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ORIGINAL ARTICLE

Phylogeny of Physarida (Amoebozoa, Myxogastria) Based on the Small-Subunit Ribosomal RNA Gene, Redefinition of *Physarum pusillum* s. str. and Reinstatement of *P. gravidum* Morgan

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ABSTRACT

Myxomycetes (also called Myxogastria or colloquially, slime molds) are worldwide occurring soil amoeboflagellates. Among Amoebozoa, they have the notable characteristic to form, during their life cycle, macroscopic fruiting bodies, that will ultimately release spores. Some 1,000 species have been described, based on the macroscopic and microscopic characteristics of their fruiting bodies. We were interested in *Physarum pusillum* (Berk. & M.A. Curtis) G. Lister, a very common species described with two variants, each bearing such morphological differences that they could represent two distinct species. In order to test this, we observed key characters in a large selection of specimens attributed to *P. pusillum,* to its synonyms (in particular *Physarum gravidum*), and to related species. In addition, the small-subunit ribosomal RNA gene was obtained from seven of these specimens. Based on these data, we provide a comprehensive phylogeny of the order Physarida (Eukaryota: Amoebozoa: Conosa: Macromycetozoa: Fuscisporidia). Morphology and phylogeny together support the reinstatement of *P. gravidum* Morgan 1896 with a neotype here designated, distinct from *P. pusillum*, here redefined.

MYXOMYCETES, also called Myxogastria or plasmodial slime molds, are worldwide occurring amoeboflagellates, although they are better known for their often ornate fruiting bodies that are visible to the naked eye (Poulain et al. 2011). They encompass c. a thousand species, worldwide distributed (Stephenson et al. 2011). Myxomycetes constitute a terminal branch in the phylum Amoebozoa (Cavalier-Smith 1998) and the main group in Macromycetozoa, along Dictyostelia and Protosporangiida (*Ceratiomyxa*) (Fiore-Donno et al. 2019; Kang et al. 2017). The main division in Myxomycetes lies between the superorders Lucisporidia and Fuscisporidia, characterized by bright and dark spore color, respectively (Fiore-Donno et al. 2010b, 2019; Kretzschmar et al. 2016). The Fuscisporidia include three orders, the basal, paraphyletic Echinosteliida, and Stemonitida paraphyletic to the monophyletic Physarida (Fiore-Donno et al. 2012; Kretzschmar et al. 2016). Physarida is characterized by the presence of lime (calcareous deposits), whose structure, either crystalline or amorphous, and its localization in different parts of the sporophore are used to distinguish the two families, Didymiidae (Didymiaceae according to the botanical code) and Physaridae (Physaraceae) (Poulain et al. 2011). The genus *Physarum*, with 144 species the largest in Myxomycetes (Lado 2005–2019), is characterized by the presence of lime in the capillitium, which is a network of fine tubules intertwined between the spores in the sporotheca; usually the nodes are filled with lime granules. In addition, the sheath surrounding the spore mass (the peridium) and the stalk can be calcareous

(Fig. 1). *Physarum* is polyphyletic (Nandipati et al. 2012), separated into two major clades intermingled with species of *Badhamia* (Erastova et al. 2013; Kamono et al. 2013; Nandipati et al. 2012). The distinction between *Badhamia* and *Physarum* resides in a capillitium entirely filled with calcareous granules, thus forming a thick network in the former. Since there is a gradual transition from species having a thin capillitium net with enlarged nodes (typical of *Physarum*) to those having a thick capillitium throughout (typical of *Badhamia*), there is no support for maintaining the two genera as hitherto circumscribed (Eliasson 2015).

Physarum pusillum (Berk. & M.A. Curtis) G. Lister was first briefly described in 1873 as a *Didymium*. The type, a specimen from South Carolina in the Kew herbarium K1492 (Lister 1911), could not be found in a recent, exhaustive search (Lado and Wrigley de Basanta 2018). In 1911, G. Lister transferred *Didymium pusillum* to the genus *Physarum* and provided a detailed and illustrated description, as a morphologically variable species with two distinct morphotypes: one with a subglobose sporotheca, and the other with a more flattened one—a form called oblate (Lister 1911) (Fig. 2). The subglobose form was associated with a capillitium with larger nodes. In the subsequent treatments, either the subglobose (Alexopoulos 1963; Emoto 1977) or the oblate form (Nannenga-Bremekamp 1974; Neubert et al. 1995) was chosen to illustrate the species. Only the latest treatise presented the two morphotypes separately (Poulain et al. 2011), highlighting also a difference in the spore ornamentation: while the spores of both morphotypes were warty and of similar size (c. 9–11 μm), in the subglobose form the spores had distinct, small clusters of darker warts, which were larger and less evident in the oblate form.

We hypothesized that the two forms of *P. pusillum* could constitute at least two distinct species. To test for this, we examined the shape of the sporotheca, the structure of the capillitium, and the spore ornamentation of herbarium samples of *P. pusillum,* its synonyms and related species. All observed specimens were differentially characterized by the general outline of the sporotheca, the spore ornamentation and the thickness of the lime in the capillitium, with no intermediate forms. We also obtained nearly complete sequences of the nuclear small-subunit ribosomal RNA gene (thereafter SSU) for seven specimens of *P. pusillum* s. l., three subglobose and four oblate morphotypes, from a wide range of geographical origins (Table 1). Phylogenetic analyses of a representative choice of sequences of Fuscisporidia supported a two-

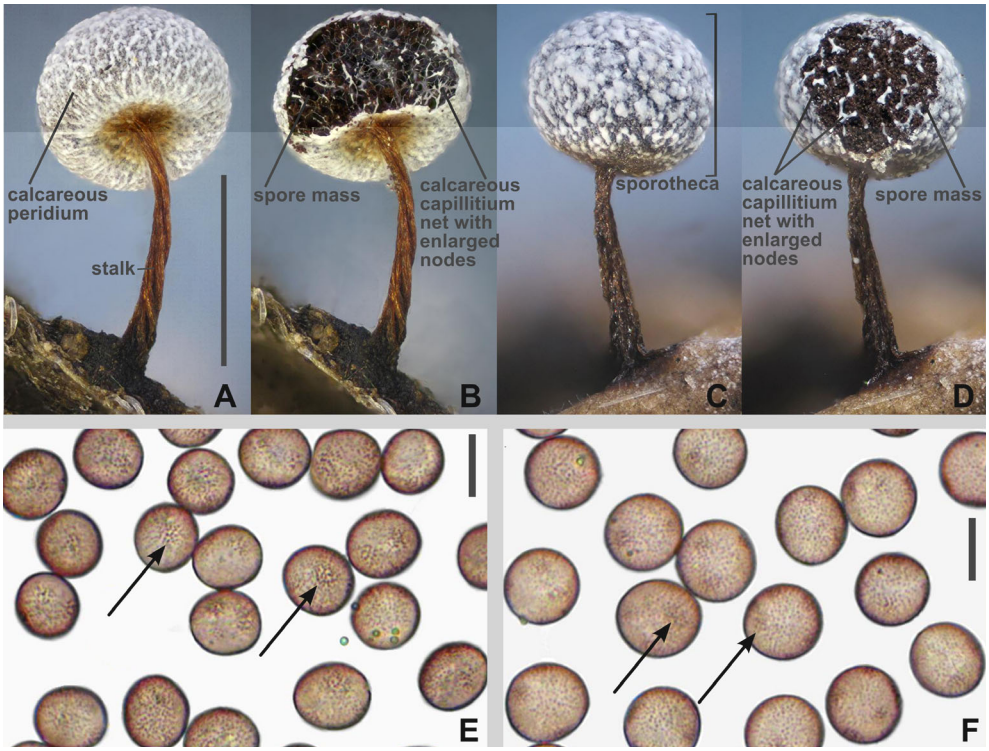

Figure 1 Illustration of the different parts of the sporophore of *Physarum pusillum* s. l. **A.** Oblate form (now *Physarum gravidum*), note the umbilicus of the sporotheca around the top of the stalk; scale bar = 0.5 mm; specimen BW2014 (Table 1 and Table S1). **B.** Same as in A, with part of the peridium removed, showing the spore mass and the capillitium. **C.** Subglobose form (now *P. pusillum* s. str.); specimen RC13120108 (Table S1). **D.** Ditto, with part of the peridium removed. **E.** Microphotograph of the spores of the specimen in A. Arrows indicate the clusters of warts. **F.** Microphotograph of the spores of the specimen as in C. Arrows indicate the clusters of warts, here smaller and darker than in E. Scale bars in E & F = 10 μm. Photograph credit: R. Cainelli.

Table 1. Specimens used in this study, herbarium number, location, collection information, and GenBank accession number of the obtained SSU sequences

Species	Herbarium #	Country	Region	Locality	Latitude	Longitude	Date of collection	Substrate	GenBank Acc. #
Physarum gravidum	MM36753	France	Savoy	Rognaix, chez Lassiaz	45.5876	6.4458	17-08-06	Dry grass piled up against North face of a wall	MK336177
P. gravidum	BW0536	France	Moselle	Eguelshardt	49.0045	7.5003	08-06-08	Dead leaves of *Alcea rosea*	MK336180
P. gravidum	BW2014	France	Alsace	Sélestat	48.2647	7.4680	19-10-15	*Urtica* and other dry plants on compost	MK336179
P. gravidum	RC16042007	USA	California	Irvine, Mason park	33.6561	−117.8133	20-04-16	*Brassica nigra*	MK336178
Physarum pusillum s. str.	RC16072004	South Africa	Hoedspruit	Kapama Southern Camp	−24.4554	31.0831	20-07-16	*Aloe marlothii*	MK336175
P. pusillum s. str.	RC14091001	Croatia	Dubrovnik-Neretva	Korčula, Lumbarda	42.9181	17.1868	10-09-14	*Agave*	MK336174
P. pusillum s. str.	BW2364	France	Alsace	Strasbourg, botanical garden	48.5833	7.7677	05-08-16	Dry twigs of *Lavandula angustifolia*	MK336176

Herbaria: MM = Marianne Meyer, BW = Bernard Woerly, RC = Renato Cainelli.

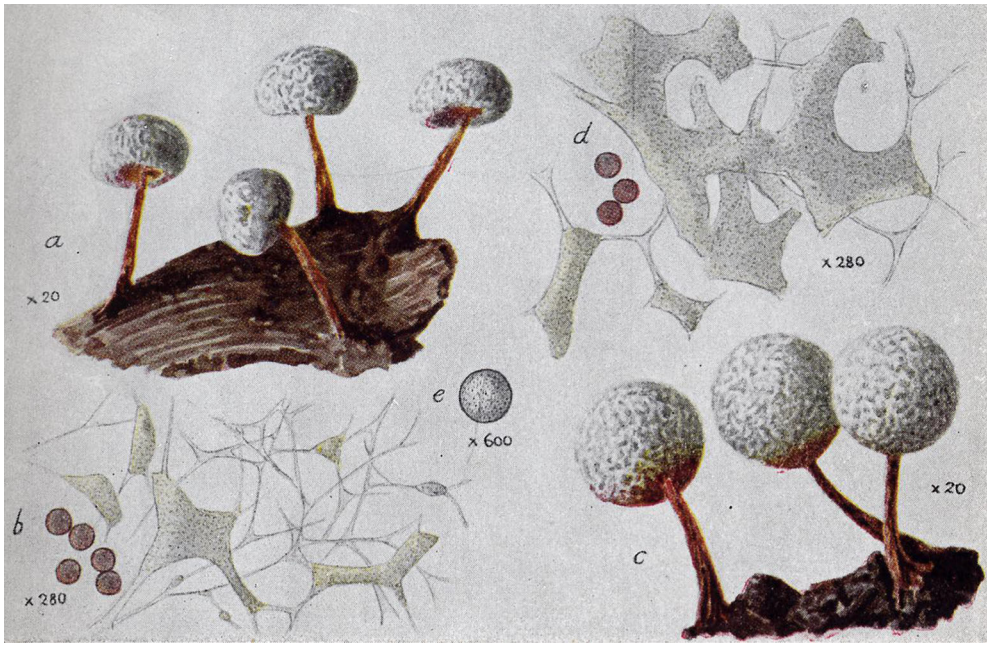

Figure 2 Reproduction of the table 43 (Lister 1911) representing the two forms of *Physarum pusillum*. **A**, **B**, and **E** represent the oblate form, with a thin capillitium with enlarged nodes and spores without clear clusters of warts. **C** and **D** represent the subglobose form with a thick capillitium—an enlarged drawing of the spores is missing. Image published more than 95 yr ago, thus in the public domain.

species hypothesis, with two distinct clades, corresponding to the two distinct morphotypes. Consequently, we reinstall as a species *Physarum gravidum* Morgan (the oblate morphotype), based on the neotype here illustrated, and redefine a *P. pusillum* s. str. (the subglobose morphotype).

MATERIALS AND METHODS

Specimens and light microscopy observations

We observed, macroscopically and microscopically, 32 recently collected specimens that were assigned to the

Table 2. Primers used in this study

	Sequence (5′–3′)	References
Forward		
S3	GATCCTGCCAGTAGTGTATGC	Fiore-Donno et al. (2008)
S4Amo	AAGGAAGGCAGCAGGCGCG	Erastova et al. (2013)
718F	TTCCAGCTCTAATAGCATACG	Fiore-Donno et al. (2008)
753F	GTTAAAACGCTCGTAGTCGGC	Fiore-Donno et al. (2008)
S12′	CAGATACCGTCGTAGTCTTAAC	Fiore-Donno et al. (2008)
S13	GAGTATGGTCGCAAGGCTG	Fiore-Donno et al. (2008)
Reverse		
SR4Dark	TGTCCTCTAATTGTTACTCGA	Fiore-Donno et al. (2012)
753R	GGTTAAAACGCTCGTAGTCGGC	Fiore-Donno et al. (2008)
SR11	GTTCGAGGGTGACCGAATTCG	Fiore-Donno et al. (2008)
SR11.5	GTGGTGAAATACGTTGACCC	This work
SR13	GGAGTATGGTCGCAAGGCTG	Fiore-Donno et al. (2008)
SR14	ACTCTTTGTGTGCCCTTCC	Fiore-Donno et al. (2012)
SR15	GTAAGTGGTGGTGCATGG	Fiore-Donno et al. (2008)
Rib2	GGTAATCGTAGGTGAACCTGC	Fiore-Donno et al. (2008)
RibB	GGTGAACCTGCAGAAGGATC	Fiore-Donno et al. (2005)

subglobose form of *P. pusillum*, and 10 to the oblate form of *P. pusillum* (= *P.* cf. *pusillum*) (Table S1). To check the identity of species synonymyzed by G. Lister, we obtained: (i) *P. gravidum* collected by Morgan, kept at the Iowa State University (ISC 403378), Ames IA, USA. It consisted of a box labeled "*P. pusillum*" containing two small boxes, both duplicates from the same substrate, dead stems of *Zea mays*; (ii) *P. "nodulosum"* Cooke & Balf., Ravenel's Fungi Americani Exsiccati No. 479 kept at Cornell University (CUP), Ithaca NY. Please note that there is some confusion about the identity of *P. "nodulosum"* Cooke & Balf. 1889 (as cited by G. Lister 1911), which is a nomen nudum (published without description, and thus shown between inverted commas). In 1899, T. McBride combined *Badhamia nodulosa* Massee to *Physarum nodulosum* (Massee) T. McBride, both taxa are considered synonyms of *P. pusillum* (Lado 2005–2019). No specimens of the *P. pusillum* synonyms could be found in the New York Botanical Garden (NY) herbarium. When no specimens could be found, we studied the protologs, all available at http://eumycetozoa.com/data/index.php (last accessed June 2019). Another synonym added by G. Lister in a later revision (Lister 1925) is *Physarum mucoroides* Schilb. 1896. From the protolog, it is unclear whether the species is a synonym of *P. pusillum*. The general outline of the sporophore does not correspond to that of *P. pusillum*, the stalk is shorter, and the sporotheca larger, while the capillitial nodes are too poorly described. No type was mentioned.

In addition, we observed the slide of *Physarum pusillopse* D.W. Mitch. & Nann.-Bremek. 1977 (the holotype is currently not available, but the slide is an isotype), plus six specimens of *P.* cf. *pusillopse*, and one specimen of *Physarum oblatum* T. Macbr. 1893; all from Nannenga-Bremekamp's collection at the herbarium of the Meise Botanic Garden (BR), Belgium.

Observations and images of macroscopic characters were obtained with a Lomo MBC-9 (St. Petersburg, Russia) and an Olympus SZX10 stereomicroscope (Tokyo, Japan) and of microscopic characters with an Optika B500Ti (Bergamo, Italy) and an Olympus BX43 microscopes. Super macro photographs were obtained on an ad hoc modified monocular microscope. All photographs were obtained by image stacking using the software CombineZP v. 1.0 (https://combinezp.software.informer.com/, last accessed Jan. 2019).

Electron microscopy observations

Scanning electron microscopy (SEM) was performed at the Meise Botanic Garden. One sporophore of each specimen was placed in a convolute of a filter paper (medium filtration rate; particle retention 5 μm; VWR, Radnor, PA), which was placed in a sample holder (stainless steel tube with meshed top and bottom) for critical point drying. The holders were submerged, respectively, for 30′ in 25% ammonia, 2 × 20′ in 70% ethanol, and 2 × 30′ in dimethoxymethane and left overnight, then 4 × 15′ in acetone; hereafter, the samples were dried in a critical point dryer (Leica EP CDP 300, Diegem, Belgium). The dried samples were mounted on aluminum specimen stubs dressed with adhesive carbon tabs and the whole was consequently coated with a layer of approximately 6 nm Pl/Pd (using Argon gas, under 0.05 mbar pressure) in a High Resolution Fine Sputter Coater for FE-SEM (JFC-2300HR Coating Unit, JEOL, Tokyo, Japan). Scanning electron microscopy was carried out with a JEOL JSM-7100FLV Field Emission SEM with a tension of 5 kV at a working distance of 6 mm.

DNA extraction, amplification, and sequencing

We selected, under a dissecting microscope, 4–6 sporophores per specimen, adjacent to each other (possibly arising from a single plasmodium), avoiding visible fungal contaminations. DNA extraction, amplification, and purification were performed as already described (Fiore-Donno et al. 2012) using the primers listed in Table 2. Sequencing was performed at the Cologne Center for Genomics (Cologne, Germany). New sequences have been submitted to GenBank under accession numbers MK336174–MK336180.

Alignment and phylogenetic analyses

The newly obtained sequences and that of other Physarida of interest were manually added to a pre-existent Fuscisporidia alignment (Fiore-Donno et al. 2012), taking into account the secondary structure as determined for *Physarum polycephalum* (Johansen et al. 1988) using BioEdit 7.2.6 (Hall 1999). A mask excluding part of the variable helices that could not be confidently aligned was created —the alignment and the mask are provided (Data S1). The final alignment comprised 100 sequences and 1,577 aligned positions, with 836 alignment patterns. The

general time reversible model (GTR) with a gamma-distributed rate of heterogeneity among sites approximated by eight categories, and a proportion of invariable sites (GTR + gamma + I) was the best-fitting evolutionary model according to jModelTest 2.1.10 (Darriba et al. 2012). Maximum likelihood analyses were performed using RAxML 8.2. (Stamatakis 2014). The best scoring ML tree was inferred from randomized starting Maximum Parsimony trees (using the -f a option and the GTRGAMMA model) and then used to report the confidence values obtained through 1,000 nonparametric bootstraps. According to the developer's recommendation, we did not include the proportion of invariable sites in the model. Bayesian inferences were conducted with MrBayes v. 3.2.6 (Huelsenbeck and Ronquist 2001) with the default settings for three million generations and sampled every 100. Convergence of the two runs (average standard deviation of split frequencies < 0.001) was reached after 530,000 generations, and the burn-in was set accordingly to 5,300. Other parameters attesting the convergence of the two runs were the potential scale reduction factors (PSRF), for all parameters ≤ 1.004.

RESULTS

Characteristics of the observed specimens

The observed specimens of *P. pusillum* and its synonyms could be assigned with confidence to one of the two morphotypes, according to the shape of the sporotheca, the aspect of the lime nodes in the capillitium and the spore ornamentation (Table S1). In her treatment of *P. pusillum*, G. Lister listed as synonyms *P. "nodulosum"* Cooke & Balf. 1881 (and its synonyms, *B. nodulosa* Massee 1889 and *Craterium nodulosum* Massee 1896), *Physarum calidris* Lister 1891, and *P. gravidum* Morgan 1896 (Lister 1911).

The syntype of *P. "nodulosum"* could be assigned with confidence to the subglobose morphotype of *P. pusillum* s. str. (Fig. S1), based on: (i) the subglobose shape of the sporotheca; (ii) the capillitial nodes larger than in *P. gravidum*; and (iii) on the spores, the very distinct small groups of darker, more robust warts among the evenly distributed ones. *Badhamia nodulosa* Massee 1889 and the associated drawing matched the subglobose morphotype; Massee did not provide a type and considered *P. nodulosum* as a synonym. Since we could observe the syntype of *P. "nodulosum,"* we assume that *B. nodulosa* is a synonym of *P. pusillum* s. str. The type of *P. calidris* Lister 1891 could not be found at BM (Lado and Wrigley de Basanta 2018). The species was drawn with a subglobose sporotheca and large capillitial nodes, thus corresponding to *P. pusillum* s. str.

Among *P. pusillum* synonyms, only the protolog of *P. gravidum* matched the oblate form. Andrew Price Morgan described a depressed-globose sporotheca with an umbilicate base, and a capillitium with slender nodes, spores 11–13 μm diameter, minutely warted (no clusters were mentioned). Our observations confirmed that the capillitium contained slender, elongated lime nodes and that the spores possessed the typical ornamentation of evenly distributed warts in combination with vague patches of darker warts.

All but one of the specimens labeled "*P.* cf. *pusillopse*" in the herbarium of the Meise Botanic Garden (BR), as well as the specimen NB13759 named *P. oblatum,* could be assigned with confidence to *P. pusillum* s. str. The specimen NB11462 had a pseudocolumella and not observable clusters of warts in the spores, and therefore, we can exclude that it was *P. pusillum* s. l. or *P. pusillopse* (Table S1).

Phylogenetic analyses

Our results were mostly congruent with published phylogenies, showing Stemonitida paraphyletic to a monophyletic Physarida. In Stemonitida, *Meriderma*, two *Comatricha* and *Stemonitis* occurred as well-supported clades, although the *Comatricha* clades appeared as paraphyletic to *Stemonitis*, contrary to the tree in Fiore-Donno et al. 2012. *Lamproderma* and allied genera were paraphyletic to Physarida, a result consistently found in every phylogeny obtained to date. Physarida was composed of Didymiidae paraphyletic to a monophyletic Physaridae. The earliest diverging clade in Physarida was *Lepidoderma* (if *L. tigrinum* is excluded, being probably a *Diderma*), in agreement with published phylogenies (Fiore-Donno et al. 2012), although with small differences: here, *Diachea subsessilis* appeared as basal to the two *Didymium* clades. Physaridae were divided into several clades, with species of *Physarum* intermingled with *Fuligo*, *Physarella,* and *Badhamia*. Their mutual relationships were mostly poorly supported, but the clades themselves were recovered with confidence.

All the sequences stemming from the specimens we attributed to the subglobose form or *P. pusillum* s. str. formed a well-supported clade (92% bootstrap, 1.0 Bayesian posterior probability—support values will be given in this order thereafter), with three other already published sequences for which no herbarium specimens were available. The four new sequences of the oblate form of *P. pusillum* (labeled as *P. gravidum*) formed a well-supported clade (100/1). The terminal clade included the following: (i) a weekly supported subclade with sequences of *P. pseudonotabile* (66/1); (ii) a weakly supported subclade including sequences of *Badhamia* spp. and *Physarum* spp., including three sequences that were labeled as *P. pusillum*.

DISCUSSION

Our phylogenetic analyses and differences in macroscopic as well as in microscopic features supported the division of *P. pusillum*, as currently accepted, into two distinct species, characterized by differences in the sporotheca shape and in the capillitium nodes, and in the spore ornamentation (Fig. 1 and Table S1). Our phylogenetic

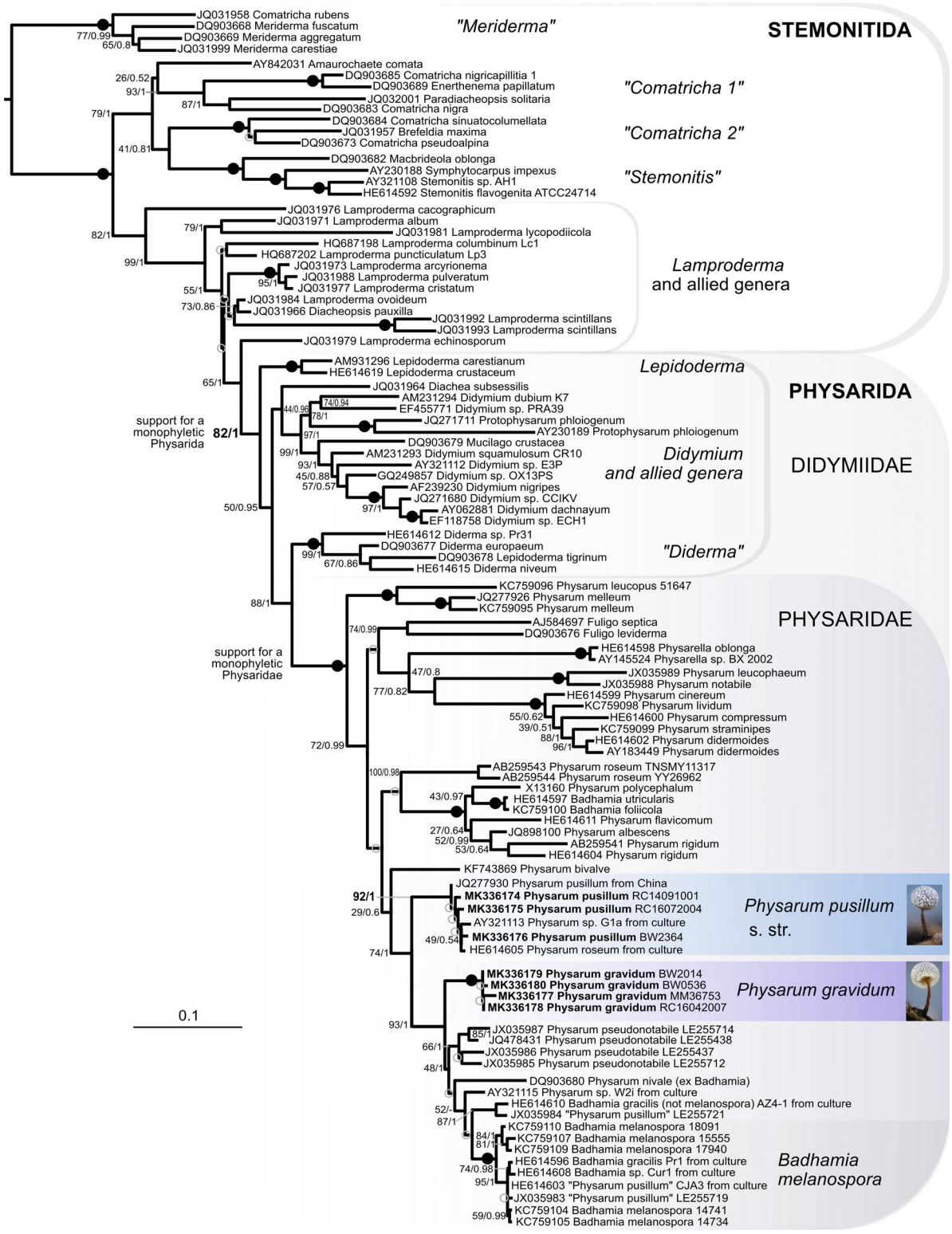

Figure 3 Small-subunit ribosomal RNA gene phylogenetic tree of Stemonitida and Physarida obtained by maximum likelihood analysis (ML) of 100 taxa and 1,577 aligned positions. The tree is rooted with *Meriderma*, according to current phylogenies. New sequences are in bold. Species names are preceded by the GenBank accession number, and to disambiguate repeated names, followed by the strain or herbarium number. ML bootstrap replicates (%) are given for each node, followed by Bayesian posterior probabilities. Black dots = maximum support in both analyses; gray circles indicate alternative topologies in the Bayesian inference and a ML bootstrap < 50%. Main taxa/groups are named, shaded when monophyletic and indicated by a bracket when paraphyletic. The scale bar indicates the fraction of substitutions per site.

analyses, including representatives of every group of Physarida for which nearly complete SSU sequences were available, showed two clearly distinct clades (Fig. 3).

The clade of *P. pusillum* s. str., the subglobose form, also included three other sequences: (i) JQ277930 named *P. pusillum*, without reference to a published article; (ii) AY321113 *Physarum* sp. G1a, previously identified as the now invalidated *Hyperamoeba* (Fiore-Donno et al. 2010a); it was obtained from an amoeba isolated from a physiotherapy bath, and no sporophores were observed (Walochnik et al. 2004); and (iii) HE614605 named *P. roseum* (Nandipati et al. 2012), obtained from a culture of J. Clark, isolated more than two decades ago. It sporulated only once and produced poorly formed but recognizable sporophores (Clark 1995). Since the sporophores are of an intense pink, it is likely that the culture in the course of the years has been mixed up or contaminated with one of the *P. pusillum* isolates by the same author. As further evidence, HE614605 did not branch with two partial sequences of *P. roseum* (Kamono and Fukui 2006).

Three sequences named "*Physarum pusillum*" did not branch in the clade that we named *P. pusillum* s. str. The sequence HE614603 (Nandipati et al. 2012) clearly stemmed from a mislabeled culture of *Badhamia melanospora*, as shown in Aguilar et al. (2014) and in this work. This sequence was later used to assign the name "*P. pusillum*" to the sequences JX035983–JX035984 of the specimens LE255719 and LE255721 by Novozhilov et al. (2013). The tree published therein included only one sequence of *Physarum* in addition to the sequences obtained by the authors: thus, the grouping of the sequence JX035984 with the sequences JX035983 and HE614603 was due to undersampling. In our tree, the sequence JX035984 ("*P. pusillum*") branches with HE614610 *Badhamia gracilis* AZ4-1 (Nandipati et al. 2012) (incorrectly named there as *B. melanospora*) from a culture of J. Clark (Clark et al. 2003). We were kindly provided with photographs of the spores of these two samples (*P. pusillum* JX035983–JX035984), and we assumed that they very likely belong to two different species of *Badhamia*, LE255719 being most probably *Badhamia melanospora*—in accordance with our phylogenetic analyses. The two sequences *Badhamia gracilis* Pr1 (HE614596) and *Badhamia* sp. Cur1 (HE614608) (Nandipati et al. 2012), from isolates of J. Clark, are most probably *B. melanospora*. We regret the occurrence in GenBank of many mislabeled sequences.

The partial sequence AB259537 *Physarum pusillum* YY-26614 (TNS) (Fiore-Donno et al. 2010a; Kamono and Fukui 2006) matched the sequences of the subglobose form, but was not included in our alignment for being too short.

Comparison with species showing similar characters

Physarum gravidum, the neotype here designed and all our specimens that we attributed to this taxon (Table S1), can be distinguished from *Physarum pusillum* s. str. by the following morphological characters. The sporotheca of *P. gravidum* is typically oblate with a flat base and an umbilicus (Fig. 1A, B, 4A, D, E), whereas *P. pusillum* has a subglobose sporotheca never umbilicate, with a more or less pronounced conical base, arising from the stalk enlarging at the top (Fig. 1C, D and Fig. S1A). Even in nearly subglobose specimens of *P. gravidum,* the basal umbilicus is always present. The capillitium of *P. gravidum* is thin and characterized by elongated fusiform to cylindrical lime nodes (Fig. 1B, 4A–G, I). The lime nodes and the capillitium of *P. pusillum* are much thicker and larger (Fig. 1D and Fig. S1A, B). Another striking difference resides in the spore ornamentation: *P. gravidum* has more or less evenly distributed warts with not well-circumscribed clusters of darker warts (Fig. 1E, 4C, H–K), while *P. pusillum* has smaller, well-defined clusters of darker warts (Fig. 1F and Fig. S1C). In addition, there could be a still unconfirmed substrate preference: *P. gravidum* could preferentially form sporophores on herbaceous substrates, while *P. pusillum* was found on both herbaceous and woody substrates. Both species were found on succulents (Table S1).

Physarum pusillopse D.W.Mitch. & Nann.-Bremek. was a species described from only one collection (Mitchell and Nannenga-Bremekamp 1977). The type in the D.W. Mitchell herbarium (DWM1868) is currently unavailable, but an isotype can be found in Nannenga-Bremekamp's collection (Meise Botanic Garden, Belgium, BR) NB9315 consisting of only one sporophore with collapsed spores in a slide in Hoyer's medium (Table S1). The type specimen, according to the original description (Mitchell and Nannenga-Bremekamp 1977), differs from *P. pusillum* by the small and rounded capillitial nodes, the shape being semiglobose with a flattened base. The slide of the isotype shows spores much darker than in our specimens (despite of colors usually progressively fading out in Hoyer's medium). The equatorial light band described was not visible. No other specimen labeled *P. pusillopse* could be found in Nannenga-Bremekamp's collection; however, we found six specimens labeled "*Physarum* cf. *pusillopse*." Their examination unambiguously revealed the characters of *Physarum pusillum* s. str. (Table S1). *Physarum oblatum* T. Macbr. is characterized by yellow pigments in the lime deposits. It usually has a stalk > 3 × the sporotheca. Less pigmented forms could be mistaken for *P. pusillum*.

Description of *Physarum gravidum* Morgan 1896

Since the protolog of *P. gravidum* was cursory and no type was designated, we provide here a more detailed description and a neotype, choosing a specimen collected by Morgan.

Sporophores stalked, up to 2 mm tall. Sporotheca covered with white lime, often orange-brown and limeless at the base, oblate, 0.4–0.6 mm diam., with flattened base, broad to narrow umbilicus around the stalk. Peridium thin transparent membrane, thickened at the base around the stalk, incrusted with white or gray-white lime, often orange-brown and limeless at the base. Stalk generally present, up to 1.3 mm tall, straight or curved, widening at the base, 0.1–0.5 mm wide, membranous, limeless, longitudinally pleated, translucent, orange-brown to red-brown, darker, and more opaque at the base (Fig. 4A, D, E).

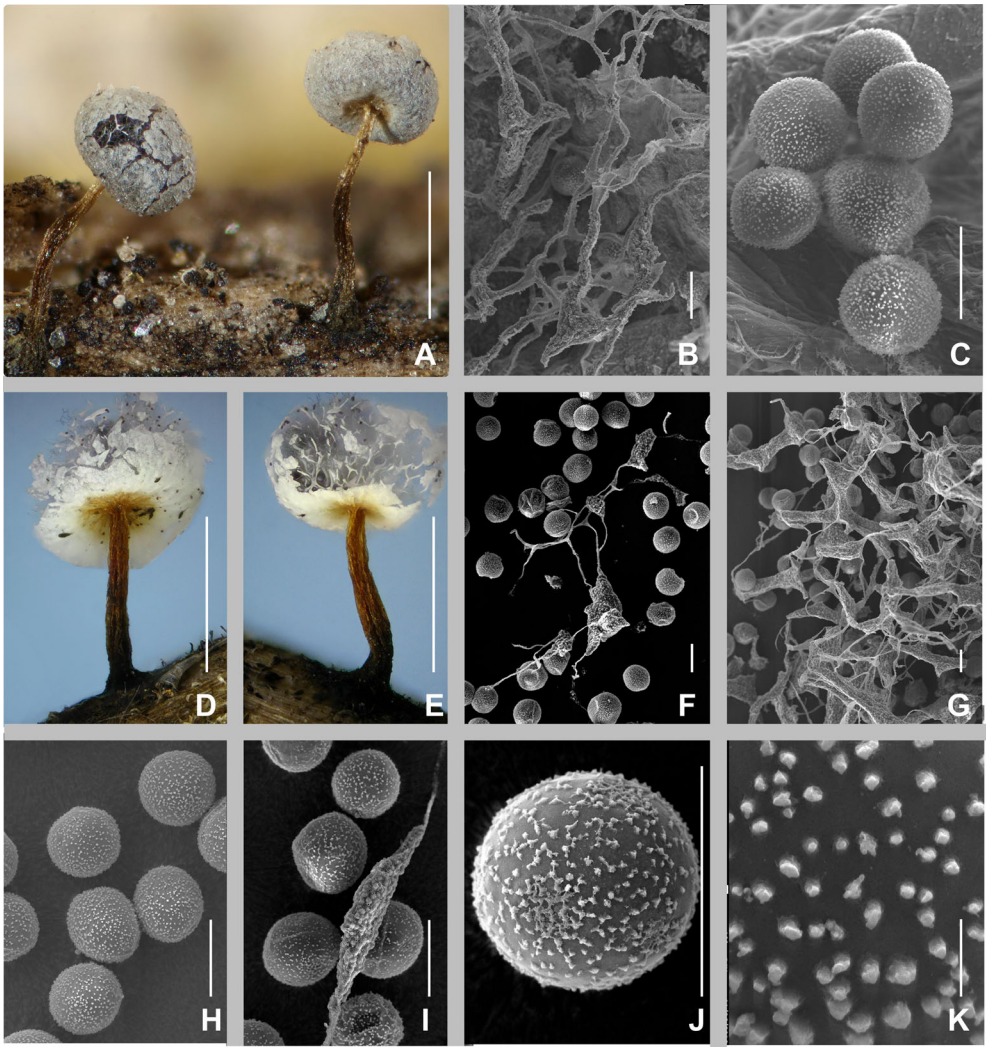

Figure 4 **A–C**. Illustration of the neotype of *Physarum gravidum* Morgan, herbarium Iowa State University 403378. A. General view of the sporophore; scale bar = 0.5 mm. B and C. SEM images, scale bars = 10 μm. C. Spores and capillitium, note the capillitium nodes filled with lime granules and the slender capillitium tubules. C. Group of spores. Photographs credit: M. de Haan. **D–K.** Illustration of *P. gravidum,* herbarium MM36753. D. General view of the sporophore. E. Same as in D, with the peridium partially removed to show the capillitium; scale bar = 0.5 mm. Photograph credit: R. Cainelli. F–K. SEM images. F and G. Spores and capillitium, note the capillitium nodes filled with lime granules and the slender capillitium tubules; scale bars = 10 μm. H and I. Spores, in I with a capillitium node. J. Higher magnification of a spore. K. Spore warts; scale bar = 1 μm. Photograph credit: M. de Haan.

Pseudocolumella absent. Capillitium consisting of a calcareous net of thin tubules (often filled with lime granules), with elongated, fusiform to cylindrical white nodes, densely filled with lime granules (Fig. 4B, F, G, I). Spores dark brown in mass. LM: brown in transmitted light, globose, (8.5–) 9–12 (–12.5) μm, ornamented with low warts often less evident on one side, with not well-delimited patches of grouped warts (Fig. 4C, H–J). SEM: low, 0.2–0.3 μm tall, broad conical warts with irregular outline, not well-delimited patches of grouped warts (Fig. 4K). Plasmodium: watery white according to G. Lister.

The protolog did not include drawings or a reference to a type specimen, but the observed specimen ISC 403378 was collected in the Miami Valley, Ohio, USA. It was well conserved and matched the protolog, including the nature of the substrate (*Z. mays*) and the provenance. On the herbarium box ISC 403378 figures the annotation "Preston, Ohio." Preston was a now disappeared town in Hamilton County, on the Miami river, visible on a 19th century map, close to Miami Whitewater Forest County Park. It is now called New Haven. This prompted us to design the specimen ISC 403378 as the neotype. We did not intent to extract DNA, because it would have been destructive and most likely not successful.

Neotype: United States of America, Ohio, Hamilton County, Crosby Township, New Haven, old locality name Preston, 39.2933, −84.7584 (approximated to 3,000 m). Date of collection not mentioned. Substrate dry stem of

Z. mays. Deposited at Iowa State University (ISC), Ames IA, herbarium number ISC 403378. MycoBank Typification #(MBT) 387483.

Habitat: decaying grasses and herbs, leaves litter, cladodes of *Opuntia*.

Distribution: cosmopolitan, France, Italy and United States of America (Ohio, California).

Redefinition of *Physarum pusillum* s. str.

Physarum pusillum s. str. (Berk. & M.A. Curtis) G. Lister, in Lister, Monogr. Mycetozoa, ed. 2, 64 (1911).

Sporophores stalked, up to 2 mm tall. Sporotheca subglobose, covered with white lime deposits, often orange-brown and limeless at the base, 0.4–0.5 mm diam., with wide funnel shaped to narrow conical base. Peridium thin transparent, colorless membrane, incrusted with lime, white or gray-white, often orange-brown and limeless at the base. Stalk generally present, up to 1.3 mm tall, straight or curved, widening at the base, 0.1–0.5 mm wide, membranous, limeless, longitudinally pleated, upper half translucent and orange-brown, lower half dark brown and opaque (Fig. 1C, D and Fig. S1A). Pseudocolumella absent. Capillitium a calcareous net of tubules often filled with lime granules with enlarged, irregular white nodes (Fig. 1C, D and Fig. S1A, B). Spores dark brown in mass. LM: brown in transmitted light, globose, (8.5–) 9–12 (–12.5) μm, ornamented with conical warts evenly distributed and with distinct denser groups of larger conical warts (Fig. 1F and Fig. S1C).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Specimens observed in this study.
Figure S1. Illustration of the syntype of *Physarum "nodulosum"* Cooke & Balf. 1881 deposited at Cornell University herbarium (CUP), without number.
Data S1. Alignment of the SSU of 100 Stemonitida and Physarida, with the first sequence a mask showing the positions kept for phylogenetic analyses.