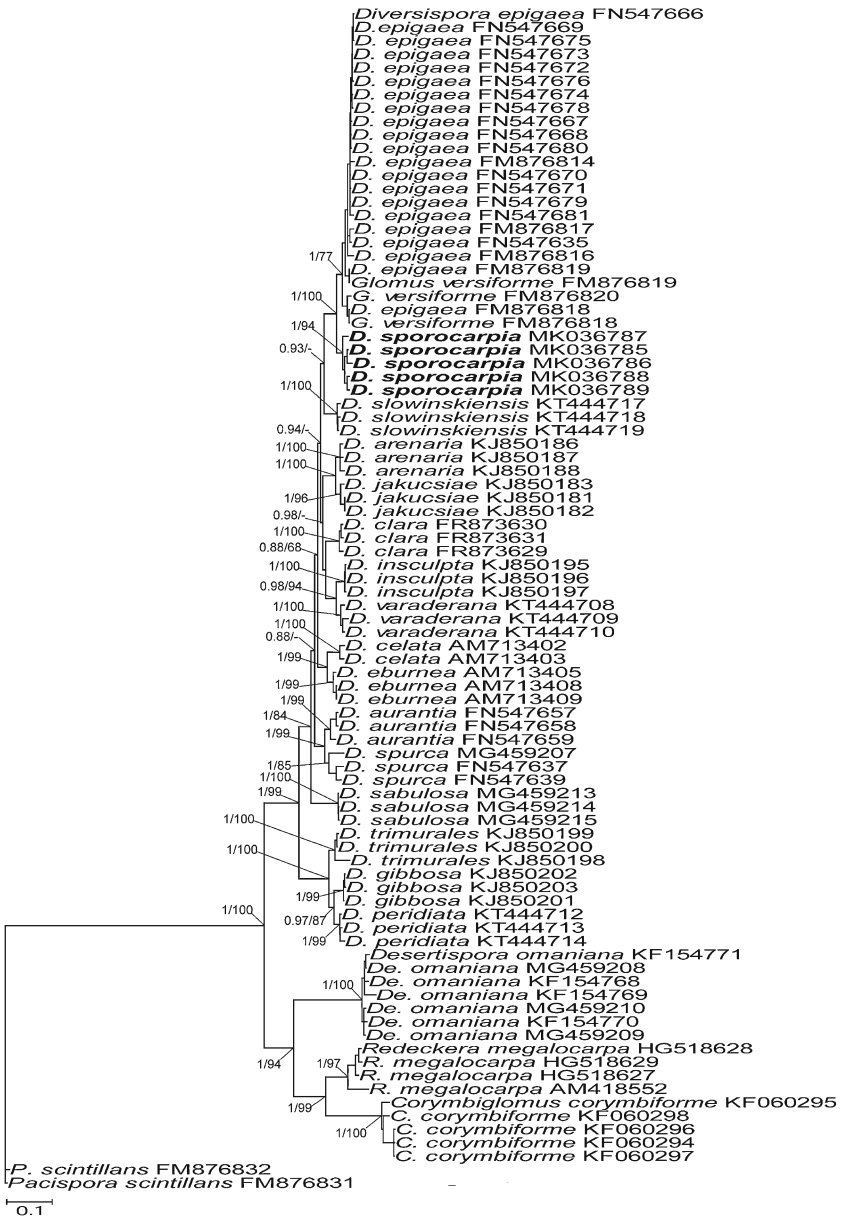



**Fig. 3** A 50% majority rule consensus phylogram produced from a Bayesian Inference analysis of SSU-ITS-LSU nrDNA sequences of *Diversispora sporocarpia* and 18 other species of four AMF genera in the family Diversisporaceae, with *Pacispora scintillans* (Pacisporaceae) as outgroup. Three sequences of *D. epigaea* are associated with the name *Glomus versiforme* according to the nomenclature used in GenBank. Bayesian Inference posterior probability values ≥ 0.50 and ML bootstrap values ≥ 50% are shown near the branches, respectively. Bar indicates 0.1 expected changes per site per branch

C, and LSU gene: TAGCGATACTCGGGTTCTTT GGGCGTACTTTCTCGCT, GCCGAAGTGTTATA GCCTC, CGTAACGGACGGGATC, as well as the RPB1 gene: TTTCATAAGTCATGTAAGCGTTCATC, TAAGCTTACGGAACTTTCTTGATATAG, ACAACCAC AAGCATTG, GGTACATCCCGGAGCC, and CAGGGATACTGGAGAG.

Specimens examined*:* See holotype and isotype specimens of *S. amazonicum* characterized below.

Genus description: Producing spores in scleroid (very hard to break), epigeous and sub-hypogeous, light- to dark-colored unorganized glomerocarps (= sporocarps) with a peridium and a gleba comprising hyphae and glomoid glomerospores (= spores) with a single subtending hypha (Figs. 7–10, 12). *Spores* hyaline; globose to subglobose; small, 35–58-μm diam; frequently ovoid (Figs. 8–16). *Spore wall* composed of two smooth layers (layers 1 and 2), of which the inner layer 2 is laminate and much thicker than the outer layer, forming the spore surface (Figs. 11–16). None of the spore wall layers stains in Melzer's reagent (Fig. 16). *Subtending hypha* hyaline, funnel-shaped with a wall consisting of two layers continuous with spore wall layers 1 and 2 (Figs. 10, 11, 14–16). *Pore* open or occluded by thickening of the subtending hyphal wall, or (rarely) occluded by a straight or slightly invaginated septum continuous with some innermost laminae of spore wall layer 2; septum, positioned at the spore base (Figs. 11, 14–16). *Germination* directly from the spore wall.

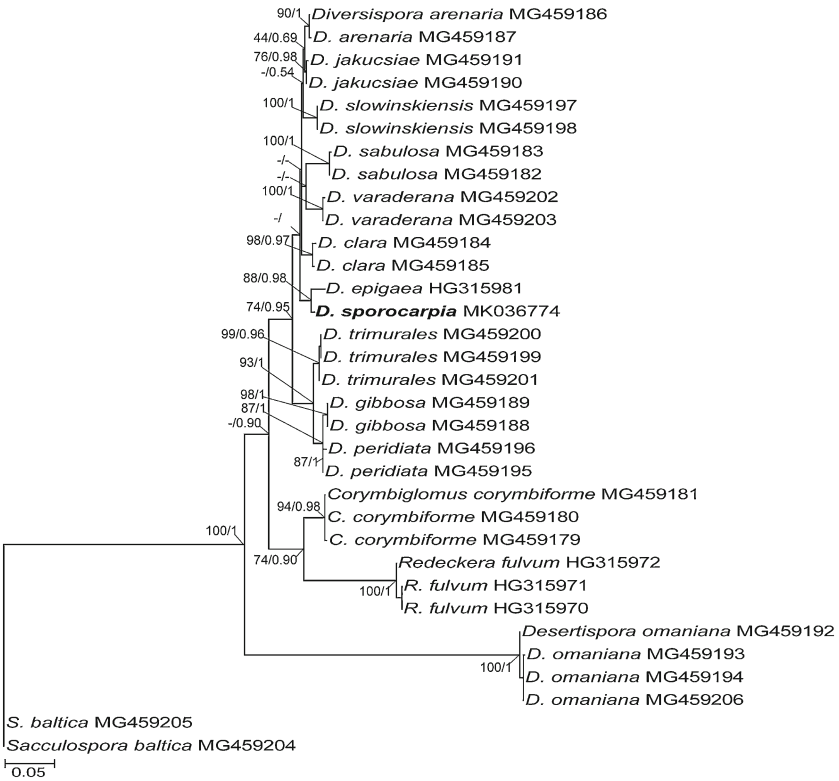



**Fig. 4** A maximum likelihood (ML) phylogram produced from analysis of RPB1 sequences of *Diversispora sporocarpia*, 13 other species of four AMF genera in the family *Diversisporaceae*, with *Sacculospora baltica* (Sacculosporaceae) as outgroup. ML bootstrap values ≥ 50% and Bayesian Inference posterior probability values ≥ 0.50 are shown near the branches, respectively. Bar indicates 0.05 expected changes per site per branch

**Description of a new species.**

***Sclerocarpum amazonicum*** Jobim, Błaszk., Niezgoda, Kozłowska & B.T. Goto, sp. nov.

**MycoBank No. MB** 828317.

***Etymology*** Latin, *amazonicum*, referring to the Amazonian ecosystems of South America, in which the species was originally found.

Specimens examined: Brazil, sporocarps found at two sites of the Adolpho Ducke Forest Reserve (02° 55′ S, 59° 59′ W),

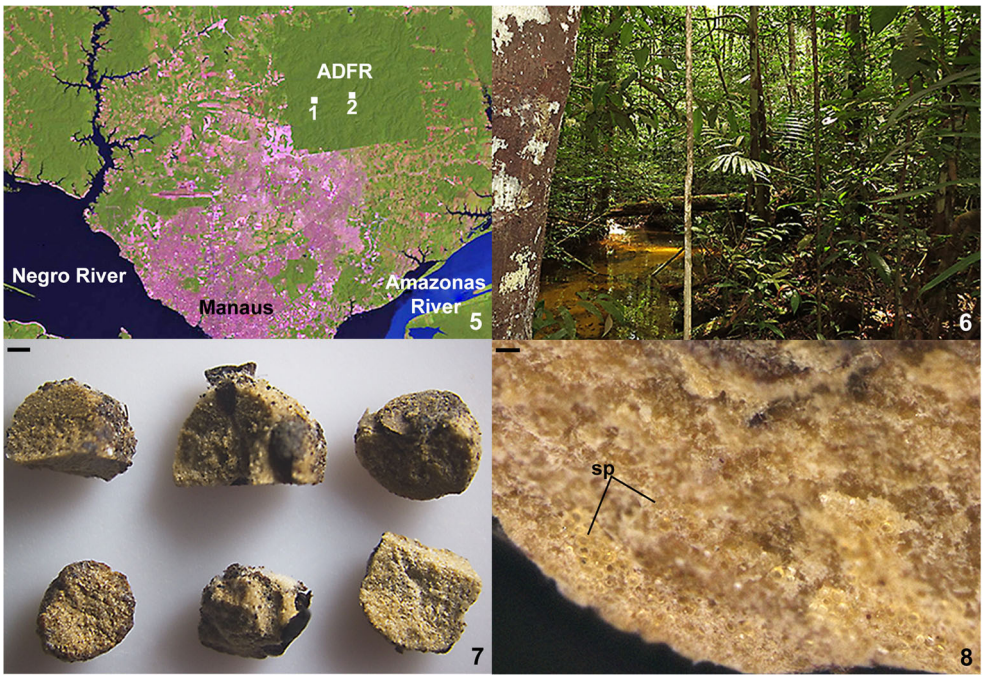



**Fig. 5–8** **5** Map showing the location of the Adolpho Ducke Forest Reserve (ADFR) and the approximate positions of the two sites in which glomerocarps (= sporocarps) of *Sclerocarpum amazonicum* were collected; map from http://ppbio.inpa.gov.br/repositorio/dados. **6** Vegetation of site 1 with dense forest and high humidity. **7** Fragments of sporocarps. **8** Sporocarpic spores (sp). Scale bar = 0.5 mm (**7**). Scale bar = 50 μm (**8**)

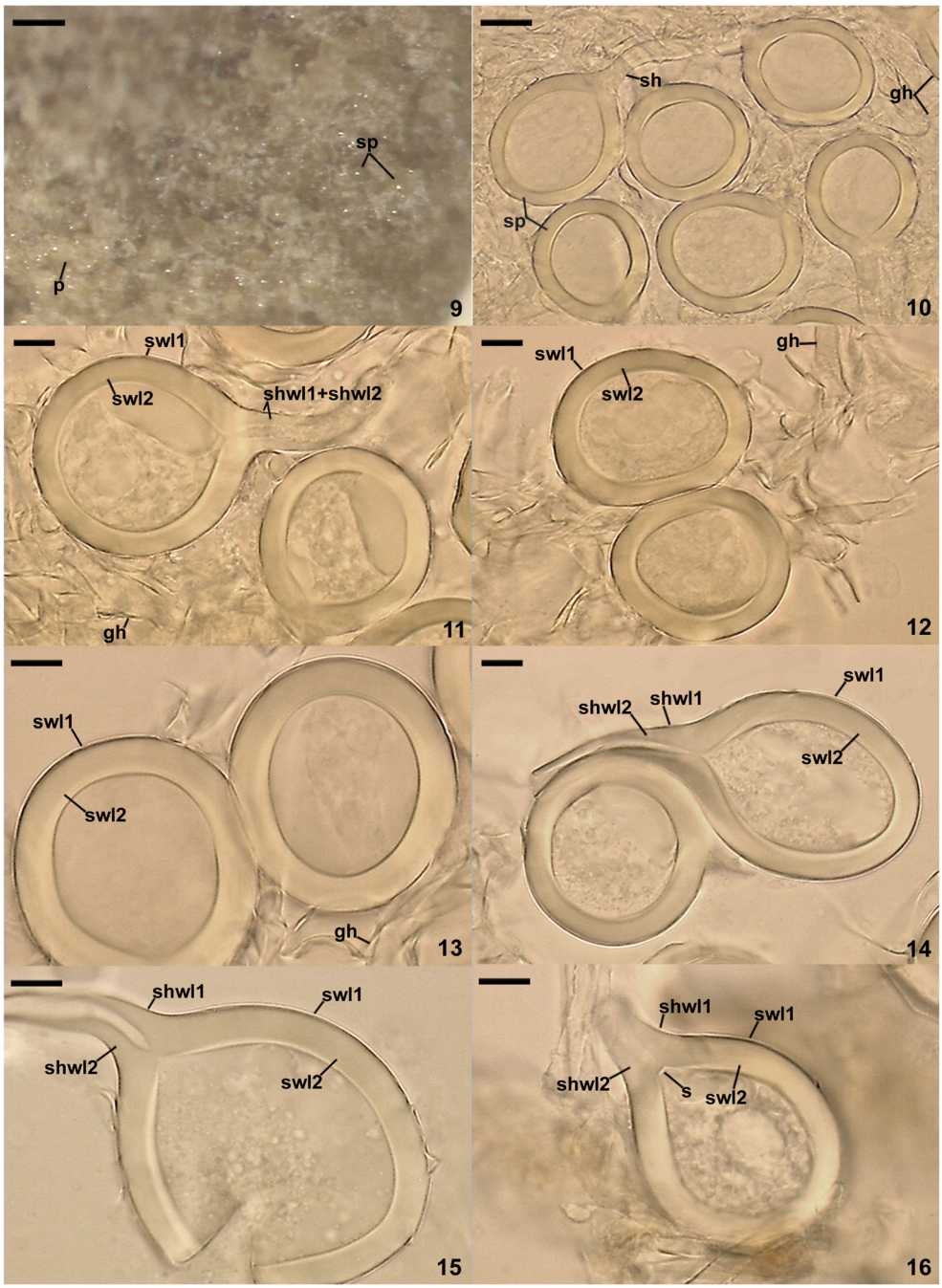



**Fig. 9–16** *Sclerocarpum amazonicum*. **9** Glomerocarps (= sporocarp) with glomerospores (= spores; sp) and peridium (p). **10** Glebal spores (sp) with subtending hyphae (sh) and glebal hyphae (gh). **11** Subtending hyphal wall layers (shwl) 1 and 2 continuous with spore wall layers (swl) 1 and 2 and glebal hyphae (gh). **12**, **13** Spore wall layers (swl) 1 and 2 and glebal hyphae (gh). **14–16** Subtending hyphal wall layers (shwl) 1 and 2 continuous with spore wall layers 1 and 2; note the pore of the subtending hypha is closed by thickened shwl2 (**14**, **15**), and it is closed by a straight septum continuous with the innermost laminae of swl2 (**16**). **9** Dry herbarium specimen. **10–15** Spores and glebal hyphae in PVLG. **16** Spore in PVLG + Melzer's reagent. **9** Light microscopy. **10–16** Differential interference microscopy. Scale bars = 100 μm (**9**), 20 μm (**10**), 10 μm (**11–16**)

located in the middle of the Amazonian Forest (Fig. 5), by K. Jobim in November 2017. Holotype. Sporocarps and a slide with spores no. UFRN-FUNGOS 3015, isotypes: a vial with a sporocarp no. 3632, slides with spores no. 3633–3643 (DEPSE), a vial with a sporocarp and slide no. INPA 280748.

*Diagnosis*: As that of *Sclerocarpum*.

*Description*: Glomerospores formed in single epigeous and subhypogeous unorganized glomerocarps. *Glomerocarps* pale yellow (3A3) to coal (3F1); irregular, (1200–)950 × 1600(−2000) μm (Figs. 7–9). *Peridium* thin, yellowish white (3A2) to pale yellow (3A3), only partially covering glomerospore conglomerations (Fig. 9). *Gleba* pale yellow (3A3) to light yellow (4A4), with hyaline; straight or branched hyphae; 5.0–10.5-μm wide, with a wall 0.8–1.3μm thick; and hundreds of glomerospores (= spores; Figs. 7–10, 12). Spores arise blastically at the tip of sporogenous hyphae (Figs. 10, 11, 14–16). *Spores* hyaline; globose to subglobose; (35–)48(−58) μm diam; frequently ovoid; 38–52 × 44–63 μm; with one subtending hypha (Figs. 8–16). *Spore wall* composed of two layers (Figs. 11–16). Layer 1, forming the spore surface, uniform (not containing visible sublayers), permanent, semi-flexible, smooth, (0.8–)1.3(− 1.5)μm thick, tightly adherent to the

upper surface of layer 2 (Figs. 11–16). Layer 2 laminate, permanent, semi-flexible, smooth, (4.8–)6.5(− 9.0)μm thick; consisting of very thin, < 0.5μm thick, laminae, tightly adherent to each other, not separating even in vigorously crushed spores (Figs. 11–16). None of the spore wall layers stains in Melzer's reagent (Figs. 16). *Subtending hypha* hyaline; straight or recurved, funnel-shaped; (11.0–)14.9(−19.0)μm wide at the spore base (Figs. 10, 11, 14–16); not braking in crushed spores. *Wall of subtending hypha* hyaline; (4.3–)7.0(−9.3)μm thick at the spore base; consisting of two layers continuous with spore wall layers 1 and 2 (Figs. 11, 14–16). *Pore* (1.2–)1.8(− 2.8)μm wide at the spore base, open or occluded by thickening of the subtending hyphal wall, or (rarely) occluded by a straight or slightly invaginated septum continuous with some innermost laminae of spore wall layer 2; septum ca. 1.5–2.3μm thick, positioned at the spore base (Figs. 11, 14–16). Spore content of hyaline oily substance. *Germination* directly from the spore wall.

**Mycorrhizal associations.** No molecular analyses were performed on roots of the plant species that grew in the places where the sporocarps of *S. amazonicum* were found. Attempts to grow *S. amazonicum* in single-species cultures with *P. lanceolata* as the host plant failed.

**Distribution and habitat.** To date, *S. amazonicum* has been found only in the Adolfo Ducke Forest Reserve located in the middle of the Amazonian Forest (Fig. 5). The geographical position and the composition of vegetation of the sites, in which *S. amazonicum* was found are characterized in the section "Materials and Methods." The soil surface of the sites where the epigeous and subhypogeous sporocarps of the fungus occurred was covered with a large amount of organic matter.

BLAST searches indicated that this species has not been recorded in the world before. The highest identities of SSU-ITS-LSU and RPB1 sequences of *S. amazonicum* compared with sequences of these two loci available in GenBank were only 90% and 88%, respectively.

***Diversispora sporocarpia*** Chachuła, Mleczko, Zubek, Niezgoda, Kozłowska, Jobim, B.T. Goto & Błaszk. sp. nov.

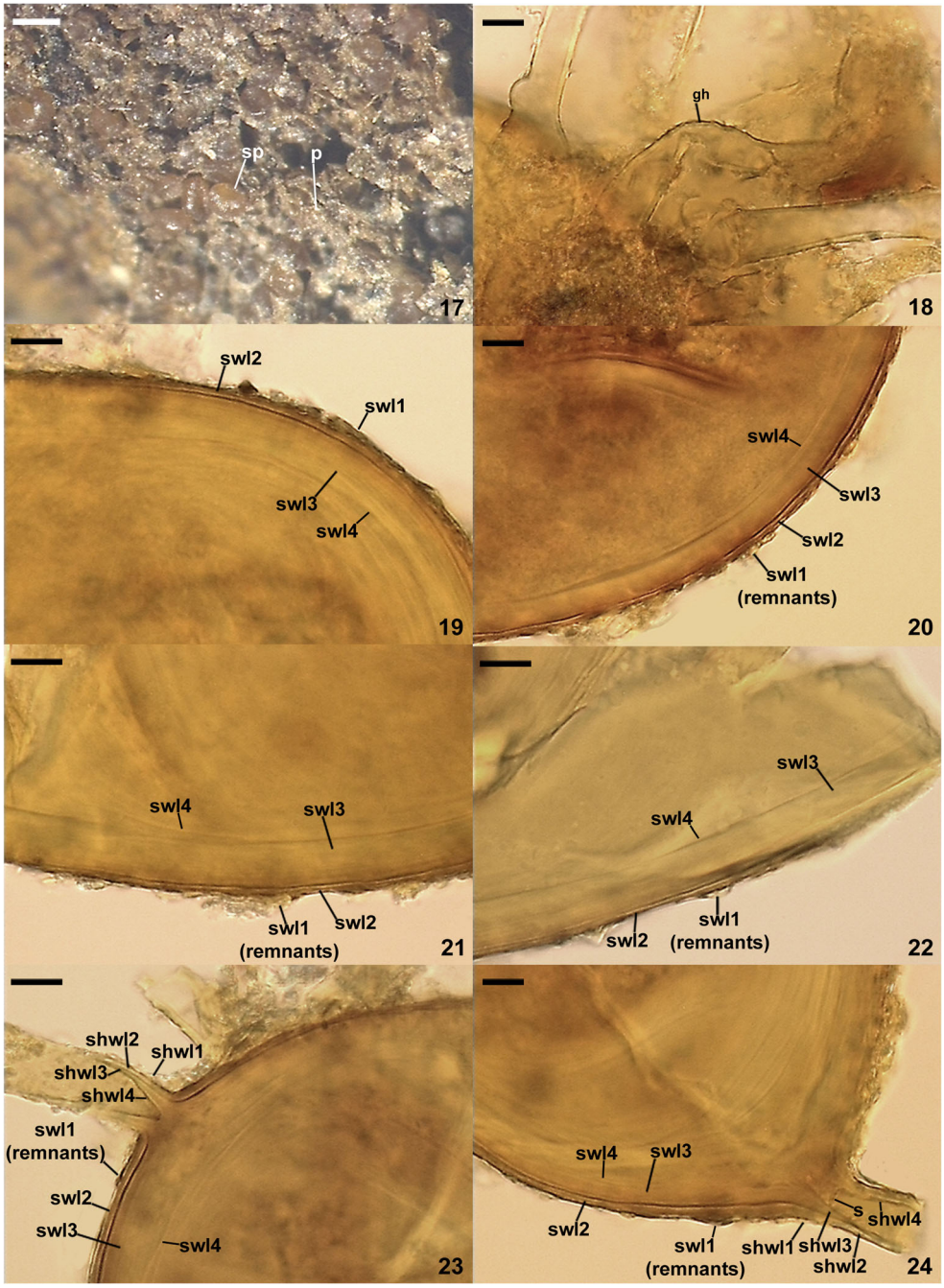
**MycoBank No. MB** 828318.

***Etymology*** Latin, *sporocarpia*, referring to the sporocarp formed by the species.

Specimens examined: Poland, sporocarp found in the Pieniny National Park (49° 24′ 56.6′ ′N, 20° 19′ 56.5′ ′E) by Piotr Chachuła on October 10, 2017. Holotype. A slide with spores no. ZT Myc 59592 (Z+ZT), isotypes: a vial with a sporocarp no. 3644 and slides with spores no. 3645–3649 (DEPSE).

*Diagnosis*: Differs from other *Diversispora* spp. in producing compact unorganized glomerocarps consisting of a peridium and a gleba comprising spores and hyphae, in the phenotypic characters of spore wall layers, and in the nucleotide composition of sequences of the SSU-ITS-LSU nrDNA region and the RPB1 gene.

*Description*: Glomerospores formed in a compact epigeous unorganized glomerocarp consisting of a peridium and a gleba. *Glomerocarp* grayish orange (6B3) to light brown(6D8); ovate; 6 × 4 mm (Fig. 17). *Peridium* thin, hyaline to light yellow (4A4), only partially covering spore conglomerations (Fig. 17). *Gleba* yellow (3A6) to light brown (5D8), with hyaline, straight or branched hyphae; 12.5–22.3μm wide, with a wall 1.0–1.5μm thick; and hundreds of spores (Figs. 17, 18). Spores arise blastically at the tip of sporogenous hyphae (Figs. 23, 24). *Spores* yellow (3A6) to light brown (5D8); usually ovoid; 110–140 × 140–190 μm; rarely globose to subglobose; (120–)136(−148)μm diam; with one subtending hypha (Figs. 17, 23, 24). *Spore wall* composed of four layers (Figs. 19, 20, 21, 22, 23, 24). Layer 1, forming the spore surface, semi-permanent, semi-flexible, smooth when intact, more or less roughened when deteriorated, rarely completely sloughed off, pale yellow (4A3), (0.8–)1.4(−2.3)μm thick when intact (Figs. 19–24). Layer 2 uniform (not divided into visible sublayers), permanent, semi-flexible, yellow (3A6) to light brown (5D8), (1.0–)1.5(−2.0)μm thick (Figs. 19–24). Layers 1 and 2 tightly adherent to and not separating from each other even in vigorously crushed spores (Figs. 19–24). Layer 3 laminate, permanent, semi-flexible, hyaline to yellowish white (4A2), (5.0–)7.6(−9.8)μm thick, consisting of very thin, < 0.5μm thick, laminae, frequently separating from each other in vigorously crushed spores; when colored, the closer to the lower surface, the sublayers of layer 3 lighten and become colorless (Figs. 19–24); layer 3 occasionally separates from the lower surface of layer 2 in vigorously crushed spores (Fig. 22). Layer 4 uniform, permanent, flexible, hyaline, 0.4–0.7μm thick, loosely associated with the lower surface of layer 3, clearly visible in slightly crushed spores, where it usually is separated from the intact laminate layer 3 (Figs. 19–24). Layers 1–4 are not staining in Melzer's reagent (Figs. 23, 24). *Subtending hypha* pastel yellow (3A4) to grayish yellow (3B8); straight or recurved, cylindrical, rarely slightly funnel-shaped; (10.2–)12.2(− 15.7)μm wide at the spore base (Figs. 23, 24); not braking in crushed spores. *Wall of subtending hypha* pastel yellow (3A4) to grayish yellow (3B8); (3.8–)4.9(− 5.5)μm thick at the spore base; consisting of four layers continuous with spore wall layers 1–4; subtending hyphal wall layer (shwl) 1 usually highly deteriorated or completely sloughed off; shwl 3 extending 6.4–16.0 μm below the spore base; shwl4 usually difficult to see (Figs. 23, 24). *Pore* (0.8–)3.0(− 5.0)μm wide at the spore base, occluded (i) by thickening of the subtending hyphal wall, (ii) by a straight or slightly invaginated septum connecting the inner surfaces of subtending hyphal wall layer 4, (iii) by both the structures, or (iv) (rarely) by a septum continuous with the innermost laminae of spore wall layer 3, rarely open; septum 1.3–5.5μm thick, positioned at or up to 7.5 μm below the spore base (Figs. 23, 24). Spore content of hyaline oily substance. *Germination* unknown.




**Figs. 17–24** *Diversispora sporocarpia*. **17** Glomerocarp (= sporocarp) with glomerospores (= spores; sp) and peridium (p). **18** Glebal hyphae (gh). **19–22** Spore wall layers (swl) 1–4. **23**, **24**. Subtending hyphal wall layers (shwl) 1–4 continuous with spore wall layers (swl) 1–4; note the pore of the subtending hypha is closed by thickened shwl3 (**23**) or it is closed by a straight septum (s) connecting the inner surfaces of shwl4 (**24**). **17** Dry herbarium specimen. **18–22** Spores in PVLG. **23**, **24** Spores in PVLG+ Melzer's reagent. **17** Light microscopy. **18–24**. Differential interference microscopy. Scale bars = 200 μm (**17**), 10 μm (**18–22**)

**Mycorrhizal associations.** No molecular analyses were performed on roots of *M. bifolium* that grew in the area where the sporocarp of *D. sporocarpia* was found. Attempts to grow *D. sporocarpia* in single-species cultures with *P. lanceolata* as the host plant failed.

**Distribution and habitat.** The forest of the Pieniny National Park (pine and spruce forest with hazel), whose geographical position and climate are characterized in section "Materials and Methods," is the sole reported location of *D. sporocarpia*. However, a BLAST search of the *D. sporocarpia* SSU-ITS-LSU sequences yielded two similar sequences with 98% identity (HE775321, HE775336), which were detected in environmental sequencing conducted in the Czech Republic (Kohout et al. 2014) and may represent the same species. There are no RPB1 sequences in GenBank that would suggest conspecificity with *D. sporocarpia*.

## Discussion

The validity of the erection of the new monospecific genus *Sclerocarpum* in the family Glomeraceae unambiguously

proved the results discussed above. First, phylogenetic analyses placed *S. amazonicum* in a clade located equivalently to other clades designated as genera in the Glomeraceae (Figs. 1, 2). Second, the divergences of the SSU-ITS-LSU sequences of *S. amazonicum* from the SSU-ITS-LSU sequences of *G. bareae*, *G. macrocarpum*, and *G. tetrastratosum*, which grouped in a sister clade in the reconstructed phylogenetic tree (Fig. 1), and the divergence of the RPB1 sequence of *S. amazonicum* from RPB1 sequences of the same three *Glomus* spp., which also positioned in a sister clade (Fig. 2), were 22.1–23.6% and 14.4–15.0%, respectively. Thus, the divergences were much or clearly larger than divergences of sequences of the same loci of other phylogenetically characterized neighbors belonging to different genera of the Glomeraceae (Błaszkowski et al. 2018a, 2018b). For example, in the Glomeraceae SSU-ITS-LSU phylogenetic tree (Fig. 1), the neighboring *Rhizoglomus irregulare* FM865555 and *Sclerocystis sinuosa* FJ461846 sequences differed by 10.5%, and the difference between the neighboring *G. tetrastratosum* KM056665 and *S. viscosum* HF548854 sequences was 20.0%. In the RPB1 tree (Fig. 2), the neighboring *Sclerocystis pubescens* HG316023 and *Rh. irregulare* HG315983 sequences differed by 14.4%.

The morphological features that clearly distinguish *S. amazonicum*, the so far sole known species of the genus *Sclerocarpum*, from representatives of other genera of the Glomeromycota that produce glomoid spores in unorganized sporocarps with a peridium are the small, hyaline, thick-walled spores and the cylindrical or funnel-shaped spore subtending hypha, whose wall layers clearly arise below the spore base and are continuous with spore wall layers (Figs. 7–16).

Surprisingly, our molecular phylogenetic analyses, which also considered many AMF species that produce hyaline, small glomoid spores, indicated that the most closely related species to *S. amazonicum* are *G.* cf. *aggregatum*, *G. bareae*, *G. macrocarpum*, and *G. tetrastratosum* (Figs. 1, 2). Of these, only *G. macrocarpum* is known to form spores in unorganized sporocarps with a peridium, although spores of the species may also be produced singly (McGee and Trappe 2002; Schüßler and Walker 2010; Błaszkowski 2012). Most importantly, spores of the latter four species are colored [pastel yellow (2A4) to yellowish brown (5D8)], are much larger (up to 4.1-fold when globose), and their spore wall consist of three to four layers, with the exception of the spore wall of *G. macrocarpum*, which is also two-layered (Berch and Fortin 1983; Błaszkowski 2012). However, spore wall layer 1 of *G. macrocarpum*, which forms the spore surface, similarly as spore wall layer 1 of the other three *Glomus* spp., is a short-lived structure that is usually mostly or completely sloughed off in mature spores, and it stains pink (11A5) to deep Magenta (14D8) in Melzer's reagent. Spore wall layer 1 of *S. amazonicum* is presumably permanent, as it was present in all specimens examined by us, and it is nonreactive in Melzer's (Figs. 11–16).

Additional species that probably belong to the genus *Sclerocarpum* are *G. pallidum* I.R. Hall, *G. segmentatum* Trappe, Spooner & Ivory, and *G. tenerum* P.A. Tandy. All three produce unorganized sporocarps with a peridium and glomoid spores. Unfortunately, the natural phylogenies of these species remain unknown and, therefore, the determination of their molecular relationship to *S. amazonicum* is impossible. However, other morphological characters prove that *S. amazonicum* differs fundamentally from the three *Glomus* spp. Spores of *G. pallidum* are colored [orange (4A3) to pale orange (5A3)], their two-layered spore wall is 1.6–2.3-fold thinner, and their spore wall layer 1 is evanescent, and frequently completely sloughed off in mature spores (Błaszkowski 2012). Moreover, the subtending hypha of *G. pallidum* spores is more uniform in shape (cylindrical to slightly flared) and, at the spore base, it is 1.6–2.2-fold narrower, and has a 2.9–6.1-fold thinner wall. Spores of *G. segmentatum* are 1.6–1.9-fold larger when globose and their spore wall consists of three layers, of which layer 1, covering the upper surface of the structural laminate layer 2, is frequently completely sloughed off in mature specimens (Oehl et al. 2011; Błaszkowski 2012). In addition, the laminate spore wall layer 2 of *G. segmentatum* occasionally stains reddish white (9A2) in Melzer's reagent (Błaszkowski 2012). Most importantly, unique to *G. segmentatum* is the formation of sporocarps composed of tough segments, of which each segment, containing crowded spores, is enclosed with a peridium (Trappe 1979; Furrazola et al. 2016). Spores of *G. tenerum* are much darker (yellow orange to brown) and 1.3–3.4-fold larger when globose (McGee and Trappe 2002).

Our molecular phylogenetic analyses showed that the closest relative of *D. sporocarpia* is *D. epigaea* (Figs. 3, 4). The novelty of *D. sporocarpia* was also confirmed by the large divergences of its SSU–ITS–LSU and RPB1 sequences when compared to *D. epigaea*, which were 3.7–9.9% and 1.5%, respectively.

Morphologically, *D. sporocarpia* also differs clearly from *D. epigaea*. The main distinctions reside in the number and phenotypic features of their spore wall components. The spore wall of *D. sporocarpia* consists of four layers (Figs. 19–24), whereas the *D. epigaea* spore wall is three-layered (Schüßler et al. 2011). Both species share the presence of a penultimate structural laminate layer and a thin, flexible innermost layer in the spore wall. However, the structural laminate layer 3 of *D. sporocarpia* is surrounded by a semi-permanent, 0.8–2.3μm thick layer 1, forming the spore surface, and by a uniform, permanent, 1.0–2.0μm thick layer 2 that directly covers the upper surface of spore wall layer 3 (Figs. 19–24). In the *D. epigaea* spore wall, the structural laminate layer is the second layer of this wall and it is covered with an evanescent outermost layer 1, which is up to 1μm thick when intact

(Schüßler et al. 2011). In addition, the subtending hypha of *D. sporocarpia* spores may be up to 1.6-fold wider at the spore base.

As we mentioned in the section "Introduction," species of AMF that produce glomoid spores in unorganized sporocarps with a peridium are one of the least-known fungal groups of the Glomeromycota. Such sporocarpic species apparently have much higher ecological (e.g., physical and biological soil properties, the composition of plant communities, temperature, humidity) and temporal requirements for fruiting than other groups of the Glomeromycota. Therefore, for herbaria and institutions with fungal collections that are interested in increasing the representation of these members of the Glomeromycota, the only solution is to pursue the collection of their fresh specimens, particularly in regions that are probably more conductive to the development of these sporocarpic species. According to Hawksworth and Rossman (1997), Hawksworth (2001), and Blackwell (2011), tropical forests are such regions, and the exploration of tropical forests have resulted in the discovery of many sporocarpic fungi (Sulzbacher et al. 2016, 2017). These include the recently newly described species of the Glomeromycota from Brazil and Cuba (Torre-Arias et al. 2017), AMF species found in these countries, but previously rarely recorded in the world (Goto and Maia 2005), as well as the new Brazilian fungus, *S. amazonicum*, characterized in this paper.

However, based on literature data and our own observations, we conclude that the Glomeromycota fungi that produce glomoid spores in unorganized sporocarps with a peridium probably have a worldwide distribution and they likely occur not only in undisturbed high-humidity habitats that are rich in organic matter, but also in highly degraded soils (McGee and Trappe 2002). It is difficult to speculate about the worldwide frequency of sporocarpic Glomeromycota. The relatively infrequent records of these fungi are likely not due to the rarity of their occurrence. Rather, they are the result of rare use of the specific and labor-intensive methods necessary to find their sporocarps. Another reason is the lack of experienced mycologists interested in deepening the knowledge of this group of fungi, which also creates a lot of difficulties in molecular studies (pers. observ.). Finally, these fungi will have little to no practical applications (e.g. as commercial inocula) because they are difficult to grow in culture and there is no certainty that all of them are typical mycorrhizal symbionts.

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**Artykuł nr 4**

| | |
|---|---|
| **Tytuł:** | Rhizoglomus dalpeae,R.maiae, and R. silesianum, newspecies |
| **Autorzy:** | a, Janusz Błaszkowski, Piotr Niezgoda<br>b, Marcin Piątek,<br>c, Franco Magurno<br>d, Monika Malicka<br>e, Szymon Zubek, Piotr Mleczko<br>f, Nourou S. Yorouf, Xochtil Margarito Vista,<br>g, Xochtil Margarito Vista, Juliana L.R.Lima , Bruno Tomio Goto |
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| **Streszczenie:** | We examined three arbuscular mycorrhizal fungi (AMF; phylum Glomeromycota) producing glomoid spores. The mode of formation and morphology of these spores suggested that they represent undescribed species in the genus *Rhizoglomus* of the family Glomeraceae. Subsequent morphological studies of the spores and molecular phylogenetic analyses of sequences of the nuc rDNA small subunit (18S), internal transcribed spacer (ITS1-5.8S-ITS2 = ITS), and large subunit (28S) region (= 18S-ITS-28S) confirmed the suggestion and indicated that the fungi strongly differ from all previously described *Rhizoglomus* species with known DNA barcodes. Consequently, the fungi were described here as new species: *R. dalpeae, R. maiae*, and *R. silesianum*. Two of these species lived hypogeously in the field in habitats subjected to strong environmental stresses. *Rhizoglomus dalpeae* originated from an inselberg located within Guineo-Sudanian transition savanna zone in Benin, West Africa, where the temperature of the inselberg rock during a 5-mo drought ranges from 40 to 60 C. *Rhizoglomus silesianum* originated from a coal mine spoil heap in Poland, whose substrate is extremely poor in nutrients, has unfavorable texture, and may heat up to 50 C. By contrast, *R. maiae* was found in more favorable habitat conditions. It produced an epigeous cluster of spores among shrubs growing in a tropical humid reserve in Brazil. Moreover, the compatibility of phylogenies of species of the family Glomeraceae reconstructed from analyses of sequences of 18S-ITS-28S and the largest subunit of RNA polymerase II (*RPB1*) gene was discussed. |
| **Słowa kluczowe:** | Arbuscular mycorrhizol fungi, Glomeromycota, molecular phylogeny, morphology, RPB1, 18S-ITS-28S nuc rDNA, 3 new taxa |

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ORIGINAL ARTICLE

# *Sieverdingia* gen. nov., *S. tortuosa* comb. nov., and *Diversispora peloponnesiaca* sp. nov. in the Diversisporaceae (Glomeromycota)

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## Abstract

Phylogenetic analyses of 18S–ITS–28S nuc rDNA sequences indicated that the arbuscular mycorrhizal fungus originally described as *Glomus tortuosum* and later transferred to the genus *Corymbiglomus* represents a separate, previously unrecognized clade at the rank of genus in the family Diversisporaceae (order Diversisporales, phylum Glomeromycota). The analyses located the clade between clades representing the genera *Desertispora* and *Redeckera*. Consequently, a new genus, *Sieverdingia*, was erected, with *S. tortuosa* comb. nov. The unique morphological feature of *S. tortuosa* is the formation of glomoid-like spores with a single-layered spore wall covered with a hyphal mantle. Importantly, the erection of *Sieverdingia* clarified the definition of *Corymbiglomus*, which currently consists of three species producing glomoid-like spores with one, three- to four-layered spore wall. The features of the innermost layer, which is hyaline, laminate, flexible to semi-flexible, indicate that it is a synapomorphy of *Corymbiglomus*. The definitions of *Corymbiglomus* and its species were emended. Moreover, the distribution of *S. tortuosa* and the three species of *Corymbiglomus* was discussed based on own studies, literature data, and molecular sequences deposited in public databases. We concluded that the distribution of *S. tortuosa* and *C. globiferum* known in environmental studies based on their partial 28S nuc rDNA sequences only may be understated because the main molecular characteristics distinguishing these species reside outside the 28S region. Finally, we described a new species in the genus *Diversispora* originating from Mediterranean dunes of the Peloponnese peninsula, Greece. The same phylogenetic analyses mentioned above indicated that the closest relative of the new species, producing dark-coloured spores, is *D. clara*, whose spores are creamy white at most.

**Keywords** Arbuscular mycorrhizal fungi · Morphology · Molecular phylogeny · 18S–ITS–28S nuc rDNA

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## Introduction

The phylum Glomeromycota C. Walker & A. Schüßler, comprising arbuscular mycorrhizal fungi (AMF), is currently represented by ca. 315 species (http://glomeromycota.wixsite.com/lbmicorrizas/cpia). About 57% of these species produce glomoid or glomoid-like spores, and the species are distributed in 25 genera belonging to nine families of the Glomeromycota. The glomoid and glomoid-like spores arise blastically at the tip of a cylindrical or funnel-shaped sporogenous hypha, like spores of *Glomus macrocarpum* Tul. & C. Tul., the type species of the genus *Glomus* Tul. & C. Tul. emend. Oehl, G.A. Silva & Sieverd. and the Glomeromycota (Clements and Shear 1931; Oehl et al. 2011), and their subcellular structure contains only one spore wall continuous with the subtending hyphal wall (Błaszkowski et al. 2018a, 2018b; Jobim et al. 2019).

The fungus originally described as *G. tortuosum* N.C. Schenck & G.S. Sm. is one of the few species (15, of which probably not all are certain species) of the Glomeromycota, which produces glomoid-like spores singly or in unorganized clusters (with randomly distributed spores), and the spores have a spore wall consisting of only one coloured layer (Schenck and Smith 1982). Based on phylogenetic analyses of sequences of the partial large subunit (28S) nuc rDNA, Błaszkowski and Chwat (2013) transferred *G. tortuosum*, along with *G. globiferum* Koske & C. Walker, to *Corymbiglomus* Błaszk. & Chwat in the family Diversisporaceae C. Walker & A. Schüßler, order Diversisporales C. Walker & A. Schüßler, and renamed the species as *C. tortuosum* (N.C. Schenck & G.S. Sm.) Błaszk. & Chwat and *C. globiferum* (Koske & C. Walker) Błaszk. & Chwat. The genus *Corymbiglomus*, with the type species *C. corymbiforme* Błaszk., was previously erected following phylogenetic analyses of partial 28S sequences of *G. corymbiforme* Błaszk. (Błaszkowski 2012). In the presented phylogenetic tree (Fig. 1; Błaszkowski and Chwat 2013), each of the species was represented by only one partial 28S sequence and the three sequences clustered in a sister clade to a clade representing the genus *Diversispora* C. Walker & A. Schüßler. Interestingly, the three-species clade consisted of two subclades, one with sequences of *C. corymbiforme* and *C. globiferum*, and the second with a sequence of *C. tortuosum*.

Unfortunately, in the analyses discussed above, no sequence of *Redeckera* spp. was included. However, the ecology and all other morphological features of *Redeckera* spp. and *C. tortuosum* differ fundamentally (Redecker et al. 2007; Schenck and Smith 1982; Oehl et al. 2011). Importantly, our preliminary phylogenetic analyses of available sequences of the partial small subunit (18S), internal transcribed spacer (ITS1–5.8S–ITS2 = ITS) and partial 28S (= 18S–ITS–28S) nuc rDNA segment or part thereof of representatives of all glomoid-like spore-producing genera of the Diversisporaceae sensu Redecker et al. (2013) suggested that *C. tortuosum* does not belong to *Corymbiglomus*, but it represents a yet undescribed genus in the Diversisporaceae, a hypothesis also postulated by Medina et al. (2014).

In the same paper, Medina et al. (2014) described a new species in *Corymbiglomus*, *C. pacificum* Oehl et al., whose spores were not surrounded by a hyphal mantle, as spores of the three species discussed above. Most importantly, the researches stated that the subcellular structure of the glomoid-like spores of *C. pacificum* consists of two spore walls and suggested that spores of *C. corymbiforme* and *C. globiferum* are also two-walled. Interestingly, *C. corymbiforme* was originally characterized (as *G. corymbiforme*) to produce spores with a single spore wall consisting of three layers (Błaszkowski 1995). The original description of *C. globiferum* (as *G. globiferum*) also testifies that its spores have one spore wall (Koske and Walker 1986). Moreover, our observations of the microphotographs of spores of *C. pacificum* published by Medina et al. (2014) suggested that spore wall 2 of *C. pacificum* sensu Medina et al. (2014) is not a separate spore wall and does not arise identically as, for example, spore wall 2 of species of *Pacispora* (Błaszkowski 2012).

Literature data and BLAST searches testify that species of *Diversispora* occur in different cultivated and natural habitats and have a worldwide distribution, as well as they suggest that many fungi of this genus remain undescribed (Sridhar and Beena 2001; Schüßler et al. 2011; Błaszkowski et al. 2015c; Symanczik et al. 2018). We extracted from a pot trap culture glomoid-like spores, whose morphological features of the subtending hypha and the spore wall at the spore base corresponded to those defined by Oehl et al. (2011) as characteristic for diversisporoid spores of *Diversispora*. In addition, other morphological characters of these spores suggested that they represent an undescribed *Diversispora* sp.

Considering the doubts and suppositions discussed above, the aims of our further studies were (i) to check the phylogenetic position of the four described species of *Corymbiglomus* among other glomoid-like spore-producing taxa of the family Diversisporaceae based on more convincing molecular information; (ii) to definitively define the subcellular structure of spores of these species; (iii) to emend the diagnostic description of the genus *Corymbiglomus*, if necessary; and (iv) to confirm the novelty of the putative undescribed *Diversispora* sp. by growing it in single-species cultures, determining its phylogenetic position among molecularly sequenced glomoid-like spore-producing members of the Diversisporaceae and by characterizing its morphology.

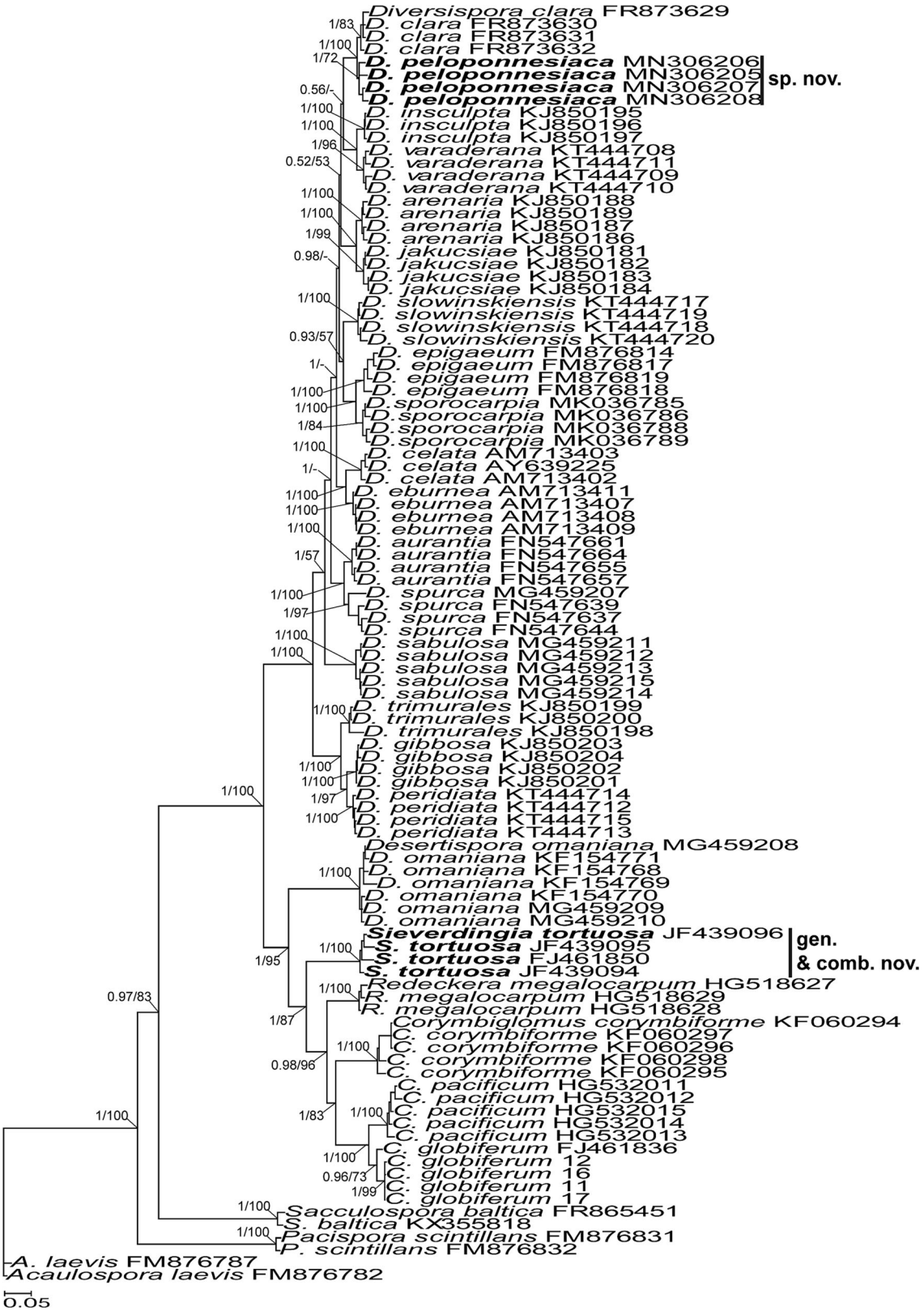



**Fig. 1** A 50% majority rule consensus phylogram inferred from a Bayesian analysis of 18S–ITS–28S nuc rDNA sequences of *Diversispora peloponnesiaca* and *Sieverdingia tortuosa*, as well as 24 other species of AMF, including *Acaulospora laevis*, *Pacispora scintillans*, and *Sacculospora baltica* as outgroup. The Bayesian posterior probabilities ≥ 0.50 and ML bootstrap values ≥ 50% are shown near the branches, respectively. Bar indicates 0.5 expected change per site per branch

## Materials and methods

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

We characterized the morphology of the fungus originally described as *G. tortuosum* based on its original description (Schenck and Smith 1982), its features described and illustrated by Dr. J.B. Morton (http://fungi.invam.wvu.edu/the-fungi/classification/diversisporaceae/diversispora/tortuosum.html), spores extracted from a pot trap culture and field-collected rhizosphere soil samples, as well as based on examination of type material (OSC#40251) used to describe the fungus by Schenck and Smith (1982). The trap culture was inoculated with the rhizosphere soil of *Ammophila breviligulata* Fern. (Poaceae Barnh.) that had colonized maritime sand dunes of New Jersey, USA. The soil sample was collected by Dr. M. Tadych in August 2000. Unfortunately, the geographical position of the sampled site was not determined. All attempts at growing the fungus in single-species cultures established from spores extracted from the trap culture failed. The rhizosphere field soils were collected from *Tabebuia roseo-alba* (Ridl.) Sandw. (Bignoniaceae Juss.) and *Guazuma ulmifolia* Lam. [Sterculiaceae (DC.) Bartl.] colonizing sand dunes of the city of Mataraca, Paraiba State, Northeast Brazil (6° 28′ 20″–6° 30′ 00″ S, 34° 55′ 50″–34° 57′ 10″ W; de Souza et al. 2013). The predominant geomorphological formation of the sampled site is clay-sand sedimentary rocks overlaid by fixed dunes, up to 100 m high. The area has a tropical rainy climate (Am type according to Koppen's classifications; Peel et al. 2007), 25 °C annual average temperature and 1700 mm average annual rainfall (Oliveira-Filho and Carvalho 1993).

Spores of *C. corymbiforme* examined in this study were extracted from field-collected rhizosphere soil samples and single-species cultures established from spores collected from trap cultures. The trap cultures were inoculated with the rhizosphere soil samples taken from *Ammophila arenaria* (L.) Link growing in dunes of the Baltic Sea. The dunes are located near the city of Świnoujście (53° 55′ 03″ N 14° 17′ 39″ E) in north-western Poland. The climate of Świnoujście is temperate, with an annual sum of rainfall and a mean annual temperature of 550 mm and 8.92 °C, respectively (https://pl.wikipedia.org/wiki/Świnoujście). All soil samples were collected by J. Błaszkowski in the years 1993–2015.

*Corymbiglomus globiferum* was characterized here based on its original description (Koske and Walker 1986), the description and pictures presented by Dr. J.B. Morton (http://fungi.invam.wvu.edu/the-fungi/classification/diversisporaceae/diversispora/globiferum.html), and spores extracted from trap cultures. The cultures were inoculated with the rhizosphere soils of *Cyperus crassipes* Vahl (Cyperaceae Juss.) and *Euploca polyphyla* (Lehm.) J.I.M. Melo & Semir (Boraginaceae Juss.) growing in sand dune soils located near the city of Galinhos and in the Reserva de Desenvolvimento Sustentável Estadual Ponta do Tubarão in the Guamaré Municipality (RDSE Ponta do Tubarão), Rio Grande do Norte State, respectively, Brazil. The *C. crassipes* soil was collected by J.N. de Paiva and K. Jobim in April 2017, and *E. polyphyla* was sampled by K.J.G da Silva and R.C. Theodoro in June and November 2015. The areas of the city of Galinhos and the RDSE Ponta do Tubarão are ca. 342 $km^2$ and 1.29 $km^2$, respectively, and both are located northwest of the city of Natal (78° 02′ S, 94° 37′ W and 5° 5′ 3.55″ S, 36° 28′ 19.13″ W, respectively). The typical biome of these areas is "restinga", which consists mainly of plant species from the families Cyperaceae and Poaceae (Oliveira Filho and Carvalho 1993). According to Koppen's classifications (Peel et al. 2007), the region has a tropical humid climate (type Afi), with an oscillating temperature of 21 °C and 30 °C (av. 26.1 °C) and an average annual rainfall between 10 and 170 mm.

The morphology of *C. pacificum* was determined in this study based on the original description of the species (Medina et al. 2014) and its holotype and isotype specimens loaned from the herbarium of ETH Zürich (Z+ZT), Switzerland.

Spores of the putative new species of *Diversispora* were originally extracted from a trap culture inoculated with the rhizosphere soil of *A. arenaria*. Subsequently, the spores were used to establish single-species cultures of the fungus. In the field, *A. arenaria* colonized Mediterranean Sea dunes of the beach Voidokoilia (36° 57′ 46″ N 21° 39′ 45″ E) located on the Peloponnese peninsula, Greece. The climate of the sampled site is warm, with an annual sum of rainfall of 493.8 mm and temperature ranging from 7–14 °C (winter) to 21–34 °C (summer) (http:/www.hnms.gr/emy/el/; https://www.meteoblue.com/en/weather/forecast/modelclimate/pylos_greece_255293). Dune soils of the site have a pH of 7.64–8.06 and are rich in Mg and Na, whose contents are ca. 74.025 and 72.343 mg/kg, respectively. The soil sample was collected by Dr. Dimitris Arrianas on July 18, 2012.

The methods used to establish trap and single-species cultures of the four species discussed here, the growing conditions of these cultures, and the methods of spore extraction and mycorrhizal staining were as those described previously (Błaszkowski et al. 2012).

### Microscopy and nomenclature

Except for *C. pacificum*, the morphological features of spores and the phenotypic and histochemical characters of spore wall layers of the other species discussed here were characterized based on at least 50–100 spores of each species mounted in water, lactic acid, polyvinyl alcohol/

lactic acid/glycerol (PVLG, Omar et al. 1979), and a mixture of PVLG and Melzer's reagent (1:1, v/v). Spores of *C. pacificum* were characterized based on the original description of this species (Medina et al. 2014) and specimens permanently mounted on microscope slides loaned from the mycological herbarium of ETH Zurich (Z+ZT), Switzerland. The preparation of spores 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), 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. The terms glomerospores and glomerocarps were used for spores and fruit bodies, respectively, produced by AMF, as Goto and Maia (2006) and Jobim et al. (2019) proposed.

Voucher specimens of *C. corymbiforme*, *C. globiferum*, the fungus originally described as *G. tortuosum*, and the new species [spores mounted in PVLG and a mixture of PVLG and Melzer's reagent (1:1, v/v) on slides] were deposited at the Herbarium of the Federal University of Rio Grande do Norte [UFRN-Fungos Herbarium; vouchers from type of *G. tortuosum* (UFRN-Fungos 3165) and *C. globiferum* (UFRN-Fungos 3166, 3167)], ETH Zurich, Switzerland (holotype of *D. peloponnesiaca*), and the Department of Ecology, Protection and Shaping of Environment (DEPSE), West Pomeranian University of Technology, Szczecin (isotypes of *D. peloponnesiaca* and specimens of the other three species mentioned above).

## Molecular phylogeny, DNA extraction, polymerase chain reaction, cloning, and DNA sequencing

Genomic DNA of *C. globiferum* and the putative *Diversispora* sp. was extracted from eight single spores of each fungus. The origin of spores of both species was presented above. Details of the treatment of the spores prior to polymerase chain reactions (PCRs), the conditions, and primers used in the PCRs to obtain 18S–ITS–28S sequences were as those described in Błaszkowski et al. (2015a, 2015b), Krüger et al. (2009), and Symanczik et al. (2014). Cloning and sequencing of PCR products to obtain 18S–ITS–28S sequences of *C. globiferum* and the potentially new *Diversispora* sp. were performed as described by Błaszkowski et al. (2015a). The sequences were deposited in GenBank (MN306205–MN306208).

Unfortunately, many attempts to obtain sequences of the largest subunit of RNA polymerase II (*RPB1*) of *C. globiferum* failed, despite nested PCRs being performed in conditions and with primers that allowed obtaining *RPB1* sequences of *C. corymbiforme* (Symanczik et al. 2018).

## Sequence alignment and phylogenetic analyses

BLAST queries indicated that our 18S–ITS–28S sequences of the diversisporoid fungus sensu Oehl et al. (2011) represent an undescribed species of *Diversispora*. Subsequently, in order to clarify the doubts regarding the molecular phylogenies of the four so far described species of *Corymbiglomus*, i.e. *C. corymbiforme*, *C. globiferum*, *C. pacificum* and *C. tortuosum*, and to determine the phylogenetic position of the new species within the genus *Diversispora*, an alignment was assembled that consisted of 93 sequences of the 18S–ITS–28S segment and eight partial 28S sequences. These sequences characterized 23 species, including our new *Diversispora* sp., belonging to four described genera of the Diversisporaceae and three species that served as outgroup. Of the eight partial 28S sequences, three characterized *C. globiferum*, *C. tortuosum*, and *Diversispora celata* C. Walker, Gamper & A. Schüßler, and five belonged in *C. pacificum*. The partial 28S sequences of *C. globiferum* and *C. tortuosum* (both treated as members of the genus *Diversispora* C. Walker & A. Schüßler; http://fungi.invam.wvu.edu/the-fungi/classification/diversisporaceae/diversispora/tortuosum.html; http://fungi.invam.wvu.edu/the-fungi/classification/diversisporaceae/diversispora/globiferum.html) were obtained from DNA of spores extracted from cultures grown at INVAM. The outgroup was represented by two 18S–ITS–28S sequences each of single species of the three most closely related families of the Diversisporaceae sensu Redecker et al. (2013), i.e. *Acaulosporaceae* J.B. Morton & Benny (represented by *A. laevis* Gerd. & Trappe), *Pacisporaceae* C. Walker et al. [*P. scintillans* (S.L. Rose & Trappe) Sieverd. et al.], and *Sacculosporaceae* Oehl et al. [*S. baltica* (Błaszk., Madej & Tadych) Oehl et al.]. Identity values of the newly obtained 18S–ITS–28S sequences of *C. globiferum* and the new *Diversispora* sp. were calculated using BioEdit (Hall 1999). With the same program, we calculated the percentage divergence of a sequence of each of the four species of *Corymbiglomus* and the *Diversispora* sp. treated separately from a directly neighbouring sequence of the closest relatives of these fungi, as well as the divergence of all analyzed sequences of each species of *Corymbiglomus* and the new *Diversispora* sp. from all sequences clustered in sister species or generic clades of these five species (Fig. 1). All comparisons were performed on sequences of the same length.

The sequence set was aligned with MAFFT v. 7 using the auto option (http://mafft.cbrc.jp/alignment/server/). 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, as described and justified in Błaszkowski et al. (2014). The phylogenetic position of the four species of *Corymbiglomus* and the new *Diversispora* sp.

within the Diversisporaceae was reconstructed from Bayesian inference (BI) and maximum likelihood (ML) phylogenetic analyses of the alignment. The BI analysis was conducted with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). The nucleotide substitution model used in this analysis was GTR, which was selected by jModelTest (Posada 2008), considering the selection of Akaike criterion. Four Markov chains were run for 5,000,000 generations, sampling every 100 steps, with a burn-in at 7500 sampled trees. The ML analysis was carried out with the raxmlGUI (Silvestro and Michalak 2012) implementation of RAxML (Stamatakis 2014) with the GTRGAMMA algorithm. A rapid bootstrap analysis with 1000 replicates was used to determine the support of branches. In both BI and ML analyses, the alignment [the nucleotide alignment plus the binary (indel) character set] was divided into four partitions, knowing that analyses of partitioned data generally increase the accuracy of phylogenetic reconstruction (Lanfear et al. 2012; Nagy et al. 2012). The generated phylogenetic tree was visualized and edited in MEGA6 (Tamura et al. 2013).

## Results

### General data and phylogeny

The analyzed sequence alignment had a length of 2063 characters, of which 838 and 755 were variable and parsimony informative, respectively. The identity values of the four 18S–ITS–28S sequences of *C. globiferum* and four 18S–ITS–28S sequences of *D. peloponnesiaca* ranged from 99.6 to 99.9% and from 97.6 to 98.9%, respectively.

Bayesian and ML analyses of the alignment generated trees, in which the position of main clades (five at the rank of genus) to each other and the species composition of sister clades of these generic clades were identical. One of these generic clades contained only sequences of the fungus originally described as *G. tortuosum* (Fig. 1), and this clade was located between the *Desertispora* and *Redeckera* clades. Another generic clade clustered sequences of *C. corymbiforme*, *C. globiferum*, and *C. pacificum*, and this clade was sister to a clade with sequences of *R. megalocarpum*. In the *Corymbiglomus* clade, *C. globiferum* and *C. pacificum* clustered in two separate sister subclades. The generic clade with *G. tortuosum* obtained full supports in both BI and ML analyses. The *Corymbiglomus* clade was fully (BI = 1.0) or strongly (ML = 83%) supported.

The divergences of the neighbouring sequences *C. tortuosum* JF439096 from *Desertispora omaniana* MG459210 and *C. tortuosum* JF439094 from *R. megalocarpum* HG518627 (Fig. 1) were 16.5% and 12.1%, respectively. The sequences *C. corymbiforme* KF060294 and *R. megalocarpum* HG518628 and *C. corymbiforme* KF060295 and *C. pacificum* HG532011 were divergent by 12.9% and 12.0%, respectively. The distance between the sequences *C. globiferum* FJ461836 and *C. pacificum* HG532013 was 5.1%.

Both BI and ML analyses of the 18S–ITS–28S sequences of our *Diversispora* confirmed our hypothesis that this fungus is an undescribed species and indicated that its sister relative is *D. clara* (Fig. 1). The clades with the new *Diversispora* sp. and *D. clara* were fully supported in the BI analysis (= 1.0), but moderately in the ML analysis (= 72% and 83%, respectively). The node connecting the two clades obtained full supports in both analyses. The divergences of the four sequences of the putative new *Diversispora* sp. from the four *D. clara* sequences (Fig. 1) ranged from 4.3 to 5.2%.

Thus, based on the data discussed above, we are fully convinced that the fungus originally described as *G. tortuosum* should represent a new genus in the Diversisporaceae and the Greek diversisporoid fungus is a new species in *Diversispora*.

## Taxonomy

### Erection of a new genus

***Sieverdingia* Błaszk., Niezgoda & B.T. Goto, gen. nov.**

MycoBank no.: MB 832298

**Type species** *Sieverdingia tortuosa* (N.C. Schenck & G.S. Sm.) Błaszk., Niezgoda & B.T. Goto

**Basionym** *Glomus tortuosum* N.C. Schenck & G.S. Sm. Mycologia 74: 83, 1982.

**Specimens examined** Brazil: Paraiba state, spores extracted from field-collected rhizosphere soils of *T. roseo-alba* and *G. ulmifolia* colonizing sand dunes of the city of Mataraca, unnumbered slides prepared by de Souza et al. (2013). USA: New Jersey, spores extracted from a trap culture inoculated with the rhizosphere soil of *A. breviligulata* growing at an unknown maritime dune site sampled by Dr. M. Tadych, *Błaszkowski J.*, (slides no.) 3027–3029 (DEPSE); Oregon State University, type material (OSC#40251) used to describe the fungus by Schenck and Smith (1982).

**Etymology** *Sieverdingia*, in honour of Dr. Ewald Sieverding, Institute for Plant Production and Agroecology in the Tropics and Subtropics, University of Hohenheim, Germany, in recognition of his important contribution to taxonomy and ecology of arbuscular mycorrhizal fungi.

**Diagnosis** Differs from other genera of the Diversisporaceae in producing glomoid-like spores individually covered with a

hyphal mantle, in having a spore wall consisting of one laminate layer and a cylindrical or slightly flared subtending hypha not inflated slightly below the spore base, as well as in having the specific sequences of the nuc rDNA ITS1: AAAATTTATATAACAAATAT and ITS2: CCTAATAT GTTATATATTATGTTTACACTTGT, ACTTGTCA TTTTAATCGATTCGTGC regions.

**Genus description** Producing glomoid-like glomerospores individually covered with a hyphal mantle, having a spore wall consisting of one laminate layer, and a subtending hypha that is cylindrical or slightly flared and is not inflated slightly below the spore base.

***Sieverdingia tortuosa* (N.C. Schenck & G.S. Sm.) Błaszk., Niezgoda & B.T. Goto, comb. nov. (Fig. 2–h)**

MycoBank no.: MB 832299

**Basionym** *Glomus tortuosum* N.C. Schenck & G.S. Sm. Mycologia 74: 83. 1982.

**Synonyms** *Corymbiglomus tortuosum* (N.C. Schenck & G.S. Sm.) Błaszk. & Chwat. Acta Mycol. 48(1):99. 2013.

**Etymology** Latin, *tortuosum* (= winding), referring to the winding pattern of hyphae in the mantle covering spores of the fungus.

**Description** Glomerospores formed singly or in loose clusters in soil (Fig. 2, b). *Spores* pale yellow (4A3) to light yellow (4A4), globose to subglobose, (140–)180(–240) μm diam., sometimes ovoid, 140–170 × 180–240 μm, with one subtending hypha; most mature spores, whether single or arranged in clusters, are surrounded individually by a hyphal mantle (Fig. 2a–h). *Clusters* globose, 280–620 μm diam., to irregular, 140–280 × 430–640 μm, with 2–6 randomly distributed spores. *Subcellular structure of spores* consists of a single-layered spore wall that is laminate, pale yellow (4A3) to light yellow (4A4), (1.2–)2.2(–2.6) μm thick; its laminae frequently separate from each other in crushed spores (Fig. 2a–h). *Subtending hypha* pale yellow (4A3) to light yellow (4A4), straight or curved, cylindrical to flared, (11.5–)13.5(–15.0) μm wide at the spore base (Fig. 2f), usually covered with the hyphal mantle and difficult to see. *Wall of subtending hypha* pale yellow (4A3) to light yellow (4A4), (1.0–)1.6(–1.9) μm thick at the spore base, composed of one layer continuous with the spore wall layer (Fig. 2f). *Pore* gradually thins with age due to thickening of the subtending hyphal wall layer; no septum was observed in the spores examined (Fig. 2f). *Hyphal mantle* 8–22 μm thick, composed of tightly interwoven, straight and branched, septate, hyaline to yellowish white (4A2), 2.7–7.8 μm wide hyphae, with walls 0.5–1.0 μm thick (Fig. 2–e, g, h), probably developing from the region of their subtending hypha. Mantle hyphae and spores not staining in Melzer's reagent. Juvenile and young spores frequently with no hyphal mantle (Fig. 2f). *Germination* through subtending hypha.

**Mycorrhizal associations** In the field, *S. tortuosa* probably lived in symbiosis with *A. breviligulata*, *Glycine max* (L.) Merr., *Ixeris repens* (L.) A. Gray, *Uniola paniculata* L., as well as with different other plant species (Schenck and Smith 1982; Blaschke 1991; Koske 1987; Cabello 2001; Oehl et al. 2003; Gai et al. 2006; Schalamuk et al. 2006; Wang et al. 2008; Goto et al. 2010; Błaszkowski 2012; de Souza et al. 2013; Srindhar and Beena 2012; Yamato et al. 2012; Jobim et al. 2016), although it was confirmed molecularly only with regard to *I. repens* (Yamato et al. 2012). In single-species cultures, *S. tortuosa* formed mycorrhiza without typical arbuscules and vesicles in roots of *Paspalum secundatum* (Walt.) Kuntze (Schenck and Smith 1982).

**Distribution and habitat** *Sieverdingia tortuosa* was originally identified associated with roots of *G. max* cultivated at the Agricultural Research Center, Live Oak, Florida, USA (Schenck and Smith 1982). Later, spores of this species were found in different dune sites of the USA (Koske 1987; Błaszkowski 2012), Brazil (de Souza et al. 2013), India and Japan (Srindhar and Beena 2012), and different cultivated and non-dune uncultivated soils of Argentina (Cabello 2001; Schalamuk et al. 2006), Brazil (Goto et al. 2010; Jobim et al. 2016), Switzerland (Oehl et al. 2003), Germany (Blaschke 1991), and China (Gai et al. 2006; Wang et al. 2008).

BLAST queries did not show any 18S–ITS–28S sequence of identity at the species level (≥ 97%) to the *S. tortuosa* 18S–ITS–28S sequences used in our analyses (Fig. 1). Instead, when the query was the *S. tortuosa* partial 28S sequence FJ461850 used by us, BLAST showed 10 environmental partial 28S sequences, whose identity to the FJ461850 sequence ranged from 97.45 to 98.87%. The sequences represented "uncultured *Diversispora*" associated with *I. repens* growing in saline coastal beach soils in Japan (Yamato et al. 2012).

## Emendation of the genus *Corymbiglomus* Błaszk. & Chwat emend. Błaszk. Niezgoda & B.T. Goto

MycoBank no.: MB 564566

**Genus description** Forming glomoid-like glomerospores individually covered with a hyphal mantle consisting of non-branched or branched hyphae with or without terminal vesiculate swellings or naked spores (without a hyphal mantle). *Spores* occurring singly or in clusters in soil. *Clusters* with several spores connected by interwoven hyphae of their

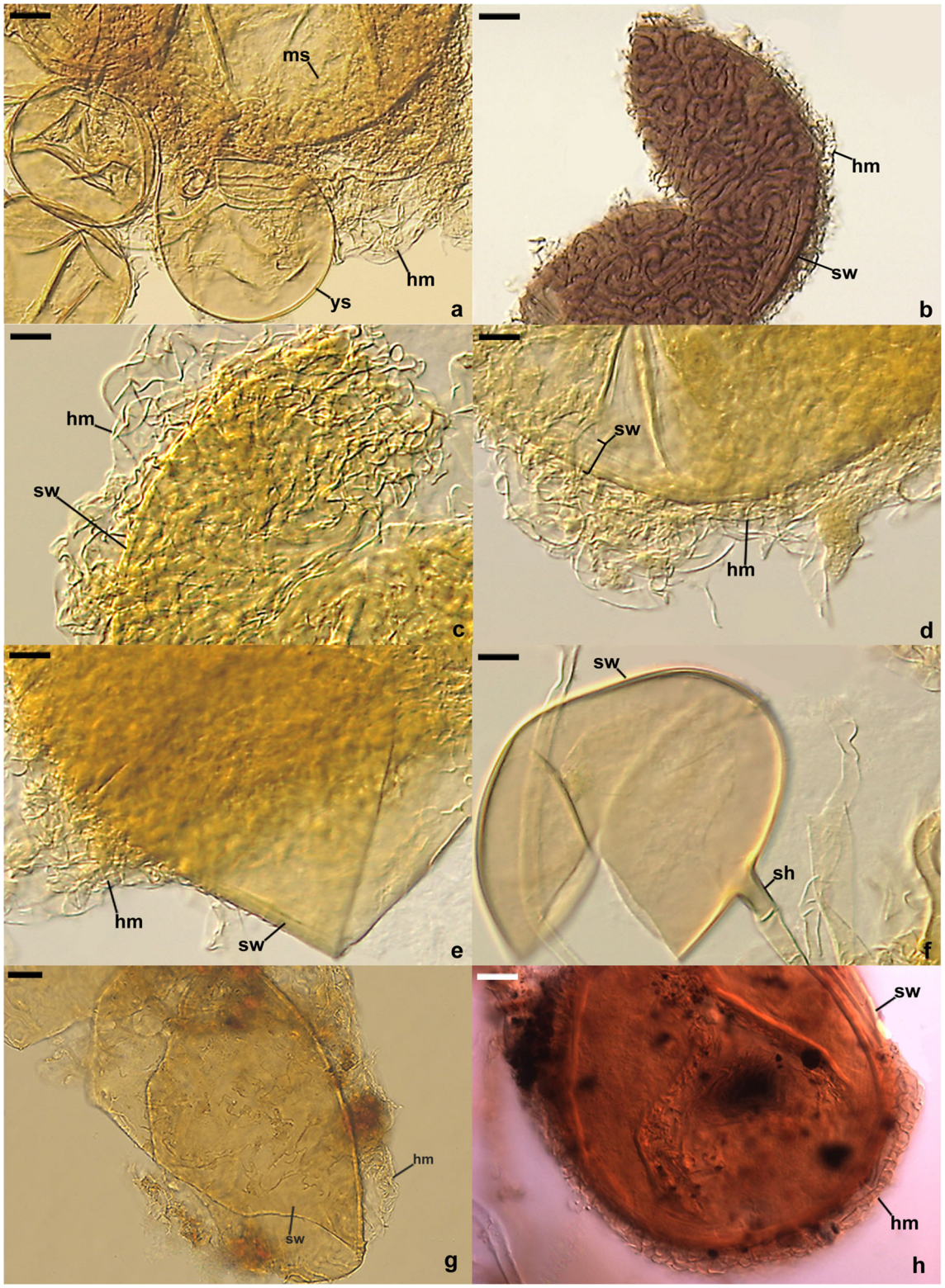



**Fig. 2** *Sieverdingia tortuosa*. **a** Cluster with a mature spore (ms) covered with a hyphal mantle (hm) and young, naked (without a mantle) spores. **b**–**e**, **g** Spores individually covered with a hyphal mantle (hm); note the single-layered, laminate spore wall (sw), whose laminae clearly separated from each other in **d**. **f** Spore wall (sw) and subtending hypha (sh) of a young, naked spore. **a**–**f** Spores from a trap culture inoculated with the rhizosphere soil of *A. arenaria* growing in USA sand dunes. **g** Spore from sand dunes of Brazil. **h** Spore from type material (OSC #40251). **b**, **c**, **g**, **h** Spores in PVLG. **a**, **d**, **e**, **f** Spores in PVLG+Melzer's reagent. **a**–**h** Differential interference microscopy. Scale bars: **a**, **b** = 20 μm, **c**–**h** = 10 μm

hyphal mantles or with two to < 20 spores arisen at the top of sporogenous hyphae dichotomously branched from a parent hypha continuous with an extraradical mycorrhizal hypha. *Subcellular structure of spores* consists of one spore wall containing three or four layers, of which the innermost layer is laminate, flexible to semi-flexible, hyaline, relatively thick, frequently readily separating from the penultimate spore wall layer above the spore base, but it is associated with the penultimate spore wall layer at the spore base and with the inner surface of the spore subtending hyphal wall at the spore base or much below. *Subtending hypha* straight or recurved, cylindrical to funnel-shaped or constricted. *Subtending hyphal wall* continuous with and coloured similarly to or slightly lighter than the spore wall. *Pore* occluded by (i) ingrowth of the innermost colourless spore wall layer (layer 3 or 4), (ii) a septum continuous with the innermost laminae of the coloured laminate spore wall layer 2, (iii) both the structures, (iv) a septum continuous with the innermost laminae of spore wall layer 2 and adherent spore wall layer 3, and (v) occasionally by thickening of spore wall layer 2; the pore sometimes seems to be open (Medina et al. 2014). *Germination* by a germ tube penetrating through the lumen of the subtending hypha and/or the spore wall (see our Fig. 3h; Medina et al. 2014).

**Type species** *Corymbiglomus corymbiforme* (Błaszk.) Błaszk. & Chwat. Glomeromycota 274. 2012.

**Other species** *Corymbiglomus globiferum* (Koske & Walker) Błaszk & Chwat.

**Basionym** *Glomus globiferum* Koske & Walker. Mycotaxon 26: 133. 1986.

*Corymbiglomus pacificum* Oehl, J. Medina, P. Cornejo, Sánchez-Castro, G.A. Silva & Palenz. Mycotaxon 127: 176. 2014.

## Emendations of *Corymbiglomus corymbiforme*, *C. globiferum*, and *C. pacificum*

### *Corymbiglomus corymbiforme* (Błaszk.) Błaszk. & Chwat emend. Błaszk. Niezgoda & B.T. Goto (Figs. 3–h and 4a, b)

MycoBank no.: MB 564567

*Glomeromycota* (Kraków): 274. 2012.

**Basionym** *Glomus corymbiforme* Błaszk. Mycologia 87(5): 732. 1995.

**Etymology** Latin, *corymbiforme*, referring to the clustered, corymbiform organization of spores in clusters of the fungus.

**Specimens examined** Poland: Glomerospores extracted from: (i) field-collected rhizosphere soils of *A. arenaria*, 6.10.1993, *Błaszkowski J.*, (slides no.) *2022* (holotype, DEPSE), *Błaszkowski J.*, *2023–2025* (isotypes, DEPSE), 1.10.1991, *Błaszkowski J.*, *2004–2010* (DEPSE), 22.09.1992, *Błaszkowski J.*, *2011–2015* (DEPSE); *Petasites spurius* (Retz.) Rchb., 6.10.1993, *Błaszkowski J.*, *2019* (DEPSE); and *Hieracium umbellatum* L., 6.10.1993, *Błaszkowski J.*, *2020–2021* (DEPSE); and (ii) single-species cultures inoculated with the rhizosphere soil of *A. arenaria*, 5.11.2018, *Błaszkowski J.*, 3660–3667 (DEPSE). All the plant species colonized dunes of the Baltic Sea located near the city of Świnoujście (53° 55′ 03″ N 14° 17′ 39″ ′E) and were sampled by J. Błaszkowski.

**Diagnosis** Differs from other species of *Corymbiglomus* in the formation of glomerospores mainly in corymbiform clusters (rarely singly), in which spores are individually covered with a hyphal mantle produced by dichotomously branched hyphae grown from spore wall layer 1 and in the nucleotide composition of sequences of the 18S–ITS–28S nuc rDNA region.

**Description** Glomerospores occurring in corymbiform clusters when formed from branched sporophores (Fig. 3, b), rarely singly in soil when produced from straight sporophores. *Clusters* globose to subglobose, (180–)336(–490) μm diam., sometimes ovoid, 180–350 × 210–500 μm, composed of 2–13 (av. 6) spores enveloped individually by a hyphal mantle (Fig. 3, b, e). *Spores* pastel yellow (3A4) to orange (6A8), globose to subglobose, (50–)142(–220) μm diam., sometimes ovoid or pyriform, 110–125 × 120–200 μm, with one subtending hypha (Fig. 3–h). *Subcellular structure of spores* of one wall with three permanent layers. Layer 1 uniform (not divided into visible sublayers), semi-rigid, smooth or slightly roughened, hyaline to deep yellow (4A8), (0.7–)1.1(–1.7) μm thick, closely attached to layer 2 (Fig. 3c–h). Layer 2 laminate, pastel yellow (3A4) to orange (6A8), (3.9–)7.0(–10.0) μm thick (Fig. 3c–h). Layer 3 laminate, semi-flexible, hyaline, (0.5–)3.4(–5.8) μm thick, usually tightly adherent to layer 2 in young and field-collected, even vigorously crushed, spores, but frequently separating from layer 2 in moderately crushed young and older spores extracted from pot cultures (Fig. 3c–h), usually with a small protrusion invaginated in the lumen of the subtending hypha (Fig. 3d, f, h). *Subtending hypha* cream (4A3) to deep orange (5A8), straight or recurvate, flared, sometimes cylindrical or constricted at the spore base, (9.8–)21.0(–31.1) μm wide at the spore base (Fig. 3e–h). *Wall of subtending hypha* cream (4A3) to deep orange (5A8), (2.2–)6.5(–13.7) μm thick, composed of three layers continuous with spore wall layers 1–3; subtending hyphal wall layer 3 present only at or far below the spore base (Fig. 3d–h). *Pore* occluded by (i) ingrowth of spore wall layer 3; (ii) a septum, (4.5–)9.6(–15.3) μm wide and (1.5–)1.7(–2.0) μm thick, continuous with the innermost lamina of spore wall layer 2; (iii) both the structures; and occasionally by (iv)

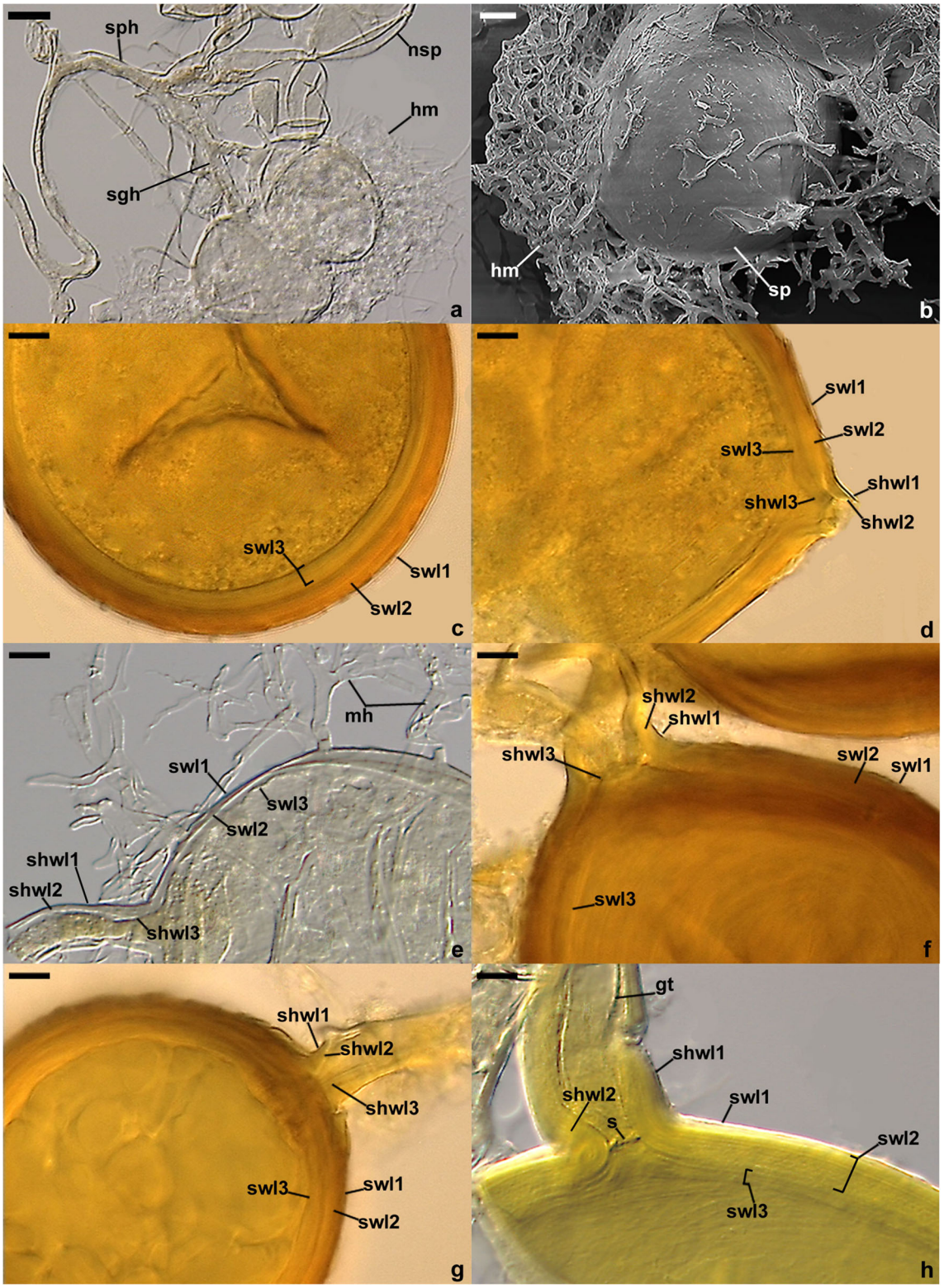



**Fig. 3** *Corymbiglomus corymbiforme*. **a** Cluster with young naked spores (nsp) and spores covered with hyphal mantle (hm); spores arisen terminally on sporogenous hyphae (sgh) dichotomously branched from sporophore (sph). **b** Spore (sp) with hyphal mantle (hm). **c** Spore wall layers (swl) 1–3; note the thick, colourless swl3. **d**, **f**–**h** Spore wall layers (swl) 1–3 continuous with subtending hyphal wall layers (shwl) 1–3; note the subtending hyphal pore closed by the invaginated swl3 in **d**, **f** and **h** and both swl3 and a septum (s) connecting the inner surfaces of the laminate swl2 in **h**; in **g**, the pore is open; a germ tube (gt) in the subtending hyphal lumen is visible in **h**. **e** Subtending hyphal wall layers (shwl) 1–3 continuous with spore wall layers (swl) 1–3 and mantle hyphae (mh) grown from swl1; note shwl3 that started developing far below the spore base. **a**, **c**, **e**–**h** Spores in PVLG. **d** Spore in PVLG+Melzer's reagent. **a**, **c**–**h** Differential interference microscopy. **b** Scanning electron microscopy. Scale bars: **a** = 20 μm, **b**–**h** = 10 μm

thickening of spore wall layer 2 (Fig. 3d, f, h), rarely open (Fig. 3e). *Mantle* (20.0–)47.5(–90.0) μm thick, consisting of a network of hyphae branching dichotomously three to four times at more or less right angles; hyphae thin-walled, hyaline to yellowish white (3A2), septate; length and diameter of branches diminishing with each successive dichotomy; initial hypha (9.8–)17.7(–27.2) μm long, (4.2–)4.9(–6.9) μm wide; developing from spore wall layer 1; final branch (13.5–)19.4(–25.0) μm long, (1.2–)1.8(–2.9) μm wide; distance between septa (14.5–)25.7(–31.4) μm; mantle frequently absent in mature spores (Fig. 3, b, e). *Sporophore* coenocytic to sparsely septate, hyaline to yellow (5A6), (10.3–)14.7(–17.5) μm wide, with a wall (1.5–)1.6(–1.7) μm thick, usually with two to three main monopodial branches, rarely straight; main branches frequently with one to ten (av. 4) monopodial second branches sometimes branched monopodially one or two times; main, second, and third branches slanted at 30–45° towards their parent hyphae; straight and branched sporophores bearing spores by swelling hyphal tips (Fig. 3). Mantle and spores not reacting in Melzer's reagent. *Germination* by a germ tube penetrating through the lumen of the subtending hypha (Fig. 3 h).

**Mycorrhizal associations** In the field, *C. corymbiforme* was probably associated with roots of *A. arenaria*, *Artemisia campestris* L., *Corynephorus canescens* (L.) P. Beauv., *Festuca polesica* Zapał., *Galium mollugo* L., *Helichrysum arenarium* (L.) Moench, *Hieracium pilosella* L., *H. umbellatum*, *Petasites spurius*, *Potentilla anserina* L., *Rosa rugosa* Thunb., *Solidago virgaurea* L., and *Viola tricolor* L. (Błaszkowski 1995, unpubl. data; Tadych and Błaszkowski 2000; Błaszkowski et al. 2002a, 2002b; Błaszkowski and Czerniawska 2006). However, no molecular analysis was performed on roots of these plant species to confirm this supposition. In single-species cultures, *C. corymbiforme* formed mycorrhiza with arbuscules and intra- and extraradical hyphae staining moderately to intensively [pale violet (17A3) to violet (17B7)] in 0.1% Trypan blue (Fig. 4a, b).

**Distribution and habitat** Glomerospores of *C. corymbiforme* were originally extracted from maritime dunes located near Świnoujście in north-western Poland (Błaszkowski 1995). Other records of this species also originate mainly from dune sites: the Słowiński National Park (Tadych and Błaszkowski 2000) and the Mierzeja Wiślana spit (Błaszkowski et al. 2002a), both belonging to Poland, dunes of the Mediterranean Sea located near Karabucak-Tuzla (Turkey), Tel-Aviv (Israel), Majorca (Spain; Błaszkowski and Czerniawska 2006; Błaszkowski, unpubl. data), and inland dunes of the Pustynia Błędowska desert (Poland, Błaszkowski et al. 2002b). In addition, *C. corymbiforme* was found in semiarid open sandy grasslands of Hungary (Takacs and Bratek 2006; Błaszkowski, unpubl. data).

Comparisons of the 18S–ITS–28S sequences of *C. corymbiforme* with sequences deposited in public databases did not show any record of this species.

### *Corymbiglomus globiferum* (Koske & C. Walker) Błaszk. & Chwat emend. Błaszk. Niezgoda & B.T. Goto (Fig. 4c–f)

MycoBank no.: MB 622179
Acta Mycol. 48(1): 99. 2013.

**Basionym** *Glomus globiferum* Koske & Walker. Mycotaxon 26: 133. 1986.

**Etymology** Latin, *globiferum* (= "sphere bearing"), referring to the spherical or near-spherical vesiculate swellings produced by mantle hyphae on the outside of spores (Koske and Walker 1986).

**Specimens examined** Brazil: Glomerospores extracted from trap cultures inoculated with the rhizosphere soils of two sand dune plant species: *C. crassipes* growing near the city of Galinhos (UFRN Fungos-3166) and *E. polyphyla* growing in the RDSE Ponta do Tubarão (UFRN-Fungos-3167), both located in the Rio Grande do Norte State.

**Diagnosis** Differs from other species of *Corymbiglomus* in the formation of single glomerospores individually covered with a hyphal mantle containing vesiculate swellings, whose hyphae grow from spore wall layer 1 and the subtending hyphal wall, as well as in the nucleotide composition of sequences of the 18S–ITS–28S nuc rDNA region.

**Description** Glomerospores hypogeous, formed singly, occasionally in clusters with two to several spores connected by interwoven hyphae of a mantle individually covering each spore. *Spores* dark orange (5A8) to brownish red (9C8), globose to subglobose, (160–)249(–320) μm diam., with one subtending hypha (Fig. 4c–f). *Subcellular structure of spores* consists of one wall composed of three layers. Layer 1 uniform (not divided into visible sublayers), semi-permanent, semi-rigid, smooth or slightly roughened, hyaline to light orange (5A4), (1.2–)1.7(–2.3) μm thick, closely attached to layer 2, occasionally slightly deteriorated in older spores (Fig. 4d–f). Layer 2 laminate, permanent, smooth, dark orange (5A8) to brownish red (9C8), (10.0–)17.1(–23.8) μm thick (Fig. 4d–f); the thickness is frequently higher in crushed spores due to the plastic properties of this layer. Layer 3 laminate, permanent, semi-flexible, hyaline, (2.0–)2.9(–4.8) μm thick, usually tightly adherent to layer 2, usually with a small protrusion invaginated in the lumen of the subtending hypha (Fig. 4d–f). *Subtending hypha* pale yellow (4A3) to reddish orange (7A6), straight or recurvate, cylindrical or slightly constricted at the spore base, rarely funnel-shaped, 15.0–27.0 μm wide at the spore base

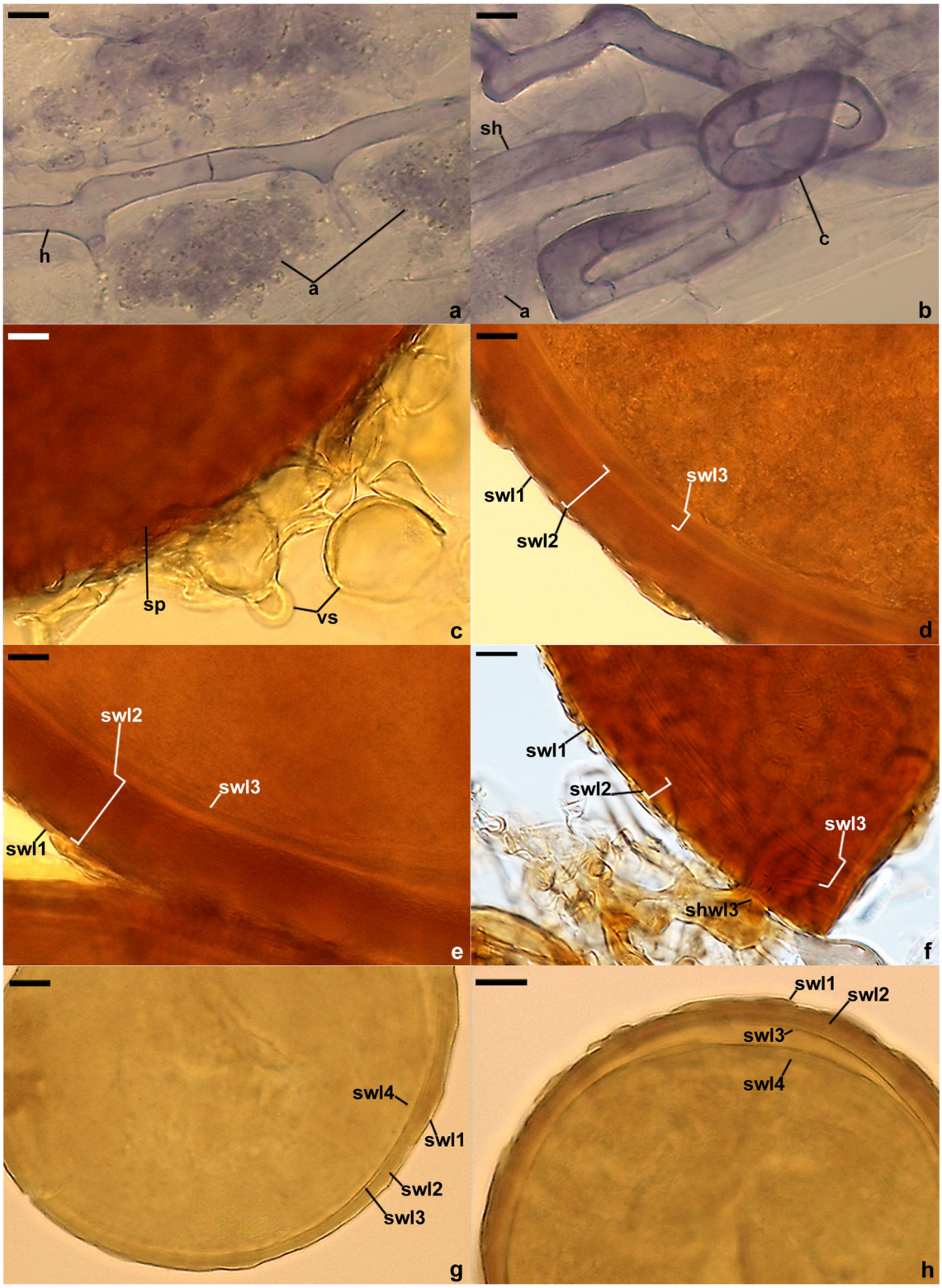



**Fig. 4** **a**, **b** *Corymbiglomus corymbiforme*. **a** Arbuscules (a) and intraradical hypha (h). **b** Arbuscule (a) and straight (sh) and coiled (c) intraradical hyphae. **c**–**f** *Corymbiglomus globiferum*. **c** Spore (s) covered with a hyphal mantle with vesiculate swellings (vs). **d**–**f** Spore wall layers 1–3; note the separated sublayers of swl3 in **e** and **f**. **g**, **h**. *Corymbiglomus pacificum*. **g**, **h** Spore wall layers (swl) 1–4. **a**, **b**, **d**, **f**–**h** Spores in PVLG. **c**, **e** Spores in PVLG+Melzer's reagent. **a**–**h** Differential interference microscopy. Scale bars: **a**–**h** = 10 μm

(Fig. 4f). *Wall of subtending hypha* pale yellow (4A3) to reddish orange (7A6), 2.0–8.0 μm thick, composed of three layers continuous with spore wall layers 1–3 (Fig. 4f). *Pore* occluded by (i) ingrowth of spore wall layer 3, (ii) a septum continuous

with the innermost lamina of spore wall layer 2, and (iii) occasionally by a granular plug. *Germination* by a germ tube penetrating through the lumen of the subtending hypha. *Mantle* 5–47 μm thick, consisting of loosely or tightly interwoven hyphae bearing numerous terminal or intercalary vesiculate swellings (Fig. 4c); hyphae coenocytic or sparsely septate, hyaline to pale yellow to yellow-brown, 5.0–50.0 μm wide, with walls (1.0–)1.5(–1.8) μm thick; swellings hyaline to pale yellow-brown, globose to ovoid, 12–75 μm diam., with a single-layered wall, 0.8–2.0 μm thick. Mantle and spores not reacting in Melzer's reagent.

**Mycorrhizal associations** In the field, *C. globiferum* probably formed mycorrhiza with *A. breviligulata* and *U. paniculata* (Koske and Walker 1986; Sylvia 1986; Sylvia and Will 1988; Wu and Sylvia 1993), as well as with *C. crassipes* and *E. polyphylla* (pers. observ.), although no molecular analysis was performed on roots of these plant species to confirm this supposition. BLAST searches indicated that, of the *C. globiferum* sequences used in our analyses (Fig. 1), only the partial 28S sequence FJ461836 showed 97.32–97.74% identity to two partial 28S sequences (AB670091, AB670092) obtained from DNA extracted from roots of *I. repens* (Yamato et al. 2012). However, the association of *C. globiferum* with *I. repens* was not confirmed when our 18S–ITS–28S sequences were used in the searches. In single-species cultures, *C. globiferum* formed mycorrhiza with *U. paniculata*, but the level of colonization was low (Sylvia and Burks 1988).

**Distribution and habitat** *Corymbiglomus globiferum* was originally characterized from glomerospores found in sand dunes at Cape May, New Jersey (Koske and Walker 1986). Sylvia (1986), Sylvia and Will (1988), and Wu and Sylvia (1993) extracted spores of this species from costal sand dunes in Florida. The specimens of this fungus used in our analyses originated from saline and no saline sand dunes located at the city of Galinhos (78° 02′ S, 94° 37′ W) and in the RDSE Ponta do Tubarão (5° 5′ 3.55″ S, 36° 28′ 19.13″ W), Brazil. In addition, Yamato et al. (2012) concluded from molecular environmental analyses that *C. globiferum* occurred in roots of *I. repens* sampled near the Tottrii sand dunes in Japan.

### *Corymbiglomus pacificum* Oehl, J. Medina, P. Cornejo, Sánchez-Castro, G.A. Silva & Palenz. emend. Błaszk. Niezgoda & B.T. Goto (Figs. 4g, h and 5a–h).

MycoBank no.: MB 805601
Mycotaxon 127: 176. 2014.

**Etymology** Latin, *pacificum*, referring to the Pacific Ocean, which is adjacent to the isolation site (Medina et al. 2014).

**Specimens examined** Slides with glomerospores permanently mounted in PVLG and PVLG+Melzer's reagent loaned from the mycological herbarium of ETH Zurich (Z+ZT, Switzerland): ZT Myc 49005–holotype (two slides), ZT Myc 49006–isotypes (six slides).

**Diagnosis** Differs from other species of *Corymbiglomus* in the formation of single glomerospores not covered with a hyphal mantle, in having spores with a four-layered spore wall, and in the nucleotide composition of sequences of the 28S nuc rDNA region.

**Description** Glomerospores hypogeous, formed singly. *Spores* bright yellow to dark yellow to rarely brownish yellow, globose to subglobose to rarely ellipsoid to irregular, (85–)95–130(–135) × (75–)85–125(–131) μm diam., with one subtending hypha (Figs. 4g, h and 5a–h). *Subcellular structure of spores* consists of one spore wall with four layers. Layer 1 evanescent to semi-permanent, subhyaline to light yellow, 0.6–1.2 μm thick, usually tightly adherent to layer 2 (Figs. 4g, h and 5a–d). Layer 2 permanent, laminate, smooth, bright yellow to dark yellow to brownish dark yellow, 1.8–3.5(–4.5) μm thick (Figs. 4g, h and 5a–d). Layer 3 permanent, uniform (not divided into visible sublayers), hyaline to dull yellow (4B3), 0.4–0.7 μm thick, usually separating from layer 3 only in vigorously crushed spores, and, therefore, difficult to see (Figs. 4g, h and 5a). Layer 4 permanent, laminate, hyaline, (2.3–)3.4(–4.7) μm thick, usually with a small protrusion invaginated in the lumen of the subtending hypha (Fig. 4g, h and 5a–d). In Melzer's reagent, only spore wall layer 4 may stain light yellow to bright dark yellow. *Subtending hypha* usually lighter than spores, light yellow (4A4) to greyish orange (5B5), straight, rarely recurvate, cylindrical, sometimes constricted at the spore base, 7.0–12.0 μm wide at the spore base (Fig. 5c, d). *Wall of subtending hypha* light yellow (4A4) to greyish orange (5B5), 2.4–5.7 μm thick at the spore base, composed of four layers continuous with spore wall layers 1–4 at the spore base, and two layers continuous with spore wall layers 1 and 2 below the spore base (Fig. 5c, d). *Pore* usually occluded by a straight or slightly curved septum, (3.2–)3.7(–4.4) μm wide and ca. 2.0 μm thick, continuous with spore wall layers 2–4 (Fig. 5d), rarely open. *Germination* by a germ tube growing from spore wall layer 4 and penetrating through the other spore wall layers.

**Mycorrhizal associations** In the field, *C. pacificum* was associated with roots of *A. arenaria* (Medina et al. 2014). Attempts to propagate this species in culture failed. Thus, morphological features of *C. pacificum* mycorrhizal structures remain unknown.

**Distribution and habitat** So far, *C. pacificum* was physically identified only among roots of *A. arenaria* growing at the mouth of Lake Budi, a saline ecosystem periodically connecting with

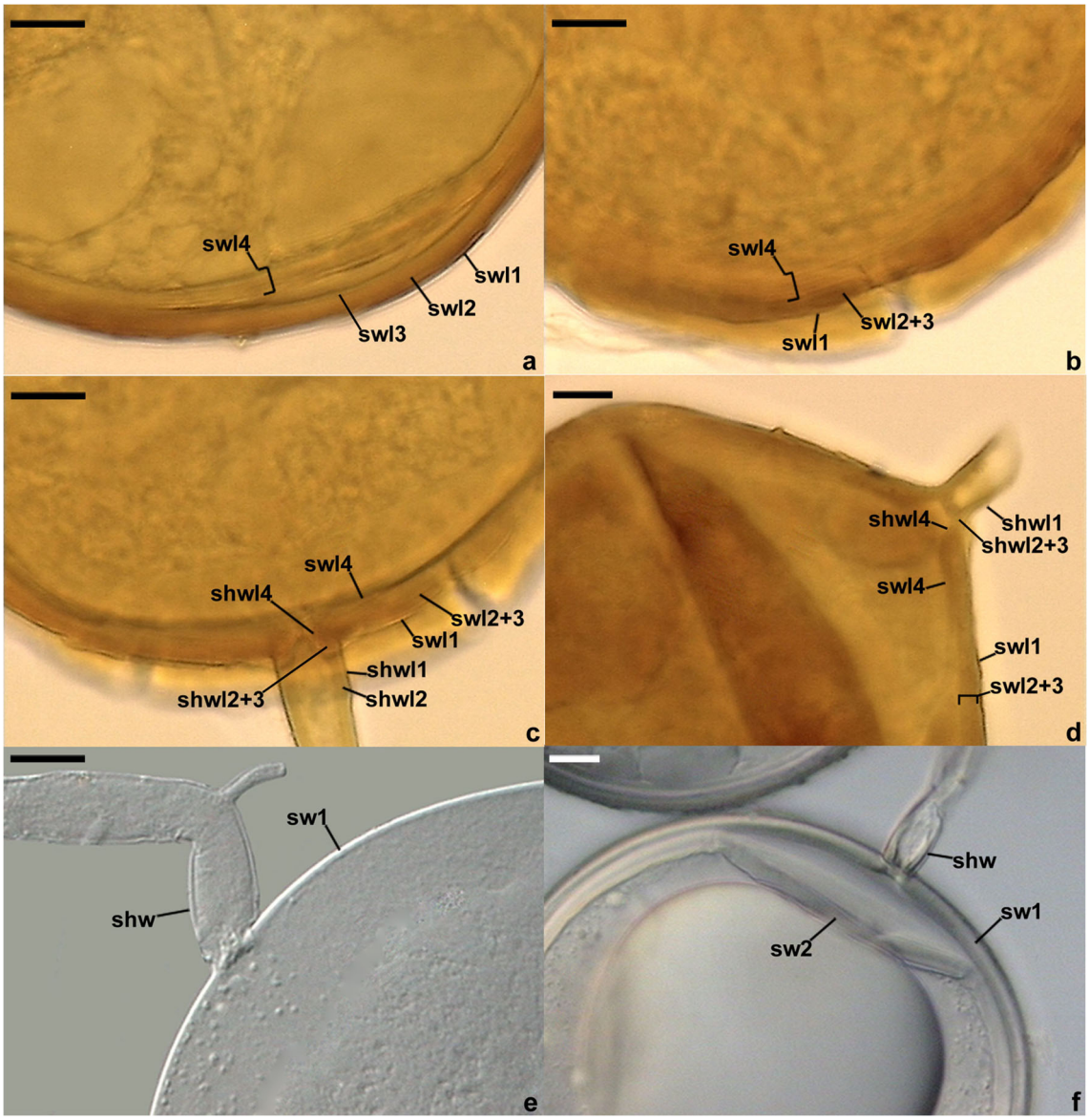



**Fig. 5** **a**–**d** *Corymbiglomus pacificum*. **a**, **b** Spore wall layers (swl) 1–4; note the laminae of the laminate swl4. **c**, **d** Spore wall layers (swl) 1–4 continuous with subtending hyphal wall layers (shwl) 1–4; note shwl4 is present only at the spore base; shwl3 probably is present, but is invisible because it is very thin and of colour similar to that of shwl2. **e**, **f** *Pacispora scintillans*. **e** Subtending hyphal wall (shw) continuous with spore wall 1 (sw1) of a juvenile specimen; note the single-walled subcellular structure of the spore. **f** Subtending hyphal wall (shw) continuous with spore wall 1 (sw1) having no physical contact with spore wall 2 (sw2) of a mature specimen. **a**–**f** Spores in PVLG. **a**–**f** Differential interference microscopy. Scale bars: **a**–**f** = 10 μm

the Pacific Ocean, located near the municipality Puerto Saavedra in La Araucanía Region (southern Chile; Medina et al. 2014). There are no available molecular data that would indicate that *C. pacificum* also occurs in other sites of the world.

## Description of a new species

***Diversispora peloponnesiaca*** **Błaszk., B.T. Goto, Orfanoudakis & Niezgoda sp. nov. (Fig. 6a–h).**

MycoBank no.: MB 832300

**Etymology** Latin, *peloponnesiaca*, referring to the Peloponnese peninsula, where this species was originally found.

**Specimens examined** Poland: Glomerospores from a single-species culture established from spores extracted from a trap culture inoculated with the rhizosphere soil of *A. arenaria* that colonized Mediterranean Sea dunes of the beach Voidokoilia (36° 57′ 46″ N 21° 39′ 45″ E) located on the Peloponnese peninsula, Greece. The field soil sample was collected by Dr. Dimitris Arrianas on July 18, 2012. Holotype. Slide with spores no. ZT Myc 60277 (Z+ZT), isotypes: slides with spores no. 3650–3659 (DEPSE).

**Diagnosis** Differs from other *Diversispora* spp. in producing glomerospores that are darkest in colour and in the nucleotide composition of sequences of the 18S–ITS–28S nuc rDNA region.

**Description** Glomerospores formed singly in soil (Fig. 6a). Spores arise blastically at the tip of sporogenous hyphae. *Spores* orange (5B8) to reddish brown (8E8) when mature, light yellow (3A5) to reddish yellow (4A7) when young; usually ovoid; 108–142 × 127–171 μm; less often globose to subglobose; (113–)139(–163) μm diam.; with one subtending hypha (Fig. 6a–f). *Spore wall* composed of three permanent layers. Layer 1, forming the spore surface, semi-flexible, smooth, deep yellow (4A8) to yellowish brown (5D8) in mature spores, hyaline in young spores, (1.3–)1.8(–2.3) μm thick (Fig. 6b–f, h). Layer 2 laminate, semi-flexible, orange (5B8) to reddish brown (8E8) in mature spores, light yellow (3A5) to reddish yellow (4A7) in young spores, (6.3–)8.4(–11.3) μm thick (Fig. 6b–f, h). Layer 3 uniform (not divided into visible sublayers), semi-flexible, hyaline to greyish yellow (4C3), (1.0–)1.1(–1.3) μm thick, usually tightly adherent to the lower surface of layer 2, occasionally separating from this layer in vigorously crushed spores (Fig. 6b–f, h). Layers 1–3 not staining in Melzer's reagent. *Subtending hypha* hyaline to greyish yellow (4B3); straight or recurved, cylindrical, rarely slightly constricted at the spore base; (9.0–)11.8(–14.5) μm wide at the spore base (Fig. 6g, h); frequently breaking in crushed spores. *Wall of subtending hypha* hyaline to greyish yellow (4B3); (1.8–)2.1(–2.3) μm thick at the spore base; consisting of one layer continuous with spore wall layer 1 (Fig. 6g, g). *Pore* (6.3–)8.9(–10.3) μm wide at the spore base, occluded by either a straight or slightly curved septum, 1.5–2.0 μm thick, connecting the inner surfaces of the subtending hyphal wall at or up to 2.5 μm below the spore base (Fig. 6g), or a curved septum continuous with spore wall layer 3 (Fig. 6h). *Germination* unknown.

**Mycorrhizal associations** In the field, *D. peloponnesiaca* probably lived in symbiosis with *A. arenaria*, although no molecular analysis was performed on roots of this plant species to confirm this hypothesis. In single-species cultures with *P. lanceolata* as the host plant, *D. peloponnesiaca* formed mycorrhiza with arbuscules, vesicles, and intraradical and extraradical hyphae that stained violet white (16A2) to dark violet (16F8) in 0.1% Trypan blue.

**Distribution and habitat** To date, *D. peloponnesiaca* was physically found in only one trap culture representing dunes of the Mediterranean beach Voidokoilia (36° 57′ 46″ N 21° 39′ 45″ E) located on the Peloponnese peninsula, Greece. BLAST queries did not show any sequence of ≥ 97% identity to the 18S–ITS–28S sequences of *D. peloponneseae*. The identity of all listed sequences was < 95.73%.

## Discussion

The molecular phylogenetic analyses discussed above fully confirmed our hypothesis that the fungus originally described as *G. tortuosum* (Schenck and Smith 1982) and later accommodated in the genus *Corymbiglomus* (Błaszkowski and Chwat 2013) should be transferred to a new genus, here described as *Sieverdingia*, in the family Diversisporaceae. Importantly, the transfer removed inconsistencies between the morphological features of spores of *C. corymbiforme*, *C. globiferum*, and *C. pacificum* versus those of *S. tortuosa* (Schenck and Smith 1982; Koske and Walker 1986; Błaszkowski 2012; Medina et al. 2014; http://fungi.invam.wvu.edu/the-fungi/classification/diversisporaceae/diversispora/tortuosum.html; http://fungi.invam.wvu.edu/the-fungi/classification/diversisporaceae/diversispora/globiferum.html). Finally, the analyses confirmed our supposition, resulting from morphological observations, that the diversisporoid spore-producing fungus that we found and grew in cultures is a new species in the genus *Diversispora* (Fig. 1).

The phylogeny of *Sieverdingia* was reconstructed from analyses of 18S–ITS–28S nuc rDNA sequences and partial sequences of the 28S gene of the so far sole species of the new genus, *S. tortuosa* (Fig. 1). The nesting of the FJ461850 partial 28S sequence obtained from spores of *S. tortuosa* grown in the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM; treated as *D. tortuosa*) between three *S. tortuosa* 18S–ITS–28S sequences obtained from spores originating from China proved that the four sequences characterize one species and strongly supported the validity of the erection of *Sieverdingia*. The accuracy and resolution power of phylogenies generally are higher when the number of analyzed molecular sequences and the amount of information contained in these sequences are higher (Redecker et al. 2013; Stockinger et al. 2014).

Currently, the living culture from which type specimens of the fungus originally described as *G. tortuosum* were extracted (Schenck and Smith 1982) does not exist (http://fungi.invam.wvu.edu/the-fungi/classification/diversisporaceae/diversispora/tortuosum.html). However, the morphology of spores of the type specimens permanently mounted on slides (B.T. Goto, pers. observ.; Fig. 2h), as well as that of spores extracted from living cultures grown by Dr. J.B. Morton in INVAM, B.T. Goto (Fig. 2g), and by J. Błaszkowski (Fig. 2a–f; Błaszkowski 2012; http://fungi.invam.wvu.edu/the-fungi/classification/diversisporaceae/diversispora/tortuosum.html; http://www.zor.zut.edu.pl/Glomeromycota/) unambiguously indicates that the spores were produced by one species, *S. tortuosa*. Our *S. tortuosa* morphologically characterized above (see the section "Erection of a new genus"; Fig. 2a–h) slightly differed in only three features from *G. tortuosum* and *D. tortuosa* described and illustrated by Schenck and Smith

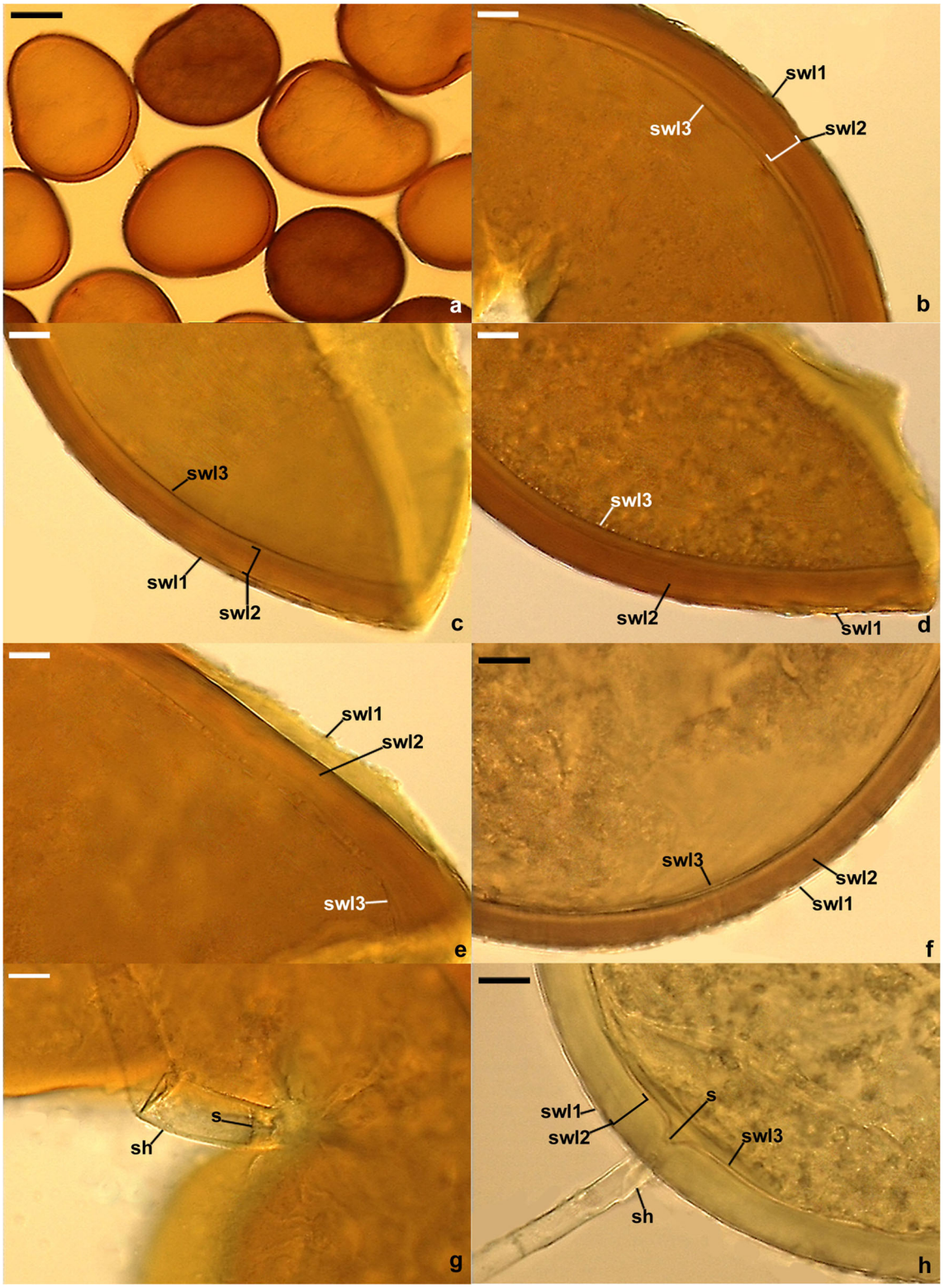



**Fig. 6** **a**–**f** *Diversispora peloponnesiaca*. **a** Intact spores. **b**–**f** Spore wall layers (swl) 1–3. **g** A straight septum (s) connecting the inner surfaces of the subtending hyphal (sh) wall located slightly below the spore base. **h** Spore wall layers (swl) 1–3, subtending hypha (sh), and a curved septum (s) continuous with swl3, closing the lumen of sh. **a** Spores in lactic acid. **b**, **c**, **e**–**h** Spores in PVLG+Melzer's reagent. **d** Spore in PVLG. **a**–**h** Differential interference microscopy. Scale bars: **a** = 50 μm, **b**–**h** = 10 μm

(1982) and Morton (http://fungi.invam.wvu.edu/the-fungi/classification/diversisporaceae/diversispora/tortuosum.html), respectively. The spores were (i) slightly lighter than those defined by Schenck and Smith (1982; yellow to dull grey-

brown) and Morton (light yellow-brown to orange brown), (ii) their spore wall was slightly thicker than 0.5–2.0 μm (Schenck and Smith 1982; no data in Morton's website), and (iii) their subtending hypha was not as wide as 20–26 μm (Schenck and Smith 1982), but the range of its width was similar to that given by Morton.

Morphologically, *S. tortuosa* is distinguished mainly by its single-layered, coloured spore wall, which is covered with a mantle consisting of straight or sinuous hyphae (Schenck and Smith 1982; Błaszkowski 2012; http://fungi.invam.wvu.edu/the-fungi/classification/diversisporaceae/diversispora/tortuosum.html; Fig. 2–h). The mantle hyphae are frequently interwoven with those of a spore or spores arising nearby, and therefore, *S. tortuosa* usually forms clusters with randomly distributed spores. Of the 15 described species of the Glomeromycota that produce glomoid or glomoid-like coloured spores with a single-layered spore wall, which do not arise radially from a central sterile hyphal plexus as spores of *Sclerocystis* spp., only spores of *S. tortuosa* and *G. convolutum* Gerd. & Trappe are individually enclosed in a hyphal mantle (Gerdemann and Trappe 1974; Schenck and Smith 1982; Błaszkowski 2012). Both species are easily distinguishable from one another based on their morphology. While spores of *S. tortuosa* arise singly or in rather loose clusters (Fig. 2a–h), those of *G. convolutum* are formed in compact glomerocarps (Gerdemann and Trappe 1974). The laminate spore wall layer of the former species is nonreactive in Melzer's reagent (Fig. 2a, d–f), and in the latter fungus it stains deep orange brown in this reagent. Finally, the oily content of *S. tortuosa* spores is colourless, whereas the oil filling *G. convolutum* spores is deep yellow. Unfortunately, we cannot support the unambiguous morphological separateness of the two species using molecular proofs because *G. convolutum* has no DNA markers sequenced and known molecular phylogeny so far, similarly as the other species of this fungal group, except for *R. fulva* (Berk. & Broome) C. Walker & A. Schüßler, *R. megalocarpa* (D. Redecker) C. Walker & A. Schüßler, and *R. pulvinata* (Henn.) C. Walker & A. Schüßler. Our research revealed that the genus *Redeckera* is a sister of *Sieverdingia* (Fig. 1), whose molecular divergence is high (by 10.5–11.9%).

The morphological analyses discussed here also confirmed our hypothesis that the genus *Corymbiglomus* should contain only species, whose a common feature is the formation of spores with a single spore wall, in which the innermost component is a relatively thick, colourless, laminate layer that frequently separates from the other spore wall layers, but usually adheres to the inner surface of the subtending hyphal wall in crushed spores (Figs. 3c, d, f–h, 4d–h and 5a–d). Such requirements meet only *C. corymbiforme*, *C. globiferum*, and *C. pacificum*. The following data presented below clearly justify our conclusion.

Species of *Pacispora* produce spores with two spore walls, of which the inner wall 2 arises de novo after the full differentiation of wall 1, forming the spore surface, and wall 2 has no physical contact with wall 1 (Fig. 5e, f; Błaszkowski 1988, 2012; Oehl and Sieverding 2004; Walker et al. 2004). Instead, Fig. H illustrating the spore base of *C. corymbiforme* (Błaszkowski 2012), pictures of *C. globiferum* (treated as *D. globifera*; http://fungi.invam.wvu.edu/the-fungi/classification/diversisporaceae/diversispora/globiferum.html), and Fig. 8 showing a crushed *C. pacificum* spore (Medina et al. 2014) clearly show that the innermost hyaline spore subcellular component [characterized by Błaszkowski (2012) and Morton (http://fungi.invam.wvu.edu/the-fungi/classification/diversisporaceae/diversispora/globiferum.html) as spore wall layer 3 in *C. corymbiforme* and *C. globiferum*, respectively, and by Medina et al. (2014) as spore wall 2 in *C. pacificum*] is associated with the spore base. Moreover, detailed examination of the subcellular structure of *C. corymbiforme* spores freshly extracted from single-species cultures, *C. globiferum* spores extracted from trap cultures and field-collected sandy soils (Błaszkowski and B.T. Goto, pers. observ.), as well as type specimens of *C. pacificum* proved that the innermost spore subcellular component consists of sublayers (laminae) and adheres to the inner surfaces of both spore wall layer 2 (in *C. corymbiforme* and *C. globiferum*) or layer 3 (in *C. pacificum*) at the spore base and the penultimate subtending hyphal wall layer, and is present either only at or far below the spore base (Figs. 3c, d, f–h, 4d–h and 5a–d), where it likely begins arising. Thus, the location of the innermost spore subcellular component in the spore subcellular structure of the three species of *Corymbiglomus* is identical to that of the innermost spore wall component of, for example, *Claroideoglomus claroideum* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler (layer 4) and *D. clara* (layer 3). Ontogenetic studies of *C. claroideum* spores revealed that the attachment of spore wall layer 4 to the inner surface of the subtending hyphal wall indicates common origin of the subtending hypha and the spore wall, and, consequently, testifies that spore wall layer 4 represents one structure, the spore wall (Stürmer and Morton 1997). Thus, although the ontogeny of the three species of *Corymbiglomus* discussed here is unknown, the attachment of the innermost spore subcellular component to the penultimate layers of the spore and subtending hyphal walls supports our conclusion that all components of the subcellular spore structure of these species also constitute one spore wall. Finally, the position of the innermost spore wall layer in the subcellular spore structure of *Corymbiglomus* spp. and, especially, its phenotypic features (a relatively thick, colourless laminate layer; Figs. 3c, d, f–h, 4d–h and 5a–d) suggest that this layer is a synapomorphy.

Interestingly, our preliminary molecular phylogenetic analyses indicated that the position of *C. corymbiforme* among the analyzed members of the Diversisporaceae (Fig. 1) changed depending on the species composition of the outgroup (data not shown). When the outgroup of the analyzed alignment was represented by two sequences of only *A. laevis* or only *S. baltica*, the position of *C. corymbiforme* was as that

depicted in Fig. 1. However, when the outgroup was two sequences of only *P. scintillans*, *C. corymbiforme* formed a two-species generic clade with *R. megalocarpum*, a species differing fundamentally in morphology, ecology, and highly distant molecularly (by 12.9%) from *C. corymbiforme* (Błaszkowski 2012; Redecker et al. 2007). Moreover, our sequence comparisons indicated the *C. corymbiforme* clade is divergent from the neighbouring *C. pacificum* clade by 11.4–13.7%, which is roughly equivalent to intergeneric divergences of other taxa of the Glomeromycota (Błaszkowski et al. 2018a, 2018b; Jobim et al. 2019). Instead, the molecular distance between the sister *C. globiferum* and *C. pacificum* clades is at the level of interspecies divergences of AMF, i.e. 4.3–6.0%. Thus, the analyses and comparisons suggest that the relationship of *C. corymbiforme* with *C. globiferum* and *C. pacificum* is not only distant but also weak, and that *C. corymbiforme* could alone occupy a generic clade. This may turn out to be true when at least one other new species will be discovered and its molecular similarity to *C. corymbiforme* will be higher than to *C. pacificum*.

Based on available own and literature data, as well as on comparisons of molecular sequences of *S. tortuosa* and the three species retained here in *Corymbiglomus* with molecular sequences of AMF deposited in public databases, we conclude that these species probably occur rather infrequently on Earth.

However, as indicated in the section "Taxonomy", the intraradical presence of *S. tortuosa* and *C. globiferum* was revealed in environmental studies only based on the partial 28S FJ461850 and FJ461836 sequences, respectively, obtained from spores of these species. Instead, BLAST searches using 18S–ITS–28S sequences of these two species, as well as *C. corymbiforme*, showed no hits. In order to explain this inconsistency, we compared the *S. tortuosa* and *C. globiferum* 18S–ITS–28S sequences with 18S–ITS–28S sequences of the other AMF contained in the phylogenetic tree showed in Fig. 1. The comparisons indicated that the molecular content particularly strongly distinguishing the three species from the other species of the tree resides outside the 28S segment. Moreover, BLAST queries using the 18S CCGAAAGG TGGCCTTTTT, ITS1 AATAATTTTTTACCCCTCCT TTTGA and ATTATATATAAATTGTATTAAAAATATAC, 5.8S-ITS2 CTAAATATTAATCGTAAAT, and ITS2 ATCTCAGATGGGTTC sequences present in all analyzed 18S–ITS–28S sequences of *C. globiferum* did not show any other member of the Kingdom Fungi. The molecular content strongly diverging *S. tortuosa* from all other representatives of the Glomeromycota and all members of the Kingdom Fungi also resides in the ITS1 and ITS2 parts of the 18S–ITS–28S sequences of this species. Thus, we conclude that the predominance of the molecular power contained in the 18S–ITS segment of the *S. tortuosa* and *C. globiferum* 18S–ITS–28S sequences is so large that in comparisons it moves these 18S–ITS–28S sequences outside the barcodes of these species originally determined by the partial 28S FJ461850 and FJ461836 sequences. Therefore, BLAST searches omitted *S. tortuosa* and *C. globiferum* when the queries were their 18S–ITS–28S sequences.

Our phylogenetic analyses showed that the closest natural (molecular) relative of *D. peloponnesiaca* is *D. clara* (Fig. 1), whose morphology is strikingly different than that of the new species (Fig. 6a–h). Compared with all described species of *Diversispora*, mature *D. peloponnesiaca* spores are darkest in colour, whereas those of *D. clara* are colourless to creamy white (Estrada et al. 2011; Błaszkowski, pers. observ.). The spore wall of both species consists of three layers, but spore wall layer 1 of *D. peloponnesiaca* is permanent (Fig. 6b–f, e), i.e. it does not deteriorate with age as spore wall layer 1 of *D. clara*, which often is absent (completely sloughed off) in mature spores. Moreover, the adherence of spore wall layers 2 and 3 of *D. peloponnesiaca* is clearly stronger than in *D. clara*. In *D. peloponnesiaca*, these spore wall layers rarely separate from one another, even in vigorously crushed spores (Fig. 6b–f, h). Instead, crushing of *D. clara* spores by applying only moderate pressure usually separates spore wall layers 2 and 3 (Błaszkowski, pers. observ.). Finally, spores of *D. peloponnesiaca* are ca. 1.5-fold larger when globose, have a ca. 1.9-fold thicker spore wall, and their subtending hypha at the spore base is 1.8–2.3-fold wider and has a 1.3–1.8-fold thicker wall.

Morphologically, *D. peloponnesiaca* most resembles *D. jakucsiae* Błaszk., T.K. Balázs & Kovács because spores of the latter species are almost as darkly coloured [reddish yellow (4A7) to brownish red (8C8)] as mature spores of *D. peloponnesiaca* and the spore wall of both species consists of three permanent layers (Balázs et al. 2015). The only characters separating the two species are the tint and thickness of spore wall layer 1, and the strength of the adherence of spore wall layers 2 and 3. In *D. jakucsiae*, spore wall layer 1 is clearly lighter [hyaline to yellowish white (4A2)] and ca. 1.7-fold thinner than in *D. peloponnesiaca*, and spore wall layer 3 is more loosely associated with the laminate spore wall layer 2 because it usually easily separates from this layer in crushed spores. Most importantly, the two species are highly divergent molecularly from one another: three clades at the species level separate *D. peloponnesiaca* from *D. jakucsiae* in our phylogenetic tree (Fig. 1).

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# New Glomeromycotan Taxa, *Dominikia glomerocarpica* sp. nov. and *Epigeocarpum crypticum* gen. nov. et sp. nov. From Brazil, and *Silvaspora* gen. nov. From New Caledonia

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Examination of fungal specimens collected in the Atlantic rain forest ecosystems of Northeast Brazil revealed many potentially new epigeous and semihypogeous glomerocarp-producing species of the phylum Glomeromycota. Among them were two fungi that formed unorganized epigeous glomerocarps with glomoid spores of almost identical morphology. The sole structure that distinguished the two fungi was the laminate layer 2 of their three-layered spore wall, which in spores of the second fungus crushed in PVLG-based mountants contracted and, consequently, transferred into a crown-like structure. Surprisingly, phylogenetic analyses of sequences of the 18S-ITS-28S nuc rDNA and the *rpb1* gene indicated that these glomerocarps represent two strongly divergent undescribed species in the family Glomeraceae. The analyses placed the first in the genus *Dominikia*, and the second in a sister clade to the monospecific generic clade *Kamienskia* with *Kamienskia bistrata*. The first species was described here as *Dominikia glomerocarpica* sp. nov. Because *D. glomerocarpica* is the first glomerocarp-forming species in *Dominikia*, the generic description of this genus was emended. The very large phylogenetic distance and the fundamental morphological differences between the second species and *K. bistrata* suggested us to introduce a new genus, here named as *Epigeocarpum* gen. nov., and name the new species *Epigeocarpum crypticum* sp. nov. In addition, our analyses also focused

on an arbuscular mycorrhizal fungus originally described as *Rhizophagus neocaledonicus*, later transferred to the genus *Rhizoglomus*. The analyses indicated that this species does not belong to any of these two genera but represents a new clade at the rank of genus in the Glomeraceae, here described as *Silvaspora* gen. nov.

**Keywords: arbuscular mycorrhizal fungi, cryptic species, morphology, molecular phylogeny, 18S-ITS-28S nuc rDNA, *rpb1***

## INTRODUCTION

In the phylum Glomeromycota, the largest group is represented by species producing glomoid spores, which arise blastically at tips of cylindrical or funnel-shaped sporogenous hyphae, as spores of *Glomus macrocarpum* (see **Supplementary Table 1** for species authors), the type species of *Glomus* and the Glomeromycota (Clements and Shear, 1931; Schüßler and Walker, 2010; Oehl et al., 2011). Of the 330 known species of the Glomeromycota, approximately 60 were originally described to form glomoid spores in epigeous or hypogeous unorganized glomerocarps, i.e., fruit bodies with randomly distributed spores inside them (Gerdemann and Trappe, 1974; Morton, 1988; Jobim et al., 2019). Importantly, of the glomerocarpic species, only seven (*Diversispora epigaea*, *Funneliformis mosseae*, *Glomus arborense*, *G. macrocarpum*, *Glomus pallidum*, *Glomus tenerum*, and *Glomus warcupii*) were managed to grow in culture, and only eight (*D. epigaea*, *Diversispora sporocarpia*, *G. macrocarpum*, *Redeckera megalocarpa*, *Redeckera fulva*, *Redeckera pulvinata*, *Sclerocarpum amazonicum*, and *Sclerocystis sinuosa*) were provided with molecular data (Jobim et al., 2019), which essentially makes this group of fungi ascribed to the Glomeromycota difficult to characterize and classify.

Among many unorganized glomerocarps, previously found from our studies in the Brazilian Northeast, there were two whose glomoid spores first seemed to be identical in morphology to each another but differed from glomoid spores of described species of the Glomeromycota. Further studies of the phenotypic features of the spores revealed very small differences and, consequently, suggested that the glomerocarps represent two semicryptic undescribed species. BLAST searchers using initial molecular data about these fungi confirmed this hypothesis and showed that these species are probably related to the genera *Dominikia* and *Kamienskia* in the family Glomeraceae.

"Crypticity" in fungal taxonomy is an aspect known for several phyla (Matute and Sepúlveda, 2019). Also, molecular analyses of some morphological species of the Glomeromycota suggest that they contain cryptic phylogenetic species, in which differences in morphology do not occur, are very small and inconclusive, or are invisible using traditional research methods and tools. Examples of such species are *Rhizoglomus irregulare* and *Septoglomus constrictum* (Stockinger et al., 2014; Błaszkowski, pers. observ.).

The disclosure of cryptic species strongly depends on the molecular markers used in phylogenetic identification. Currently, of the many markers that have been used in phylogenetic studies of the Glomeromycota (Błaszkowski et al., 2018a,b, 2019a,b; Jobim et al., 2019; Magurno et al., 2019), those comprising the partial small subunit (18S), internal transcribed spacer (ITS1-5.8S-ITS2 = ITS), and partial large subunit (28S) nuc rDNA segment (= 18S-ITS-28S), as well as the largest subunit of RNA polymerase II (*rpb1*) gene, have the highest resolution power and characterize the highest proportion of described species within the Glomeromycota (Krüger et al., 2012; Stockinger et al., 2014; Al-Yahya'ei et al., 2017). The first marker allowed to separate, for example, *Rhizoglomus dunense* from *Rhizoglomus clarum*, whose morphologies are almost identical, but sequence divergence is very high (13.3%; Al-Yahya'ei et al., 2017). The sequence resolution of the *rpb1* gene is higher and indicating that *R. irregulare* likely contains at least one cryptic species (Stockinger et al., 2014) and strongly confirming the uniqueness of *R. dunense* with a dissimilarity of 8.1% from *R. clarum*. According to literature data and our analyses, 18S-ITS-28S and *rpb1* sequences show interspecies differences when they differ by ≥3% and approximately 1%, respectively (Krüger et al., 2012; Stockinger et al., 2014; Błaszkowski et al., 2019b; Corazon-Guivin et al., 2019b). The large proportion of species provided with molecular data contained in the two markers allows both to compare a relatively large number of species and to better define the phylogenetic divergences between species belonging to different higher taxonomic ranks. Moreover, it is widely recognized that the concatenation of sequences of the ribosomal RNA-coding genes with sequences of protein-coding genes significantly increases phylogenetic resolving power for revealing relationships between analyzed taxa (Miadlikowska et al., 2014).

The genera *Dominikia* and *Kamienskia* were erected in the phylum Glomeromycota mainly due to phylogenetic analyses of 18S-ITS-28S sequences of six species originally described in the genus *Glomus* and the newly described *Dominikia disticha* (Błaszkowski et al., 2015a). The type species of these new genera became *Dominikia minuta* and *Kamienskia bistrata*. The main characters shared by the two fungi were hyaline or pale-colored, small spores (10- to 65-µm diameter when globose) formed in hypogeous clusters, which were produced in pot trap cultures, but were not found in the field-collected rhizosphere samples used to inoculate the cultures. Therefore, it was suggested that species of *Dominikia* and *Kamienskia* sporulate rarely or seasonally and were not found in the field soils because their delicate spores quickly break down before soil sampling (Błaszkowski et al., 2015a).

Later, new species of *Dominikia* and *Kamienskia* were described, whose spores were either (i) similar in color and size (*Dominikia lithuanica*, *Dominikia litorea*, *Kamienskia divaricata*), (ii) darker-colored (*Dominikia aurea*, *Dominikia bernensis*, *Dominikia compressa*, *Dominikia duoreactiva*,

*Dominikia emiratia*), (iii) larger (*D. compressa*, *D. duoreactiva*, *D. emiratia*), and (iv) formed mainly singly (*Dominikia difficilevidera*) (Oehl et al., 2014, 2015; Błaszkowski et al., 2015b, 2016, 2018c; Al-Yahya'ei et al., 2017) compared to the spores of the species listed above.

Recently, *Kamienskia perpusilla* and *K. divaricata* were transferred to a newly erected genus, *Microkamienskia* with a newly described species, *Microkamienskia peruviana* (Corazon-Guivin et al., 2019a), and *D. litorea* and *D. emiratia* became members of the new genera *Microdominikia* and *Orientoglomus*, respectively (Corazon-Guivin et al., 2019b). In addition, Corazon-Guivin et al. (2019b) described a new, monospecific genus, *Nanoglomus*, with *Nanoglomus plukenetiae*, sp. nov., with very similar morphology to *K. perpusilla*, but phylogenetically closely related to species of clades previously treated as *Dominikia*. All the taxa were erected mainly based on phylogenetic analyses of 18S-ITS-28S sequences and divergences of the sequences from other most closely related species, although Corazon-Guivin et al. (2019b) also showed morphological differences in the spore wall and subtending hypha between these taxa.

All described species of *Dominikia* and *Kamienskia* and their closest molecular relatives possess morphological characters distinguishing them from each other. However, none of these species has a morphological synapomorphy that would clearly demonstrate a generic separateness.

Recently, a new AMF, namely, *Rhizophagus neocaledonicus*, was described, which in the field lived in symbiosis with metallophytes in ultramafic soil in New Caledonia (Crossay et al., 2018). It was soon reassigned to the genus *Rhizoglomus*, as *Rhizoglomus neocaledonicum* (Turrini et al., 2018), because the generic name *Rhizophagus* should not be assigned to any species in the Glomeromycota (Sieverding et al., 2014). The assignment of this species into the genus *Rhizoglomus* (treated as *Rhizophagus*) was made based on a maximum likelihood phylogenetic analysis of 18S-ITS-28S sequences, even though the *Rhizoglomus* clade with the basally positioned *R. neocaledonicum* obtained only 49% bootstrap support. Moreover, the genetic divergence of *R. neocaledonicum* from the six *Rhizoglomus* species considered in the analysis was in the order of values separating clades of the Glomeromycota at the rank of genus. The sister species to the *Rhizoglomus* clade was a member of the genus *Sclerocystis*, *S. sinuosa* (Crossay et al., 2018). Molecular phylogenetic analyses performed by Turrini et al. (2018) confirmed the basal position and strong genetic divergence of *R. neocaledonicum* in the *Rhizoglomus* clade. However, they did not include *S. sinuosa* in their analyses. Further phylogenetic analyses of 18S-ITS-28S sequences of all *Rhizoglomus* species with available DNA barcodes and representatives of the other genera of the Glomeraceae accommodated *R. neocaledonicum* between the recently described monospecific genus *Halonatospora*, represented by *Halonatospora pansihalos*, and *S. sinuosa*, of which the latter was located basally to the other species of *Rhizoglomus* (Błaszkowski et al., 2018b, 2019b). Thus, the information presented above suggested that the New Caledonian fungus does not belong to *Rhizoglomus* but represents a new clade at the rank of genus in the Glomeraceae.

Therefore, the aims of this study were (i) to document that the two Brazilian fungi are new species of the Glomeromycota, (ii) to determine the molecular phylogenetic position of these fungi among their generic relatives of known phylogenies, and (iii) to describe in detail the morphology of these fungi. Moreover, we tested the hypothesis that *R. neocaledonicum* is a member of an undescribed genus in the Glomeraceae using already available and newly collected morphological and molecular data about this species.

## MATERIALS AND METHODS

### Origin of Study Material

The glomerocarps of the two fungi were sampled by K. Jobim at Parque das Trilhas, belonging to the Serra de Baturité Environmental Protection Area (Baturité EPA; 4°16′36.8″ S, 38°56′20.4″ W; 865 m above sea level) in the Guaramiranga municipality. The area covers 32,690 hectares located in the Northeast portion of the State of Ceará, 90 km from Fortaleza (Governo do Ceará, 2018) and is part of the Atlantic Forest. It houses a rich biodiversity with a high degree of endemism and includes a high percentage of preserved remnants of the Atlantic Forest that constitute a refuge for species originating from humid habitats, such as the Atlantic and Amazon forests (Figueiredo and Barbosa, 1990; Girão, 2006). In this region, annual mean temperatures are 19–22°C, and the average annual sum of rainfalls is 1,500 mm (Governo do Ceará, 2018). The high rainfalls, the altitude range between 100 and 1,000 m above sea level, and the exposure to humid air masses render the Baturité EPA one of the most humid areas in the State of Ceará (Governo do Ceará, 2018). The glomerocarps of these species, tentatively named "Species 1" and "Species 2," were found in July 2018 and April 2018, respectively.

### Collection of Glomerocarps, Establishment, and Growth of Single-Species Cultures

The glomerocarps of the two fungi were found employing the methodology used by Jobim et al. (2019) for collecting glomerocarpic species. Single-species pot cultures for each of the collected fungi were established by inoculating sterile growth substrate with fragments of the glomerocarps, of which each fragment contained at least 100 spores. The plant host used in the pot cultures was *Plantago lanceolata* L. The composition of the growth substrate, the methods of culture establishment, inoculation, and growing conditions were as those described by Błaszkowski et al. (2012).

### Extraction of Spores and Staining of Mycorrhizal Structures

The spore conglomerations used for the establishment of single-species cultures were extracted from the glomerocarps by means of a preparation needle under a dissecting microscope. Attempts at extracting spores from the single-species cultures of the two fungi were performed by a method characterized

by Błaszkowski et al. (2015b). Roots of *P. lanceolata* from the single-species cultures were stained following the protocol of Błaszkowski (2012).

## Microscopy and Nomenclature

Morphological features of spores and phenotypic and histochemical characters of spore wall layers of the two fungi were characterized based on at least 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, vol/vol). The preparation of spores for study and photography were as those described previously (Błaszkowski, 2012; Błaszkowski et al., 2012). Types of spore wall layers are those defined by Walker (1983) and Błaszkowski (2012). 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; see **Supplementary Table 1**. The terms "glomerospores" and "glomerocarps" were used for spores and fruit bodies 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, vol/vol) on slides] were deposited at UFRN hebarium (Brazil; holotypes on slides and dry glomerocarps in vials) and in the Laboratory of Plant Protection, Department of Shaping of Environment (LPPDSE; isotypes on slides and dry glomerocarps in vials), West Pomeranian University of Technology in Szczecin, Poland.

## DNA Extraction, Polymerase Chain Reaction, Cloning, and DNA Sequencing

Genomic DNA of the two potentially new species was extracted separately from fragments of glomerocarps containing approximately 20 spores. Details on spores preparation prior to polymerase chain reaction (PCR), PCR conditions, and primers used to obtain 18S-ITS-28S amplicons were as described in Krüger et al. (2009), Błaszkowski et al. (2014), and Symanczik et al. (2014), respectively.

The *rpb1* sequences of *Rhizoglomus dalpeae* were obtained by amplification with primers designed by Stockinger et al. (2014) following the recommended conditions. We used the same DNA, from which 18S-ITS-28S sequences had been obtained (Błaszkowski et al., 2019b). The first PCR with DNA of this species was performed with the primers RPB1-DR160mix (a, b, c, d) and RPB1-HS2680GPr, whereas the second PCR with RPB1-HS189GPf and RPB1-DR1210r.

The *rpb1* sequences of "Species 1" and "Species 2" were amplified with a nested approach. The primer set consisted of two forward primers, RPB1-3F and RPB1-4F, on the third and fourth exon, respectively, and two reverse primers, RPB1-5R and RPB1-5RN, on the fifth exon of the gene (**Table 1**). The *rpb1* sequences of *R. neocaledonicum* and *Rhizoglomus silesianum* were amplified using the primer RPB1-4F1, designed on the fourth exon to match all the *rpb1* sequences available for the Glomeraceae (with a maximum tolerance of one mismatch), in combination with RPB1-5R. The three forward primers were used at a final concentration = 200 nM, whereas the concentration of reverse primers was higher (**Table 1**) to compensate the gap in the Tm predicted using Oligo/Analyzer[1]. DreamTaq DNA Polymerase (Thermo Fisher) was used for the amplifications in 20 μL final volume according to the manufacturer's specifications, adding $MgCl_2$ 3 mM and bovine serum albumin 0.5 $\mu g\ \mu L^{-1}$ as final concentrations only in the PCR on raw DNA. The thermal cycling was as follows: 5 min initial denaturation (95°C), 40 cycles (30 cycles in the nested PCR) of 30-s denaturation (95°C), 30-s annealing, elongation at 72°C (**Table 1**), and 5-min (72°C) final elongation.

Cloning and sequencing of the PCR products were as those described by Błaszkowski et al. (2015a). The sequences were deposited in GenBank (MW507148–57, MW541060–67).

## Sequence Alignment and Phylogenetic Analyses

Comparative analyses of 18S-ITS-28S and *rpb1* sequences of the two fungi, using BLAST, confirmed our initial hypotheses that the fungi of our study are undescribed species of the Glomeromycota (see section "Introduction"). In particular, these analyses indicated that "Species 1" belongs to *Dominikia*, and the closest relative of "Species 2" is *K. bistrata*. Three sequence alignments were created in order (*i*) to determine the position of the new *Dominikia* sp. among its generic relatives, (*ii*) to assess the robustness of the relationship of the second new species with *K. bistrata*, and (*iii*) to test the hypothesis expressed previously (Błaszkowski et al., 2019b) and here that *R. neocaledonicum* should represent a new genus. The first alignment consisted

[1]https://eu.idtdna.com

**TABLE 1 |** Primers characteristics and PCR conditions used for the amplification of the *rpb1* sequences.

| Primer | Nucleotide sequence (5′–3′) | Position on the sequence | In combination with | Expected amplicon length | Ta°C | t elong. |
|---|---|---|---|---|---|---|
| RPB1-3F | GTC TTC GTG CAG TTT GGG A | 727–745 | RPB1-5R [1 μM] | ≈1,410 bp | 55 | 1 min 30 s |
| RPB1-4F | CTA GGC CTG ATT GGA TGA T | 1,204–1,222 | RPB1-5RN [500 nM] | ≈870 bp | 54 | 1 min |
| RPB1-4F1 | GCT CGT CCT GAT TGG ATG A | 1,203–1,221 | RPB1-5R [1 μM] | ≈935 bp | 55 | 1 min |
| RPB1-5R | ACG ATT TGT TTT GGT ACC AT | 2,119–2,138 | | | | |
| RPB1-5RN | TTC ATC TCA TCA CCA TCA A | 2,048–2,066 | | | | |

*The position of primers is given based on sequence of Rhizoglomus intraradices HG316020.1. The final concentration of the reverse primers is provided for each combination, as well as the expected product size, the temperature of annealing (Ta), and the time of elongation (t elong).*

of sequences of the 18S-ITS-28S nuc rDNA region or part thereof, which characterized all sequenced species of *Dominikia*, *Kamienskia*, and *Rhizoglomus*, as well as representative species of all other genera of the Glomeraceae *sensu* Schüßler and Walker (2010), Oehl et al. (2011), Błaszkowski et al. (2015a, 2018a,b), Corazon-Guivin et al. (2019a,b,c), Jobim et al. (2019), and Wijayawardene et al. (2020), except for the genus *Simiglomus* (a total of 15 genera; **Figure 1**). The alignment contained 139 sequences of 48 species of the Glomeraceae (including our two new species), of which the *Rhizoglomus natalense* KJ210824 and KJ210826 sequences represented the 28S locus only. To increase the phylogenetic signal from *S. sinuosa*, the 18S-ITS-28S sequence, not available in the GenBank database, was obtained by assembling the overlapping sequences AJ133706 (18S), AJ437106 (18S-ITS-28S), and FJ461846 (28S) generated from the same isolate MD126. The second alignment consisted of *rpb1* sequences from all species of the 18S-ITS-28S alignment that have been provided with *rpb1* sequences (**Figure 2**). This alignment included 75 sequences of 29 species in 14 genera of the Glomeraceae (including our two new species). The third alignment contained concatenated 18S-ITS-28S and *rpb1* sequences in the same number and species composition as in the *rpb1* alignment (**Figure 3**). In all three alignments, the outgroup was represented by seven sequences of four species of the family Entrophosporaceae. Data about the origin of the sequences used are presented in **Supplementary Table 2**.

Identity values of the 18S-ITS-28S and *rpb1* sequences of the two new species were calculated separately for each species using BioEdit (Hall, 1999). With the same program, we calculated the sequence divergence of these new species and *R. neocaledonicum* from sequences of their closest relatives (**Figures 1**, **2**). All comparisons were performed on sequences of the same length.

Indels were coded as binary characters by means of FastGap 1.2 (Borchsenius, 2009), with the possibility to code missing data to be recognized by the phylogenetic inference programs. The coded binary character sets were added to the respective nucleotide alignments, as described in Błaszkowski et al. (2014). The 18S-ITS-28S, *rpb1*, and 18S-ITS-28S + *rpb1* alignments were aligned separately with MAFFT 7 using the auto option[2].

In order to reconstruct the phylogenetic positions of the two new species among sequenced species of the Glomeraceae, as well as to confirm our hypothesis on the phylogeny of *R. neocaledonicum*, Bayesian inference (BI) and maximum likelihood (ML) phylogenetic analyses of the alignments were performed via CIPRES Science Gateway 3.1 (Miller et al., 2010). GTR + G was predicted as best substitution model for the DNA partitions in the BI analysis by using jModelTest2 (Darriba et al., 2012). For the indel partition, F81 model was chosen as suggested in the MrBayes manual. Four Markov chains were run over 1 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, using a maximum likelihood/1,000 rapid bootstrapping run, was computed with RAxML 8.2.12 (Stamatakis, 2014) using the GTRGAMMA algorithm. To improve the accuracy of phylogenetic reconstruction (Lanfear et al., 2012; Nagy et al., 2012), in both BI and ML analyses, the 18S-ITS-28S alignment was divided into four partitions: 18S, ITS, 28S, and the binary (indel) character set. Two partitions (gene and indel set) were used for the *rpb1* alignment. The same partitioning scheme was used in the 18S-ITS-28S + *rpb1* alignment with concatenated sequences, resulting in six partitions totally. We assumed that clades were supported when BI posterior probability and ML bootstrap support values were ≥0.95 and ≥70%, respectively. The phylogenetic trees obtained in the analyses were visualized and rooted in Archaeopteryx.js[3].

# RESULTS

## General Data and Phylogeny

Data about the numbers of base pairs as well as variable and parsimony informative sites of the 18S-ITS-28S, *rpb1*, and 18S-ITS-28S + *rpb1* alignments are presented in **Table 2**. The identity values of the 18S-ITS-28S sequences of "Species 1" and "Species 2" (five sequences each) were 98.6 and 99.4%, respectively, and the identity values of the *rpb1* sequences of these species (two sequences each) were 99.1%. The identities of the three 18S-ITS-28S and two *rpb1* sequences of *Rhizoglomus neocaledonicum* were 99.5 and 99.7%, respectively.

In the 18S-ITS-28S and 18S-ITS-28S + *rpb1* trees, the topologies of the family Glomeraceae clade, reconstructed from BI and ML analyses, were identical (**Figures 1**, **3**). Additionally, for all trees (**Figures 1–3**), the branches of the outgroup Entrophosporaceae clade received full or strong BI and ML support. Instead, the topologies of the *rpb1* Glomeraceae clade slightly differed depending on the algorithm used in the analyses. The differences regarded only the positions of the monospecific genera *Microdominikia* and *Orientoglomus* (**Figure 2**). In the *rpb1* BI tree, *Microdominikia litorea* was a basal clade to the clades with *Orientoglomus emiratium* and *Dominikia* spp. Instead, in the *rpb1* ML tree, the relationships between *Orientoglomus*, *Microdominikia*, and *Dominikia* clades were not supported (**Figure 2**).

In all trees, 18S-ITS-28S, *rpb1*, 18S-ITS-28S + *rpb1*, "Species 1" (=*Dominikia glomerocarpica*) clustered in a sister clade to the clade with *D. aurea*, and "Species 2" (= *Epigeocarpum crypticum*) populated a sister clade to the clade with *K. bistrata* (**Figures 1–3**). Both BI and ML analyses of all three alignments supported the tie between "Species 1" and *D. aurea* (BI = 0.99–1.0; ML = 74–97%; **Figures 1–3**). Instead, the node connecting "Species 2" with *K. bistrata* obtained moderate or strong supports in BI and ML analyses of the 18S-ITS-28S (0.99/88) and 18S-ITS-28S + *rpb1* (1/96) alignments (**Figures 1**, **3**), but neither BI nor ML analysis of the *rpb1* alignment supported (0.68/52) the tie between these species (**Figure 2**).

In the 18S-ITS-28S and 18S-ITS-28S + *rpb1* trees, the clades with "Species 1" and "Species 2" obtained full supports in both analyses, and in the *rpb1* tree, the supports for both species were

[2]http://maft.cbre.jp/alignment/server/

[3]https://sites.google.com/site/cmzmasek/home/software/archaeopteryx-js

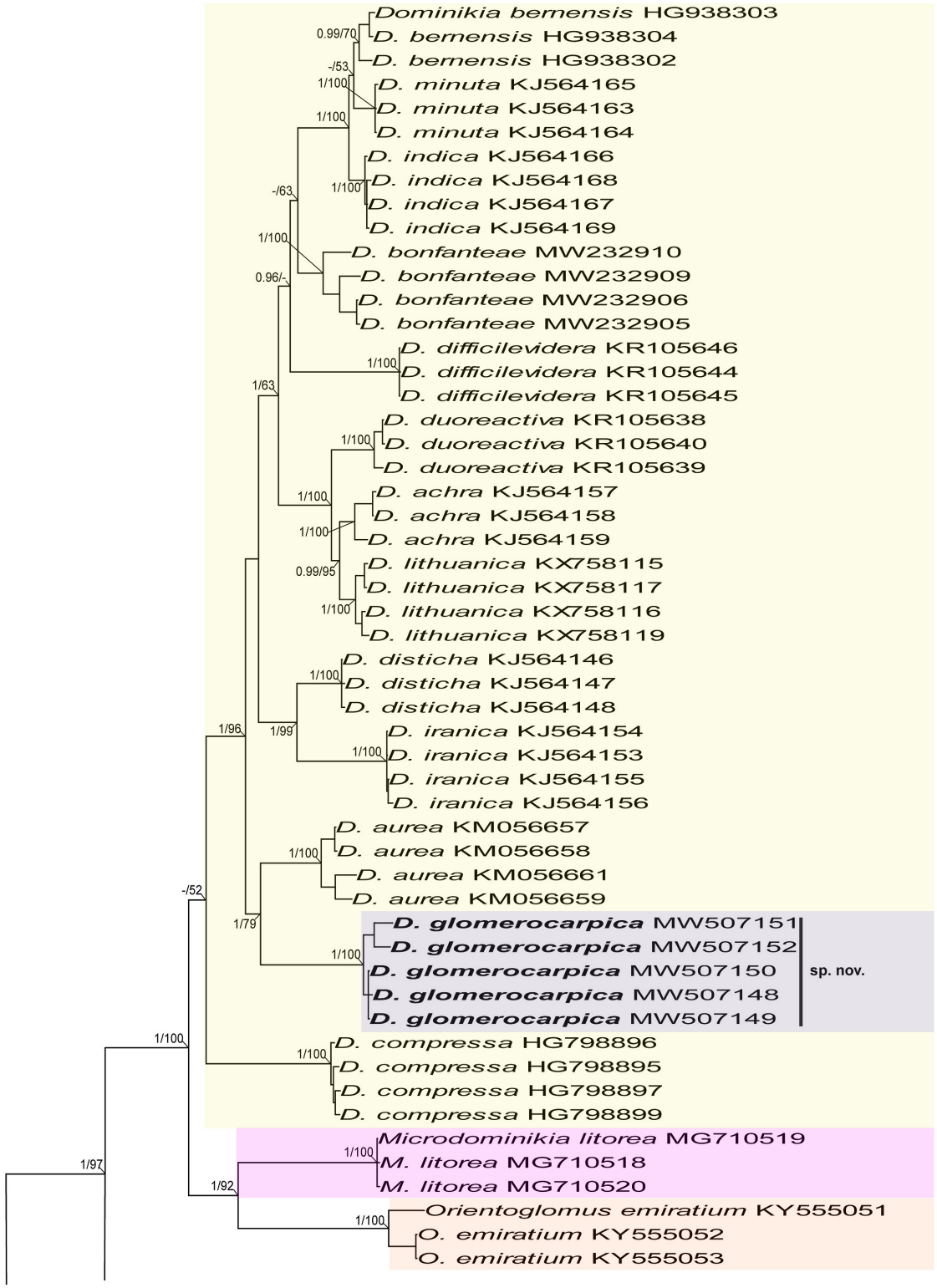



**FIGURE 1 |** Continued

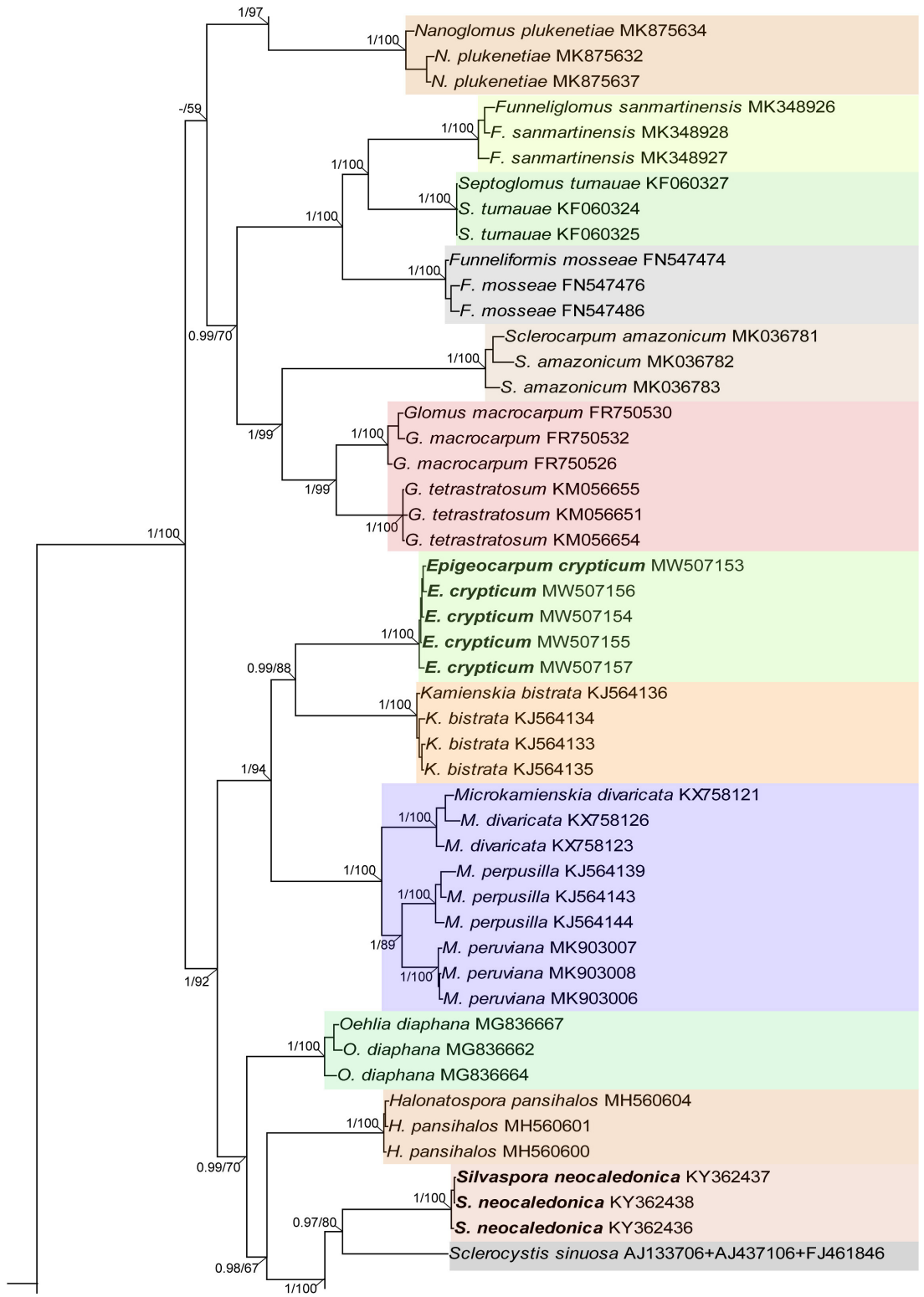



**FIGURE 1 |** Continued

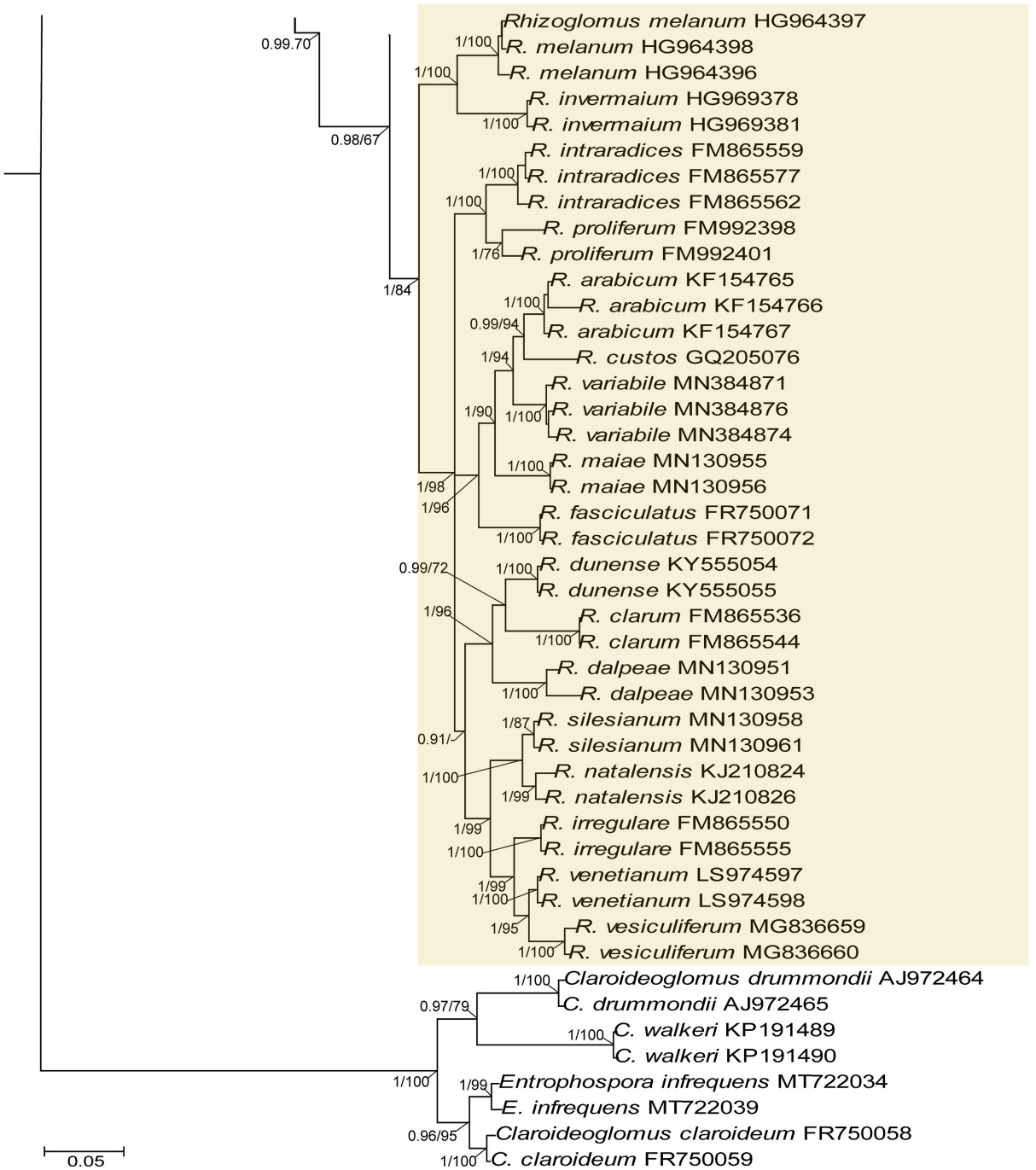



**FIGURE 1 |** A 50% majority rule consensus phylogram inferred from a Bayesian inference analysis of 18S-ITS-28S nuc rDNA sequences of *Dominikia glomerocarpica* and *Epigeocarpum crypticum*, as well as 50 other species of AMF, including *Claroideoglomus claroideum*, *Claroideoglomus drummondii*, *Claroideoglomus walkerii*, and *Entrophospora infrequens* as outgroup. The Bayesian posterior probabilities ≥0.90 and ML bootstrap values ≥50% are shown near the branches, respectively. Bar indicates 0.05 expected change per site per branch.

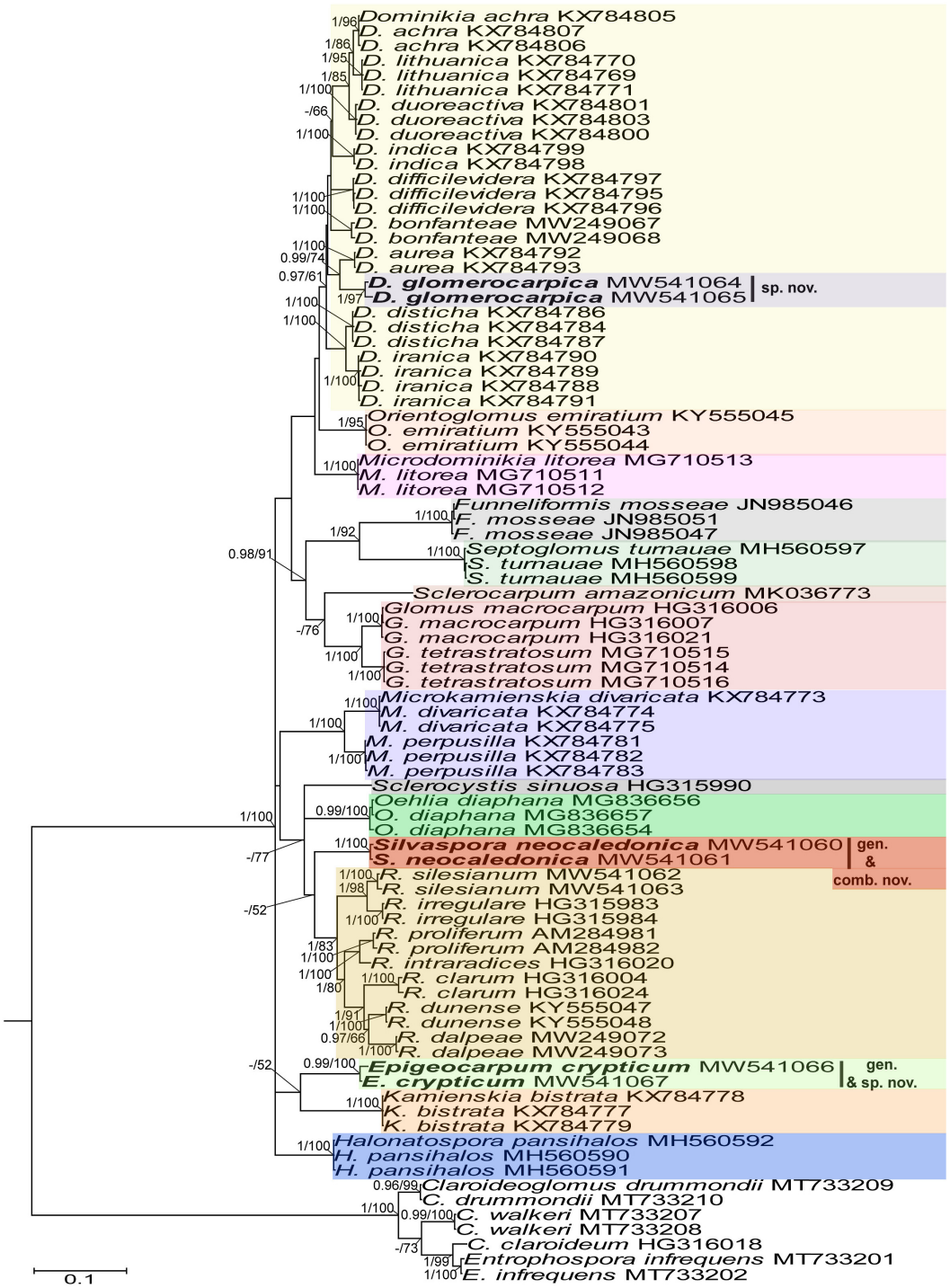



**FIGURE 2 |** A 50% majority rule consensus phylogram inferred from a Bayesian inference analysis of *rpb1* sequences of *D. glomerocarpica* and *E. crypticum*, as well as 34 other species of AMF, including *C. claroideum*, *C. drummondii*, *C. walkerii*, and *E. infrequens* as outgroup. The Bayesian posterior probabilities ≥0.90 and ML bootstrap values ≥50% are shown near the branches, respectively. Bar indicates 0.1 expected change per site per branch.

full or high (BI = 1.0, ML = 97% for "Species 1" and BI = 0.99, ML = 100% for "Species 2"; **Figures 1**, **3**).

The *R. neocaledonicum* (=*Silvaspora neocaledonica*) clade in the 18S-ITS-28S, *rpb1*, and 18S-ITS-28S + *rpb1* trees obtained full supports in both BI and ML analyses (**Figures 1–3**). In the 18S-ITS-28S and 18S-ITS-28S + *rpb1* trees, *R. neocaledonicum* was placed in a sister clade to *S. sinuosa* (**Figures 1**, **3**). The node linking the two species was moderately supported in both BI and

**TABLE 2 |** Characteristics of the sequence alignments analyzed.

| Name of alignment | Number of sequences | Number of species | Number of base pairs | Number of variable sites | Number of parsimony informative sites |
|---|---|---|---|---|---|
| 18S-ITS-28S | 147 | 52 | 2,492 | 1,094 | 978 |
| *rpb1* | 86 | 36 | 2,841 | 1,390 | 1,195 |
| 18S-ITS-28S + *rpb1* | 86 | 36 | 4,074 | 2,350 | 2,067 |

ML analyses of the 18S-ITS-28S alignment (BI = 0.97, ML = 80%), whereas only the BI analysis of the 18S-ITS-28S + *rpb1* alignment moderately (BI = 0.97) supported this association (**Figures 1**, **3**). The closest neighbors of the *R. neocaledonicum*–*S. sinuosa* clade were the generic clades *Halonatospora* and *Rhizoglomus*, respectively. In the *rpb1* tree, *R. neocaledonicum* sequences clustered basally with weak support (BI = 0.77, ML = 52) to the *Rhizoglomus* spp. clade (**Figure 2**).

The molecular distances between "Species 1" and *D. aurea*, calculated based on comparisons of their 18S-ITS-28S and *rpb1* sequences, were 8.5–10.2% and 4.3–4.8%, respectively. The divergences of the 18S-ITS-28S and *rpb1* sequences of "Species 2" and *K. bistrata* were 14.5–15.6% and 11.0–11.6%, respectively. The 18S-ITS-28S and *rpb1* sequences of *R. neocaledonicum* and *S. sinuosa* differed by 22.7–22.9% and 11.4%, respectively. The 18S-ITS-28S and *rpb1* sequences of *R. neocaledonicum* and *Rhizoglomus* spp. differed by 12.5–18.5% and 10.8–13.0%, respectively.

Considering the results of the phylogenetic analyses and comparisons of sequences of the two species found in northeast Brazil and the AMF originally described as *Rhizophagus neocaledonicus* discussed above, as well as the morphological distinctiveness of these species from their closest phylogenetically relatives, below we characterize the two species from Brazil as *D. glomerocarpica* sp. nov. and *E. crypticum* gen. nov. et sp. nov. In addition, we emend the morphological description of and transfer *Rhizophagus neocaledonicum* to a new genus, *Silvaspora* gen. nov., with *S. neocaledonica* comb. nov. Because *D. glomerocarpica* is the first glomerocarp-forming species in *Dominikia*, the definition of this genus was emended.

# Taxonomy

## Erection of New Genera

*Epigeocarpum* Błaszk., B.T. Goto, Jobim, Niezgoda & Marguno, gen. nov.

MycoBank No. MB838879.

*Etymology*: Latin, *Epigeocarpum*, *Epigeo* (=soil surface) and *carpum* (=fruit body), referring to the habitat, in which the type species of the new genus formed glomerocarps (=sporocarps).

*Type species*: *E. crypticum* Jobim, Błaszk., Niezgoda, Magurno & B.T. Goto

*Diagnosis*: Differs from *K. bistrata*, the phylogenetically closely related species of the monospecific genus *Kamienskia*, in (*i*) producing compact, epigeous, yellow-colored glomerocarps with a peridium and hyaline to light yellow, glomoid spores having a three-layered spore wall, of which the laminate layer 2 is relatively thick compared to the small spores size, contracts and, consequently, transfers into a crown-like structure in spores crushed in PVLG-based mountants and stains in Melzer's reagent; (*ii*) the possession of a septum separating the spore subtending hyphal lumen from the spore interior; and (*iii*) the nucleotide composition of sequences of the 18S-ITS-28S nuc rDNA region and the *rpb1* gene.

*Genus description*: Producing glomoid glomerocarps in a compact epigeous unorganized glomerocarp. Spores hyaline to light yellow (4A4), usually globose to subglobose, 34–46 μm diameter, with a spore wall consisting of three permanent, smooth layers. Spore wall layer (swl) 2 laminate, usually transferring into a crown-like structure due to contracting in spores crushed in PVLG and PVLG + Melzer's reagent. Only swl 2 stains in Melzer's reagent. Subtending hypha funnel-shaped with a wall continuous with swl 1 and 2 and an open pore at the spore base; the channel connecting the lumen of the subtending hypha with the interior of spores closed by a septum continuous with swl 3; the septum usually positioned at half the thickness of swl 2; the subtending hyphal lumen gradually narrowing in maturing spores due to thickening of subtending hyphal wall layer 2.

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

MycoBank number MB838881.

*Type species*: *S. neocaledonica* (D. Redecker, Crossay & Cilia) Błaszk., Niezgoda, B.T. Goto, Crossay & Magurno, comb. nov. MycoBank MB838882.

*Specimens examined*: 3,768–3,772 (LPPDSE).

*Etymology*: *Silvaspora*, in honor of Dr. Gladstone Alves da Silva, Departamento de Micologia, CCB, Universidade Federal de Pernambuco, Brazil, in recognition of his important contribution to taxonomy and ecology of arbuscular mycorrhizal fungi.

*Diagnosis*: Differs from other genera in the Glomeraceae mainly in the nucleotide composition of sequences of the 18S-ITS-28S nuc rDNA region and the *rpb1* gene.

*Genus description*: Forming colored glomoid glomerospores with a spore wall consisting of three layers, of which only layer 1, forming the spore surface, is impermanent and hyaline to brightly colored. Subtending hypha colored similarly to the spore wall, cylindrical, slightly funnel-shaped, or constricted at the spore base, with a pore occluded due to thickening subtending hyphal wall layer 2 continuous with the laminate spore wall layer 2, rarely slightly open. Forming mycorrhiza with arbuscules, vesicles, and hyphae staining dark in trypan blue.

## Emendation of the Genus *Dominikia* Błaszk., Chwat & Kovács Emend. Błaszk., Niezgoda, Magurno, Jobim & B.T. Goto

*Genus description*: Fungi forming glomoid-like spores in soil or epigeous glomerocarps (=sporocarps). Hypogeous spores usually produced in unorganized (with random distribution), loose to




**FIGURE 3 |** A 50% majority rule consensus phylogram inferred from a Bayesian inference analysis of 18S-ITS-28S + *rpb1* sequences of *D. glomerocarpica* and *E. crypticum*, as well as 34 other species of AMF, including *C. claroideum*, *C. drummondii*, *C. walkerii*, and *E. infrequens* as outgroup. The Bayesian posterior probabilities ≥0.90 and ML bootstrap values ≥50% are shown near the branches, respectively. Bar indicates 0.1 expected change per site per branch.

compact clusters, rarely singly, sometimes inside roots. Epigeous sporocarps compact with hundreds to thousands of randomly distributed glomerospores. Spores hyaline to yellow brown, small, 17–87 μm diameter when globose. Spore wall with two to three layers. Layer 1, forming the spore surface, mucilaginous, short-lived, or unit (not divided into sublayers), semipermanent to permanent, only slightly or not deteriorating with age. Layer 2 unit or laminate, permanent. Layer 3 flexible to semiflexible,

permanent. Layers 1 and 3 staining or not staining in Melzer's reagent. Spore subtending hypha cylindrical to funnel-shaped with a pore open or occluded by a septum (i) continuous with the innermost lamina(e) of the laminate spore wall layer 2 or 3, (ii) connecting the inner surfaces of the innermost subtending hyphal wall layer, (iii) continuous with the flexible to semiflexible innermost spore wall layer, and (iv) sometimes as in (iii) and still by another underlying septum connecting the inner surfaces of the innermost subtending hyphal wall layer. Forming vesicular–arbuscular or only arbuscular mycorrhiza staining dark in Trypan blue.

*Type species*: *Dominikia minuta* (Błaszk., Tadych & Madej) Błaszk., Chwat & Kovács Mycotaxon 76, 189. 2000.

*Other accepted species. Dominikia achra* (Błaszk., D. Redecker, Koegel, Schützek, Oehl & Kovács) Błaszk., Chwat & Kovács.

*Basionym*. *Glomus achrum* Błaszk., D. Redecker, Koegel, Schützek, Oehl & Kovács, Botany 87, 262. 2009.

*Dominikia aurea* (Oehl & Sieverd.) Blaszk. Chwat, G.A. Silva & Oehl.

*Basionym*. *Glomus aureum* Oehl & Sieverd. J. Appl. Bot., Angew. Bot. 77, 111. 2003.

*Dominikia bernensis* Oehl, Palenz., Sánchez-castro & G.A. Silva.

*Dominikia bonfanteae* Magurno, Niezgoda, B.T. Goto & Błaszk.

*Dominikia compressa* (Sieverd. Oehl, Palenz., Sánchez-Castro & G.A. Silva) Oehl, Palenz., Sánchez-Castro & G.A. Silva.

*Basionym*. *Glomus compressum* Sieverd., Oehl, Palenz., Sánchez-Castro & G.A. Silva, Nova Hedwigia 99, 433. 2014.

*Dominikia difficilevidera* Błaszk., Góralska & Chwat.

*Dominikia disticha* Błaszk., Chwat & Kovács.

*Dominikia duoreactiva* Błaszk., Góralska & Chwat.

*Dominikia indica* (Błaszk., Wubet & Harikumar) Błaszk., G.A. Silva & Oehl.

*Basionym*. *Glomus indicum* Błaszk., Wubet & Harikumar, Botany 88, 134. 2010.

*Dominikia iranica* (Błaszk., Kovács & Balázs) Błaszk., Chwat & Kovács.

*Basionym*. *Glomus iranicum* Błaszk., Kovács & Balázs, Mycologia 102, 1457. 2010.

*Dominikia lithuanica* Błaszk., Chwat & Góralska.

## Description of New Species and a New Combination

*Dominikia glomerocarpica* Jobim, Błaszk., Niezgoda, Magurno & B.T. Goto, sp. nov. **Figures 4A–H**.

MycoBank No. MB838883.

*Etymology*: Latin, *glomerocarpica*, referring to the glomerocarps (=sporocarps) produced by the species.

*Specimens examined*: Brazil, Parque das Trilhas, belonging to the Serra de Baturité Environmental Protection Area (4°16′36.8″ S, 38°56′20.4″ W; 865 m above sea level) in the Guarapiranga municipality, Ceará State, a glomerocarp found by K. Jobim in July 2018. Holotype. A fragment of a glomerocarp and a slide with spores no. UFRN-Fungos 3282, isotypes: a vial with a fragment of a glomerocarp no. 3802 and slides with spores no. 3773–3782 (LPPDSE).

*Diagnosis*: Differs from *D. aurea*, the sister phylogenetic relative, in (i) the habitat of production and the features of the structure forming the spore conglomeration; (ii) spore color, the composition of the spore wall and the phenotypic and biochemical properties of the spore wall layers; (iii) the characters of the septum separating the spore subtending hyphal lumen from the spore interior; and (iv) the nucleotide composition of sequences of the 18S-ITS-28S nuc rDNA region and the *rpb1* gene.

*Description*: Glomerospores formed in a compact epigeous glomerocarp. *Glomerocarp* yellowish gray (4B2) to grayish yellow (4B3); approximately 3.30 × 3.38 mm (**Figure 4A**). *Peridium* thin, hyaline to pale gray (1B1), only partially covering glomerospores' conglomerations. *Gleba* yellowish white (4A2) to grayish yellow (4B3), with hyaline; straight or branched hyphae; (1.6−)3.2(−4.8) μm wide, with a wall 0.8–1.1 μm thick; staining pinkish white (7A2) to pastel red (9A6) in Melzer's reagent; glomerocarp hosting hundreds of glomerospores (=spores; **Figures 4B–H**). *Spores* arise blastically at tips of sporogenous hyphae (**Figures 4C,D,F–H**). Spores hyaline to light yellow (4A5); globose to subglobose; (30−)39(−46) μm diameter; rarely ovoid; 34–39 μm × 40–46 μm; with one subtending hypha (**Figures 4B–H**). *Spore wall* composed of three permanent, smooth layers (**Figures 4C,E–H**). Layer 1, forming the spore surface, uniform (not containing visible sublayers), semiflexible, hyaline, (0.8−)1.2(−1.5) μm thick, tightly adherent to the upper surface of layer 2 (**Figures 4C,E–H**). Layer 2 laminate, semiflexible, hyaline to light yellow (4A5), (1.8−)8.2(−10.5) μm thick; consisting of very thin, <0.5 μm thick, laminae, tightly adherent to each other, not separating even in vigorously crushed spores; not contracting in spores crushed in PVLG-based mountants (**Figures 4C,E–H**). Layer 3 flexible, hyaline, approximately 1.0 μm thick, usually slightly contracting and separating from the lower surface of layer 2 in intact spores and spores crushed in PVLG, 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 (**Figures 4C,E–H**). In Melzer's reagent, only spore wall layer 2 stains pastel red (9B7) to brownish violet (11D8; **Figure 4H**). *Subtending hypha* hyaline to light yellow (4A5); straight or recurved, funnel-shaped; (4.0−)5.8(−8.5) μm wide at the spore base (**Figures 4C–H**); not braking in crushed spores. *Wall of subtending hypha* hyaline to light yellow (4A5); (2.0−)2.5(−3.2) μm thick at the spore base; consisting of two layers continuous with spore wall layers 1 and 2 (**Figure 4H**). *Pore* (0.6−)0.9(−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 usually positioned at half the thickness of swl 2; the subtending hyphal lumen gradually narrowing in maturing spores due to thickening of subtending hyphal wall layer 2 (**Figures 4C,E–G**). Spore content of hyaline oily substance. *Germination* unknown.

*Mycorrhizal associations*: no molecular analyses were performed on roots of the plant species that grew in the place where the glomerocarp of *D. glomerocarpica* was found.

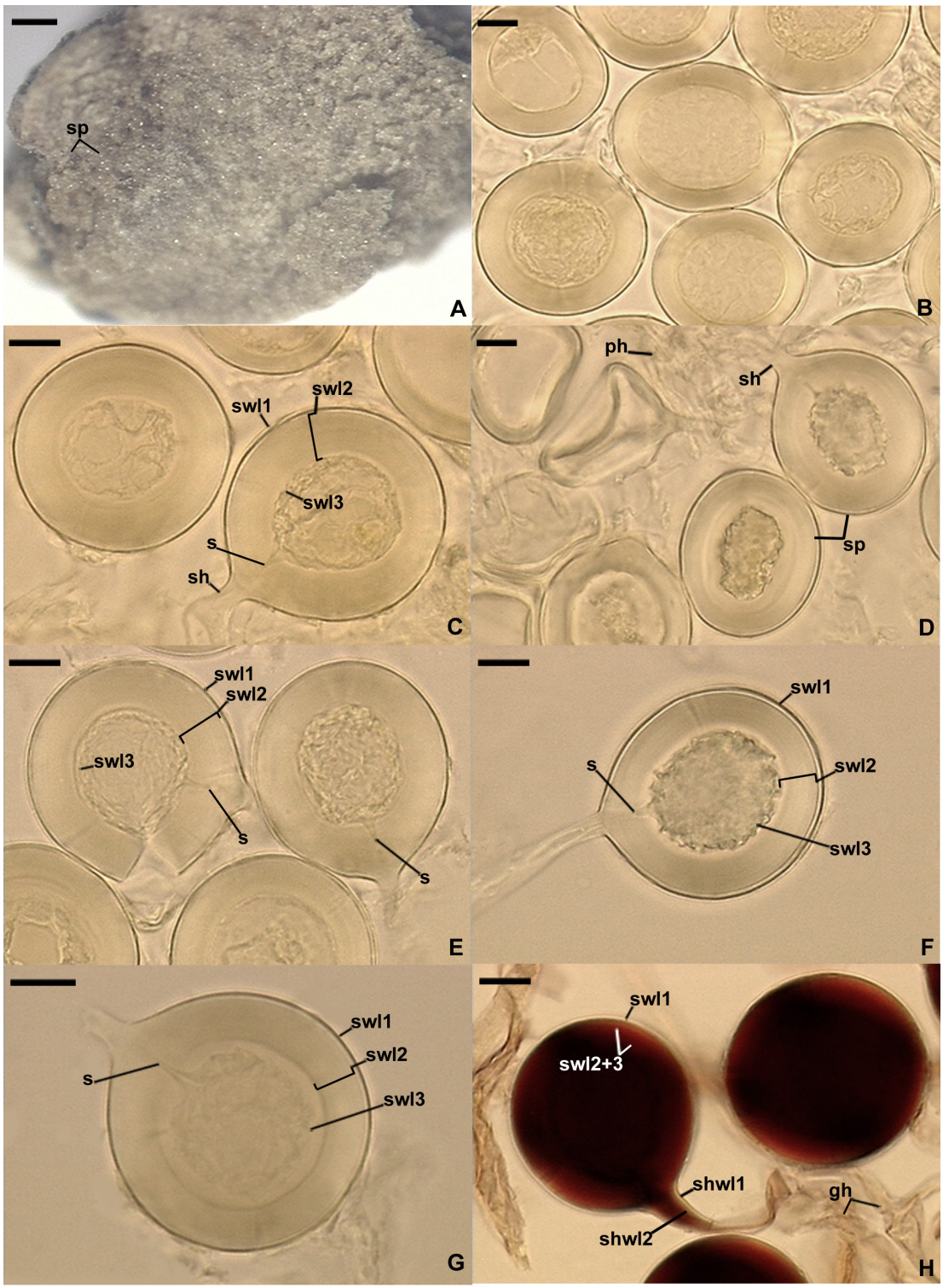



**FIGURE 4 |** *Dominikia glomerocarpica*. **(A)** Glomerocarp (=sporocarp) with glomerospores (=spores; sp). **(B)** Intact spores. **(C–G)** Intact and crushed spores with a three-layered spore wall (swl1–3) and a subtending hypha (sh); note that swl2 did not change in shape in crushed spores, and swl3 usually slightly contracted and separated from the lower surface of the laminate swl2 in intact and crushed spores and formed a septum (s) located at approximately half the length of the channel connecting the subtending hyphal lumen with the spore interior; peridial hyphae (ph) are visible in **(D)**. **(H)** Intact spores with swl1–3, subtending hyphal wall layers (shwl) 1 and 2, and glebal hyphae (gh) mounted in PVLG + Melzer's reagent. **(A)** Dry specimen. **(B–G)** Spores in PVLG. **(H)** Spores in PVLG + Melzer's reagent. **(A)** Light microscopy. **(B–H)** Differential interference microscopy. Scale bars: **(A)** = 200 μm, **(B–H)** = 10 μm.

Attempts to grow *D. glomerocarpica* in single-species cultures with *P. lanceolata* as host plant failed.

*Distribution and habitat*: the Parque das Trilhas, being part of the Serra de Baturité Environmental Protection Area in Brazil, is so far the sole known site of occurrence of *D. glomerocarpica*. The geographic location and climate of this site were characterized in Section "Materials and Methods."

BLAST searches indicated that *D. glomerocarpica* has not been found in other environments around the word before. The highest identities of 18S-ITS-28S and *rpb1* sequences of this species compared with sequences of the two loci deposited in GenBank were only 93.78 and 95.84%, respectively.

*Epigeocarpum crypticum* Jobim, Błaszk., Niezgoda, Magurno & B.T. Goto, sp. nov. **Figures 5A–H**.

MycoBank No. MB838880.

*Etymology*: Latin, *crypticum*, referring to the very small and hidden morphological differences compared to the very large differences in the DNA nucleoid composition between *E. crypticum* and its closest molecular relative, *K. bistrata*.

*Specimens examined*: Brazil, Parque das Trilhas, belonging to the Serra de Baturité Environmental Protection Area (4°16′36.8″ S, 38°56′20.4″ W; 865 m above sea level) in the Guarapiranga municipality, Ceará State, a glomerocarp found by K. Jobim in April 2018. Holotype: A fragment of a glomerocarp and a slide with spores no. UFRN-Fungos 3283, isotypes: a vial with a fragment of a glomerocarp no. 3803 and slides with spores no. 3783–3801 (LPPDSE).

*Diagnosis*: As that regarding the genus *Epigeocarpum* (see above).

*Description*: Glomerospores formed in a compact epigeous glomerocarp. *Glomerocarp* yellowish white (4A2) to grayish yellow (4B3); 1.65 mm × 1.69 mm (**Figure 5A**). *Peridium* thin, yellowish white (3A2) to pale yellow (3A3), only partially covering glomerospores' conglomerations (**Figure 5A**). *Gleba* yellowish white (4A2) to light yellow (4A5), with hyaline; straight or branched hyphae; (2.2−)5.1(−7.5) μm wide, with a wall 0.6–1.8 μm thick; staining reddish white (7A2) to grayish red (8B6) in Melzer's reagent; glomerocarp hosting hundreds of glomerospores (=spores; **Figures 5A–H**). *Spores* arise blastically at tips of sporogenous hyphae. Spores hyaline to light yellow (4A4); globose to subglobose; (34−)40(−46) μm diameter; rarely ovoid; 34–44 μm × 40–51 μm; with one subtending hypha (**Figures 5B–H**). *Spore wall* composed of three permanent, smooth layers (**Figures 5B–H**). Layer 1, forming the spore surface, uniform (not containing visible sublayers), semiflexible, hyaline, (0.8−)1.5(−2.0) μm thick, tightly adherent to layer 2 in spores immersed in water, but frequently slightly separated from the upper surface of layer 2, even in intact spores, due to contracting of layer 2 in PVLG-based mountants (**Figures 5B–H**). Layer 2 laminate, semiflexible, hyaline to light yellow (4A4), (2.0−)8.0(−12.5) μm thick; consisting of very thin, <0.5 μm thick, laminae, tightly adherent to each other, not separating even in vigorously crushed spores; usually transforming into a crown-like structure due to contracting in spores crushed in PVLG and PVLG + Melzer's reagent (**Figures 5C–H**). Layer 3 flexible, hyaline, approximately 1.0 μm thick, usually slightly contracting and separating from the lower surface of layer 2 in intact spores and spores crushed in PVLG, 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 (**Figures 5C–E,G,H**). In Melzer's reagent, only spore wall layer 2 stains reddish white (7A2) to brownish violet (11D8; **Figures 5F,H**). *Subtending hypha* hyaline to light yellow (4A4); straight or recurved, funnel-shaped; (4.4−)6.6(−9.0) μm wide at the spore base (**Figures 5C,D,F–H**); not braking in crushed spores. *Wall of subtending hypha* hyaline to light yellow (4A4); (2.0−)2.8(−3.3) μm thick at the spore base; consisting of two layers continuous with spore wall layers 1 and 2 (**Figures 5C,F,G,H**). *Pore* (0.6−)1.1(−1.6) 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 usually positioned at half the thickness of swl 2; the subtending hyphal lumen gradually narrowing in maturing spores due to thickening of subtending hyphal wall layer 2 (**Figure 5G**). Spore content of hyaline oily substance. *Germination* unknown.

*Mycorrhizal associations*: No molecular analyses were performed on roots of the plant species that grew in the place where the glomerocarp of *E. crypticum* was found. Attempts to grow *E. crypticum* in single-species cultures with *P. lanceolata* as host plant failed.

*Distribution and habitat*: The physical presence of *E. crypticum* was so far found only in Parque das Trilhas. The geographic location of the park and other characteristics of the habitat, in which the glomerocarp of *E. crypticum* was discovered, are described in *Distribution and habitat* regarding *D. glomerocarpica* (see above).

BLAST's searches suggested that *E. crypticum* was also associated with roots of native plants growing in a petroleum-polluted soil of the Amazon region of Ecuador. The identity of the 28S MH504057 sequence compared to the 18S-ITS-28S sequences of *E. crypticum* was 98.5%. The identity of *E. crypticum rpb1* sequences compared to those listed by BLAST was only ≤92.7%.

*Silvaspora neocaledonica* (D. Redecker, Crossay & Cilia) Błaszk., Niezgoda, B.T. Goto, Crossay & Magurno, comb. nov. **Figures 6A–D**.

MycoBank number MB838882.

*Basionym*: *Rhizophagus neocaledonicus* D. Redecker, Crossay & Cilia, Mycol. Prog. 17, 739. 2018.

*Synonym*: *Rhizophagus neocaledonicum* (D. Redecker, Crossay & Cilia) Oehl, Turrini & Giovann. Mycol. Prog. 17, 1218. 2018.

*Description*: Glomerospores formed singly and in loose clusters of 2–3 in soil, roots, and in root organ culture. *Spores* dark chestnut to coffee brown [orange (6C7) to brown (6E8)]; usually globose to subglobose; (61–)75(−90) μm diameter; sometimes oblong to irregular; 68–82 μm × 95–113 μm; with one subtending hypha (**Figures 6A–C**). *Spore wall* composed of three layers (**Figures 6A,B**). Layer 1, forming the spore surface, evanescent, short-lived, hyaline to yellowish white (4A2), 1.0–2.5 μm thick, usually highly deteriorated or completely sloughed off in mature spores. Layer 2 laminate, permanent, dark brown [orange (6C7) to brown (6E8)], (4.2–)6.1(−8.8) μm thick. Layer 3 uniform (without visible sublayers), bright brown [golden yellow

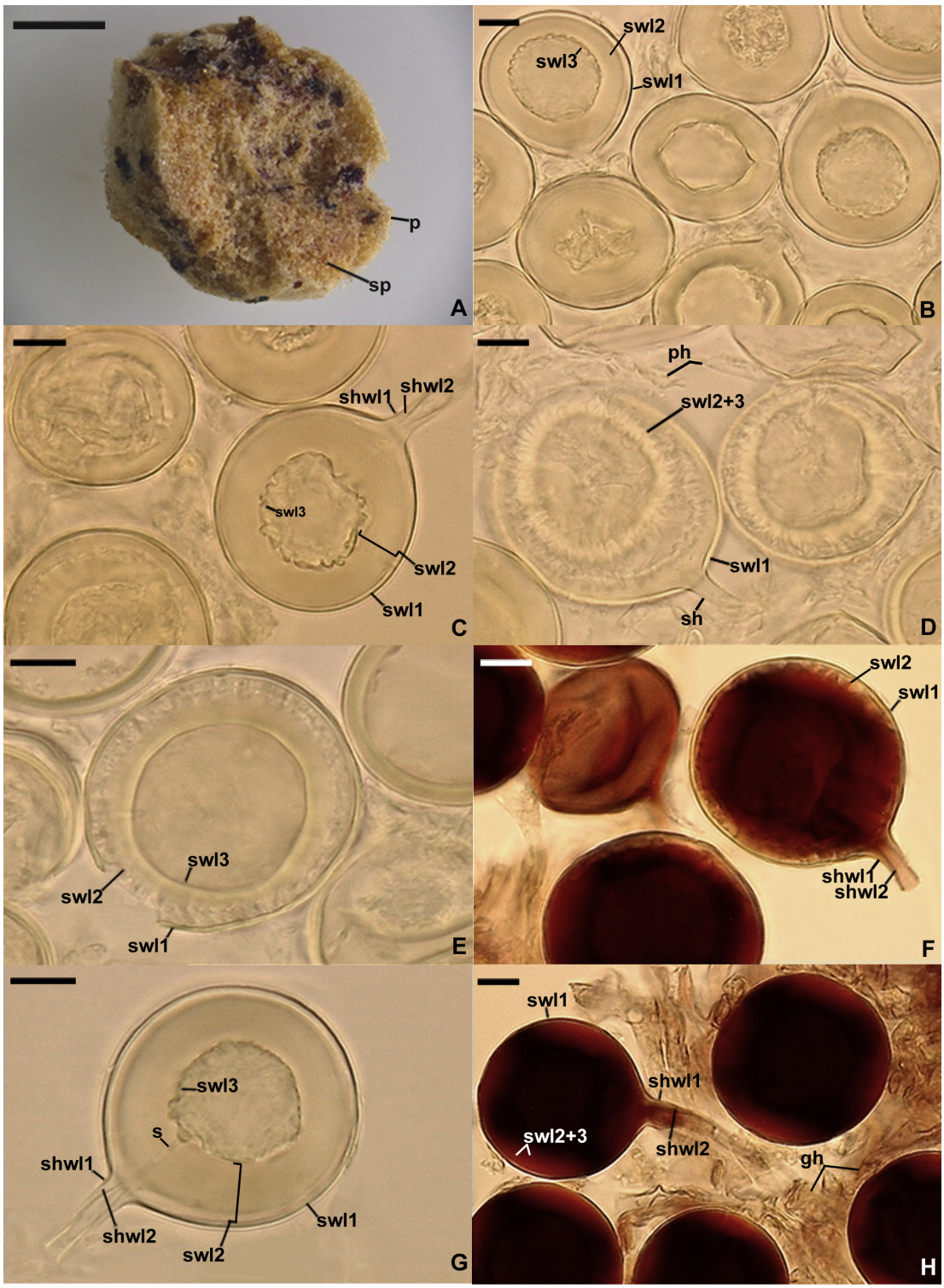



**FIGURE 5 |** *Epigeocarpum crypticum*. **(A)** Glomerocarp (=sporocarp) with glomerospores (=spores; sp). **(B)** Spores on which no pressure was applied; note that spore wall layer (swl) 2 slightly contracted and separated from the lower surface of swl1, and swl3 only slightly separated from the lower surface of swl2. **(C–G)** Spore wall layers (swl) 1–3 and subtending hyphal wall layers (shwl) 1 and 2 of spores on which pressure was applied; note that the laminate swl2 transferred into a crown-like structure, and swl3 usually slightly contracted and separated from the lower surface of swl2 and formed a septum (s) located at approximately half the length of the channel connecting the subtending hyphal lumen with the spore interior; peridial hyphae (ph) are visible in **(D)**. **(H)** Intact spores with swl1–3, shwl1 and 2, and glebal hyphae (gh) mounted in PVLG + Melzer's reagent. **(A)** Dry specimen. **(B–E,G)** Spores in PVLG. **(F,H)** Spores in PVLG + Melzer's reagent. **(A)** Light microscopy. **(B–H)** Differential interference microscopy. Scale bars: **(A)** = 200 μm, **(B–H)** = 10 μm.

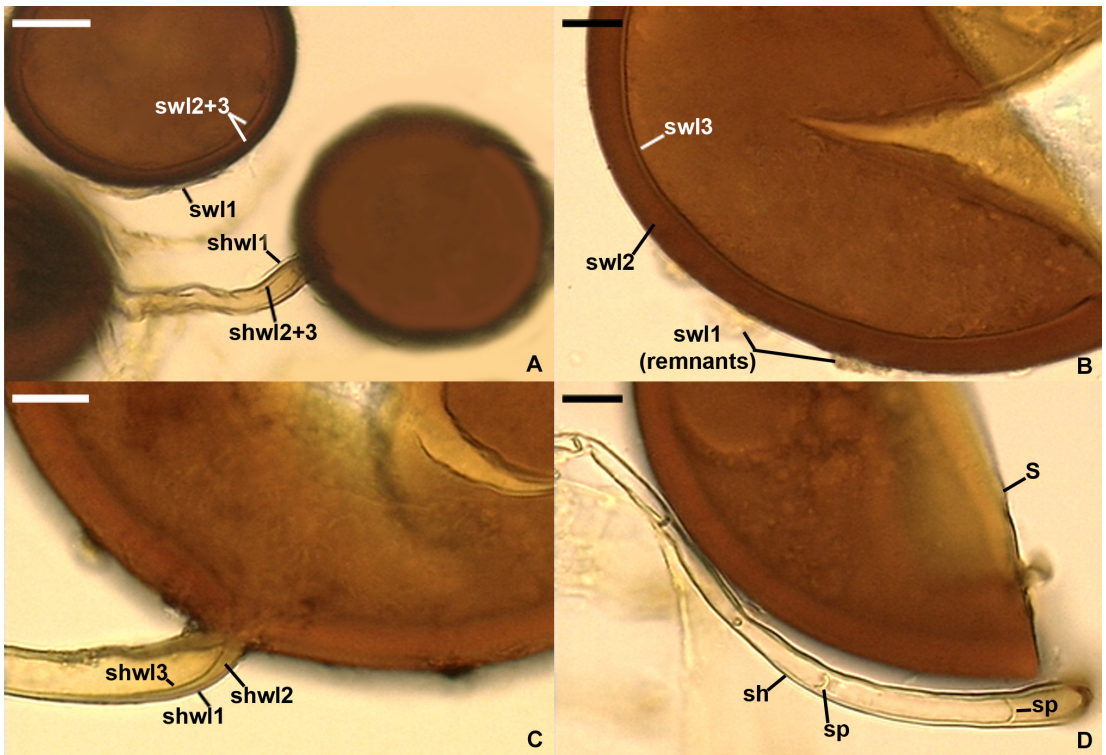



**FIGURE 6 |** *Silvaspora neocaledonica*. **(A)** Spores with a spore wall (sw) consisting of three layers (swl1–3). **(B)** Spore wall layers (swl) 1–3; swl1 is almost completely sloughed off. **(C)** Subtending hyphal wall layers (shwl) 1–3. **(D)** Spore (s) and subtending hypha (sh) with two septa (sp) indicated. **(A,B,D)** Spores in PVLG. **(C)** Spores in PVLG + Melzer's reagent. **(A–D)** Differential interference microscopy. Scale bars: **(A)** = 20 μm, **(B–D)** = 10 μm.

(5B7) to orange (6B7)], 0.8–1.2 μm thick, usually tightly adherent to the lower surface of layer 2, even in vigorously crushed spores, difficult to detect and characterize due to the similarity in appearance to and almost inseparability from the innermost laminae of the laminate layer 2. None of spore wall layers 1–3 stains in Melzer's reagent. *Subtending hypha* pale orange (5A3) to light orange (5A4); cylindrical, slightly funnel-shaped, or slightly constricted at the spore base; (6.2−)8.3(−11.6) μm wide at the spore base (**Figures 6A,C,D**). *Wall of subtending hypha* pale orange (5A3) to light orange (5A4); (1.6−)2.8(−5.4) μm thick at the spore base; consisting of three layers continuous with spore wall layers 1–3; subtending hyphal wall (shwl) layer 3 usually is difficult to see because it is thin, similarly colored, and tightly adherent to shwl2 (**Figures 6A,C**). *Pore* usually occluded due to thickening of spore wall layer 2 and subtending hyphal wall layer 2 (**Figures 6C,D**), rarely slightly open at the spore base; the subtending hyphal lumen occasionally with some transverse septa widely distributed along the subtending hypha (**Figure 6D**). *Germination* through subtending hypha. *Mycorrhiza* with arbuscules, vesicles, and hyphae staining lightly to dark in trypan blue.

*Notes*: The description presented above was prepared based on the original description of this species and examination of its spores provided by T. Crossay. We added color names of spores and spore wall layers determined from Kornerup and Wanscher (1983) to compare them with the color of the subtending hypha, whose pigmentation was not characterized in the original description of *S. neocaledonica*. Also, we changed some measurement values when they differed from the range of measurements given by Crossay et al. (2018) and replaced the name of spore wall layer 1, originally determined as mucilaginous, into evanescent. A mucilaginous spore wall layer *sensu* Stürmer and Morton (1997) stains in Melzer's reagent, whereas spore wall layer 1 of *S. neocaledonica* does not react in this reagent. Finally, we significantly extended the characterization of the subtending hypha compared to that originally published.

*Specimens examined*: 3768–3772 (LPPDSE).

*Distribution and habitat*: To date, this species is known only from the type locality: Noumea, in the rhizosphere soil of *Alphitonia neocaledonica* (Schltr.) Guillaumin growing in an ultramafic *maquis* in the New Caledonia (Crossay et al., 2018). BLAST searches of 18S-ITS-28S and *rpb1* sequences indicated that *S. neocaledonica* has not been recorded in other regions of the world.

## DISCUSSION

As we expected based on exploratory morphological and molecular phylogenetic analyses, our extended phylogenetic analyses of sequence alignments containing representatives of

all genera of the Glomeraceae, using 18S-ITS-28S and *rpb1* data, unambiguously and strongly confirmed our hypothesis that the two glomerocarps found in Northeast Brazil are two new species of the Glomeromycota (**Figures 1–3**). Surprisingly, these morphologically almost identical fungi represent strongly phylogenetically divergent taxa in the Glomeraceae, one belonging to *Dominikia* and the second neighboring the monospecific genus *Kamienskia* (**Figures 1–3**). The divergences of 18S-ITS-28S and *rpb1* sequences between the new species, *D. glomerocarpica* and *E. crypticum*, were 18.5–19.0% and 11.0–11.8%, respectively.

Another interesting finding in our analyses was that the sister species of *D. glomerocarpica* was *D. aurea* (**Figures 1–3**), which presents significant morphological differences. *D. glomerocarpica* is distinguished morphologically by (i) the formation of a compact, epigeous glomerocarp with a peridium; (ii) the production of small, colorless to light yellow (4A4), glomoid spores; (iii) the possession of a spore wall consisting of three permanent, smooth layers, of which the colorless to light yellow (4A4) laminate layer 2 is distinctly thick compared to the spore size and stains dark in Melzer's reagent; and (*iv*) the formation of an invaginated septum continuous with spore wall layer 3, which is usually located at half the length of the channel connecting the lumen of the spore subtending hypha with the spore interior (**Figures 4A–H**).

*D. aurea* was originally described to produce hypogeous, peridium-free, 450–1,200 μm × 510–1,600 μm diameter, glomerocarps (called sporocarps), consisting of tightly packed spores. However, the microphotographs presented in the article of Oehl et al. (2003) and our specimens testify that *D. aurea* spores arise mainly in compact, hypogeous clusters (Błaszkowski, 2012) rather than in typical unorganized glomerocarps, as the much larger, epigeous glomerocarp with a peridium formed by *D. glomerocarpica*. Also, we often extracted loose clusters with few spores and single spores of *D. aurea*. Moreover, *D. aurea* spores are much darker colored and never are colorless at maturity, have a 1.2- to 2.4-fold thinner and only two-layered spore wall, of which layer 1 is impermanent, usually completely sloughed off in mature spores, and stains in Melzer's reagent. Finally, the pore of the spore subtending hypha is closed by a curved septum, which is continuous with the innermost laminae of the laminate spore wall layer 2 and is located at or up to 5 μm below the spore base (Oehl et al., 2003; Błaszkowski, 2012). The clear distinctiveness of *D. aurea* and *D. glomerocarpica* also proved the large molecular divergences between their 18S-ITS-28S and *rpb1* sequences, which were 8.5–10.2% and 4.3–4.8%, respectively.

Another surprising finding of our efforts to establish the taxonomic status of the Brazilian fungi was the placement of the previously named "Species 2" in a sister clade to the monospecific, generic clade represented by *K. bistrata* (**Figures 1–3**). However, the two species differ fundamentally in morphology and are separated from one another by a very large molecular distance (11.0–15.5%). Therefore, we erected a new genus for "Species 2," named *Epigeocarpum*, typified by *E. crypticum*, despite the fact that the morphological characters distinguishing and clearly separating this species from *K. bistrata* are shared by other members of the Glomeraceae, as discussed below.

The distinctive morphological features of *E. crypticum* are (i) the production of small, colorless or light yellow (4A5), glomoid spores in a compact, epigeous glomerocarp with a peridium; (ii) a spore wall composed of three permanent, smooth layers, of which the much thicker laminate layer 2 stains dark in Melzer's reagent and, most importantly, contracts and transfers into a crown-like structure in spores crushed in PVLG and PVLG + Melzer's reagent; and (iii) the formation of a septum by spore wall layer 3 of characters and location similar to those of the septum of *D. glomerocarpica* spores (**Figures 5A–H**).

*K. bistrata* does not produce epigeous glomerocarps with a peridium, but its spores are grouped in loose to compact hypogeous clusters (Błaszkowski et al., 2009a; Błaszkowski, 2012). Most importantly, the spore wall of *K. bistrata* is 2.2- to 4.4-fold thinner than the *E. crypticum*'*s*, consists of only two colorless layers, of which the uniform layer 1 is thicker than the laminate layer 2, none of these layers stains in Melzer's reagent, and the channel connecting the lumen of the spore subtending hypha with the spore interior is not closed by any septum.

As mentioned above, one of the distinctive features of the two here newly described species is the location of the septum separating the lumen of the spore subtending hypha from the spore interior (**Figures 4C,F,G**, **5G**). The septum usually occurs at half the thickness of the laminate spore wall layer 2 as similarly described for *Rhizoglomus maiae* (Błaszkowski et al., 2019b), whereas for all other glomoid spore-producing species, the septum is formed at or slightly below the spore base. Moreover, *R. maiae* also produces compact, epigeous clusters with spores, whose spore wall structure and phenotypic properties of its components are like those of *D. glomerocarpica* and *E. crypticum*, except for the contractility of spore wall layer 2 of *E. crypticum* (**Figures 5D–F**). Thus, the location of the septum in these three species, belonging to three far related genera of the Glomeraceae, clearly proves that this feature is phylogenetically uninformative.

The formation of the septum much above the spore base in *D. glomerocarpica*, *E. crypticum*, and *R. maiae* probably is associated with the spore wall ontogeny and particularly with the differentiation of the distinctively thick laminate spore wall layer 2 by these fungi. As ontogenetic studies of spores of *Claroideoglomus claroideum* revealed, the innermost spore wall layer 4, forming an invaginated septum at or slightly below the spore base, arises after the full differentiation of the laminate spore wall layer 3 (Stürmer and Morton, 1997). Most likely, a similar sequence of events occurs during the development of the spore wall of *D. glomerocarpica*, *E. crypticum*, and *R. maiae*. However, the laminate spore wall layer 3 of *C. claroideum* is a relatively thin component of the entire spore wall, and consequently, the full differentiation of this layer is faster than that of the much thicker laminate spore wall layer 2 of the three main species discussed above. Therefore, the formation of the invagination of spore wall layer 4 of *C. claroideum*, shaped by the available space (the channel connecting the spore subtending hyphal lumen with the spore interior) in which the development of this layer is initiated, starts relatively earlier and closer to the spore base.

Another structure distinguishing *E. crypticum* is the contracting laminate spore wall layer 2, which takes the shape of a crown in spores crushed in PVLG-based mountants (**Figures 5D–F**). A spore wall layer of similar properties was also found in *Microkamienskia perpusilla*, originally described as *Glomus perpusillum* (Błaszkowski et al., 2009b), although the contracting spore wall layer 2 of *M. perpusilla* is a unit structure (not divided into visible sublayers) and does not transform into a crown following crushing of spores in PVLG-based mountants. Therefore, it seems that the contractility of spore wall layer 2 of *E. crypticum* is also not a generic synapomorphy of the new genus *Epigeocarpum*.

In summary, the sole characters convincingly rendering the genus *Epigeocarpum* unique are those residing in its DNA. Importantly, the genetic differences between the 18S-ITS-28S (14.5–15.6%) and *rpb1* (11.0–11.6%) sequences of *E. crypticum* and *K. bistrata* are similar to those between sequences of directly neighboring species of most of the genera present in the tree depicted in **Figures 1**, **2**. Several examples of pairwise comparisons are displayed in **Supplementary Table 3**. Of the described species producing unorganized glomerocarps with glomoid spores, whose molecular phylogeny is uncertain or unknown, *D. glomerocarpica* and *E. crypticum* most closely resemble *Glomus microcarpum* and *Glomus nanolumen*; the spores of these species are similar in color and size. The main differences readily separating the four species reside in their spore wall structure, the phenotypic and histochemical features of the spore wall components, and the characters of the pore connecting the subtending hyphal lumen with the spore interior.

*Glomus microcarpum* was originally characterized as having one, thick spore wall, which was also found by Gerdemann and Trappe (1974) and Berch and Fortin (1984). Instead, Błaszkowski (2012) defined and illustrated this species as producing spores with a spore wall consisting of two layers: a short-lived, quickly sloughing off, or semipermanent, slowly decomposing, thin layer 1, forming the spore surface, and a permanent, thick, laminate layer 2, staining dark red in Melzer's reagent. Similar specimens identified as *G. microcarpum* with a spore wall as that characterized by Błaszkowski (2012) were presented by Oehl et al. (2011) and repeatedly found in Northeast Brazil (unpublished data). Of the Glomeromycota species grown in culture, only *Sieverdingia tortuosa* produced spores with a one-layered spore wall (Błaszkowski et al., 2019a), and in most of glomoid and glomoid-like spore-producing species, their laminate spore wall layer is covered with a relatively thin, usually short-lived, rarely permanent layer (Oehl et al., 2011; Błaszkowski, 2012). In field-collected spores, this outer spore wall layer is usually strongly or completely sloughed off, and therefore, it is difficult to detect or no longer present. On top of our knowledge, *G. microcarpum* has not yet been grown in culture, which makes impossible to examine spores at different developmental stages. However, regardless of the number of layers existing in the spore wall of the true *G. microcarpum*, the structure of the spore wall and the phenotypic properties of its components in *D. glomerocarpica* and *E. crypticum* (**Figures 4B–H**, **5B–H**) differ clearly from those characterized above for *G. microcarpum*. The sequence identity, obtained by BLAST, of 18S-ITS-28S sequences of *E. crypticum* compared to 38 18S-ITS-28S sequences of *G. microcarpum* deposited in GenBank is only 87.5–92.02%. Unfortunately, no data were published about the morphology of the "donor" of the sequences ascribed to *G. microcarpum*. Thus, there is an urgent need for unambiguous morphological and molecular characterization of *G. microcarpum*, one of the first two described species currently classified in the phylum Glomeromycota and until 2010 regarded as the type species of *Glomus* (e.g., Gerdemann and Trappe, 1974).

Regarding *G. nanolumen*, its glomerocarps do not contain a peridium, and most importantly, their spores have a two-layered spore wall only, of which the laminate layer 2 is not uniform in thickness but differs two to three times in its different regions, and none of these layers stains in Melzer's reagent (Koske and Gemma, 1989; Błaszkowski, 2012). Moreover, the subtending hypha of *G. nanolumen* is 1.6- to 2.5-fold wider at the spore base, and the channel connecting the subtending hyphal lumen with the spore interior is open.

Three evidences unambiguously justified the erection of the new monospecific genus *Silvaspora* in the Glomeraceae for the New Caledonian fungus, originally described as *R. neocaledonicus* (Crossay et al., 2018) and here renamed *S. neocaledonica*. First, our phylogenetic analyses of the 18S-ITS-28S and 18S-ITS-28S + *rpb1* alignments, containing sequences of all sequenced *Rhizoglomus* species, placed the New Caledonian fungus in a clade at the rank of genus, and importantly, the clade was located in the sister relation to the genus *Sclerocystis*, which was outside the *Rhizoglomus* clade (**Figures 1**, **3**). Thus, assigning the New Caledonian fungus to *Rhizoglomus* would make this genus polyphyletic. Moreover, although the analyses of *rpb1* sequences clustered the New Caledonian fungus with *Rhizoglomus* spp., this grouping did not obtain either BI or ML support (**Figure 2**). Second, the divergences of 18S-ITS-28S and *rpb1* sequences of the New Caledonian fungus from those of *S. sinuosa* were 22.7–22.9% and 11.4%, respectively. Thus, these divergence values were similar to the sequence divergences of other neighboring genera in the Glomeraceae (see above). Third, the mode of formation and morphology of spores of *S. sinuosa* differ fundamentally from those of *S. neocaledonica* (Gerdemann and Bakshi, 1976; Błaszkowski, 2012; Crossay et al., 2018; pers. observ.). All *Sclerocystis* species *sensu* Schüßler and Walker (2010) produce organized glomerocarps, in which glomoid-like spores arise radially from a central sterile plexus of mycelium, and the spores have a one-layered spore wall. Instead, *S. neocaledonica* produces spores singly or in clusters of 2 to 3, and their spore wall consists of three layers (**Figures 6A–D**; Crossay et al., 2018; pers. observ.).

We did not include into our phylogenetic analyses the monospecific genus *Simiglomus* with *Simiglomus hoi* because it 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, and the origination of these sequences is uncertain, as stated by Redecker et al. (2013). Thus, further studies are needed to clarify the status of *Simiglomus* in the Glomeraceae.

The known glomerocarpic species probably constitute a small part of those existing in nature, mainly due to the difficulty in finding and characterizing these fungi. Collecting glomerocarps by raking and searching the upper soil layer and buried plant fragments is much more time-consuming and tiring than the

wet-sieving and decanting method (Gerdemann and Nicolson, 1963) commonly used to find specimens of the Glomeromycota. Interestingly, Gerdemann and Trappe (1974) stated that finding glomerocarps is also related to the acquisition of a so-called hypogeous instinct. Attempts to grow glomerocarpic fungi in culture rarely succeeded, and therefore their characterization, defined only on the basis of field-collected specimens, may lack accurateness. Besides, the morphological and molecular characterization of these fungi may be impossible due to the degradation of their physical structures and DNA (pers. observ.). Importantly, the few described glomerocarpic species with known molecular phylogeny belong to various taxonomic groups in the Glomeromycota, and frequently they miss genus-specific morphological characters. Moreover, molecular phylogenetic analyses have shown that glomerocarpic-like fruit bodies with glomoid-like spores are also produced by fungal taxa not belonging to the Glomeromycota (Yamamoto et al., 2020). Also, the trophic status of glomerocarpic and glomerocarpic-like species is unclear, particularly that of epigeocarpic species. In order to know the real representativeness of glomerocarpic species in the Glomeromycota and determine their trophic and taxonomic status, the collection of specimens of this group of fungi and the study of their ecology and phylogeny should be significantly intensified.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) are as follows: GenBank MW507148-MW507157, MW541060-MW541067, and MW249072-MW249073.

## AUTHOR CONTRIBUTIONS

BG, FM, JB, PN, and SZ were responsible for conceptualization and funding acquisition. BG, FM, JB, and PN were responsible for data acquisition. FM, JB, PN, TB, and WB performed analyses. JB and FM wrote the first draft. BG, EM, FM, JB, KJ, LC, PN, RM, PM, SZ, TC, and WB wrote, reviewed, and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.655910/full#supplementary-material

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DGfM

ORIGINAL ARTICLE

# New taxa in *Glomeromycota*: *Polonosporaceae* fam. nov., *Polonospora* gen. nov., and *P. polonica* comb. nov.

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## Abstract

Phylogenetic analyses of sequences of the nuc rDNA small subunit (18S), internal transcribed spacer (ITS1-5.8S-ITS2 = ITS), and large subunit (28S) region (= 18S-ITS-28S), as well as sequences of this region concatenated with sequences of the largest subunit of RNA polymerase II (*RPB1*) gene, proved that the species originally described as *Acaulospora polonica* (phylum *Glomeromycota*) represents a new genus and a new family of the ancient order Archaeosporales, here introduced into the *Glomeromycota* under the names *Polonospora* and *Polonosporaceae*, respectively. The phylogenetic analyses and BLASTn queries also indicated that the *Polonosporaceae* with *P. polonica* comb. nov. still contains several morphologically undescribed taxa at the ranks of genus and species, which have a worldwide distribution.

**Keywords** Arbuscular mycorrhizal fungi · 18S-ITS-28S nuc rDNA and 18S-ITS-28S + *RPB1* molecular phylogenies · *Archaeosporales*

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## Introduction

Among 58 species so far described in the genus *Acaulospora* (see Online Resource 1 for species authors), family *Acaulosporaceae*, is *A. polonica*, accommodated in this genus because forming spores laterally directly on the neck of a sporiferous saccule (Gerdemann and Trappe 1974; Błaszkowski 1988; 2012), as similarly described for *A. laevis*, the type species of *Acaulospora*. However, several morphological characters of *A. polonica* spores did not fit those of most *Acaulospora* species. All typical *Acaulospora* species produce spores with a colored spore wall (spore wall 1), forming the spore surface, and two colorless inner walls (spore walls 2 and 3). Spore wall 1 consists of two to three layers, and its laminate layer in many species is ornamented. Spore wall 2 usually contains two tightly adherent thin (< 1 µm thick), smooth, flexible to semi-flexible layers, and occasionally is one-layered. Spore wall 3 consists of two layers, of which layer 1 is thin and ornamented with fine granules, rarely smooth. Layer 2 in most species is thin when mounted in water but becomes plastic, strongly thickening in lactic acid-based mountants, e.g., in polyvinyl alcohol/lactic acid/glycerol (PVLG, Omar et al. 1979), and stains dark in Melzer's reagent. Only in few species, this layer remains thin in PVLG and stains faintly or does not stain in Melzer's.

The subcellular spore structure of *A. polonica* also consists of three walls, but spore wall 1 remains hyaline to white through its entire life cycle and is smooth (Błaszkowski 1988, 2012). However, the fundamental differences between *A. polonica* and typical *Acaulospora* species reside in the composition, as well as in the phenotypic and histochemical properties of spore wall 3. In *A. polonica*, spore wall 3 is also two-layered, but the outer layer 1 is relatively thick, coriaceous sensu Walker (1986), and smooth. Spore wall 3 layer 2 is flexible to semi-flexible and does not thicken in PVLG. In addition, none of spore wall 3 layers 1 and 2 stains in Melzer's reagent.

Of members of the *Glomeromycota*-producing spores laterally on the neck of a sporiferous saccule, named acaulosporoid spores (Sieverding and Oehl 2006; Oehl et al. 2011a), the features of spore wall 3 of *A. polonica* most resemble those of the innermost spore wall 2 of all species of the genera *Ambispora* and *Archaeospora*, for example, *Am. leptoticha* and *Ar. trappei*, originally described as *A. gerdemannii* and *A. trappei*, respectively (Nicolson and Schenck 1979; Morton and Redecker 2001; Sieverding and Oehl 2006; Bills and Morton 2015).

Thus, *A. polonica* spores have a spore wall 2 with phenotypic features similar to spore wall 2 of typical *Acaulospora* species and a spore wall 3, whose characters do not fit those of spore wall 3 of other *Acaulospora* species but share characters of spore wall 2 of acaulosporoid spores of *Ambispora* and *Archaeospora* species. Another feature linking *A. polonica*, typical *Acaulospora* species, and species of *Archaeospora* but separating these species from *Ambispora* species is the place where acaulosporoid spores are formed. Spores of *Acaulospora* species and *Ar. trappei* arise directly on the neck of a sporiferous saccule, whereas spores of *Ambispora* species develop at the tip of a short branch of the neck. Moreover, *Ambispora* species also produce glomoid spores at tips of cylindric or funnel-shaped sporogenous hyphae, as *Glomus macrocarpum* and *Archaeospora* species also form glomoid and entrophosporoid spores (Morton and Redecker 2001; Oehl et al. 2011a). The latter arise inside the neck of a sporiferous saccule, as in a species originally described as *G. infrequens* (Hall 1977) and later renamed *Entrophospora infrequens* (Ames and Schneider 1979), as well as in some species described in *Entrophospora* and then transferred to *Acaulospora* (Kaonongbua et al. 2010). All specimens of *A. polonica* found by us so far had acaulosporoid spores only (Błaszkowski 2012). Finally, species of *Ambispora* and *Archaeospora* are distinguished by their mycorrhizal structures, which stain faintly, much lighter than those of typical *Acaulospora* species, or do not stain at all in Trypan blue (Morton and Redecker 2001).

Currently, species of *Ambispora* and *Archaeospora* are classified in the families *Ambisporaceae* and *Archaeosporaceae*, respectively, both belonging to the order *Archaeosporales*, that represent ancient members of the *Glomeromycota* (Morton and Redecker 2001) and molecularly are strongly divergent from the generic *Acaulospora* clade (Oehl et al. 2011b; Online Resource 8).

Despite possessing the unique morphological characters discussed above, the true placement in the *Glomeromycota* of the species currently included into *Ambispora* and *Archaeospora* was possible only based on phylogenetic analyses of their molecular sequences (Morton and Redecker 2001; Oehl et al. 2011a). Also, the morphological divergences of *A. polonica* from typical *Acaulospora* species, as well as the morphological similarities and differences of *A. polonica* to species of *Ambispora* and *Archaeospora*, suggest that *A. polonica* does not belong to *Acaulospora* but should represent an undescribed clade located among basal clades of the *Glomeromycota*. Unfortunately, the determination of the placement of this clade based on morphology of *A. polonica* spores only has not been possible using current morphological approaches.

*Acaulospora polonica* has so far been found physically very rarely in the field and has not been grown in culture. Only recently, we found *A. polonica* abundantly sporulating in pot trap cultures. Therefore, the aims of the studies discussed here were (*i*) to grow *A. polonica* in single-species

cultures, (*ii*) to check whether the morphological and histochemical properties of its spores were correctly characterized by Błaszkowski (1988, 2012), (*iii*) to characterize mycorrhizal structures of this species, and (*iv*) to determine whether this species forms spore morphotypes other than acaulosporoid, as well as (*v*) to perform relevant molecular phylogenetic analyses to confirm our hypothesis that this species belongs to an undescribed clade located among basal clades of the *Glomeromycota* and to determine its position within this phylum.

## Materials and methods

### Origin of study material

Spores of *A. polonica* were originally extracted from a trap pot culture inoculated with field-collected rhizosphere soil and root fragments of *Rosa rugosa* inhabiting maritime dunes of the Baltic Sea. The spores were used to establish single-species pot cultures. The plant used for the trap and single-species cultures was *Plantago lanceolata*. The field inoculum was collected near the village of Kuźnica (54° 44′ 11″ N 18° 34′ 47″ E) located on the Hel Peninsula in northern Poland by J. Błaszkowski 21 Aug 2017.

### Establishment and growth of trap and single-species cultures

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 in Błaszkowski et al. (2012). The only exception was the source of the supplemental lighting. We used LEDDY Retro Fit tube lamps located ca. 40 cm above the cultures. The photosynthetic photon flux density measured at the level of the upper surface of the growing substrate was ca. 40 µmol $m^2$ $s^{-1}$. The exposure time was 12 h a day. Ten to 20 spores of uniform morphology stored in water for ca. 2 weeks at 4 °C in a refrigerator were used to establish single-species cultures.

### Microscopy and nomenclature

Morphological features of spores and the phenotypic and histochemical characters of spore wall layers of *A. polonica* were characterized based on at least 100 spores mounted in water, lactic acid, PVLG, and a mixture of PVLG and Melzer's reagent (1:1, v/v). The preparation of spores for study and photography was as those described previously (Błaszkowski 2012; Błaszkowski et al. 2012). Types of spore wall layers are those defined by Błaszkowski (2012) and Walker (1983). Color 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. The term glomerospores was used for spores produced by AMF, as proposed by Goto and Maia (2006).

Voucher specimens of the proposed new taxon [spores permanently mounted in PVLG and a mixture of PVLG and Melzer's reagent (1:1, v/v) on slides] are deposited at Z+ZT (ETH Zurich, Switzerland: ZT Myc 64,926 and 64,927) and in the Laboratory of Plant Protection, Department of Shaping of Environment (LPPDSE), West Pomeranian University of Technology in Szczecin, Poland (holotype, isotypes).

### DNA extraction, PCR conditions, cloning, and DNA sequencing

Genomic DNA of *A. polonica* was extracted from eight samples, each consisting of 5−10 spores crushed with the end of a preparation needle on sterile microscope slides observed under a dissecting microscope. Details of the treatment of the spores prior to polymerase chain reaction (PCR), the conditions, and primers used in the PCR to obtain 18S-ITS-28S sequences of the fungus were as those described in Błaszkowski et al. (2014, 2015b), Krüger et al. (2009), and Symanczik et al. (2014). *RPB1* sequences of *P. polonica* were obtained by amplification with primer RPB1-4F1 in combination with RPB1-5R, as described in Błaszkowski et al. (2021).

*RPB1* sequences of *Ar. schenckii*, *Innospora majewskii*, and *Paraglomus laccatum* were obtained by amplification with primers designed by Stockinger et al. (2014) following the recommended conditions. We used the same DNA, from which 18S-ITS-28S sequences had been obtained (Błaszkowski et al. 2017). The first PCR was performed with primers RPB1-Ac and RPB1-DR2160r, while the second PCR with RPB1-Ac and RPB1-DR1210r+RPB1-DR1210r_Aca_div.

Cloning and sequencing of the PCR products to obtain both types of sequences were as those described by Błaszkowski et al. (2015a). The sequences were deposited in GenBank with the accession numbers MZ359654−MZ359660 and MZ362263−MZ362271.

### Sequence alignment and phylogenetic analyses

BLASTn' searches, using 18S-ITS-28S sequences of *A. polonica*, showed that closest relatives of the species are uncultured fungi of the *Archaeosporales*. To determine the placement of *A. polonica* among morphologically and molecularly characterized members of the *Archaeosporales*, as well as among the uncultured fungi indicated by BLASTn, two alignments were assembled: 18S-ITS-28S and 18S-ITS-28S+*RPB1*. The 18S-ITS-28S alignment consisted

of 60 sequences of the 18S-ITS-28S nuc rDNA region or 28S gene that characterized five species of *Archaeospora* and four species of *Ambispora*, *A. polonica*, and *Geosiphon pyriformis* (*Geosiphonaceae*), as well as 20 sequences of uncultured members of the *Archaeosporales* identified in eleven countries of the world. The outgroup was twelve sequences of members of the *Paraglomerales*: four species of the genus *Paraglomus*, *Innospora majewskii* (*Paraglomeraceae*), and *Pervetustus simplex* (*Pervetustaceae*). In the 18S-ITS-28S + *RPB1* alignment, containing all sequences of the 18S-ITS-28S alignment, 15 *RPB1* sequences were concatenated with 18S-ITS-28S sequences of eight species of the *Archaeosporales* and *Paraglomerales*, all so far provided with sequences of this gene, except for *Ar. trappei* (reasons for this exception are exposed in the "Discussion" section). The *RPB1* sequences covered part of the fourth and fifth exon of the gene and the intron in between.

Before concatenation, each sequence set was aligned separately with MAFFT 7, using E-INS-i as iterative refinement method (http://mafft.cbrc.jp/alignment/server/). Indels were coded for the 18S-ITS-28S alignment as binary characters by means of FastGap 1.2 (Borchsenius 2009), with the possibility to code missing data to be recognized by the phylogenetic inference programs. Sequences from the two alignments were then manually concatenated to produce the 18S-ITS-28S + *RPB1* alignment. The alignments are shown in Online Resources 2 and 3.

The sequences of uncultured members of the *Archaeosporales* were selected as follows: about 450 sequences were downloaded from a BLASTn search using sequences of *A. polonica* as queries. The sequences were ranging from 99 to 80% of identity with the query. After alignment with MAFFT 7, a shared portion of the 28S gene (ca 550 bp), spanning approximately between the primer sites 28G1 and LSUBr (da Silva et al. 2006; Krüger et al. 2009), was used for clustering the sequences in OTUs in Mothur v.1.44.3 (Schloss et al. 2009), using 0.04 as distance cutoff. Pilot analysis on the same 28S portion from species in the *Archaeosporales* detected the cutoff value = 0.04 as the best choice to cluster at species level and cutoff value = 0.1 to cluster at genus level. Singletons were not considered for further analysis, resulting in twenty OTUs for 364 sequences. Full-length sequences representative of each OTUs were then used in phylogenetic analysis. A summary of the OTUs composition and the geographical origin of the sequences is shown in Online Resources 4 and 5.

Identity values of the 18S-ITS-28S and *RPB1* sequences of *A. polonica* were calculated separately using BioEdit (Hall 1999). With the same program, we calculated the percentage sequence divergence of this species from sequences of its closest relatives (Fig. 1, Online Resource 6). All comparisons were performed on sequences of the same length.

The phylogenetic position of *A. polonica* among sequenced species and other potential members of the *Archaeosporales* was reconstructed based on Bayesian inference (BI) and maximum likelihood (ML) phylogenetic analyses of the alignments, performed via CIPRES Science Gateway 3.3 (Miller et al. 2010). GTR + G + I was chosen as substitution model for the DNA partitions for both BI and ML analysis (Abadi et al. 2019). For the indel partition, F81 model was chosen, as suggested by MrBayes manual. Four Markov chains were run over one million generations in MrBayes 3.2 (Ronquist et al. 2012), sampling every 1000 generations, with a burn-in at 3000 sampled trees. The ML phylogenetic tree inference, using a maximum likelihood/1000 rapid bootstrapping run, was computed with RAxML 8.2.12 (Stamatakis 2014). To improve the accuracy of phylogenetic reconstruction (Lanfear et al. 2012; Nagy et al. 2012), in both BI and ML analyses, the 18S-ITS-28S alignment was divided into six partitions: 18S, ITS1, 5.8S, ITS2, 28S, and the binary (indel) character set. The same partitioning scheme was used in the 18S-ITS-28S + *RPB1* alignment with five additional partitions: for the two exons, separate partitions were applied for the first two and for the third codon positions, while a single partition was applied to the intron. In the analysis of the resulting trees, we assumed that clades were supported when BI posterior probability and ML bootstrap support values were ≥ 0.95 and ≥ 70%, respectively. In addition, the 18S-ITS-28S and 18S-ITS-28S + *RPB1* trees were compared based on three measures: (*i*) the number of species clades supported with BI ≥ 0.95 and ML ≥ 70%, (*ii*) mean supports of nodes with BI ≥ 0.95 and ML ≥ 70%, and (*iii*) the amount of resolution of each tree. The mean supports of nodes were the sums of BI ≥ 0.95 and ML ≥ 70% supports divided by the number of nodes with BI ≥ 0.95 and ML ≥ 70% present in each tree. The amount of resolution is the number of nodes with BI ≥ 0.95 and ML ≥ 70% divided by the number of all nodes. The phylogenetic trees obtained in the analyses were visualized and rooted in Archaeopteryx.js (https://sites.google.com/site/cmzmasek/home/software/archaeopteryx-js).

## Results

### General data and phylogeny

In this study, 92 sequences of the 18S-ITS-28S rDNA region or 28S rDNA gene and 15 sequences of the *RPB1* gene were analyzed. Of these, 16 were new (7 18S-ITS-28S and 9 *RPB1*). The 18S-ITS-28S and 28S sequences represented 17, and those of *RPB1* eight species of the *Archaeosporales* ingroup, including the species originally described as *A. polonica*, and the *Paraglomerales* outgroup. These sequences were part of two alignments (18S-ITS-28S and 18S-ITS-28S + *RPB1*) that were analyzed using BI and ML algorithms.

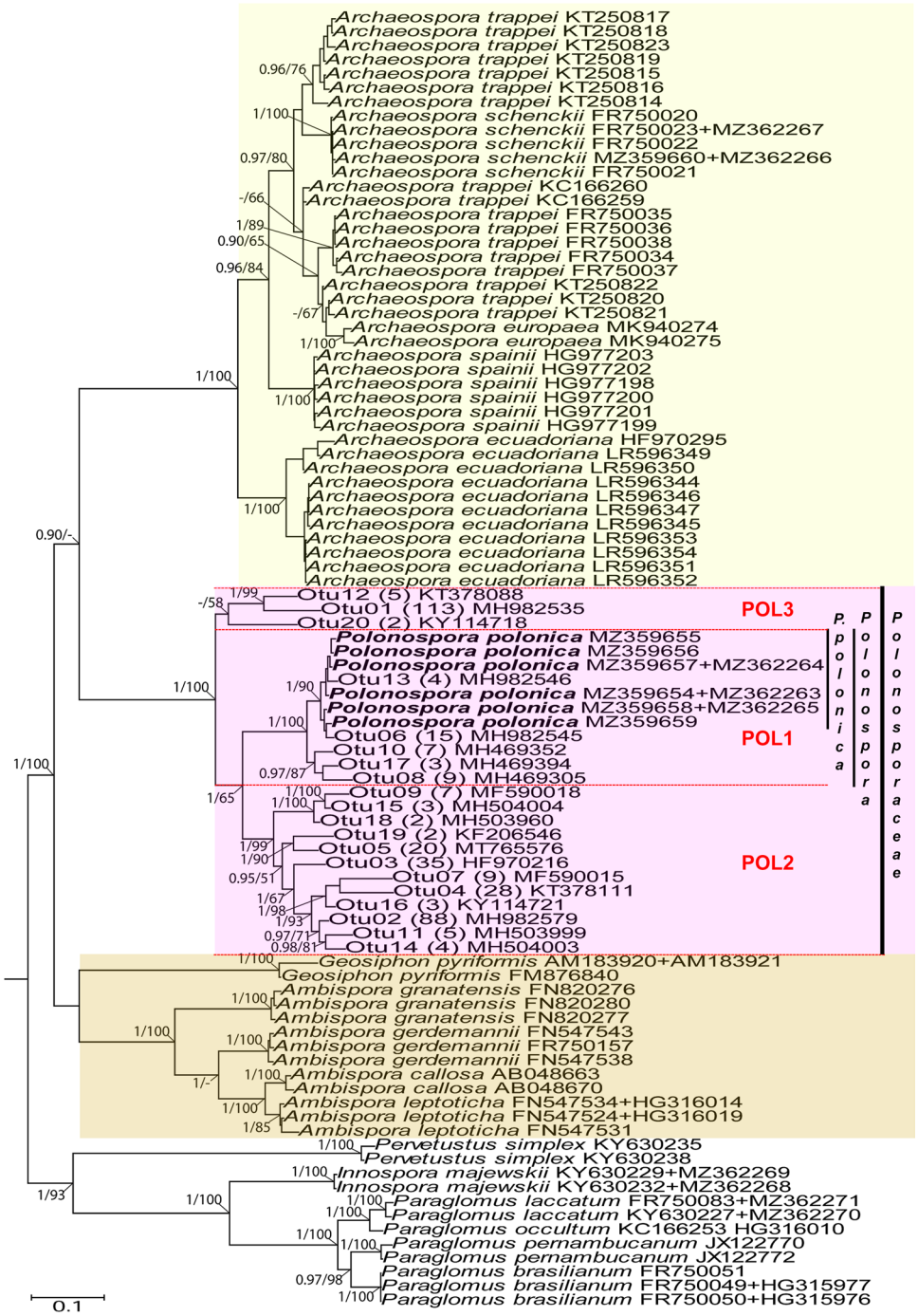



**Fig. 1** A 50% majority rule consensus phylogram inferred from a Bayesian inference analysis of 18S-ITS-28S + *RPB1* sequences of *Polonospora polonica*, 10 species of the *Archaeosporales*, 20 OTUs representative sequences from environmental studies (the number of sequences clustered in each OTU is shown between brackets), and six species of the *Paraglomerales* serving as outgroup. Families in the *Archaeosporales*, including the new *Polonosporaceae*, are highlighted by colored boxes. The three distinct clades (POL1–3) in the *Polonosporaceae* are separated by red dashed lines. The Bayesian posterior probabilities ≥ 0.90 and ML bootstrap values ≥ 50% are shown near the branches, respectively. Bar indicates 0.1 expected change per site per branch

**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 |
|---|---|---|---|---|---|
| 18S-ITS-28S | 92 | 17 | 2474 | 1120 | 992 |
| 18S-ITS-28S + *RPB1* | 92 | 17 | 3496 | 1549 | 1385 |

Data about the numbers of base pairs as well as variable and parsimony-informative sites of the 18S-ITS-28S and 18S-ITS-28S + *RPB1* alignments are presented in Table 1. The identity values of the six 18S-ITS-28S and three *RPB1* sequences of *A. polonica* were 96.9–98.9% and 99.3%, respectively.

The topologies of the trees generated in BI and ML analyses of the 18S-ITS-28S and 18S-ITS-28S + *RPB1* alignments were identical for both the *Archaeosporales* and *Paraglomerales* clades (Fig. 1, Online Resource 6). Also, the measures we used to compare these trees were very similar (Online Resource 7). Because it is widely recommended to reconstruct fungal phylogenies from multiple unlinked loci (Matheny 2005; Miadlikowska et al. 2014; Stadler and Weber 2021), here were discuss the phylogeny of *A. polonica* mainly based on trees generated in analyses of the 18S-ITS-28S + *RPB1* alignment (Fig. 1).

Sequences of *A. polonica* clustered in a new clade at the rank of family in a sister position to the *Archaeosporaceae* clade, but this association was weakly supported in the BI analysis only (Fig. 1, Online Resource 6). The new family clade consisted of three subclades (named POL1, 2, and 3) at the rank of genus. POL1 and POL2 formed a sister relationship with good support only in the BI analysis. POL1 consisted of two sister clades, of which one contained sequences of *A. polonica* and sequences obtained in environmental studies conducted in China, Georgia, Japan, Portugal, and the USA (Michigan and Massachusetts). The sister clade to *A. polonica* contained only environmental sequences. These were obtained from studies performed in China, Japan, and Portugal, and clustered in three OTUs representing potentially new species. POL2, sister to the generic clade with *A. polonica*, hosted the highest number of environmental sequences, clustering in 12 OTUs representing several potentially new species. POL2 was also the most geographically widespread; its available environmental sequences originated from Australia, China, Europe, and South and North America (see Online Resource 5 for details). The third generic clade contained environmental sequences from China, Czech Republic, and Norway. The new family clade obtained full supports in both BI (= 1.0) and ML (= 100%) analyses. Also, both analyses strongly supported the generic clades POL1 and POL2. Instead, the generic clade POL3 was supported only in the BI analysis of the 18S-ITS-28S alignment. The species clade with *A. polonica* obtained strong supports in both BI (= 1.0) and ML (= 90.0%) analyses.

Of the five species of the *Archaeosporaceae* clade, the closest relative of *A. polonica* was *Ar. ecuadoriana*. The 18S-ITS-28S sequence divergence between these species was 24.5–28.0%.

Considering the results of the phylogenetic analyses and comparisons of sequences described above, we transferred *A. polonica* to the *Archaeosporales* and accommodated it in two newly erected taxa, *Polonosporaceae* fam. nov. and *Polonospora* gen. nov., as *P. polonica* comb. nov. In addition, we presented an updated morphological description of *P. polonica* and its possible distribution.

## Taxonomy

### Erection of a new family, genus, and combination

***Polonosporaceae*** Błaszk., Niezgoda, B.T. Goto, Magurno, fam. nov.

**MycoBank**: MB840255.

***Type***: *Polonospora* Błaszk., Niezgoda, B.T. Goto, Magurno.

***Etymology***: Latin, *Polonosporaceae*, *Polono* (= from Poland) and *sporaceae* (= forming spores), referring to Poland, in which spores of the new family were originally found.

***Diagnosis***: Differs from the *Archaeosporaceae* and *Ambisporaceae* in the number of spore walls (three-walled vs. two-walled) and the nucleotide composition of sequences of the 18S-ITS-28S nuc rDNA region and the *RPB1* gene.

***Description***: Forming hypogeous single acaulosporoid glomerospores (= spores) directly on the neck of a sporiferous saccule (Fig. 2a, b). Spores hyaline to white (1A1), usually globose to subglobose, 80–115 µm diam, with three spore walls (spore walls 1–3; Fig. 2a–h). Spore wall 1 consisting of a short-lived, evanescent, thin layer, continuous with the wall of the sporiferous saccule, and a permanent, laminate, thicker layer (Fig. 2c–h). Spore wall 2 composed of one permanent, flexible to semi-flexible layer (Fig. 2c–h). Spore wall three permanent, coriaceous, 1.8–3.6-µm thick, composed of two tightly adherent layers (Fig. 2c–h). Only spore wall 1 layer 2 sometimes stains reddish white (7A2) in Melzer's reagent.

***Distribution and habitat***: Worldwide, with records of physical specimens and environmental sequences originating from Australia, Czech Republic, China, Georgia, Japan, Norway, Poland, Portugal, and North and South Americas (Online Resource 5). Associated with roots of plants growing in dunes, forests, grasslands, hydrocarbon-polluted sites, tropical forested and cultivated soils, and wetland ecosystems.

***Polonospora*** Błaszk., Niezgoda, B.T. Goto, Magurno, gen. nov.

**MycoBank**: MB840256.

***Type***: *Polonospora polonica* (Błaszk.) Błaszk., Niezgoda, B.T. Goto, Magurno.

***Etymology***: As that for the *Polonosporaceae* (see above).

***Diagnosis***: As that of *Polonosporaceae* (see above).

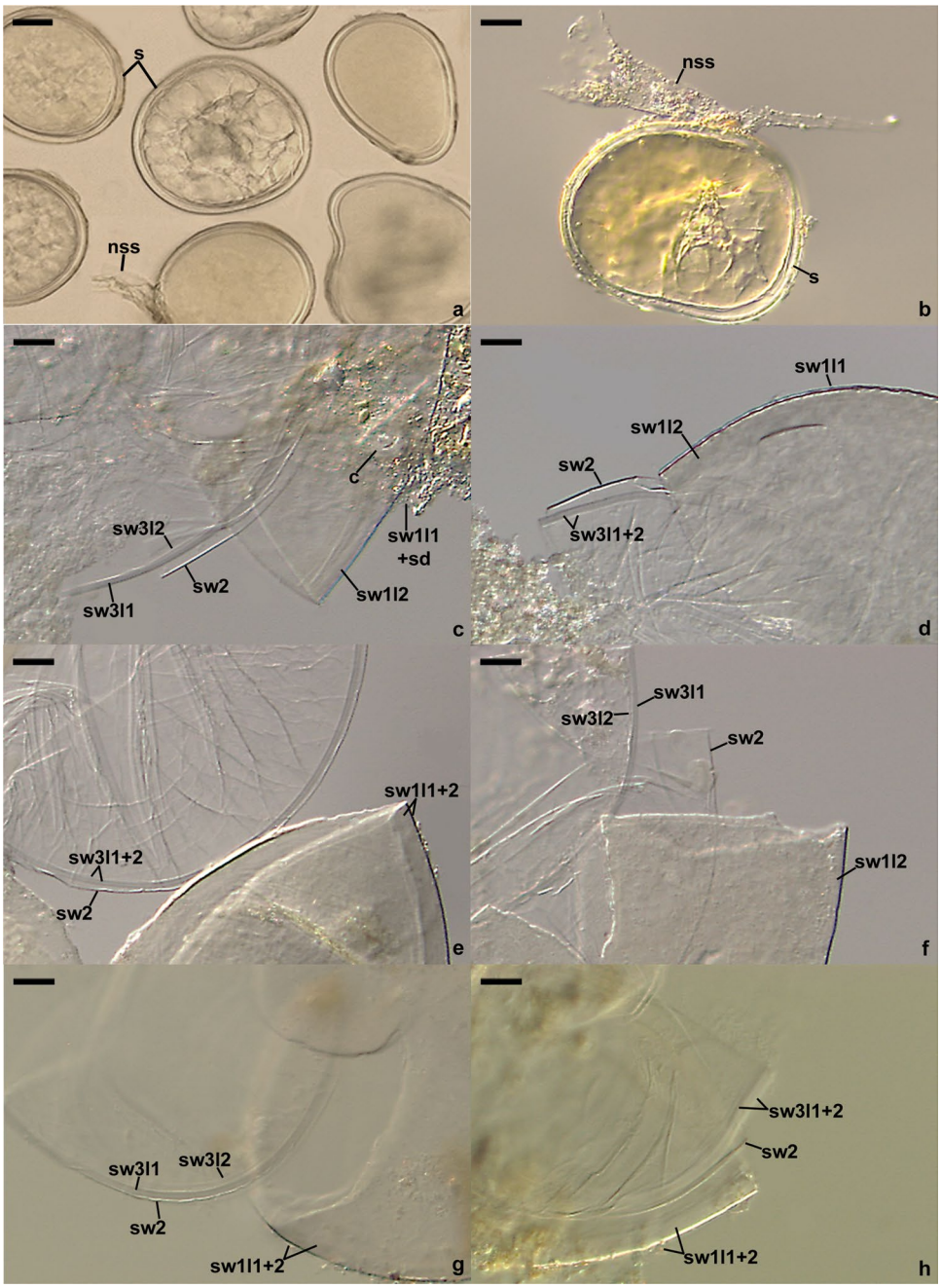



**Fig. 2** *Polonospora polonica*. **a**, **b**. Intact spores (s) with the neck of a sporiferous saccule (nss; remnants). **c**–**h**. Spore wall 1 (sw1) layers 1 and 2, spore wall 2 (sw2), and spore wall 3 (sw3) layers 1 and 2; soil debris (sd) covering the upper surface of sw1l1 and cicatrix (c) are indicated in **c**. **a**–**f**. Spores in PVLG. **g**, **h**. Spores in PVLG + Melzer's reagent. **a**–**h**. Differential interference microscopy. Scale bars: **a**, **b** = 20 µm, **c**–**h** = 10 µm

***Distribution and habitat***: Worldwide, with records of physical specimens and environmental sequences originating from China, Georgia, Japan, Lithuania, Poland, Portugal, and the USA (Online Resource 5). Associated with roots of plants growing in gardens, dunes, forests, and highly contaminated sites (Gai et al. 2006; Błaszkowski 2012).

***Specimens examined***: 95–136, 3804–3821, LPPDSE.

***Polonospora polonica*** (Błaszk.) Błaszk., Niezgoda, B.T. Goto, Magurno, comb. nov. Figure 2a–h.

**MycoBank**: MB840257.

***Basionym***: *Acaulospora polonica* Błaszk., Karstenia 27, 38. 1988.

***Description***: Glomerospores (= spores) formed singly in soil, arise laterally from the neck of a sporiferous saccule continuous with an extraradical mycorrhizal hypha (Fig. 2a, b). *Spores* hyaline to white (1A1), glistening, globose to subglobose, (80–)94(–115) µm diam, with three spore walls (Fig. 2a–h). *Spore wall 1* with two layers (Fig. 2c–h). Layer 1 evanescent, hyaline, up to 1.5-µm thick, continuous with the wall of the sporiferous saccule, usually highly

deteriorated or completely sloughed in mature spores. Layer 2 permanent, laminate, smooth, hyaline to white (1A1), (3.3–)4.7(–5.5) µm thick. *Spore wall 2* consists of one, permanent, flexible to semi-flexible, (0.8–)1.1(–1.3) µm thick, hyaline layer (Fig. 2c–h). *Spore wall 3* composed of two permanent, hyaline, smooth layers (Fig. 2c–h). Layer 1 semi-flexible, (1.3–)1.8(–2.3) µm thick. Layer 2 flexible to semi-flexible, 0.5–1.3-µm thick, usually tightly adherent to the lower surface of layer 1 and difficult to see. In Melzer's reagent, only spore wall 1 layer 2 sometimes stains reddish white (7A2). *Germination orb* not found. *Sporiferous saccule* hyaline, globose to subglobose, 60–90 µm diam; neck 40–70 µm long, tapering from 10.0–17.5 µm diam at the saccule to 8.0–12.5 µm diam at the point of spore attachment. *Saccule wall* a hyaline, smooth, 0.5–1.0-µm thick layer. Saccule collapsing at maturity and usually detached in mature spores. *Cicatrix* a slightly raised collar when seen in cross view, circular, 6–9 µm diam, when observed in plain view.

***Mycorrhizal association***: In the field, produced spores in rhizosphere of, e.g., *Ammophila arenaria*, *Chamaecyparis lawsoniana*, *Corynephorus canescens*, *Juncus balticus*, *Juniperus communis*, *R. canina*, and *Thuja occidentalis* (Błaszkowski 1988, 1993a, b, 1994, unpubl. data). Abundantly sporulated in trap cultures inoculated with rhizosphere soil and root fragments of *R. rugosa*, whose host plant was *P. lanceolata*. Many attempts at growing *P. polonica* in single-species cultures failed.

***Distribution and habitat***: Worldwide, with records of physical specimens and environmental sequences originating from China, Georgia, Japan, Lithuania, Poland, Portugal, and the USA (Online Resource 5). Associated with roots of plants growing in gardens, dunes, forests, and highly contaminated sites (Gai et al. 2006; Błaszkowski 2012).

***Specimens examined***: **Holotype**. POLAND. Hel, ca 200 m from the Baltic Sea, under *Thuja occidentalis*, 21.08.1985, *Błaszkowski J.*, 95, LPPDSE; 96–136 (**isotypes**), LPPDSE; Kuźnica, under *R. rugosa*, 21.08.2017, *Błaszkowski J.*, 3810–3821, LPPDSE. LITHUANIA. Kuronian Spit, Parnidos dune, under *A. arenaria*, 15.09.2013, *Błaszkowski J.*, 3804–3809, LPPDSE.

## Discussion

The results of molecular phylogenetic analyses and comparisons of sequences discussed above confirmed our hypothesis that the species originally described as *A. polonica* (Błaszkowski 1988) does not belong to *Acaulospora* but represents a new genus in a new family of the *Archaeosporales*, here introduced into the *Glomeromycota* under the names *Polonospora* and *Polonosporaceae*, respectively. Moreover, our analyses indicated that (*i*) the *Polonosporaceae* consists of three groups at the rank of genus: *Polonospora*, typified by *P. polonica* comb. nov, and AMF known from molecular environmental studies only (Fig. 1, Online Resources 4, 5) and (*ii*) the *Polonosporaceae* with *P. polonica* and the yet undescribed taxa have a worldwide distribution (Online Resource 5). Unfortunately, our attempts at growing *P. polonica* in single-species cultures failed. Therefore, the characters of mycorrhizal structures of this species and its potential ability to produce spore morphotypes other than acaulosporoid remain unknown.

Our POL1, POL2, and POL3 correspond to the Arch3, Arch2, and Arch1 clades, respectively, of an undescribed family in the *Archaeosporales* recognized by Kolaříková et al. (2021) based on analyses of environmental sequences. The Arch1 clade did not receive full support in our analysis, but with the present data, it is not possible to establish if it could represent more than one genus. Notably, considering the full-length sequences of the three OTUs in the clade, one is highly divergent (by 13.0–15.5%) from the other two.

Our analyses indicated a moderate sister relationship of the *Polonosporaceae* to the *Archaeosporaceae*, whereas the Kolaříková et al.'s (2021) informal family was a sister to a clade formed by the *Ambisporaceae* and *Geosiphonaceae*. This could result from the differences in the phylogenetic approaches. In Kolaříková et al. (2021), only sequences of the unpartitioned 18S-ITS-28S nuc rDNA locus were employed in the BI and ML analyses, while in the present analysis, a partitioning scheme was applied to the alignment containing 18S-ITS-28S sequences concatenated with those of the unlinked protein-coding *RPB1* gene. Furthermore, the sequence set used in the present study consisted of sequences of the *Archaeosporales* and *Paraglomerales* only (the latter used as outgroup). The sequence set analyzed by Kolaříková et al. (2021) additionally contained sequences of the *Diversisporales* and *Glomerales*. Finally, twenty OTUs, representing 364 environmental sequences, and six sequences belonging to *P. polonica* were used to represent the new family *Polonosporaceae*, while in Kolaříková et al. (2021), only six OTUs and four sequences from another study (Melo et al. 2018) were used. The genetic diversity of sequence data, the resolution power of their components, the method of analyzing, and the taxonomic composition of the analyzed set may affect the reliability of fungal phylogenies (Matheny 2005; Redecker et al. 2013; Miadlikowska et al. 2014).

We did not concatenate any 18S-ITS-28S sequence of *Ar. trappei* with the sole available *RPB1* sequence of this species (HG315988) obtained from the *Ar. trappei* INVAM CR401B isolate (Stockinger et al. 2014) because in public data bases, there is no 18S-ITS-28S sequence obtained from this isolate. Schüßler and Walker (2019) demonstrated that the phylogenetic position of *Ar. trappei* is unknown. In our 18S-ITS-28S and 18S-ITS-28S + *RPB1* trees, sequences ascribed to *Ar. trappei* were also accommodated in three clades separated by *Ar. europaea* and *Ar.*

*schenckii* (Fig. 1, Online Resource 6). No living cultures of ex-type material of *Ar. trappei* are available (Schüßler and Walker 2019). Thus, to unambiguously determine the phylogenetic position of *Ar. trappei* and other members of this genus within the *Archaeosporaceae*, an epitype of this species has to be designated based on spores obtained from material collected from the area, where *Ar. trappei* was originally found (Ames and Linderman 1976).

Apart from *Ar. trappei*, the *Glomeromycota* contains many other species, whose natural phylogeny is uncertain or unknown (Schüßler and Walker 2010; Błaszkowski 2012; Redecker et al. 2013; Kolaříková et al. 2021; pers. observ.). One of them is *A. gedanensis*, whose morphological features (Błaszkowski 1988, 2012) suggest belonging to an undescribed taxon related to members of the *Ambisporaceae*. As *P. polonica*, *A. gedanensis* was also originally found in soils of the Hel Peninsula.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11557-021-01726-4.

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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 Błaszkowski, Bruno Tomio Goto, Franco Magurno; writing—review and editing: Janusz Błaszkowski, Bruno Tomio Goto, Franco Magurno, Monika Malicka, Edward Meller, Paweł Milczarski, Sylwia Uszok, Piotr Niezgoda, and Szymon Zubek; funding acquisition: Bruno Tomio Goto, Piotr Niezgoda, Szymon Zubek; resources: Janusz Błaszkowski, Franco Magurno, Piotr Niezgoda; Supervision: Janusz Błaszkowski. All authors read and approved the final manuscript.

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

**Code availability** Not applicable

## Declarations

**Conflict of interest** The authors declare no competing interests.

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**Artykuł 8**

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

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As a result of phylogenomic, phylogenetic, and morphological analyses of members of the genus *Claroideoglomus*, four potential new glomoid spore-producing species and *Entrophospora infrequens*, a new order, Entrophosporales, with one family, Entrophosporaceae (=Claroideoglomeraceae), was erected in the phylum Glomeromycota. The phylogenomic analyses recovered the Entrophosporales as sister to a clade formed by Diversisporales and Glomeraceae. The strongly conserved entrophosporoid morph of *E. infrequens*, provided with a newly designated epitype, was shown to represent a group of cryptic species with the potential to produce different glomoid morphs. Of the four potential new species, three enriched the Entrophosporales as new *Entrophospora* species, *E. argentinensis*, *E. glacialis*, and *E. furrazolae*, which originated from Argentina, Sweden, Oman, and Poland. The fourth fungus appeared to be a glomoid morph of the *E. infrequens* epitype. The physical association of the *E. infrequens* entrophosporoid and glomoid morphs was reported and illustrated here for the first time. The phylogenetic analyses, using nuc rDNA and *rpb1* concatenated sequences, confirmed the previous conclusion that the genus *Albahypha* in the family Entrophosporaceae sensu Oehl et al. is an unsupported taxon. Finally, the descriptions of the

Glomerales, Entrophosporaceae, and *Entrophospora* were emended and new nomenclatural combinations were introduced.

KEYWORDS

**arbuscular mycorrhizal fungi, *Claroideoglomus*, four new taxa, morphology, new combinations, nuc rDNA, phylogenomic and phylogenetic taxonomy, *rpb1***

## Introduction

The genus *Entrophospora* with the type species *E. infrequens* was erected in the family Endogonaceae based on a species originally described as *Glomus infrequens* (Hall, 1977), but later recognized to form spores differently (inside the neck of a sporiferous saccule; Ames and Schneider, 1979) than species producing glomoid spores sensu Morton and Redecker (2001). Glomoid spores arise at the tips of sporogenous hyphae as in *G. macrocarpum*, the type species of the genus *Glomus* and the phylum Glomeromycota, including arbuscular mycorrhizal fungi (AMF; Clements and Shear, 1931; Schüßler and Walker, 2010; Oehl et al., 2011a). Subsequently, *Entrophospora* was enlarged by *E. schenckii* (Sieverding and Toro, 1987) and *E. baltica* (Błaszkowski et al., 1998), producing two-walled spores with a thick, smooth wall 2 as in *E. infrequens*, as well as *E. colombiana* (Schenck et al., 1984), and *E. kentinensis* (Wu et al., 1995) having spores with three spore walls, of which the innermost wall 3 is relatively thin and usually ornamented ("beaded"), as in most *Acaulospora* species.

Morton and Benny (1990) transferred the entrophosporoid [name coined by Sieverding and Oehl (2006)] species mentioned above to the family Acaulosporaceae in the order Glomales. Sieverding and Oehl (2006) accommodated *E. baltica* and *E. infrequens* in a newly erected family, Entrophosporaceae, in the class Glomeromycetes; *E. colombiana* and *E. kentinensis* in a new genus, *Kuklospora*, in the Acaulosporaceae; and *E. schenckii* in *Intraspora* gen. nov. in the family Archaeosporaceae sensu Morton and Redecker (2001). Schüßler and Walker (2010) introduced Claroideoglomeraceae fam. nov. with *Claroideoglomus* gen. nov. into the Glomerales (orthographically corrected Glomales; Schüßler et al., 2001). The type species of the monogeneric Claroideoglomeraceae, *Claroideoglomus*, was *C. claroideum* (Schüßler and Walker, 2010), originally described as *Glomus claroides* (Schenck and Smith, 1982). Entrophosporaceae and *Entrophospora* were recognized as taxa of uncertain systematic position in the order Diversisporales, *Kuklospora* synonymized with *Acaulospora*, and *I. schenckii* renamed as *Archaeospora schenckii*. Kaonongbua et al. (2010), based on a phylogeny reconstructed from nuc 28S rDNA (28S) sequences, also rejected *Kuklospora* as a valid monophyletic group, therefore, integrated it into *Acaulospora*, and concluded that the mode of spore formation is not a genus-specific character.

Oehl et al. (2011b) synonymized Claroideoglomeraceae with Entrophosporaceae based on phylogenetic analyses of nuc 18S rDNA (18S) and 28S sequences of *E. infrequens*, three species of *Claroideoglomus*, other representatives of Glomerales, and members of eleven (in the 18S analyses) and nine (28S) other previously described families of Glomeromycota. The genus *Claroideoglomus* remained in Entrophosporaceae, and *Entrophospora* with *E. infrequens* became the type genus of this family. The same analyses also prompted Oehl et al. (2011b) to transfer *C. drummondii* and *C. walkeri*, originally described as *G. drummondii* and *G. walkeri* (Błaszkowski et al., 2006), to a newly erected genus, *Albahypha*, with *A. drummondii* and *A. walkeri*, and *G. viscosum* to a new genus, *Viscospora*, in the Entrophosporaceae. In addition, Oehl et al. (2011b) accommodated *E. baltica* in Sacculosporaceae fam. nov., as *S. baltica* in the Diversisporales.

Except for the introduction of the Sacculosporaceae, Redecker et al. (2013) rejected the other changes discussed above and resurrected Claroideoglomeraceae. They found that both 18S and 28S sequences of *E. infrequens* nest among sequences of *Claroideoglomus*, and the incongruence between morphological and molecular characters of these taxa could not be explained from either dataset.

The main argument for rejecting *Albahypha* was linked to analyses conducted by Krüger et al. (2012), which indicated sequences of *A. drummondii* and *A. walkeri* clustering among *Claroideoglomus* sequences. In addition, Redecker et al. (2013) underlined that the generic clade *Albahypha* in Oehl's et al. (2011b) 28S tree was not supported and, therefore, rendered *Claroideoglomus* paraphyletic.

In Schüßler and Walker's (2010) classification, Claroideoglomeraceae was a sister taxon of Glomeraceae in the Glomerales. Two phylogenies have recently been published suggesting other relationships of Claroideoglomeraceae within Glomeromycota sensu Redecker et al. (2013). Beaudet et al. (2018), using spore transcriptomic data for analyses of nine taxa from seven families, recovered *Claroideoglomus* as sister to *Ambispora* and *Paraglomus,* belonging to the orders Archaeosporales and Paraglomerales, respectively. Montoliu-Nerin et al. (2021), using single nuclei sequencing to obtain new genomes of 15 species belonging to seven families, indicated

that Claroideoglomeraceae represents an autonomous, well-supported group, sister to a clade formed by Glomeraceae and Diversisporales.

Species of *Claroideoglomus*, Glomeraceae, and *Paraglomus* produce solely glomoid spores, while *Ambispora* species are dimorphic, forming acaulosporoid and glomoid morphs (Bills and Morton, 2015). Of members of the genera of the Diversisporales, only those of *Corymbiglomus*, *Desertispora*, *Diversispora*, *Sieverdingia*, *Redeckera*, and *Pacispora* produce glomoid spores (Oehl et al., 2011c; Błaszkowski, 2012; Symanczik et al., 2018; Błaszkowski et al., 2019). Glomoid spore-producing species of the genera mentioned above have one-walled spores, except for species of *Pacispora* and *Paraglomus*, which produce spores with two spore walls (Mello et al., 2013).

Schüßler and Walker (2010) and Oehl et al. (2011a) stated that the spores of *Claroideoglomus* species are morphologically distinguished by the composition of their spore wall and the phenotypic features of the spore wall components, as well as the characters of the spore subtending hypha at the spore base. According to Schüßler and Walker (2010), the spore wall of the members of *Claroideoglomus* contains a flexible, thin, colorless innermost layer. Oehl et al. (2011a) distinguished *Claroideoglomus* spores as having conspicuously funnel- or bill-shaped and colorless subtending hyphae regardless of whether the spore wall is colored or not. However, the above-mentioned morphological features not only characterize all *Claroideoglomus* species but also define glomoid spores of members of several other genera of Glomeromycota, for example, *Diversispora* species and *Orientoglomus emiratium*, a member of Glomeraceae (Błaszkowski et al., 2022). So, a reliable separation of *Claroideoglomus* species from other Glomeromycota species producing glomoid spores based exclusively on their morphology would be biased.

The data presented above confirm the widely accepted opinion that a more accurate identification and classification of AMF, including the Claroideoglomeraceae/Entrophosporaceae members, needs to be based mainly on reliable molecular data. Short sequences are usually the source of weak signals, resulting in insignificant support values for topologies of phylogenetic trees (Redecker et al., 2013). Even full-length 28S sequences failed to resolve closely related AM fungal species (Redecker et al., 2013).

The following hypotheses might explain the cause of the divergent conclusions about the Claroideoglomeraceae/Entrophosporaceae, as well as the phylogenies of *E. infrequens* and the two species considered as *C./A. drummondii* and *C./A. walkeri*. First, as suggested by Montoliu-Nerin et al. (2021), the conclusions of Beaudet et al. (2018) were biased by the low number of taxa analyzed. Second, we considered the vague phylogenies of *E. infrequens*, *C./A. drummondii,* and *C./A. walkeri* that resulted from the sequence under-sampling of the taxa and the use of sequences with too low taxonomic resolution. Therefore, we assumed that (i) genome-scale phylogenetic analyses will shed light on the phylogenetic placement of the family Claroideoglomeraceae/Entrophosporaceae and (ii) increased sampling and the use of sequences covering the 18S (partial), ITS1-5.8S-ITS2, and 28S (partial) nuc rDNA (=45S) segment concatenated with partial sequences of the largest subunit of RNA polymerase II (*rpb1*) will clarify the *E. infrequens*, *C./A drummondii,* and *C./A. walkeri* phylogenies. Sequences of each of the two loci generally separated even very closely related species (Krüger et al., 2012; Kohout et al., 2014; Stockinger et al., 2014). However, phylogenies reconstructed from concatenated sequences of the two unlinked loci (45S+*rpb1*) were more robust and revealed relationships unexposed when sequences of these two loci were analyzed separately (Błaszkowski et al., 2019, 2021a, 2015b,c; Yu et al., 2022).

We grew *E. infrequens* and four potential new glomoid spore-producing AMF species in trap and single-species cultures. The morphological characteristics of these glomoid fungi, particularly the colorless subtending hyphae of their pigmented spores, suggested that they may represent *Albahypha*/*Claroideoglomus* or *Diversispora*.

The aims of this study were (i) to determine which of the known hypotheses about the status and relationship of Claroideoglomeraceae/Entrophosporaceae is reliable, (ii) to clarify the position of *E. infrequens* in the Glomeromycota with the support of *rpb1* as an additional phylogenetic marker, (iii) to characterize in detail the morphology of the four potentially new AMF species and determine their phylogenetic positions among sequenced species of *Albahypha*/*Claroideoglomus* based on 45S and *rpb1* sequences, and (iv) to settle the conflict over the taxonomic status of *Albahypha* using previously available and newly generated morphological and molecular (45S and *rpb1*) data.

# Materials and methods

## Origin of the study material

*Entrophospora infrequens* and the potential new species, initially named *Claroideoglomus* 1–4, were characterized based on spores extracted from single-species pot cultures. These cultures were established from spores extracted from trap pot cultures inoculated with field-collected mixtures of rhizosphere soils and root fragments of the following plant species. The field host of *E. infrequens* was *Juniperus communis* L. growing in a pine forest on inland sand dunes of Kampinos National Park (52°19′ N, 20°45′ E), Poland. The field sample was collected by J. Błaszkowski on 26 July 1986.

The field hosts of *Claroideoglomus* 1 were *Deschampsia flexuosa* (L.) Trin and *Poa rigidifolia* Steud. These plants grew in a steppe community located in northern Tierra del Fuego (Mendoza et al., 2011) on a complex of moraine deposits and

glacial-outwash plains dominated by grasses of *Chiliotrichum diffusum* (G. Forst.) Kuntze, *D. flexuosa*, *Empetrum rubrum* Vahl ex Willd., *Festuca gracillima* Hook., and *P. rigidifolia* (Collantes et al., 1999).

The regional climate is influenced by the proximity of the Atlantic Ocean (Paruelo et al., 1998). The mean air temperature is about 0°C in July (winter) and reaches 10°C in January (summer). The annual rainfall ranges from about 300 to 400 mm along a NE–SW gradient and is evenly distributed throughout the year. The field sample was collected by R. Mendoza, Museo Argentino de Ciencias Naturales (MACN-CONICET) "Bernardino Rivadavia," Buenos Aires, Argentina, during the period from 1 to 15 March 2009.

*Claroideoglomus* 2 was harbored in the field within an initial community consisting of cryptogamic species and the following vascular plants: *F. vivipara* (L.) Sm., *Po. alpina* L., *Salix herbacea* L., *S. polaris* Wahlenb., and *Silene acaulis* (L.) Jacq. This community grew in a glacier foreland of Isfallglaciarän located in Tarfala valley, N Sweden (67°54′58 N, 18°35′ E). The average annual air temperature and the annual sum of rainfall in this site are 3.4°C and 503.3 mm, respectively (The Swedish Agency for Marine and Water Management, available at: www.smhi.se). The soil contained 19.28 and 3.17% of silt and clay, respectively, and its chemical properties were as follows: pH (in $H_2O$), 6.3; $P_2O_5$ (mg/100 g), 4.63; Na (cmol/kg), 0.04; N (%), 0.00; and C (%), 0.08. The field sample was collected by Paulina Wietrzyk-Pełka and Michał Węgrzyn (Institute of Botany of the Jagiellonian University) on 23 July 2019.

The field inoculum containing *Claroideoglomus* 3 was collected under an initial community consisting of *Limonium sinuatum* (L.) Mill. This community grew in a Solonetz Gley sodic soil, which was hydromorphic, compact, and contained high levels of surface salt deposits. The community was located at Fortuna, Murcia, Spain (38°06′ N, 1°06′ W). The mean annual air temperature and the annual sum of rainfall in this site are 19.6°C and 300 mm, respectively. The soil contained 20.28 and 53% of silt and clay, respectively, and its chemical properties were as follows: pH (in $H_20$), 8.5; $P_2O_5$ (mg/100 g), 2.1, $CaCO_3$ (%), 12.0; $Mg_2$ (cmol/kg), 2967.04; K (cmol/kg), 2955; Na (cmol/kg), 1829.4; N (%), 0.90; and C (%), 0.08. The field sample was collected by Félix Fernández in November 2009.

*Claroideoglomus* 4 was isolated from samples collected in the Sultanate of Oman and in Poland. In Oman, *Claroideoglomus* 4 was associated with roots of *Prosopis cineraria* (L.) Druce. It grew in an undisturbed habit adjacent to a date palm (*Phoenix dactylifera* L.) plantation of an experimental station (22°14′ N, 59°10′ E) belonging to the Ministry of Agriculture, Fisheries and Water Resources of Oman. Summer temperatures exceed 48°C (Glennie and Singhvi, 2002). Based on the aridity index defined by the United Nations Environmental Program (UNEP, 2006), this site is characterized as Hyperarid (AI < 0.05), with annual rainfall not exceeding 100 mm (Fisher and Membery, 1998). The soil of this site was sandy loam with the following chemical properties: pH (in $H_2O$), 8.2; contents of P (mg $kg^{-1}$), 41.4; N (%), < 0.1; and organic matter (%), 1.5 (Al-Yahya'ei et al., 2011). The field sample was collected by Mohamed N. Al-Yahya'ei in August 2006.

The site in Poland where *Claroideoglomus* 4 was found is a field near Lubliniec in the Silesian Upland (50°37′ N, 18°43′ E) from where *Dominikia bonfanteae* was isolated (Błaszkowski et al., 2021b). Details about plant community and the physico-chemical properties of soil are available in Malicka et al. (2020). The climate of the region, where the study material was collected, is characterized by Błaszkowski et al. (2019).

Spores of the species originally described as *G. walkeri* and *C. hanlinii*, the latter of which was also included in our analyses (see below), originated from single-species cultures grown by J. Błaszkowski.

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

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

## Microscopy and nomenclature

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

Voucher specimens of the proposed new species [spores permanently mounted in PVLG and a mixture of PVLG and Melzer's reagent (1:1, v/v) on slides] were deposited at 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 for obtaining 45S and *rpb1* sequences was extracted from eight single spores of *E. infrequens* and each of the four potential new *Claroideoglomus* species. Details of the treatment of 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 species are available in Krüger et al. (2009), Symanczik et al. (2014), and Błaszkowski et al. (2015b,c).

To obtain *rpb1* sequences of these five species, as well as *C. hanlinii*, *C. luteum*, and the species originally described as *G. walkeri*, a new set of primers was developed. The primer set consisted of two forward primers (RPB1-3F: GTC TTC GTG CAG TTT GGG A; RPB1-4F: CTA GGC CTG ATT GGA TGA T) and two reverse primers (RPB1-5R: ACG ATT TGT TTT GGT ACC AT; RPB1-5RN: TTC ATC TCA CCA TCA A). Characteristics of the primers and the PCR conditions are available in Błaszkowski et al. (2021a). To increase the chance to obtain a successful amplification of unknown sequences, the following approach was used to design the primers. All the possible 19-21 bases oligos obtained from the *rpb1* sequence available for *C. claroideum* were assessed according to the following criteria: (i) a perfect match with the *C. etunicatum* and *C. claroideum* sequences, (ii) at least one C/G in the 3′ end, and (iii) the highest number of matches among all the AMF *rpb1* sequences available (about 200) with maximum three mismatches tolerated if not in the last five residues at the 3′ end. Candidate oligos were evaluated with OligoAnalyzer for Tm and the presence of secondary structures.

Cloning and sequencing of *rpb1* PCR products were performed identically to those used to obtain 45S sequences (see above). Both 45S and *rpb1* sequences were deposited in GenBank (45S: MH590060, MH590061, MT722021–MT722024, ON950363–ON950390, and MT722034–MT722038; *rpb1*: MT733201–MT733210 and ON938320–ON938327).

## Phylogenomic analyses

Previous genome and transcriptome data phylogenies have shown two conflicting topologies with respect to Claroideoglomeraceae (Beaudet et al., 2018; Montoliu-Nerin et al., 2021). Therefore, we compiled three different datasets based on previously published data (**Supplementary Table 1**). The first dataset included data used by Beaudet et al. (2018) and Montoliu-Nerin et al. (2021), the second dataset included only Beaudet et al.'s (2018) data, and the third dataset included only genomes from the Montoliu-Nerin et al. (2021) analyses. Single-copy orthologs (SCOs) were identified with OrthoMCL (Li et al., 2003) using default settings, and only SCOs that were present in at least 50% of the taxa were included in the phylogenetic analyses. Individual SCOs were aligned with MAFFT 7.407 (Katoh and Standley, 2013). Poorly aligned regions were trimmed with trimAl 1.4.1 (Capella-Gutiérrez et al., 2009) with a gap threshold of 0.1. The individual alignments were concatenated using the script geneStitcher.py (Ballesteros and Hormiga, 2016). An ML phylogeny was inferred with IQ-TREE 2.0 (Nguyen et al., 2015) using ModelFinder (Kalyaanamoorthy et al., 2017) and searching for the best partition scheme. In addition, the individual SCO alignments were used to infer individual gene trees with IQ-TREE and then a phylogenetic inference with ASTRAL-III 5.7.3 (Zhang et al., 2018) was performed. The dataset that included only genome data used by Montoliu-Nerin et al. (2021) was then used to test the hypothesis that Claroideoglomeraceae is sister to Diversisporales and Glomeraceae. For this, we evaluated the support among individual gene trees for alternative branching orders and carried out a polytomy test in ASTRAL-III (Sayyari and Mirarab, 2018).

## Phylogenetic analyses

To clarify the status of *Albahypha* and *Entrophospora*, as well as to infer the position of the four potential new species, two sequence datasets were produced. The first consisted of sequences of the 45S segment, or part thereof, of our *E. infrequens* and the AMF, initially recognized as new species of *Claroideoglomus*, as well as all other sequenced species of these genera sensu Oehl et al. (2011b). *Claroideoglomus hanlinii*, a species described later (Błaszkowski et al., 2015a), was also included. The second dataset contained *rpb1* sequences available for the species of the first dataset and sequences from the genome of *C. candidum*. The sequences covered part of the fourth and fifth exons of the *rpb1* gene and the intron between them. In each of these datasets, the outgroup was represented by *Diversispora* species, and the datasets were aligned separately using MAFFT 7 with the E-INS-i strategy. Subsequently, the two alignments were used to manually produce a concatenated alignment, 45S+*rpb1*. The 45S sequences lacking the *rpb1* part were not removed from the alignment. An additional alignment was produced as follows: 45S+*rpb1*_G with three *Glomus* instead of *Diversispora* species in the outgroup to test the impact of this outgroup representing Glomeraceae, the sister family of Claroideoglomeraceae in the Glomerales sensu Schüßler and Walker (2010), on the topology and measures used in comparison of the trees generated in the analyses (described below).

The phylogenetic position of *Albahypha*, *E. infrequens*, and the four potential new species was reconstructed based

on Bayesian inference (BI) and maximum likelihood (ML) phylogenetic analyses of the 45S, *rpb1*, 45S+*rpb1*, and 45S+*rpb1*_G 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, and 28S. Five additional *rpb1* partitions were added in the alignments with the concatenated genes, as described in Błaszkowski et al. (2021b).

As suggested by Abadi et al. (2019), in both BI and ML analyses, GTR+I+G was chosen as the nucleotide substitution model for each nucleotide partition. 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 compared to those where GTR+I+G was used.

Four Markov chains were run over two million generations in MrBayes 3.2 (Ronquist et al., 2012), sampling every 2,000 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/1,000 bootstrapping run and an ML estimated proportion of invariable sites and base frequencies. The alignments and tree files were deposited as **Supplementary material**.

We assumed that clades were supported when the Bayesian posterior probabilities and the ML bootstrap values were ≥0.95 and ≥70%, respectively. The generated 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 three measures, referred to the ingroup, which are as follows: (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 by dividing the number of significantly supported internal branches by the size of the ingroup (Thorley and Wilkinson, 2000).

To detect other possible findings of the potential new species, their 45S sequences were used as queries in BLASTn to retrieve more than 300 nucleotide sequences from GenBank. The sequences were selected according to the percentage of identity > 96.5, with at least one of the queries. The possible relatedness with any of the four species was assessed using an evolutionary placement algorithm-based (EPA) approach, performed with RAXML 8.2.10 (Berger et al., 2011). The sequences were aligned in MAFFT together with the 45S dataset, and the 45S+*rpb1* ML tree was used as reference support for the phylogenetic affiliation. GTRGAMMA model, the maximum likelihood estimation for the base frequencies, and the partition scheme previously described for the 45S alignment were used for the phylogenetic inference. The jplace output was used in Gappa (Czech et al., 2020) for the placement mass (likelihood weight ratio) accumulation of the placements of each sequence upward from the reference tree with the threshold = 0.9.

# Results

## General data and phylogeny

The first dataset used for the phylogenomic analyses, which included data from Beaudet et al. (2018) and Montoliu-Nerin et al. (2021), contained 78 SCOs and recovered Claroideoglomeraceae as sister to Diversisporales and Glomeraceae (**Supplementary Figure 1**). Since Beaudet et al. (2018) showed Claroideoglomeraceae to be sister to Ambisporaceae and Paraglomeraceae based on analyses of spore transcriptomic data, we produced and analyzed a dataset that contained only these data. The analysis yielded 122 SCOs, and the phylogeny showed again Claroideoglomeraceae as sister to Diversisporales and Glomeraceae (**Supplementary Figure 2**). Finally, to obtain a higher number of SCOs and to perform a topology test, we excluded the Beaudet et al.'s (2018) dataset and analyzed only AM genome data used by Montoliu-Nerin et al. (2021). For this dataset, we obtained 1,260 SCOs present in at least 50% of the taxa, and the phylogenomic analysis also recovered Claroideoglomeraceae as sister to Diversisporales and Glomeraceae (**Figure 1**). Other phylogenomic analyses on every genome dataset consistently recovered Glomerales as polyphyletic, with Claroideoglomeraceae as sister to Diversisporales and Glomeraceae (data not shown). The quartet gene frequencies inferred with ASTRAL supported this relationship (q1 = 0.43, q2 = 0.26, q3 = 0.31) (**Figure 2**).

The phylogenetic position of *Albahypha*, *Claroideoglomus*, *E. infrequens*, and the four potential new species was reconstructed based on 115 sequences of the 45S segment or part thereof (28S) and 36 *rpb1* sequences. Of these, 57 were newly obtained (39 45S, 18 *rpb1*). The 45S and *rpb1* sequences characterized 14 and 10 species, respectively, of *Albahypha*/*Claroideoglomus*, including our four new species, as well as three *Diversispora* (*Glomus*) species that served as an outgroup. These sequences were used to prepare four alignments (45S, *rpb1*, 45S+*rpb1*, and 45S+*rpb1*_G) for BI and ML analyses. Data about the numbers of variable and parsimony informative sites of each alignment are presented in **Table 1**.

Four phylogenetic trees summarizing the BI and ML analyses were reconstructed, named 45S, *rpb1*, 45S+*rpb1*, and 45S+*rpb1*_G (**Figure 3** and **Supplementary Figures 3–5**, respectively). Small differences that occurred in the topologies of the trees were described below. In all trees, the number of supported species clades and the mean support values of species clades and nodes were identical or similar (**Supplementary Table 2**). Overall, the trees with concatenated genes performed slightly better than the 45S tree.

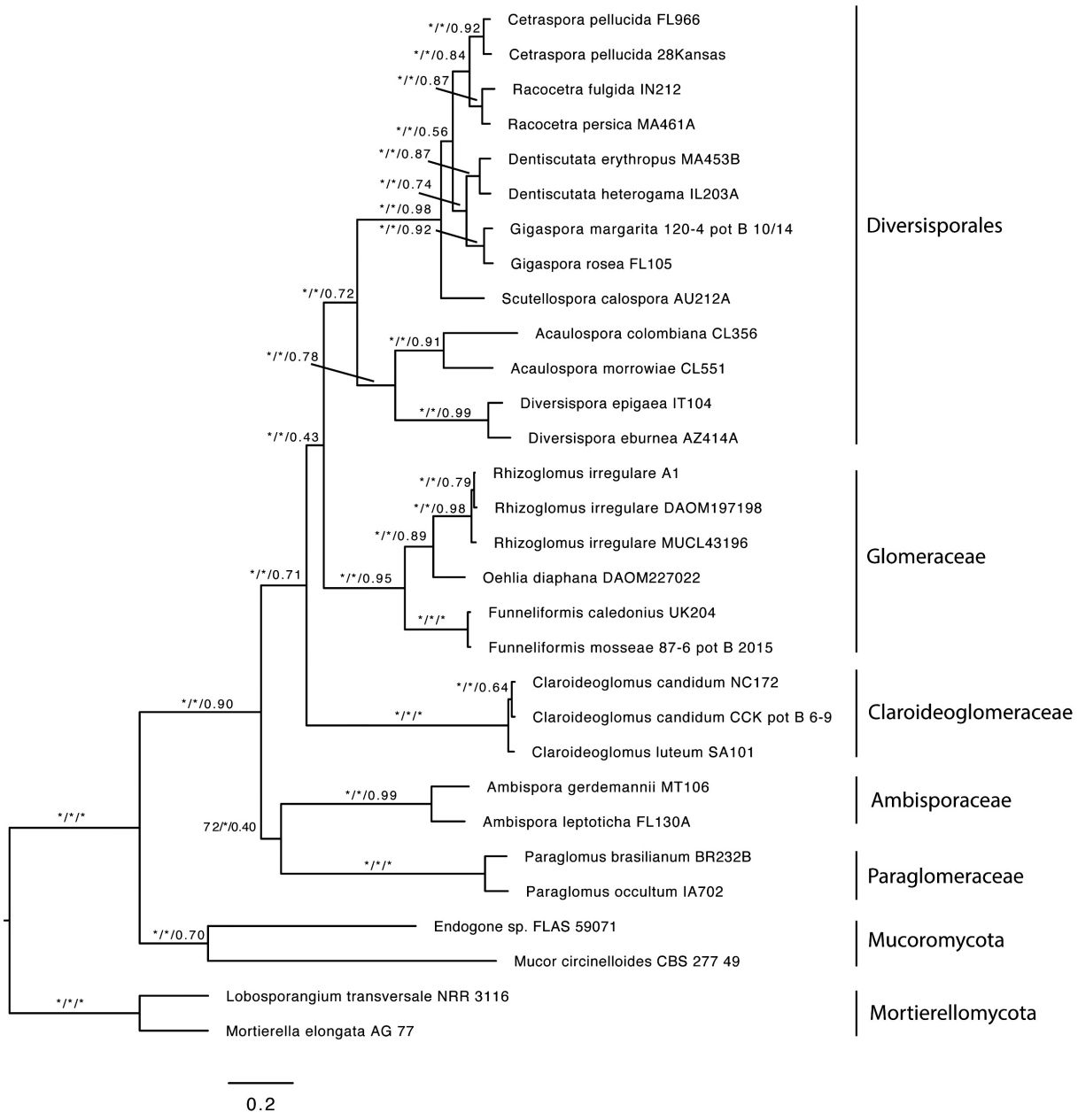



FIGURE 1
Best Maximum Likelihood phylogeny inferred with IQ-TREE from a concatenated alignment of 1260 single copy orthologs shared by at least 50% of the taxa. The same topology was recovered with ASTRAL III. Values near the branches correspond to bootstrap support (BS) from the ML analysis, local posterior probabilities (LPP), and quartet support from the ASTRAL analysis (BS/LPP/quartet support). Asterisks indicate maximum support (100 or 1.0). Mucoromycota and Mortierellomycota were used as outgroups. Bar indicates 0.2 expected change per site per branch.

In all trees, the clades with *Claroideoglomus* 1–4 obtained full or almost full BI (= 0.99–1.0) supports (Figure 3 and Supplementary Figures 3–5). Also, the ML supports of these species in all trees were significant (≥70%), and most of them were very high or full.

In all trees, the glomoid *Claroideoglomus* 3 formed a mixed clade with the entrophosporoid *E. infrequens*, whose 45S and *rpb1* sequences were obtained from spores originating from our collection (Figure 3 and Supplementary Figures 3–5). In the 45S+*rpb1* and 45S trees, the glomoid *Claroideoglomus* 2 also grouped with *E. infrequens* (Figure 3 and Supplementary Figure 3), whose 28S sequences were obtained by Oehl et al. (2011b); no *rpb1* sequence of the fungus was available (pers. comm.). Hereafter, the two mixed clades will be referred to as *Claroideoglomus* 2 and *Claroideoglomus* 3.

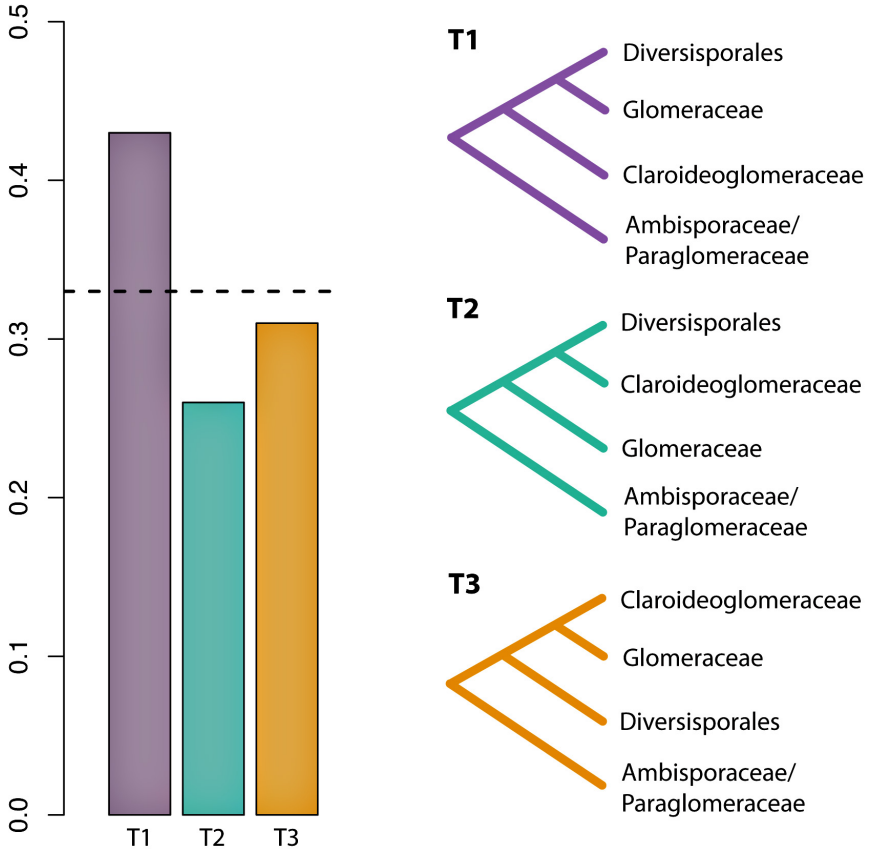



FIGURE 2
Evaluation of support among 1260 individual gene trees for alternative hypotheses of the phylogenetic placement of Claroideoglomeraceae. Bar graphs represent the gene tree quartet frequencies for three possible branching orders. The dashed horizontal line indicates the expectation of a hard polytomy.

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 | 106 | 14 | 1700 | 707 | 609 |
| *rpb1* | 27 | 10 | 948 | 291 | 270 |
| 45S+*rpb1* | 106 | 14 | 2648 | 998 | 879 |
| 45S+*rpb1*_G | 106 | 14 | 2665 | 975 | 864 |

In all trees, *Claroideoglomus* 1 and *Claroideoglomus* 3 occupied autonomous phylogenetic positions, except for the 45S tree, which showed an unsupported sister relationship of *Claroideoglomus* 1 with *C. etunicatum* (Figure 3 and Supplementary Figures 3–5). *Claroideoglomus* 2 formed a clade with *C. candidum* and six of the eleven analyzed sequences of *C. claroideum* in the 45S (not supported), 45S+*rpb1*, and 45S+*rpb1*_G trees. *Claroideoglomus* 4 was placed either in a strongly supported autonomous clade (in the 45S+*rpb1* and *rpb1* trees) or a BI-supported clade sister to *C. drummondii* (45S, 45S+*rpb1*_G). However, the ML supports of this sister relationship were insignificant (45S) or slight (45S+*rpb1*_G; ML = 76%).

Four other 28S sequences ascribed to *E. infrequens* formed a clade with or were accommodated in the neighborhood of *C. hanlinii* or clustered in an autonomous clade; none of the positions obtained support (Figure 3 and Supplementary Figures 3, 5). Although *Claroideoglomus* 4, *C. drummondii*, and *C. walkeri* formed a supported group corresponding to the genus *Albahypha* sensu Oehl et al. (2011b) in the 45S+*rpb1*_G tree, the clade comprising the other analyzed species was not supported (Supplementary Figure 5).

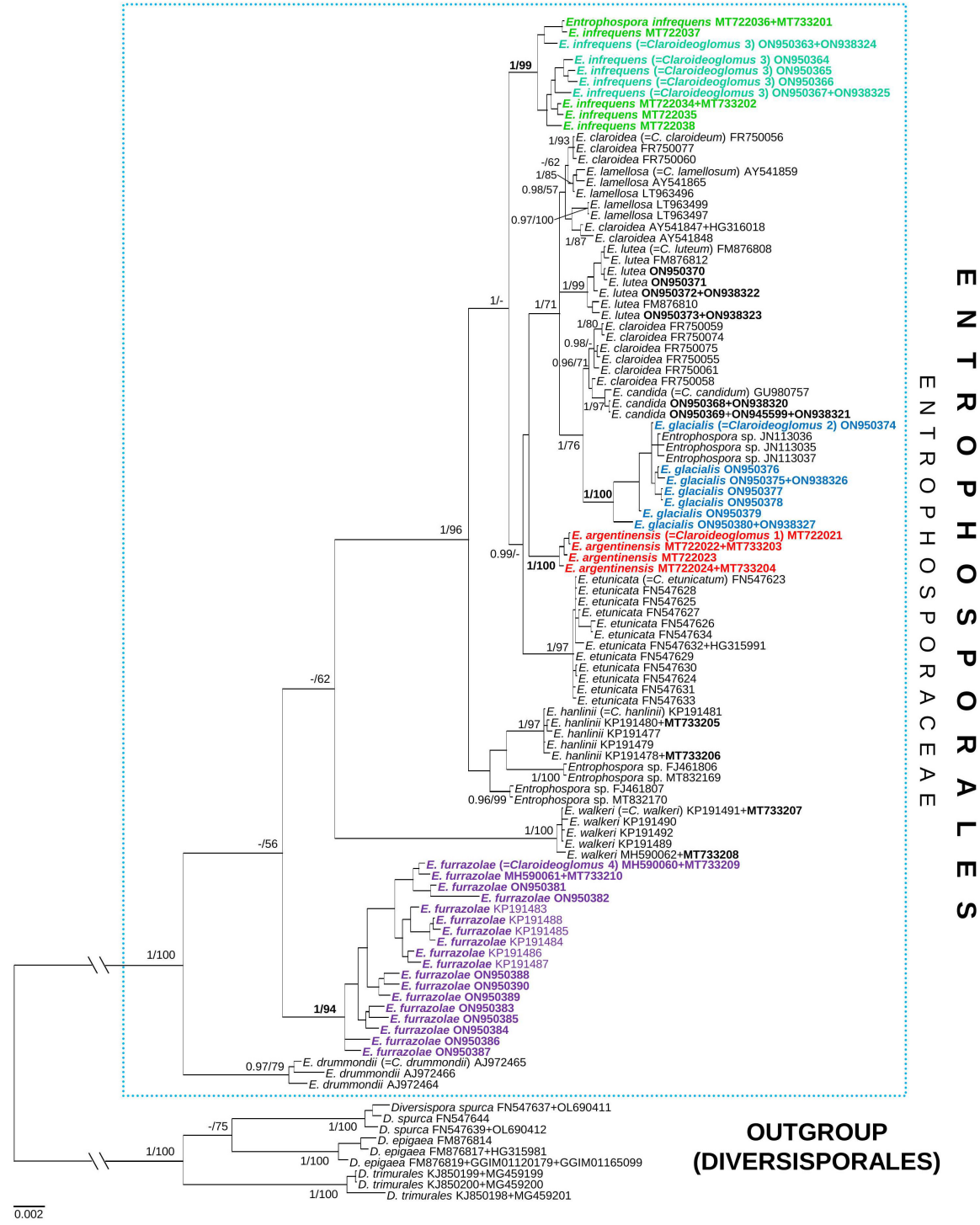



FIGURE 3

A 50% majority-rule consensus tree from the Bayesian analysis of 45S nuc rDNA sequences concatenated with *rpb1* sequences of *Claroideoglomus* 1, 2, 4 (newly described as *Entrophospora argentinensis*, *E. glacialis*, and *E. furrazolae*, respectively), *Claroideoglomus* 3 (a glomoid morph of the *E. infrequens* epitype), eight other species of *Claroideoglomus* sensu C. Walker and A. Schüßler, and three *Diversispora* species serving as outgroup. The former species names included in Entrophosporales are reported between brackets. The new species and the accession numbers of the sequences obtained in this study are in bold. The Bayesian posterior probabilities ≥0.90 and ML bootstrap values ≥50% are shown near the branches, respectively. Bar indicates 0.002 expected change per site per branch. The two basal branches were shortened to 20% in length to improve visibility (indicated by //).

# Taxonomy

The results of the phylogenomic and phylogenetic analyses described above prompted us (i) to propose a new order, Entrophosporales, for Entrophosporaceae in place of Claroideoglomeraceae, (ii) to emend the descriptions of Glomerales, Entrophosporaceae, *Entrophospora*, and *E. infrequens,* (iii) to designate an epitype of *E. infrequens* with the morphology and phylogeny determined from analyses of the entrophosporoid *E. infrequens* morph and the glomoid morph preliminary named *Claroideoglomus* 3, which are grown in our collection, (iv) to introduce new nomenclatural combinations, and (v) to describe *Claroideoglomus* 1, 2, and 4 as *E. argentinensis* sp. nov., *E. glacialis* sp. nov., and *E. furrazolae* sp. nov., respectively.

## Description of a new order

**Entrophosporales** Błaszk., Sánchez-García, B.T. Goto, and Magurno, **ord. nov**.

MycoBank MB846043

*Type family*: Entrophosporaceae (Oehl and Sieverd.), emend. Błaszk., Sánchez-García, B.T. Goto, and Magurno.

*Diagnosis*: Producing entrophosporoid and glomoid spores or only one of these morphs. Entrophosporoid spores formed singly within the necks of sporiferous saccules, in soil, rarely in roots. Spores with two spore walls. Spore wall 1, forming the spore surface, composed of two short-lived to semi-permanent layers continuous with the neck and sporiferous saccule wall layers and a permanent, laminate, pigmented layer continuous with a wall of a cylindric to a funnel-shaped structure supporting the wall of the part of the neck directly connected with the sporiferous saccule and the wall of the saccule at its base; not extending into the part of the neck located distally to the sporiferous saccule, hence spores have only one persistent cicatrix, proximal to the sporiferous saccule, created when the opening connecting the interior of the spore with the lumen of the sporiferous saccule neck is closed by a plug made of the contents of the spore when fully formed; layer 1 stains dark in Melzer's reagent. Spore wall 2, having no physical contact with spore wall 1, composed of three, hyaline, smooth layers: two thin, flexible layers enclosing a much thicker, semi-flexible layer; none of the layers stains in Melzer's reagent. Glomoid spores formed at tips of sporogenous hyphae either branched from the sporiferous saccule wall and/or the sporiferous saccule neck of the entrophosporoid morph or continuous with extraradical mycorrhizal hyphae, occasionally intercalarily inside hyphae, rarely in roots, usually with one subtending hypha. Extraradical spores produced singly, rarely in clusters with few spores. Spores with one spore wall consisting of two to five layers; when multilayered, the innermost layer frequently is flexible to semi-flexible, <1.0 μm thick, colorless or brightly colored, and loosely associated with the inner surface of the penultimate, usually laminate, layer. One or two spore wall layers may stain in Melzer's reagent. Subtending hypha with a hyaline, rarely brightly colored wall, always strikingly much lighter than the spore wall in colored spores, frequently conspicuously funnel- or bill-shaped at the spore base; subtending hyphal wall composed of layers continuous with spore wall layers. Pore closed by a septum connecting the inner surfaces of either the subtending hyphal wall or the spore wall, or septa connecting both these structures, at or slightly below the spore base, and frequently additionally by a septum continuous with the innermost flexible to semi-flexible spore wall layer. Entrophosporoid and glomoid species producing mycorrhiza with arbuscules, vesicles, as well as intra- and extraradical hyphae staining dark in Trypan blue.

## Emendation of Glomerales

Glomerales J.B. Morton and Benny, emend. Błaszk., B.T. Goto, and Magurno.

*Type family*: Glomeraceae Piroz. and Dalpé.

*Type genus*: *Glomus* Tul. and C. Tul. emend. Oehl, G.A. Silva, and Sieverd.

*Other genera*: *Dominikia* Błaszk., Chwat, and Kovács, *Microdominikia* Oehl, Corazon-Guivin, and G.A. Silva, *Kamienskia* Błaszk., Chwat, and Kovács, *Microkamieskia* Corazon-Guivin, G.A. Silva, and Oehl, *Orientoglomus* G.A. Silva, Oehl, and Corazon-Guivin, *Nanoglomus* Corazon-Guivin, G.A. Silva, and Oehl, *Septoglomus* Sieverd., G.A. Silva, and Oehl, *Rhizoglomus* Sieverd., G.A. Silva, and Oehl (= *Rhizophagus*), *Oehlia* Błaszk., Kozłowska, Niezgoda, B.T. Goto, and Dalpé, *Halonatospora* Błaszk., Niezgoda, B.T. Goto, and Kozłowska, *Sclerocarpum* B.T. Goto, Błaszk., Niezgoda, Kozłowska, and Jobim, *Epigeocarpum* Błaszk., B.T. Goto, Jobim, Niezgoda, and Magurno, *Funneliformis* C. Walker and A. Schüssler emend. Oehl, G.A. Silva, and Sieverd., *Funneliglomus* Corazon-Guivin, G.A. Silva, and Oehl, *Silvaspora* Błaszk., Niezgoda, B.T. Goto, Crossay, and Magurno, *Sclerocystis* Berk. and Broome.

*Diagnosis*: Spores formed at tips of sporogenous hyphae, occasionally intercalarily inside hyphae, and in roots, usually with one subtending hypha. Extraradical spores produced singly, in clusters with few to dozens of spores, compact unorganized glomerocarps (= sporocarps) with randomly distributed spores or organized glomerocarps with spores born terminally from hyphae developed radially from a central plexus of hyphae. Glomerocarps with or without a peridium. Spores with one spore wall consisting of one laminate layer or many layers; when multilayered, the innermost layer usually is laminate and thicker than the others. Subtending hypha with a wall concolorous with or slightly lighter than the spore wall, funnel-shaped, cylindrical, or constricted at the spore base; subtending hyphal wall composed of layers continuous with spore wall layers. Pore closed by thickening spore wall and

subtending hyphal wall toward the center of the subtending hyphal lumen connecting it with the spore interior, a septum connecting the inner surfaces of the subtending hyphal wall slightly below the spore base, or the innermost spore wall layer at or slightly above the spore base. Mycorrhiza with arbuscules, vesicles, as well as intra- and extraradical hyphae staining dark in Trypan blue.

## Emendation of Entrophosporaceae, *Entrophospora*, *Entrophospora infrequens*, and new combinations

**Entrophosporaceae** (Oehl and Sieverd.), emend. Błaszk., Sánchez-García, B.T. Goto, and Magurno. = Entrophosporaceae Oehl and Sieverd., emend. Oehl, Sieverd., Palenz., and G.A. Silva. Mycotaxon 117:306, 2011. = Claroideoglomeraceae C. Walker and A. Schüßler. The *Glomeromycota* – a species list:21. 2010.

*Type genus*: *Entrophospora* R.N. Ames and R.W. Schneid., emend. Błaszk., Sánchez-García, Fernández, B.T. Goto, and Magurno.

*Diagnosis*: As that of Entrophosporales.

***Entrophospora*** R.N. Ames and R.W. Schneid., emend. Błaszk., Sánchez-García, Fernández, B.T. Goto, and Magurno. = *Albahypha* Oehl, G.A. Silva, B.T. Goto, and Sieverd., Mycotaxon 117:308. 2011. = *Claroideoglomus* C. Walker and A. Schüßler, emend. Oehl, Sieverd., B.T. Goto, and G.A. Silva, Mycotaxon 117:308. 2011. = *Claroideoglomus* C. Walker and A. Schüßler, The *Glomeromycota* – a species list:21. 2010.

*Type species*: *Entrophospora infrequens* (I.R. Hall) R.N. Ames and R.W. Schneid., emend. Błaszk., Sánchez-García, Fernández, B.T. Goto, and Magurno.

*Epitype*: Entrophosporoid morph, POLAND. 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 *J. communis* from a pine forest in inland sand dunes of Kampinos National Park (52°19′ N, 20°45′ E), July 1986, J. Błaszkowski (slide with spores no. 668–674, **isoepitypes** slides with spores no. 3870–3872, LPPDSE; 45S and *rpb1* sequences: MT722034–MT722038 and MT733201–MT733202, respectively, GenBank).

Glomoid morph, SPAIN. FORTUNA, MURCIA. 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 *L. sinuatum* from an initial community (38°06'57 N, 1°06'W), November 2009, F. Fernández (**epitype** slide with spores no. ZT Myc 0066907, **isoepitypes** slides with spores no. 3839–3846, LPPDSE); 45S and *rpb1* sequences: ON950364–ON950367 and ON938324–ON938325, respectively, GenBank.

*Diagnosis*: As that of Entrophosporales.

***Entrophospora infrequens*** R.N. Ames and R.W. Schneid., emend. Błaszk., Sánchez-García, Fernández, B.T. Goto, and Magurno.

*Diagnosis*: Differs from other *Entrophospora* species forming glomoid spores in spore morphometric features, phenotypic and histochemical properties of spore wall layers, as well as in nucleotide composition of sequences of the 45S nuc rDNA region and the *rpb1* gene.

*Description*: Producing entrophosporoid and glomoid glomerospores (= spores), or only one of these morphs. Entrophosporoid spores formed in soil, singly, arise blastically inside the neck of a sporogenous saccule (**Figures 4A–H**); golden yellow (5B8) to brownish orange (7C7); globose to subglobose; (95–)135(–175) μm diam, with two cicatrices. *Spores* with two spore walls having no physical contact with one another. Spore wall 1 composed of three layers. Layer 1, forming the spore surface, evanescent, hyaline, (1.5–)2.5(–4.5) μm thick, staining crayfish red (9B8) to cerise (12C8) in Melzer's reagent, highly deteriorated or completely sloughed off in mature and older spores. Layer 2, semi-permanent, smooth, 2.8–6.0 μm thick, slowly degrading with age. Layer 3, permanent, laminate, golden yellow (5B8) to brownish orange (7C7); (1.5–)2.5(–4.5) μm thick, on the upper surface ornamented with tooth-shaped outgrowths, 1.8–3.8 × 1.0–3.0 μm, with a central depression in the upper surface. Layers 1 and 2 continuous with the neck and sporiferous saccule wall layers. Layer 3 continuous with a wall of a cylindric to funnel-shaped structure supporting the wall of the part of the neck directly connected with the sporiferous saccule and the wall of the saccule at its base; not extending into the part of the neck located distally to the sporiferous saccule. Spore wall 2 consisting of three permanent, hyaline layers. Layer 1, flexible, <0.5 μm thick, usually tightly adherent to layer 2, and, hence, frequently difficult to see. Layer 2, flexible to semi-flexible, coriaceous sensu Walker (1986), (5.5–)7.8(–10.0) μm thick. Layer 3 flexible, ∼0.5 μm thick, rarely separating from the lower surface of layer 2 in even vigorously crushed spores. None of the spore wall 2 layers 1–3 stains in Melzer's reagent. *Cicatrices.* Visible as two scars in the region of contact between the spore and the saccule neck. A scar proximal to the saccule is a slightly depressed area, circular, 13.5–27.0 μm diam, to ellipsoidal, 13.5–23.0 × 16.0–27.0 μm, when seen in a plane view. A scar distal to the saccule is circular, 11.0–14.5 μm diam when observed in a plane view; it is usually completely sealed in mature spores and, therefore, invisible. *Sporiferous saccule* hyaline, consisting of a cylindric to slightly funnel-shaped neck, 21–33 wide, and a globose to egg-shaped, 100–170 × 130–190 μm, saccule, usually detached from mature spores. *Sporiferous saccule wall* composed of spore wall 1 layers 1–3. *Germination* unknown. *Mycorrhiza*. In single-species cultures with *P. lanceolata* and *Pueraria phaseoloides* (Roxb.) Benth. as the host plants, formed mycorrhiza with arbuscules, vesicles, and hyphae staining clearly in Trypan blue (Sieverding and Oehl, 2006; pers. observ.).

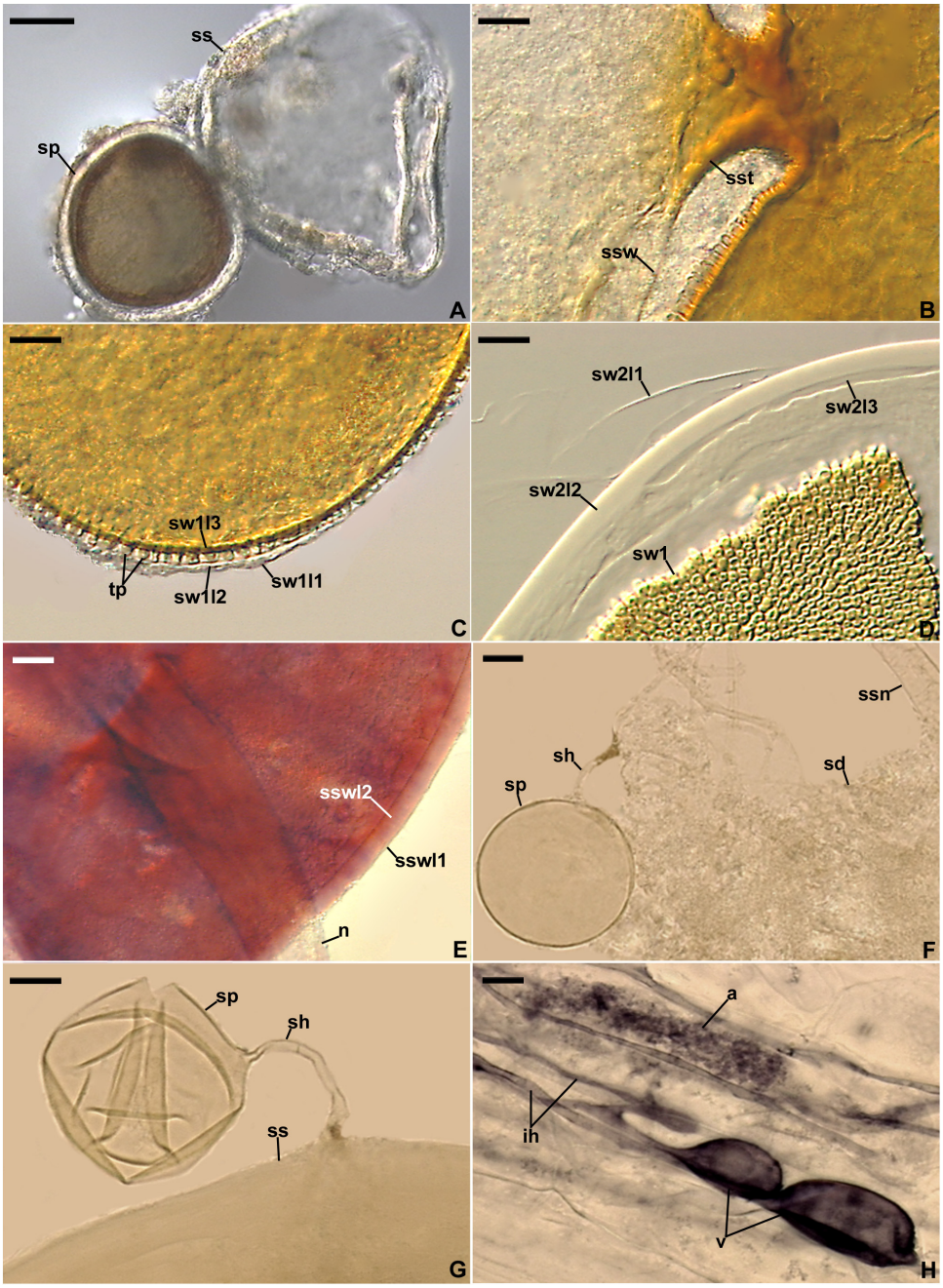



FIGURE 4
*Entrophospora infrequens*. **(A)** Entrophosporoid morph with spore (sp) formed inside the sporiferous saccule (ss). **(B)** Funnel-shaped structure, continuous with spore wall 1 layer 3, supporting the sporiferous saccule wall (ssw). **(C)** Spore wall 1 layers (sw1l) 1–3; swl1 is almost completely sloughed off; tooth-shaped projections (tp) in cross-view are visible. **(D)** Spore wall 1 (sw1) and spore wall 2 layers (sw2l) 1–3. **(E)** Sporiferous saccule wall layers (sswl) 1 and 2, and the neck (n) of sporiferous saccule. **(F)** Juvenile glomoid spore (sp) with subtending hypha (sh) developed from the sporiferous saccule neck (ssn); soil debris (sd) are indicated. **(G)** Juvenile glomoid spore (sp) with subtending hypha (sh) developed from sporiferous saccule (ss). **(H)** Mycorrhiza with arbuscule (a), vesicles (v), and intraradical hyphae (ih). **(A–D,F–H)** Spores and mycorrhizal structures in PVLG. **(E)** Sporiferous saccule in PVLG+Melzer's reagent. **(A–H)** Differential interference microscopy. Scale bars: **(A)** = 50 μm, **(B–H)** = 10 μm.

Glomoid spores formed in soil, singly or in loose clusters with 2–3 spores, arise blastically at tips of sporogenous hyphae (Figures 5A,B,D–F) either branched from the sporiferous saccule wall and/or the sporiferous saccule neck of the entrophosporoid morph (Figures 4F,G), or continuous with extraradical mycorrhizal hyphae, occasionally intercalarily. *Spores* subhyaline to pastel yellow (3A4); globose to subglobose; (20–)50(–76) μm diam; rarely ovoid; 36–64 × 41–69 μm;

with one subtending hypha (Figures 4F,G, 5A–F). *Spore wall* composed of two permanent layers (Figures 5B–F). Layer 1, forming the spore surface, uniform (not containing visible sublayers), semi-flexible, hyaline to yellowish white (3A2), (0.6–)1.1(–2.0) μm thick, tightly adherent to the upper surface of layer 2, rarely slightly deteriorated in its upper part (Figures 5B–F). Layer 2 laminate, semi-flexible, hyaline to pastel yellow (3A4), (1.2–)2.0(–3.4) μm thick; consisting of very thin, <0.5 μm thick, laminae, tightly adherent to each other (Figures 5B-F). In Melzer's reagent, only layer 2 turns yellow (3A6; Figures 5E,F). *Subtending hypha* hyaline to pastel yellow (3A4); straight or recurved, usually slightly funnel-shaped, rarely cylindrical or slightly constricted at the spore base; (5.6–)7.1(–9.2) μm wide at the spore base (Figures 5A,B,D–F); not braking in crushed spores. *Wall of subtending hypha* subhyaline to pastel yellow (3A4); (1.4–)1.7(–2.8) μm thick at the spore base; consisting of two layers continuous with spore wall layers 1 and 2; subtending hyphal wall layer 2 formed up to 5.2 μm below the spore base (Figures 5D–F). *Pore* (2.0–)3.0(–3.8) μm wide at the spore base, usually open, rarely closed by a curved septum, 0.6–1.7 μm thick, continuous with spore wall layer 2 in mature spores (Figures 5B,D–F). Spore content of hyaline oily substance. *Germination* unknown. *Mycorrhiza*. In single-species cultures with *P. lanceolata* as the host plant, formed mycorrhiza with arbuscules, vesicles, as well as intra- and extraradical hyphae that stained dark [pale violet (16A3) to grayish violet (16E5)] in 0.1% trypan blue (Figures 4H, 5G,H).

*Ecology and distribution*: Entrophosporoid morph found associated with roots of many cultivated and uncultivated plant species growing in soils highly differing in pH and the content of nutrients, organic matter, and water (Sieverding and Oehl, 2006; Błaszkowski, 2012). Glomoid morph physically connected with an entrophosporoid morph grown in a single-species culture with the host plant *P. lanceolata* (Figures 4F,G) and probably lived in arbuscular mycorrhizal symbiosis with roots of *L. sinuatum* in the field, but no molecular analysis was performed to confirm this hypothesis. Reported as *C.* cf. *claroideum*, *E. infrequens*, Glomeromycota sp., and uncultured *Glomus* from Czech Republic, France, Peru, Poland, Spain, Switzerland, and USA (Supplementary Table 3).

## New combinations

***Entrophospora candida*** (Furrazola, Kaonongbua, and Bever) Błaszk., Niezgoda, B.T. Goto, and Magurno, **comb. nov**.

MycoBank MB836245

≡ *Glomus candidum* Furrazola, Kaonongbua, and Bever. Mycotaxon 113: 103. 2010 (basionym).

≡ *Claroideoglomus candidum* (Furrazola, Kaonongbua, and Bever) Oehl, G.A. Silva, and Sieverd. Mycotaxon 116:106. 2011.

***Entrophospora claroidea*** (N.C. Schenck and G.S. Sm.) Błaszk., Niezgoda, B.T. Goto, and Magurno, **comb. nov**.

MycoBank MB836246

≡ *Glomus claroideum* N.C. Schenck and G.S. Sm., Mycologia 74:84. 1982 (basionym).

≡ *Claroideoglomus claroideum* (N.C. Schenck and G.S. Sm.) C. Walker and A. Schüßler. The *Glomeromycota* – a species list:21. 2010.

***Entrophospora drummondii*** (Błaszk. and Renker) Błaszk., Niezgoda, B.T. Goto, and Magurno, **comb. nov**.

MycoBank MB836247

≡ *Glomus drummondii* Błaszk. and Renker. Mycological Research 110:559. 2006 (basionym).

≡ *Claroideoglomus drummondii* (Błaszk. and Renker) C. Walker and A. Schüßler. The *Glomeromycota* – a species list:22. 2010.

≡ *Albahypha drummondii* (Błaszk. and Renker) Sieverd., Oehl, B.T. Goto, and G.A. Silva. Mycotaxon 117:308. 2011.

***Entrophospora etunicata*** (W.N. Becker and Gerd.) Błaszk., Niezgoda, B.T. Goto, and Magurno, **comb. nov**.

MycoBank MB836248

≡ *Glomus etunicatum* W.N. Becker and Gerd. Mycotaxon 6:29. 1977 (basionym).

≡ *Claroideoglomus etunicatum* (W.N. Becker and Gerd.) C. Walker and A. Schüßler. The Glomeromycota – a species list:22. 2010.

***Entrophospora hanlinii*** (Błaszk., Chwat, and Góralska) Błaszk., Niezgoda, B.T. Goto, and Magurno, **comb. nov**.

MycoBank MB836249

≡ *Claroideoglomus hanlinii* Błaszk., Chwat, and Góralska. Mycological Progress 14:7. 2015 (basionym).

***Entrophospora lamellosa*** (Dalpé, Koske, and Tews) Błaszk., Niezgoda, B.T. Goto, and Magurno, **comb. nov**.

MycoBank MB836250

≡ *Glomus lamellosum* Dalpé, Koske, and Tews. Mycotaxon 43:289. 1992 (basionym).

≡ *Claroideoglomus lamellosum* (Dalpé, Koske, and Tews) C. Walker and A. Schüßler. The Glomeromycota – a species list:22. 2010.

***Entrophospora lutea*** (L.J. Kenn., J.C. Stutz, and J.B. Morton) Błaszk., Niezgoda, B.T. Goto, and Magurno, **comb. nov**.

MycoBank MB836251

≡ *Glomus luteum* L.J. Kenn., J.C. Stutz, and J.B. Morton. Mycologia 91:1090. 1999 (basionym).

≡ *Claroideoglomus luteum* (L.J. Kenn., J.C. Stutz, and J.B. Morton) C. Walker and A. Schüßler. The Glomeromycota – a species list:22. 2010.

***Entrophospora walkeri*** (Błaszk. and Renker) Błaszk., Niezgoda, B.T. Goto, and Magurno, **comb. nov**.

MycoBank MB836252

≡ *Glomus walkeri* Błaszk. and Renker. Mycological Research 110:563. 2006 (basionym).

≡ *Claroideoglomus walkeri* (Błaszk. and Renker) C. Walker and A. Schüßler. The *Glomeromycota* – a species list:22. 2010.

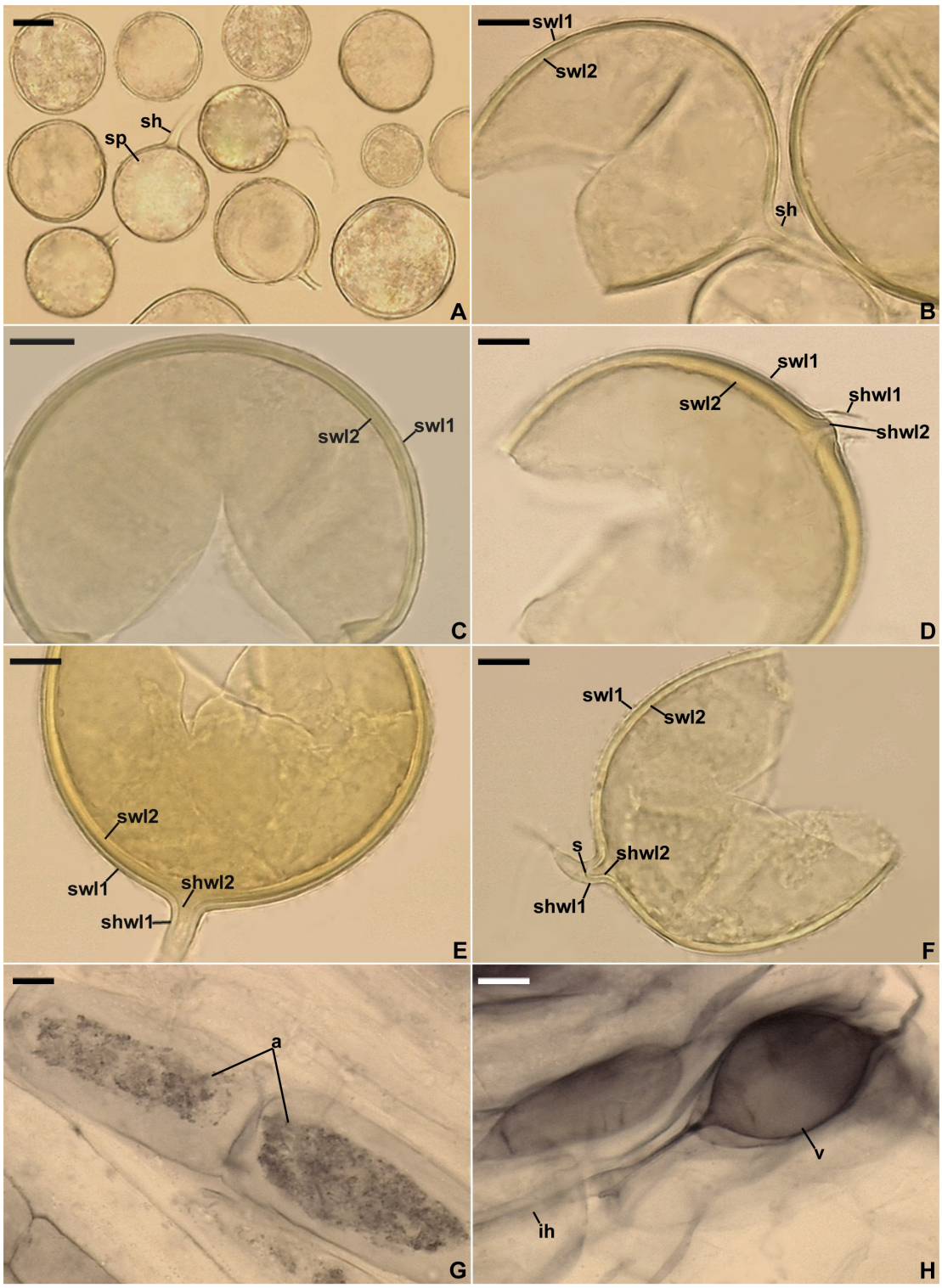



FIGURE 5
*Entrophospora infrequens*. **(A)** Intact glomoid spores (sp) with subtending hyphae (sh). **(B–F)** Spore wall layers (swl) 1 and 2 continuous with subtending hyphal wall layers (shwl) 1 and 2; a septum (s) continuous with swl2 in the lumen of the sh is indicated in **(F)**. **(G,H)** Arbuscules (a), intraradical hyphae (ih), and vesicles (v) in roots of *Plantago lanceolata* stained in 0.1% Trypan blue. **(A–D,G,H)** Spores and mycorrhizal structures in PVLG. **(E,F)** Spores in PVLG+Melzer's reagent. **(A–H)** Differential interference microscopy. Scale bars: **(A)** = 20 μm, **(B–H)** = 10 μm.

≡ *Albahypha walkeri* (Błaszk. and Renker) Sieverd., Oehl, B.T. Goto, and G.A. Silva. Mycotaxon 117:309. 2011.

## Description of new species

***Entrophospora argentinensis*** Błaszk., B.T. Goto, Magurno, Niezgoda, and Cabello, **sp. nov**.

Figures 6A–H, 7A,B.

MycoBank MB836244

*Typification*: ARGENTINA. TIERRA DEL FUEGO: 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 *D. flexuosa* and *Po. rigidifolia* from a steppe community (Mendoza et al., 2011), 1–15 Mar 2009, R. Mendoza (**holotype** slide with spores no. ZT Myc 61117, **isotypes** slides with spores no. 3694–3699, LPPDSE); 45S and *rpb1* sequences: MT722021–MT722024 and MT733203–MT733204, respectively, GenBank.

*Etymology*: *argentinensis* (Latin), referring to Argentina, where the species was originally found.

*Diagnosis*: Differs from other species of *Entrophospora* in having a laminate spore wall layer uneven in thickness in its different regions and nucleotide composition of sequences of the 45S nuc rDNA region and the *rpb1* gene.

*Description*: Glomerospores (= spores) glomoid, formed singly in soil (Figure 6A). Spores arising blastically at the tips of sporogenous hyphae (Figures 6G,H). *Spores* yellowish white (3A2) to dark yellow (4C8); globose to subglobose; (45–)95(–115) μm diam, rarely ovoid; 85–115 × 100–140 μm; with one subtending hypha (Figures 6A–H). *Spore wall* composed of three layers (Figures 6B–D,F–H). Layer 1, forming the spore surface, evanescent, semi-flexible, smooth in young spores, becoming roughened with age, occasionally completely sloughed off in older spores, hyaline to yellowish white (4A2), (0.8–)1.1(–1.5) μm thick when intact (Figures 6B–D,F–H). Layer 2 permanent, laminate, semi-flexible, yellowish white (3A2) to dark yellow (4C8), uneven in thickness when observed in a cross view, (1.0–)3.8(–7.5) μm thick in thinner regions, (2.4–)6.0(–10.0) μm thick in thicker regions; therefore, the lower edge of this layer is wavy, when observed in a plan view; thinner regions visible as circular, (1.2–)5.0(–13.0) μm diam or ellipsoidal, 5.2–7.0 × 7.4–14.8 μm, more or less evenly distributed lighter patches (Figures 6B–H); in young spores, layer 2 frequently uniform in thickness (Figures 6F,H); layer 2 with birefringent properties in polarized light, where smaller or larger areas, despite yellow-colored, turn almost black (Figures 6C,G). Layer 3 permanent, uniform (not divided into visible sublayers), semi-flexible, hyaline to light yellow (4A4), 0.8–1.3 μm thick, usually tightly adherent to the lower surface of layer 2, occasionally separating from layer 2 in vigorously crushed spores (Figures 6F,H). Layers 1–3 do not stain in Melzer's reagent (Figure 6B). *Subtending hypha* yellowish white (3A2) to dark yellow (4C8) near the spore base, hyaline below the pigmented portion; straight or recurved, cylindrical or slightly funnel-shaped, rarely slightly constricted at the spore base; (6.2–)7.8(–8.8) μm wide at the spore base (Figures 6G,H); not braking in crushed spores. *Wall of subtending hypha* yellowish white (3A2) to dark yellow (4C8) up to 9.2 μm below the spore base, then hyaline; (2.0–)3.4(–4.8) μm thick at the spore base; consisting of three layers continuous with spore wall layers 1–3; subtending hyphal wall layer (shwl) 1 usually highly deteriorated or completely sloughed off even in young spores; shwl 3 present only at the spore base or extending up to 2.8 μm below the spore base (Figures 6G,H). *Pore* (0.8–)1.5(–3.4) μm wide at the spore base, occluded by a straight or curved septum continuous with a few innermost laminae of spore wall layer 2 and spore wall layer 3; septum 0.8–3.4 μm wide, 1.0–2.0 μm thick, positioned at or up to 4.2 μm below the spore base (Figures 6G,H). *Germination* unknown.

*Ecology and distribution*: In the field, associated with roots of *D. flexuosa* and *Po. rigidifolia* in Tierra del Fuego, Argentina. Forming mycorrhiza with arbuscules, vesicles, as well as intraradical and extraradical hyphae in single-species cultures with *P. lanceolata* as the host (Figures 7A,B); structures stained violet white (16A2) to deep violet (16E8) in 0.1% Trypan blue. Found as *Glomus* cf. *claroideum* associated with a spore of *E. infrequens* grown in trap culture inoculated with soil from an agricultural grassland of Switzerland and as trap-cultured glomeromycotan spore in the USA (Supplementary Table 3).

***Entrophospora glacialis*** Zubek, Niezgoda, B.T. Goto, Magurno, and Błaszk., **sp. nov.**

Figures 8A–H.

MycoBank MB846045

*Typification*: SWEDEN. TARFALA VALLEY: 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 *F. vivipara*, *Po. alpina*, *S. herbacea*, *S. polaris*, and *Si. acaulis* from a glacier foreland of Isfallglaciarän (67°54′ N, 18°35′ E), 23 July 2019, P. Wietrzyk-Pełka and M. Węgrzyn (**holotype** slide with spores no. ZT Myc 0066908, **isotypes** slides with spores no. 3847–3859, LPPDSE); 45S and *rpb1* sequences: ON950374–ON950380 and ON938326–ON938327, respectively, GenBank.

*Etymology*: Latin, *glacialis*, referring to the glacial habitat, in which this new species was originally found.

*Diagnosis*: Differs from other *Entrophospora* species with glomoid spores in producing spores with a spore wall consisting of five permanent layers and nucleotide composition of sequences of the 45S nuc rDNA region and the *rpb1* gene.

*Description*: Glomerospores (= spores) glomoid, formed singly in soil, arise blastically at tips of sporogenous hyphae (Figures 8A–H) continuous with extraradical mycorrhizal hyphae. *Spores* yellowish white (4A2) to yellowish brown (5E8); globose to subglobose; (40–)91(–129) μm diam; rarely

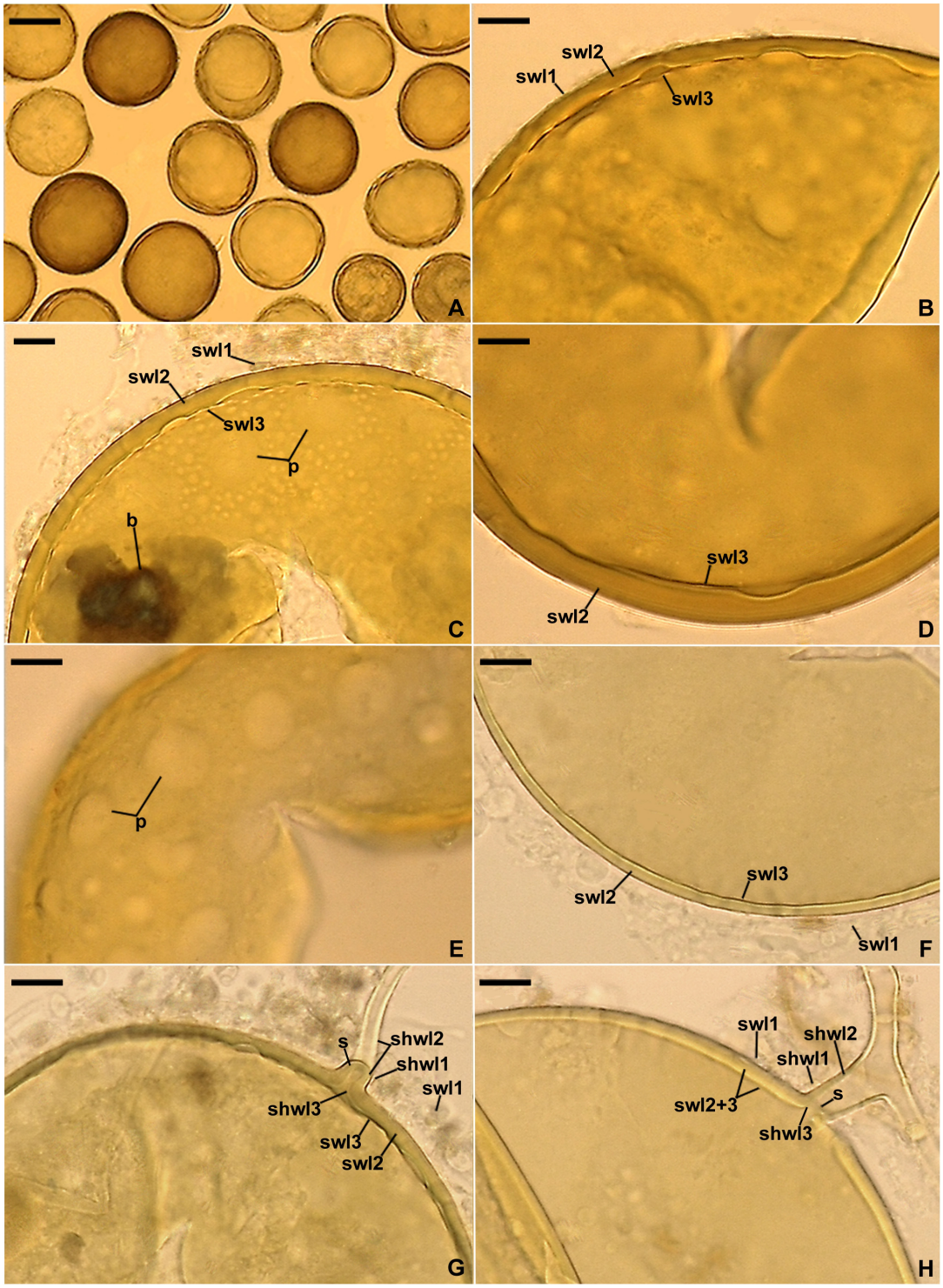



FIGURE 6
*Entrophospora argentinensis*. **(A)** Intact spores. **(B–D,F)** Spore wall layers (swl) 1–3; note the differences in thickness of the laminate swl2 in mature spores depicted in **(B–D)** and the equal thickness of swl2 in a young spore presented in **(F)**, as well as the birefringent (b) properties of layer 2 in polarized light visible in **(C)**. **(E)** Circular and ellipsoidal lighter patches (p) formed by the thinner areas of the laminate spore wall layer 2 seen in a plan view. **(G,H)** Subtending hyphal wall layers (shwl) 1–3 continuous with spore wall layers (swl) 1–3; note the highly deteriorated swl1 and a septum (s) in the subtending hyphal lumen formed by shwl2 and 3 continuous with swl2 and 3. **(A,D–H)** Spores in PVLG. **(B)** Spore in PVLG+Melzer's reagent. **(A–H)** Differential interference microscopy. Scale bars: **(A)** = 50 μm, **(B–H)** = 10 μm.

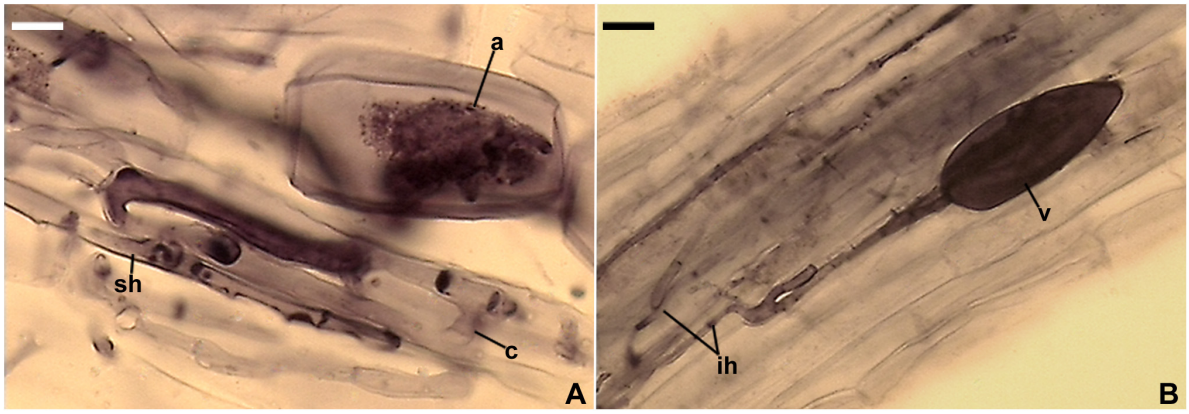



FIGURE 7
Mycorrhizal structures of *Entrophospora argentinensis* in roots of *Plantago lanceolata* stained in 0.1% Trypan blue. **(A)** Arbuscule (a), coiled (c) and straight (sh) intraradical hyphae. **(B)** Vesicle (v) and intraradical hyphae (ih). **(A,B)** In PVLG. **(A,B)** Differential interference microscopy. Scale bars: **(A)** = 10 μm, **(B)** = 20 μm.

ovoid; 77–101 × 90–123 μm; with one subtending hypha (Figures 8A–H). *Spore wall* composed of five permanent layers (Figures 8B–H). Layer 1, forming the spore surface, uniform (not containing visible sublayers), semi-flexible, hyaline to yellowish white (4A2), (0.8–)1.3(–2.0) μm thick, tightly adherent to the upper surface of layer 2, occasionally with small, local thickenings rendering the spore surface slightly wavy when observed in a cross-section (Figures 8B–H). Layer 2 uniform, semi-flexible, yellowish white (4A2), (1.0–)1.8(–2.6) μm thick (Figures 8B–H). Layer 3 uniform, semi-flexible, hyaline to yellowish white (4A2), (2.0–)4.1(–9.0) μm thick (Figures 8B–H). Layer 4 laminate, semi-flexible, yellowish white (4A2) to yellowish brown (5E8), (2.4–)3.6(–5.0) μm thick, consisting of very thin, < 0.5 μm thick, laminae, tightly adherent to each other (Figures 8B–H). Layer 5 uniform, flexible to semi-flexible, (1.0–)1.1(–1.2) μm thick, usually slightly separating from the lower surface of the laminate layer 4 (Figures 8B–H). None of the spore wall layers stains in Melzer's reagent. *Subtending hypha* hyaline to yellowish white (4A2); straight or recurved, usually slightly funnel-shaped, rarely cylindrical or slightly constricted at the spore base; (8.4–)10.8(–14.2) μm wide at the spore base (Figures 8A–H); not braking in crushed spores. *Wall of subtending hypha* hyaline to yellowish white (4A2); (3.8–)4.8(–6.4) μm thick at the spore base; consisting of five layers continuous with spore wall layers 1–5; subtending hyphal wall layers 4 and 5 formed up to 6.1 μm below the spore base (Figures 8A–H). *Pore* (1.0–)1.4(–2.0) μm wide at the spore base, usually open, rarely closed by a curved septum, 0.7–1.1 μm thick, continuous with spore wall layers 4 and 5 (Figures 8G,H). Spore content of hyaline oily substance. *Germination* unknown.

*Ecology and distribution*: In the field, present under an initial community consisting of cryptogamic species, as well as *F. vivipara*, *Po. alpina*, *S. herbacea*, *S. polaris*, and *Si. acaulis* in a glacier foreland of Sweden. No molecular analysis was performed to reveal which of these species harbored *E. glacialis*. In single-species cultures with *P. lanceolata* as the host plant, *E. glacialis* formed mycorrhiza with arbuscules, vesicles, as well as intra- and extraradical hyphae that stained dark [pale violet (16A2) to deep violet (16D8)] in 0.1% trypan blue. Found as *C. claroideum*, *C.* cf. *claroideum*, *E. infrequens*, *G. luteum*, Glomeromycota sp., and uncultured *Glomus* in cultivated and uncultivated sites, including submerged and contaminated habitats, in Brazil, China, Japan, Netherlands, Norway, Poland, Switzerland, and USA (Supplementary Table 3).

***Entrophospora furrazolae*** Magurno, Niezgoda, B.T. Goto, and Błaszk., **sp. nov.**

Figures 9A–H.

MycoBank MB 846048

*Typification*: POLAND. LUBLINIEC, SILESIAN UPLAND: 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 *Po. trivialis* from a plant community in the unpolluted shore of Kokotek pond II (50°37′ N, 18°43′ E), July 2016, Monika Malicka (**holotype** slide with spores no. ZT Myc 0066909, **isotypes** slides with spores no. 3860–3869, LPPDSE); 45S and *rpb1* sequences: MH590060, MH590061, ON950381–ON950390, and MT733209–MT733210, respectively, GenBank.

*Etymology*: Latin, *furrazolae*, in honor of Eduardo Furrazola, in recognition of his important contribution to studies of arbuscular mycorrhizal fungi. This recognition comes after the premature death caused by COVID-19 in 2021.

*Diagnosis*: Differs from other *Entrophospora* species with glomoid spores in formation of spores with a spore wall containing three permanent layers and two layers staining in Melzer's reagent, as well as in nucleotide composition of sequences of the 45S nuc rDNA region and the *rpb1* gene.

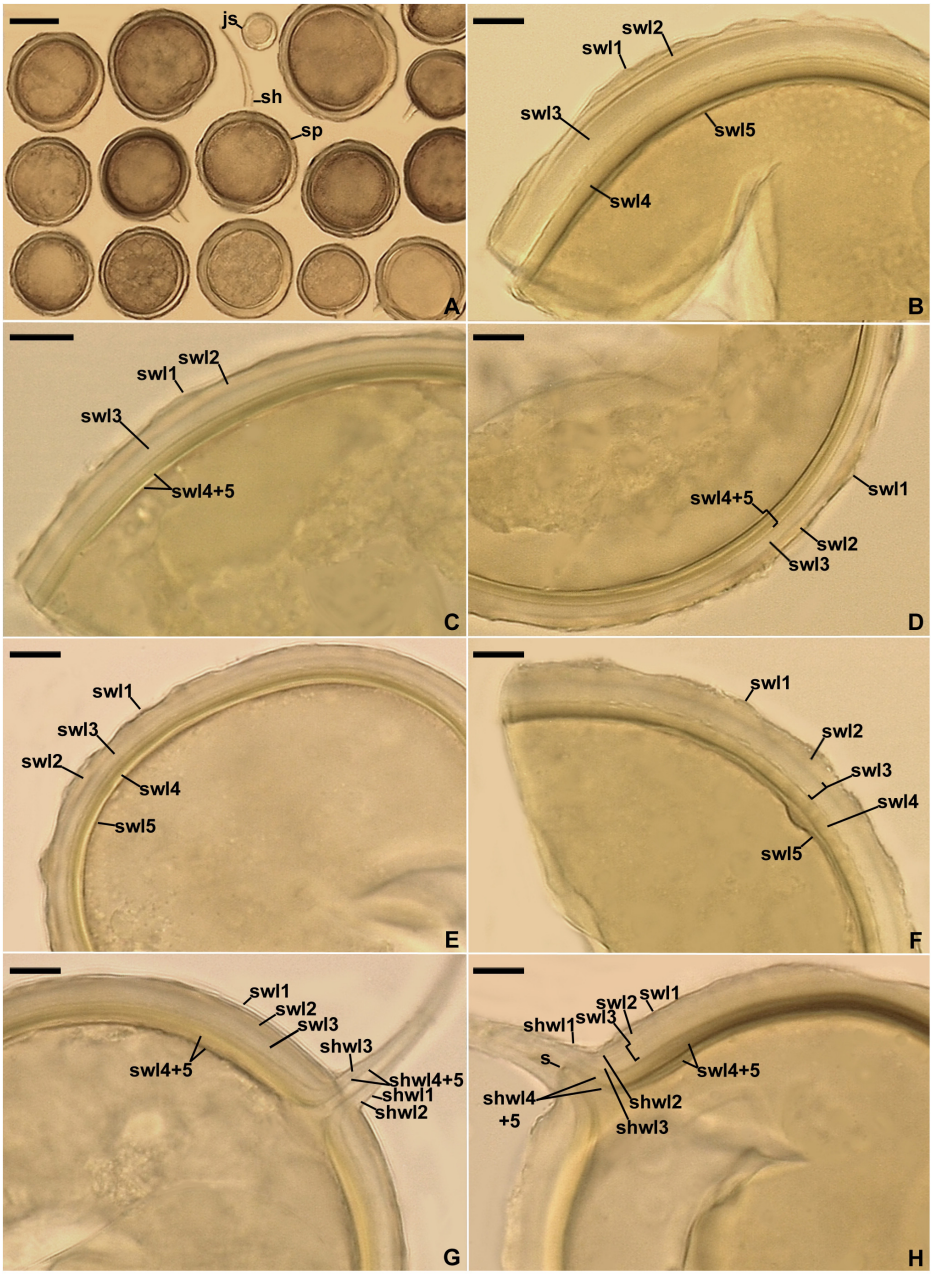



FIGURE 8
*Entrophospora glacialis*. **(A)** Intact spores (sp) with subtending hyphae (sh); juvenile spore (js) is indicated. **(B–F)** Spore wall layers (swl) 1–5. **(G,H)** Subtending hyphal wall layers (shwl) 1–5 continuous with spore wall layers (swl) 1–5; a septum (s) continuous with swl5 is indicated in **(H)**. **(A,E,G,H)** Spores in PVLG. **(B–D,F)** Spores in PVLG+Melzer's reagent. **(A–H)** Differential interference microscopy. Scale bars: **(A)** = 50 μm, **(B–H)** = 10 μm.

*Description*: Glomerospores (=spores) glomoid, formed singly in soil, arise blastically at tips of sporogenous hyphae (Figures 9A,C,G,H) continuous with extraradical mycorrhizal hyphae. *Spores* hyaline to yellowish white (4A2); globose to subglobose; (60–)72(–87) μm diam; rarely ovoid; 50–57 × 60–74 μm; with one subtending hypha (Figures 9A–H). *Spore wall* composed of four layers (Figures 9B–F,H). Layer 1, forming the spore surface, mucilaginous, short-lived, flexible, hyaline, (0.6–)0.8(–1.0) μm thick, usually highly deteriorated or completely sloughed off in mature spores (Figures 9B–F,H). Layer 2 uniform (without visible sublayers), permanent, flexible to semi-flexible, hyaline, (0.8–)1.0(–1.6) μm thick, tightly adherent to the upper surface of layer 3, not separating from this layer in even vigorously crushed spores (Figures 9B–F,H).

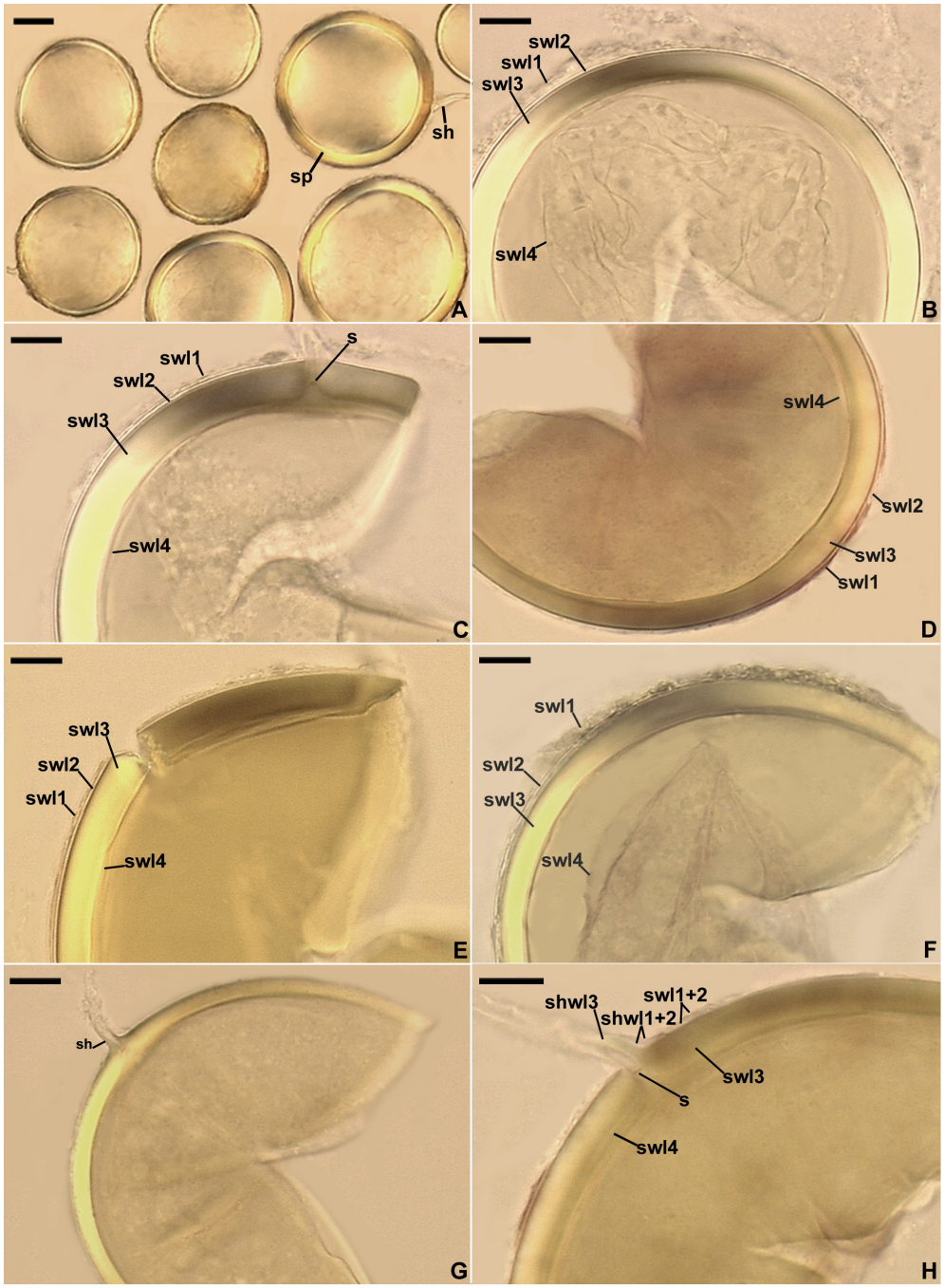



FIGURE 9
*Entrophospora furrazolae*. **(A)** Intact spores (sp) with subtending hyphae (sh). **(B–F)** Spore wall layers (swl) 1–4; a septum (s) continuous with swl4 is indicated in **(C)**. **(G,H)** Subtending hypha (sh) with subtending hyphal wall layers (shwl) 1–3; a septum (s) continuous with swl4 is indicated in **(H)**. **(A–C)** Spores in PVLG. **(D–H)** Spores in PVLG+Melzer's reagent. **(A–H)** Differential interference microscopy. Scale bars: **(A)** = 20 μm, **(B–H)** = 10 μm.

Layer 3 laminate, permanent, semi-flexible, hyaline to yellowish white (4A2), (2.4–)6.4(–10.2) μm thick, consisting of very thin, <0.5 μm thick, laminae, tightly adherent to each other (Figures 9B–F,H); layer 3 has birefringent properties in polarized light, in which smaller or larger colorless or yellowish white (4A2) fragments of this layer turn almost black (Figures 9B,C,E,F). Layer 4 flexible, permanent, hyaline, (0.6–)0.8(–1.0) μm thick, usually separating from the lower surface of the laminate layer 3 in even moderately crushed spores (Figures 9B–F,H). In Melzer's reagent, spore wall layers

1 and 4 frequently stain reddish white (10A2) to grayish rose (11B4) and reddish white (8A2) to pastel pink (11A3), respectively, but these staining reactions do not appear in all spores (**Figures 9D–H**). *Subtending hypha* hyaline; straight or recurved, usually slightly funnel-shaped, rarely cylindrical or slightly constricted at the spore base; (3.6–)5.3(–7.4) µm wide at the spore base (**Figures 9A,C,G,H**); frequently braking at the base of crushed spores. *Wall of subtending hypha* hyaline; (1.6–)2.3(–3.5) µm thick at the spore base; consisting of three layers continuous with spore wall layers 1–3; subtending hyphal wall layer (shwl) 1 usually highly deteriorated or completely sloughed off in mature spores, thereby difficult to see or invisible; shwl2 usually present only at the spore base, rarely starting developing up to ca. 6 µm below the spore base; shwl3 gradually thickening beginning from ca. 9 µm below the spore base (**Figure 9H**). *Pore* (0.8–)1.0(–1.4) µm wide at the spore base, closed by a curved septum, 0.6–1.0 µm thick, continuous with spore wall layer 4; the septum usually located at the center of the laminate spore wall layer 3 when observed in a cross section (**Figures 9C–H**). Spore content of hyaline oily substance. *Germination* unknown.

*Ecology and distribution*: In Oman, *E. furrazolae* probably lived in the field in arbuscular mycorrhizal symbiosis with *Pr. cineraria* in a site characterized by hyper aridity, but no molecular analysis was performed to confirm this hypothesis. In Poland, *E. furrazolae* was associated with *Po. trivialis* in an unpolluted natural site, as indicated by NGS sequencing of partial 28S PCR amplicons obtained from DNA extracted from rhizosphere soil and root samples of this plant species (Malicka et al., 2022). In single-species cultures with *P. lanceolata* as the host plant, *E. furrazolae* formed mycorrhiza with arbuscules and intra- and extraradical hyphae that stained bluish-white (20A2) to light blue (20A5) in 0.1% trypan blue. Found as *C. drummondii*, as well as uncultured Claroideoglomeraceae, *Glomus*, and Glomeromycota in undisturbed and degraded soils of Czech Republic, China, Spain, and Switzerland (**Supplementary Table 3**).

## Discussion

Overall, the results of phylogenomic, phylogenetic, and morphological analyses presented in this study led to the following statements. First, the glomoid spore-producing species of the genus *Claroideoglomus* with the type species *C. claroideum* in the monogeneric family Claroideoglomeraceae sensu Schüßler and Walker (2010) represent a new order in the Glomeromycota, here described as Entrophosporales. Second, the entrophosporoid morph morphologically conspecific with *E. infrequens* represents a group of cryptic and potential dimorphic species widely distributed in the world. Third, our hypothesis was confirmed: the glomoid spore-producing AMF, preliminary named as *Claroideoglomus* 1, 2, and 4, originating from Argentina, Poland, Oman, and Sweden, are actually new species, described here as *E. argentinensis*, *E. glacialis*, and *E. furrazolae*. Finally, Redecker et al.'s (2013) conclusion that *Albahypha* is an unsupported taxon was confirmed (**Figure 3** and **Supplementary Figures 3–5**).

Previous phylogenomic analyses have suggested that the order Glomerales is polyphyletic (Beaudet et al., 2018; Montoliu-Nerin et al., 2021), but the phylogenetic position of Claroideoglomeraceae was not clearly established. When data from Beaudet et al. (2018) and Montoliu-Nerin et al. (2021) were combined, we recovered the same topology as in Montoliu-Nerin et al. (2021). The topology, showing Claroideoglomeraceae sister to Diversisporales and Glomeraceae, was consistently recovered in all other analyses, even when only the Beaudet et al.'s (2018) data were used (**Figure 1** and **Supplementary Figures 1**, **2**). However, when combining all the data, we were able to extract only 78 SCOs present in at least 50% of the taxa (vs. 1260 SCOs), which could be due to the low genome completeness of the transcriptomic data presented in Beaudet et al. (2018).

In all phylogenomic analyses, we included only a few members of Mortierellomycota and Mucoromycota as outgroups. This was because the phylogenetic relationship among the three phyla was not the focus of the present work, but rather the phylogenetic placement of the former Claroideoglomeraceae with respect to the rest of Glomeromycota. This (i) resulted in better taxonomic resolution at the phylum level (Glomeromycota), (ii) provided consistent support for the phylogenetic placement of the former Claroideoglomeraceae as sister to a clade formed by Diversisporales and Glomeraceae, and, consequently, (iii) supported the need to transfer the Claroideoglomeraceae from Glomerales to a new order, Entrophosporales. The name of the order followed the synonymization of Claroideoglomeraceae with Entrophosporaceae because *E. infrequens* was described before *C. claroideum* (Ames and Schneider, 1979; Schenck and Smith, 1982). Through this, the order Glomerales remained with one family, Glomeraceae. Furthermore, the comparisons of the phylogenetic trees reconstructed from analyses with concatenated genes using *Glomus* (Glomerales after emendation) or *Diversispora* (Diversisporales) as alternative outgroups, despite some differences in the topology (**Figure 3** and **Supplementary Figures 3**, **5**), did not lead to different conclusions about the status of the new species, and *Albahypha* was not a valid generic taxon.

Both 45S, 45S+*rpb1*, and 45S+*rpb1*_G trees showed a group of taxa, formerly *Claroideoglomus* sensu Oehl et al. (2011b), characterized by short branches and a few deep-supported nodes (**Figure 3** and **Supplementary Figures 3**, **5**), a pattern that resembles an evolutionary radiation event, and a group of three sister taxa, referable to the former *Albahypha* sensu Oehl et al. (2011b), characterized by longer branches and different relationships depending on the dataset used. In the former

*Claroideoglomus* clade, the species *E. claroidea* and *E. lamellosa* were always unresolved, as also shown in Delavaux et al. (2022) for the former species, a situation coherent with the evolutionary radiation hypothesis. The *Albahypha* clade occurred only in the 45S+*rpb1*_G phylogeny, where the former *Claroideoglomus* clade was affected by insufficient BI and ML supports. The presence of a supported *Albahypha* clade in the 45S+*rpb1*_G phylogeny probably resulted from long-branch attraction (LBA) rather than true close relationships.

Our research significantly enriched the knowledge of Glomeromycota by showing the dimorphism and cryptic nature of the species called *E. infrequens,* previously known only for its entrophosporoid morph with a distinctive and uniform morphology. First, both nuc rDNA and *rpb1* sequences of one of the analyzed morphs, here designated as an epitype of *E. infrequens* from spores found in Poland, clustered with sequences of a Spanish glomoid morph preliminary named *Claroideoglomus* 3 (**Figure 3** and **Supplementary Figures 3–5**). The sequences from the two isolates were obtained in different years and from two different laboratories, so we ruled out the possibility that our findings could be biased by PCR contamination. Second, sequences of a Swedish glomoid morph (*Claroideoglomus* 2/*E. glacialis*) formed a mixed clade with sequences obtained from *E. infrequens* identified in Switzerland (Oehl et al., 2011b). Third, the FJ461806, FJ461807, MT832169, and MT832170 sequences of *E. infrequens* originating from the U.S. Indiana and California states were placed in unresolved positions of the trees. Finally, the entrophosporoid morph of the *E. infrequens* epitype was shown to be physically connected with glomoid spores (**Figures 4F,G**). Consequently, the discovery of the dimorphic and cryptic nature explained the early inexplicable localization of sequences obtained from this entrophosporoid morph in different phylogenetic tree clades and unraveled the inconsistent opinion on the taxonomic status of *E. infrequens* sensu Ames and Schneider (1979).

The dimorphic behavior of AMF has been observed almost exclusively in acaulosporoid spore-producing species of the genera *Ambispora* and *Archaeospora*, whose second morph were glomoid spores (Goto et al., 2008; Palenzuela et al., 2011; Bills and Morton, 2015; Oehl et al., 2019). The exceptions were *Ar. ecuadoriana*, which formed acaulosporoid, entrophosporoid, and glomoid spores (Schüßler and Walker, 2019), as well as *Am. callosa* sensu Walker et al. (2007), synonymized with *Am. leptoticha* (Bills and Morton, 2015), whose phylogeny was reconstructed from analyses of DNA originating from the only known glomoid morph of this species, originally described as *G. callosum* (Sieverding, 1988).

According to Lücking et al. (2021), cryptic speciation may be caused by the delay of phenotypic divergence relative to phylogenetic divergence because, e.g., (i) species may have diverged too recently to have also diverged in phenotype and (ii) the forces at work were selective and prevented phenotypes from diverging. This may explain the phenotypic indistinctness of the entrophosporoid morph representing different glomoid spore-producing *Entrophospora* species (**Figures 3**, **5–8** and **Supplementary Figures 3–5**). On the other hand, the massive construction of entrophosporoid spores, consisting of two thick walls with four permanent components, may have acted as a physical barrier that prevented the production of other entrophosporoid phenotypes due to the influence of the external environment.

Dimorphism is not the persistent behavior of dimorphic AMF species which, for undefined reasons, produced either both morphs (e.g., acaulosporoid and glomoid by *Am. leptoticha*) or only one of them (glomoid by another strain of *Am. leptoticha*) even over many propagation cycles (Bills and Morton, 2015). More (1998) concluded that the dimorphism in even extensively studied fungal groups was influenced or governed by a wide range of unspecific metabolic and environmental factors.

Thus, the cryptic nature and the dimorphic abilities of the entrophosporoid morph implied treating the glomoid spore-producing species of the genus *Claroideoglomus* sensu Schüßler and Walker (2010) as members of the newly emended genus *Entrophospora*, in accordance with the new combinations introduced in the section "Taxonomy".

Like the phylogenetic analyses, morphological comparisons also showed that the relationships between the three new glomoid spore-producing *Entrophospora* species and the glomoid morph of the epitype of *E. infrequens* versus all previously sequenced *Entrophospora* species are bland or very weak. None of the known *Entrophospora* species produces spores (i) with a laminate spore wall layer, which is uneven in thickness in its different regions, as in *E. argentinensis* (**Figures 6B–E,G**), (ii) so strongly resembling spores of species of *Dominikia* and related genera in their frequent formation in loose clusters, appearance, size, pigmentation, spore wall structure, as well as in the phenotypic and histochemical properties of components of this wall (Błaszkowski et al., 2021b), as the glomoid spores of *E. infrequens* (**Figures 4F,G**, **5A–F**), (iii) with a spore wall consisting of five permanent layers, as in *E. glacialis* (**Figures 8B–H**), and (iv) with a spore wall containing three permanent layers, of which two stain in Melzer's reagent, except for *E. furrazolae* (**Figures 9B–F,H**).

The poorly and ambiguously supported relationship of *E. glacialis* to *E. claroidea* and *E. candida* (**Figure 3** and **Supplementary Figures 3**, **5**) somewhat confirms their morphological similarity. Spores of *E. glacialis* and *E. claroidea* (i) are surrounded by a halo produced by colorless or slightly colored spore wall layers surrounding the main structural, much darker colored, laminate spore wall layer and (ii) have a spore wall with an innermost layer that is flexible to semi-flexible, colorless, thin, and usually separates from the lower surface of the laminate layer in crushed spores (**Figures 8A–H**; Błaszkowski et al., 2021b). However, in *E. claroidea,* the halo is produced by two impermanent layers, frequently highly or completely sloughed off in mature

specimens, of which the outermost layer 1, forming the spore surface, stains in Melzer's reagent. The innermost *E. claroidea* spore wall component is layer 4. Instead, in *E. glacialis* the halo is formed by three permanent layers (layers 1–3), remaining intact even in old spores, and none of the five-layered spore wall layers reacts in Melzer's (**Figures 8A–H**). In addition, *E. claroidea* spores are clearly lighter in color, not reaching the shade of brown of *E. glacialis* spores at maturity.

Spores of *E. candida* also have a halo when all spore wall layers are present (Furrazola et al., 2010). However, according to the original description, spores of this species compared to those of *E. glacialis* are much lighter (white, rarely pale yellow) and 1.2- to 2.2-fold larger when globose, have only a two-layered and ∼ 1.4-fold thinner spore wall, as well as may have an up to 1.5-fold wider subtending hypha at the spore base.

Although unsupported, the appearance of a sister relationship of *E. argentinensis* to *E. etunicata* in the 45S tree (**Supplementary Figure 3**) and the neighborhood of these species in the 45S+*rpb1*, *rpb1*, and 45S+*rpb1*_G trees (**Figure 3** and **Supplementary Figures 4**, **5**) call for a comparison of their morphology. As mentioned above, none of the glomoid spore-producing *Entrophospora* species, including *E. etunicata*, has a laminate spore wall layer that is uneven in thickness (Błaszkowski, 2012; Błaszkowski et al., 2015a). In addition, the spore wall of *E. etunicata* consists of two layers only, lacking the semi-flexible spore wall layer 3 of *E. argentinensis* (**Figures 6B–D,F–H**), and the spore wall layer 1 of *E. etunicata* stains in Melzer's reagent (Becker and Gerdemann, 1977; Stürmer and Morton, 1997; Błaszkowski, 2012). In *E. argentinensis*, none of the spore wall layers reacts in this reagent (**Figure 6B**). Moreover, the pore of the spore subtending hypha of *E. etunicata* is closed by a septum continuous with the innermost laminae (sublayers) of the laminate spore wall layer 2, and that of *E. argentinensis* is closed by the innermost laminae of the laminate spore wall layer 2 and a septum continuous with the innermost spore wall layer 3 (**Figures 6G,H**). Finally, globose *E. etunicata* spores may be 1.4-fold larger.

Morphologically, *E. furrazolae* may be confused with *E. drummondii*, a sister species indicated in the 45S and 45S+*rpb1*_G trees (**Figure 3** and **Supplementary Figure 3**). In both species, the flexible, thin innermost spore wall layer, forming a septum in the subtending hypha, stains in Melzer's reagent (**Figures 9D–H**; Błaszkowski et al., 2006; Błaszkowski, 2012), a property unknown in other glomoid spore-producing species. However, in *E. drummondii*, this staining reaction occurs only in spore wall layer 3, and in *E. furrazolae* it appears in spore wall layer 4, absent in the spore wall of the former species, and spore wall layer 1 (**Figures 9D–H**). In addition, the spore wall of *E. drummondii* is 1.4- to 1.9-fold thinner, and the mature spores of this species are always slightly darker colored.

We omitted in our analyses the genus *Viscospora* included in the Entrophosporaceae defined by Oehl et al. (2011b) because (*i*) its sole species, *V. viscosa*, originally described as *G. viscosum* (Walker et al., 1995), is provided with only one 18S nuc rDNA sequence and (*ii*) Redecker et al. (2013) found that *V. viscosa* belongs to the genus *Septoglomus*. However, morphologically *V. viscosa* does not match any *Septoglomus* species. Therefore, more robust molecular proofs are needed to clarify the phylogenetic position of *V. viscosa*. In addition, our analyses included the MT733209 and MT733210 *rpb1* sequences erroneously ascribed to *C.* cf. *drummondii* by Błaszkowski et al. (2021a) instead of to the new *E. furrazolae*.

We recommend for the upcoming works involving species in the Entrophosporales that (i) *E. infrequens*-like culture collections should be checked carefully to detect the possible presence of dimorphism, (ii) the morphology of *E. infrequens*-like and/or glomoid-like spores should be carefully described to highlight any possible difference not yet detected, and (iii) descriptions of new species based on *E. infrequens*-like cultures should be based on strong molecular data.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI MH590060, MH590061, MT722021–MT722024, ON950363–ON950390, MT722034–MT722038, MT733201–MT733210, and ON938320–ON938327.

## Author contributions

JB, MS-G, BG, LC, FM, MM, PM, PN, FF, MC, AV, MNA, WB, and SZ: material preparation, data collection, and analysis. JB, MS-G, BG, LC, and FM: conceptualization. JB, MS-G, BG, FM, and PN: methodology. JB, MS-G, BG, FM, MM, PM, PN, SS, RM, and SZ: formal analysis, investigation, and writing—review and editing. JB, BG, and FM: writing—original draft preparation. BG, FM, PN, and SZ: funding acquisition. JB, FM, and PN: resources. JB: writing first draft of the manuscript and supervision. All authors commented on previous versions of the manuscript, contributed to the study conception and design, read, and approved the final manuscript.

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## Conflict of interest

Authors FF and AV were employed by R&D Department, Symborg SL. Author LC was employed by Mycorrhizal Applications LLC at Bio-Research and Development Growth Park.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.962856/full#supplementary-material

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## OŚWIADCZENIE WSPÓŁAUTORA

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**3.** Oświadczam, że w pracy ,,Jobim K., **Błaszkowski J.**, Niezgoda P., Kozłowska A., Zubek Sz., Mleczko P., Chachuła P., Ishikawa N. K., Goto B. T. 2019. New sporocarpic taxa in the phylum Glomeromycota: *Sclerocarpum amazonicum* gen. et sp. nov. in the family Glomeraceae (Glomerales) and *Diversispora sporocarpia* sp. nov. in Diversisporaceae (Diversisporales). Mycological Progress 18, 369–384, doi: https://doi.org/10.1007/s11557-018-01462-2

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**4.** Oświadczam, że w pracy ,,**Błaszkowski J.**, Niezgoda P., Piątek M., Magurno F., Malicka M., Zubek Sz., Mleczko P., Yorou N. S., Jobim K., Vista X. M., Lima J. L. R., Goto B. T. 2019. *Rhizoglomus dalpeae*, *R. maiae*, and *R. silesianum*, new species". Mycologia 6, 965–980, doi: 10.1080/00275514.2019.1654637

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filogenetycznych, interpretacji części wyników badań, statystycznym ich opracowaniu, współtworzeniu manuskryptu i finalnej korekcie manuskryptu po recenzji co określam jako 24% wkładu w przygotowanie ww. publikacji.

**5.** Oświadczam, że w pracy ,,**Błaszkowski J.**, Niezgoda P., de Paiva J. N., da Silva K. J. G., Theodoro R. C., Jobim K., Orfanoudakis M., Goto B. T. 2019. Sieverdingia gen. nov., S. tortuosa comb. nov., and Diversispora peloponnesiaca sp. nov. in the Diversisporaceae (Glomeromycota). Mycological Progress 18, 1363–1382, doi: https://doi.org/10.1007/s11557-019-01534-x''

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**6.** Oświadczam, że w pracy ,,**Błaszkowski J.**, Jobim K., Niezgoda P., Meller E., Malinowski M., Milczarski P., Zubek Sz., Magurno F., Casieri L., Bierza W., Błaszkowski T., Crossay T., Goto B. T. 2021. New glomeromycotan taxa, *Dominikia glomerocarpica* sp. nov. and *Epigeocarpum crypticum* gen. nov. et sp. nov. from Brazil, and *Silvaspora* gen. nov. from New Caledonia''. Frontiers in Microbiology 12, 655910. doi: 10.3389/fmicb.2021.655910

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**7.** Oświadczam, że w pracy ,,**Błaszkowski J.**, Niezgoda P., Meller E., Milczarski P., Zubek Sz., Malicka M., Uszok S., Casieri L., Goto B. T., Magurno F. 2021. New taxa in Glomeromycota: Polonosporaceae fam. nov., *Polonospora* gen. nov., and *P. polonica* comb. nov. ''. Mycological Progress 20, 941–951, doi: 10.1007/s11557-021-01726-4

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**8.** Oświadczam, że w pracy ,,**Błaszkowski J.**, Niezgoda P., Zubek Sz., Meller E., Milczarski P., Malinowski R., Malicka M., Uszok S., Goto B. T., Bierza W., Casieri L., Magurno F. 2022. Three new species of arbuscular mycorrhizal fungi of the genus *Diversispora* from maritime dunes of Poland''. Mycologia 114, 453–466, doi:10.1080/00275514.2022.2030081

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**9.** Oświadczam, że w pracy ,,**Błaszkowski J.**, Sánchez-García M., Niezgoda P., Zubek Sz., Fernández F., Vila A., Al-Yahya'ei M. N., Symanczik S., Milczarski P., Malinowski R., Cabello M.,

Goto B. T., Casieri L., Malicka M., Bierza W., Magurno F. 2022. A new order, Entrophosporales, and three new *Entrophospora* species in Glomeromycota". Frontiers in Microbiology 13, 962856, doi:10.3389/fmicb.2022.962856

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**OŚWIADCZENIE WSPÓŁAUTORA**

**CO-AUTHOR STATEMENT**

Oświadczam, że w pracy ,,Błaszkowski J., Niezgoda P., de Paiva J. N., da Silva K. J. G., Theodoro R. C., Jobim K., Orfanoudakis M., Goto B. T. 2019. Sieverdingia gen. nov., S. tortuosa comb. nov., and Diversispora peloponnesiaca sp. nov. in the Diversisporaceae (Glomeromycota). Mycological Progress 18, 1363–1382, doi: https://doi.org/10.1007/s11557-019-01534-x''

mój udział polegał na współautorstwie koncepcji badań, zebraniu materiału badawczego, interpretacji uzyskanych wyników, oraz redagowaniu tego artykułu, co określam jako 2% wkładu w przygotowanie ww. publikacji.

I declare that, as a co-author of the article, Błaszkowski J., Niezgoda P., de Paiva J. N., da Silva K. J. G., Theodoro R. C., Jobim K., Orfanoudakis M., Goto B. T. 2019. *Sieverdingia* gen. nov., S. tortuosa comb. nov., and *Diversispora peloponnesiaca* sp. nov. in the Diversisporaceae (Glomeromycota). Mycological Progress 18, 1363–1382, doi: https://doi.org/10.1007/s11557-019-01534-x'', I participated in the preparation of the research concept, collecting research material, interpreting the obtained results, and editing this article. My contribution to the preparation of this publication was 2%.



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**OŚWIADCZENIE WSPÓŁAUTORA**

**1.** Oświadczam, że w pracy ,, Błaszkowski J., Jobim K., Niezgoda P., Meller E., Malinowski R., Milczarski P., Zubek Sz., Magurno F., Casieri L., Bierza W., **Błaszkowski T.**, Crossay T., Goto B. T. 2021. New glomeromycotan taxa, *Dominikia glomerocarpica* sp. nov. and *Epigeocarpum crypticum* gen. nov. et sp. nov. from Brazil, and *Silvaspora* gen. nov. from New Caledonia". Frontiers in Microbiology 12, 655910. doi: 10.3389/fmicb.2021.655910

mój udział polegał na współautorstwie koncepcji badań, interpretacji uzyskanych wyników, oraz redagowaniu tego artykułu, co określam jako 2% wkładu w przygotowanie ww. publikacji.

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**OŚWIADCZENIE WSPÓŁAUTORA**

**CO-AUTHOR STATEMENT**

**1.** Oświadczam, że w pracy ,,Błaszkowski J., Sánchez-García M., Niezgoda P., Zubek Sz., Fernández F., Vila A., Al-Yahya'ei M. N., Symanczik S., Milczarski P., Malinowski R., **Cabello M.**, Goto B. T., Casieri L., Malicka M., Bierza W., Magurno F. 2022. A new order, Entrophosporales, and three new Entrophospora species in Glomeromycota. Frontiers in Microbiology 13, 962856, doi:10.3389/fmicb.2022.962856’’

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Signature

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## OŚWIADCZENIE WSPÓŁAUTORA

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Podpis



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**OŚWIADCZENIE WSPÓŁAUTORA**

**CO-AUTHOR STATEMENT**

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I declare that, as a co-author of the article "Błaszkowski J., Kozłowska A., Niezgoda P., **Goto B.T.**, Dalpé Y. 2018. A new genus, Oehlia with Oehlia diaphana comb. nov. and an emended description of Rhizoglomus vesiculiferum comb. nov. in the Glomeromycotina. Nova Hedwigia 107 (3-4), 501-518. doi: 10.1127/nova_hedwigia/2018/0488", I participated in the preparation of the research concept, collecting research material, interpreting the obtained results, and editing this article. My contribution to the preparation of this publication was **8**%.

**2.** Oświadczam, że w pracy ,,Błaszkowski J., Niezgoda P., **Goto B. T.**, Kozłowska A. 2018. Halonatospora gen. nov. with H. pansihalos comb. nov. and Glomus bareae sp. nov. (Glomeromycota; Glomeraceae). Botany 96: 737–748. dx.doi.org/10.1139/cjb-2018-0107''

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**3.** Oświadczam, że w pracy ,,Jobim K., Błaszkowski J., Niezgoda P., Kozłowska A., Zubek Sz., Mleczko P., Chachuła P., Ishikawa N. K., **Goto B. T.** 2019. New sporocarpic taxa in the phylum Glomeromycota: Sclerocarpum amazonicum gen. et sp. nov. in the family Glomeraceae (Glomerales) and Diversispora sporocarpia sp. nov. in Diversisporaceae (Diversisporales). Mycological Progress 18, 369–384, doi: https://doi.org/10.1007/s11557-018-01462-2''

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Glomeromycota: Sclerocarpum amazonicum gen. et sp. nov. in the family Glomeraceae (Glomerales) and Diversispora sporocarpia sp. nov. in Diversisporaceae (Diversisporales). Mycological Progress 18, 369–384, doi: https://doi.org/10.1007/s11557-018-01462-2'', I participated in the preparation of the research concept, collecting research material, interpreting the obtained results, and editing this article. My contribution to the preparation of this publication was **5%**.

**4.** Oświadczam, że w pracy „Błaszkowski J., Niezgoda P., Piątek M., Magurno F., Malicka M., Zubek Sz., Mleczko P., Yorou N. S., Jobim K., Vista X. M., Lima J. L. R., **Goto B. T.** 2019. Rhizoglomus dalpeae, R. maiae, and R. silesianum, new species. Mycologia 6, 965–980, doi: 10.1080/00275514.2019.1654637''

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**5.** Oświadczam, że w pracy ,,Błaszkowski J., Niezgoda P., de Paiva J. N., da Silva K. J. G., Theodoro R. C., Jobim K., Orfanoudakis M., **Goto B. T.** 2019. Sieverdingia gen. nov., S. tortuosa comb. nov., and Diversispora peloponnesiaca sp. nov. in the Diversisporaceae (Glomeromycota). Mycological Progress 18, 1363–1382, doi: https://doi.org/10.1007/s11557-019-01534-x''

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I declare that, as a co-author of the article "XXX", I participated in the preparation of the research concept, collecting research material, interpreting the obtained results, and editing this article. My contribution to the preparation of this publication was **5%**.

**6.** Oświadczam, że w pracy ,,Błaszkowski J., Jobim K., Niezgoda P., Meller E., Malinowski M., Milczarski P., Zubek Sz., Magurno F., Casieri L., Bierza W., Błaszkowski T., Crossay T., **Goto B. T.** 2021. New glomeromycotan taxa, Dominikia glomerocarpica sp. nov. and Epigeocarpum crypticum gen. nov. et sp. nov. from Brazil, and Silvaspora gen. nov. from New Caledonia. Frontiers in Microbiology 12, 655910. doi: 10.3389/fmicb.2021.655910''

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I declare that, as a co-author of the article ,,Błaszkowski J., Jobim K., Niezgoda P., Meller E., Malinowski M., Milczarski P., Zubek Sz., Magurno F., Casieri L., Bierza W., Błaszkowski T., Crossay T., **Goto B. T.** 2021. New glomeromycotan taxa, Dominikia glomerocarpica sp. nov. and Epigeocarpum crypticum gen. nov. et sp. nov. from Brazil, and Silvaspora gen. nov. from New Caledonia. Frontiers in Microbiology 12, 655910. doi: 10.3389/fmicb.2021.655910'', I participated in the preparation of the research concept, collecting research material, interpreting the obtained results, and editing this article. My contribution to the preparation of this publication was **2%**.

**7.** Oświadczam, że w pracy ,,Błaszkowski J., Niezgoda P., Meller E., Milczarski P., Zubek Sz., Malicka M., Uszok S., Casieri L., **Goto B. T.**, Magurno F. 2021. New taxa in Glomeromycota: Polonosporaceae fam. nov., Polonospora gen. nov., and P. polonica comb. nov. Mycological Progress 20, 941–951, doi: 10.1007/s11557-021-01726-4'' 

mój udział polegał na współautorstwie koncepcji badań, zebraniu materiału badawczego, interpretacji uzyskanych wyników, oraz redagowaniu tego artykułu, co określam jako **3%** wkładu w przygotowanie ww. publikacji.

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**8.** Oświadczam, że w pracy ,,Błaszkowski J., Niezgoda P., Zubek S., Meller E., Milczarski P., Malinowski R., Malicka M., Uszok S., **Goto B. T.**, Bierza W., Casieri L., Magurno F. 2022. Three new species of arbuscular mycorrhizal fungi of the genus Diversispora from maritime dunes of Poland. Mycologia 114, 453–466, doi:10.1080/00275514.2022.2030081''

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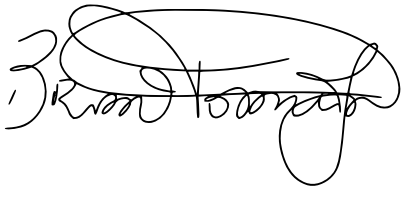
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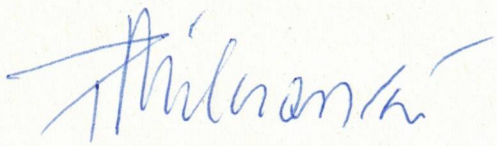
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mój udział polegał na zbiorze części materiału w terenie, współautorstwie koncepcji badań, interpretacji części wyników badań, co określam jako 2% wkładu w przygotowanie ww. publikacji.

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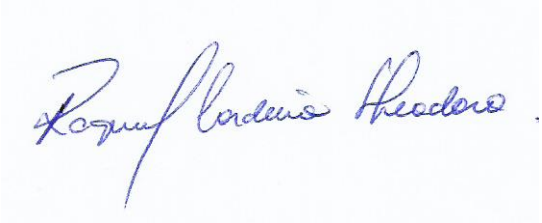
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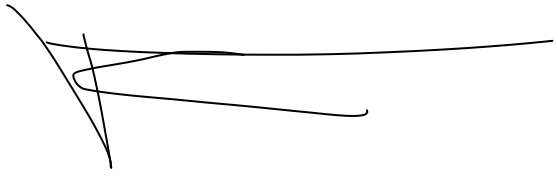
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I declare that, as a co-author of the article ,,Błaszkowski J., **Jobim K.**, Niezgoda P., Meller E., Malinowski M., Milczarski P., Zubek Sz., Magurno F., Casieri L., Bierza W., Błaszkowski T., Crossay T., Goto B. T. 2021. New glomeromycotan taxa, Dominikia glomerocarpica sp. nov. and Epigeocarpum crypticum gen. nov. et sp. nov. from Brazil, and Silvaspora gen. nov. from New Caledonia. Frontiers in Microbiology 12, 655910. doi: 10.3389/fmicb.2021.655910'', I participated in the preparation of the research concept, collecting research material, interpreting the obtained results, and editing this article. My contribution to the preparation of this publication was **5%**.

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**OŚWIADCZENIE WSPÓŁAUTORA**

**CO-AUTHOR STATEMENT**

**1.** Oświadczam, że w pracy ,,Błaszkowski J., Niezgoda P., Piątek M., **Magurno F.**, Malicka M., Zubek Sz., Mleczko P., Yorou N. S., Jobim K., Vista X. M., Lima J. L. R., Goto B. T. 2019. Rhizoglomus dalpeae, R. maiae, and R. silesianum, new species. Mycologia 6, 965–980, doi: 10.1080/00275514.2019.1654637''

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**2.** Oświadczam, że w pracy ,,Błaszkowski J., Jobim K., Niezgoda P., Meller E., Malinowski M., Milczarski P., Zubek Sz., **Magurno F.**, Casieri L., Bierza W., Błaszkowski T., Crossay T., Goto B. T. 2021. New glomeromycotan taxa, Dominikia glomerocarpica sp. nov. and Epigeocarpum crypticum gen. nov. et sp. nov. from Brazil, and Silvaspora gen. nov. from New Caledonia. Frontiers in Microbiology 12, 655910. doi: 10.3389/fmicb.2021.655910''

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**3.** Oświadczam, że w pracy ,,Błaszkowski J., Niezgoda P., Meller E., Milczarski P., Zubek Sz., Malicka M., Uszok S., Casieri L., Goto B. T., **Magurno F.** 2021. New taxa in Glomeromycota: Polonosporaceae fam. nov., Polonospora gen. nov., and P. polonica comb. nov. Mycological Progress 20, 941–951, doi: 10.1007/s11557-021-01726-4''

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**5.** Oświadczam, że w pracy ,,Błaszkowski J., Sánchez-García M., Niezgoda P., Zubek Sz., Fernández F., Vila A., Al-Yahya'ei M. N., Symanczik S., Milczarski P., Malinowski R., Cabello M., Goto B. T., Casieri L., Malicka M., Bierza W., **Magurno F.** 2022. A new order, Entrophosporales, and three new Entrophospora species in Glomeromycota. Frontiers in Microbiology 13, 962856, doi:10.3389/fmicb.2022.962856''

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**1.** Oświadczam, że w pracy ,, Błaszkowski J., Niezgoda P., Piątek M., Magurno F., **Malicka M.**, Zubek Sz., Mleczko P., Yorou N. S., Jobim K., Vista X. M., Lima J. L. R., Goto B. T. 2019. *Rhizoglomus dalpeae*, *R. maiae*, and *R. silesianum*, new species". Mycologia 6, 965–980, doi: 10.1080/00275514.2019.1654637

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**3.** Oświadczam, że w pracy ,, Błaszkowski J., Niezgoda P., Zubek S., Meller E., Milczarski P., Malinowski R., **Malicka M.**, Uszok S., Goto B. T., Bierza W., Casieri L., Magurno F. 2022. Three new species of arbuscular mycorrhizal fungi of the genus *Diversispora* from maritime dunes of Poland". Mycologia 114, 453–466, doi:10.1080/00275514.2022.2030081

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mój udział polegał na współautorstwie koncepcji badań, interpretacji części wyników badań, co określam jako 2% wkładu w przygotowanie ww. publikacji.

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**OŚWIADCZENIE WSPÓŁAUTORA**

**1.** Oświadczam, że w pracy ,,Błaszkowski J., **Kozłowska A.**, Niezgoda P., Goto B.T., Dalpé Y. 2018. A new genus, *Oehlia* with *Oehlia diaphana* comb. nov. and an emended description of *Rhizoglomus vesiculiferum* comb. nov. in the Glomeromycotina. Nova Hedwigia 107 (3-4), 501-518. doi: 10.1127/nova_hedwigia/2018/0488"

mój udział polegał na współautorstwie koncepcji i metodyki badań, interpretacji części wyników badań, co określam jako 20% wkładu w przygotowanie ww. publikacji.

**2.** Błaszkowski J., Niezgoda P., Goto B. T., **Kozłowska A.** 2018. *Halonatospora* gen. nov. with *H. pansihalos* comb. nov. and *Glomus bareae* sp. nov. (Glomeromycota; Glomeraceae). Botany 96: 737–748. dx.doi.org/10.1139/cjb-2018-0107

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**3.** Jobim K., Błaszkowski J., Niezgoda P., **Kozłowska A.**, Zubek Sz., Mleczko P., Chachuła P., Ishikawa N. K., Goto B. T. 2019. New sporocarpic taxa in the phylum Glomeromycota: *Sclerocarpum amazonicum* gen. et sp. nov. in the family Glomeraceae (Glomerales) and *Diversispora sporocarpia* sp. nov. in Diversisporaceae (Diversisporales). Mycological Progress 18, 369–384, doi: https://doi.org/10.1007/s11557-018-01462-2

mój udział polegał na współautorstwie koncepcji i metodyki badań, interpretacji części wyników badań, co określam jako 2% wkładu w przygotowanie ww. publikacji.

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