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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.

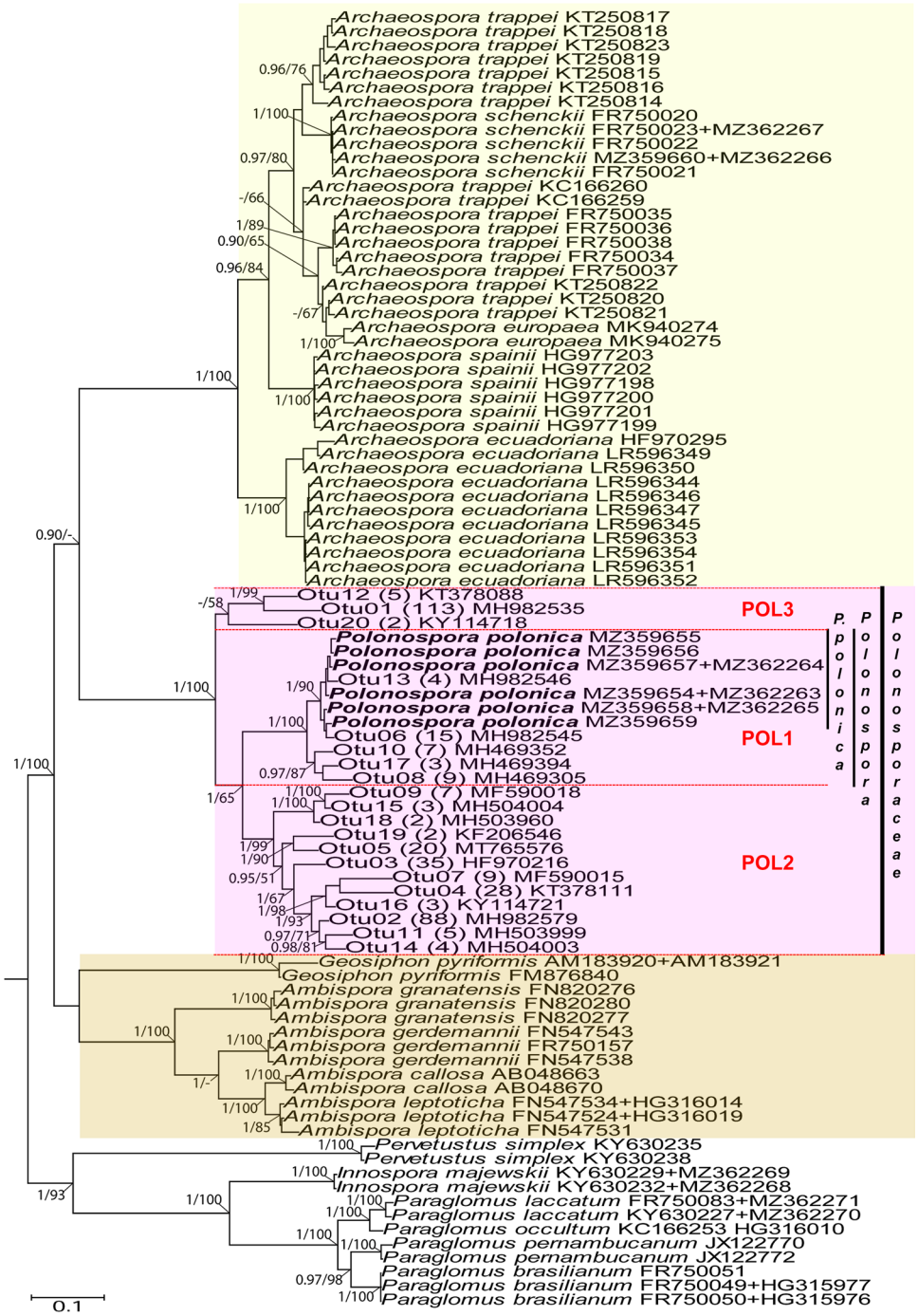

Fig. 1 A 50% majority rule consensus phylogram inferred from a Bayesian inference analysis of 18S-ITS-28S + *RPB1* sequences of *Polonospora polonica*, 10 species of the *Archaeosporales*, 20 OTUs representative sequences from environmental studies (the number of sequences clustered in each OTU is shown 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).

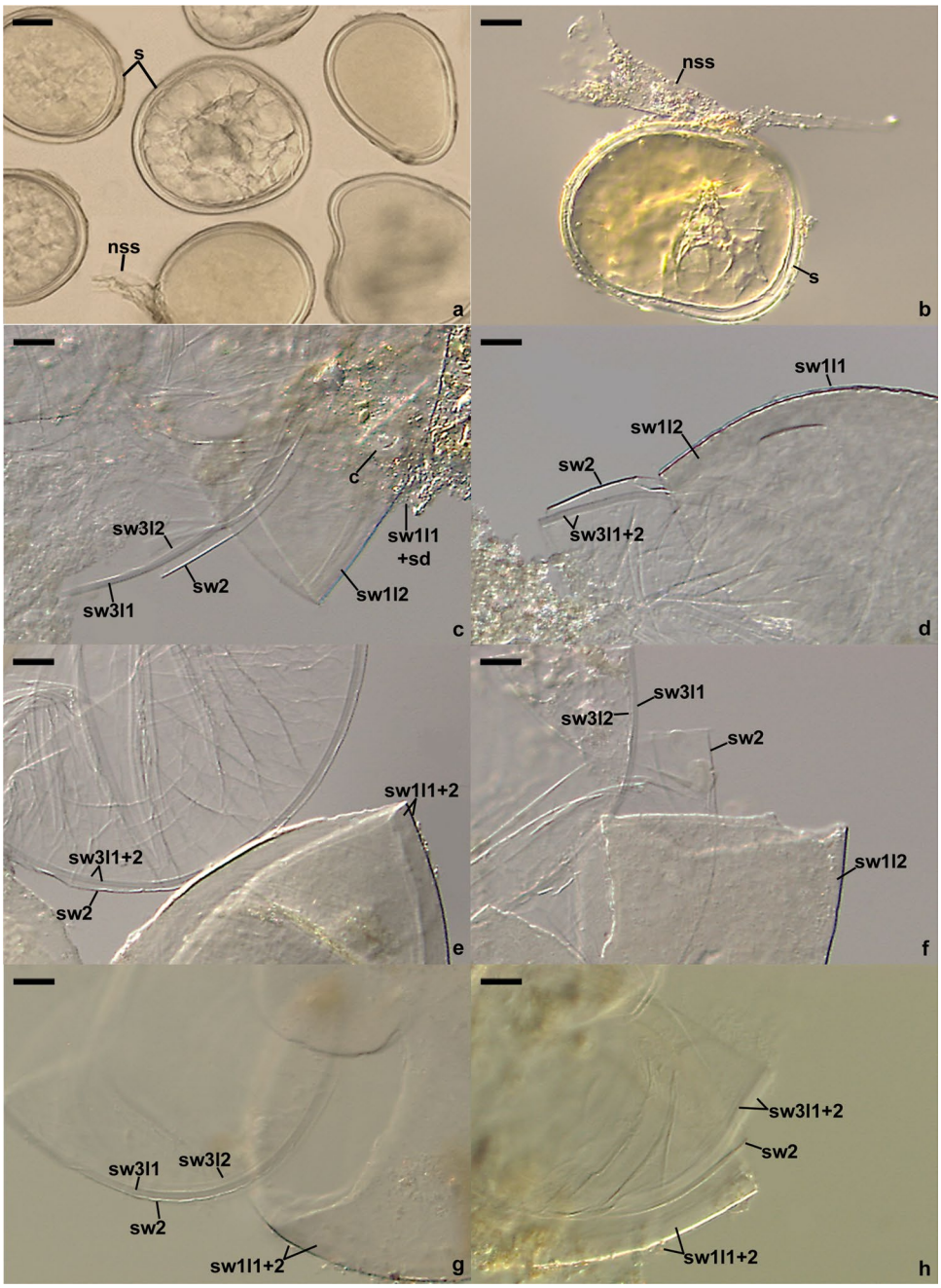

Fig. 2 *Polonospora polonica*. **a**, **b**. Intact spores (s) with the neck of a sporiferous saccule (nss; remnants). **c**–**h**. Spore wall 1 (sw1) layers 1 and 2, spore wall 2 (sw2), and spore wall 3 (sw3) layers 1 and 2; soil debris (sd) covering the upper surface of sw1l1 and cicatrix (c) are indicated 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.

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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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