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Multigene phylogeny of the order *Physarales* (*Myxomycetes*, *Amoebozoa*): shedding light on the dark-spored clade

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Key words

α-Tub
classification
EF-1α
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mtSSU
Nannengaella
new taxa
nomenclature
nSSU
systematics
taxonomy

Abstract The class *Myxomycetes* consists of free-living protists characterised by their complex life cycle, which includes both microscopic (amoebae, flagellates and cists) and macroscopic stages (spore-bearing fruiting bodies, sclerotia, and plasmodia). Within it, the order *Physarales*, with more than 450 recognised species, constitutes the largest group. Although previous studies have shown the polyphyly of some of the traditionally accepted genera, its internal phylogenetic relationships have remained uncertain so far, and together with the lack of data for some key species, it prevented any taxonomic and nomenclatural revisions. We have compiled a substantially expanded dataset in terms of both taxon sampling and molecular data, including most of the genera described to date and four unlinked DNA regions, for which we provide partial sequences: nSSU, *EF-1α*, *α-Tub*, and mtSSU, analysed through maximum likelihood and Bayesian methods. Our results confirm that the family *Didymiaceae* is paraphyletic to the rest of *Physarales*. Within *Didymiaceae* s.lat., the recent reinstatement of the genus *Polyschismium* for most species traditionally ascribed to *Lepidoderma*, except for the type (Ronikier et al. 2022), is further supported here, as well as the definite inclusion of the genus *Mucilago* in *Didymium* and *Lepidoderma* s.str. (*L. tigrinum*) in *Diderma* (Prikhodko et al. 2023). Additionally, the genus *Diachea* is redefined to include some species previously treated in *Physaraceae* (*Craterium* spp. with true columella). Within the monophyletic family *Physaraceae*, most genera are recovered as polyphyletic, suggesting that they should be no longer accepted as currently defined. However, the lack of resolution of some relationships within *Physaraceae* prevents us from resuscitating or creating several new genera to mitigate polyphyly. Among the well-defined groups with clear molecular signatures, we propose two taxonomic and nomenclatural changes at generic level: 1) a new genus, *Nannengaella*, is proposed for a major clade containing *Physarum globuliferum* and other species with heavily calcified sporophores and, often, a true calcareous columella; 2) *Lignydium* is resurrected for the clade containing *Fuligo muscorum*. Additionally, *Trichamphora* is suggested as the correct name for the clade containing *Physarum pezizoideum*. The taxonomy and nomenclature of some provisional genera, currently synonymous with *Fuligo* and *Physarum*, are disentangled, and we provide a comprehensive and updated nomenclatural conspectus that can be used when better resolved phylogenies are obtained. In total, 22 new combinations are proposed in different genera. A provisional key to the genera of the order is also provided.

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INTRODUCTION

The class *Myxomycetes*, often called plasmodial slime molds or myxogastrids, is a monophyletic group (Cavalier-Smith et al. 2015, Fiore-Donno et al. 2019) of free-living protists comprising more than 1 050 species (Lado 2005–2023), being among the largest groups within *Amoebozoa* (Stephenson & Schnittler 2017). These organisms present a complex life cycle that includes microscopic and macroscopic vegetative stages (amoebae-flagellates and multinucleate plasmodia, respectively), resistance forms (cysts and sclerotia), and fruiting bodies, also referred to as sporophores, producing internal spores. These sporophores exhibit dramatic morphological differences among species. *Myxomycetes*, also known as Mycetozoa (De Bary 1859, 1864, Rostafiński 1874, 1875, 1876, Lister 1894, 1911, 1925), are amoeboid protists, regarded as 'protozoan fungal analogues'. They are not related to fungi, as proved in multiple independent molecular studies performed during the last decades (Baldauf & Doolittle 1997, Cavalier-Smith et al. 2015, Kang et al. 2017), although, due to the appearance of their ephemeral fructifications and their sexual reproduction via spores, they were included in the kingdom Fungi for centuries (Ainsworth 1973, Alexopoulos & Mims 1979), and their nomenclature is under the International Code of Nomenclature for algae, fungi and plants, ICN (Turland et al. 2018).

Based on distinctive spore colours and pigments, Lister (1925) classified myxomycete species into *Lamprosporales* (spores not violet brown or purplish grey) and *Amaurosporales* (spores violet-brown or purplish grey), a concept recently updated with the recognition of two major clades: bright- and dark-spored *Myxomycetes*, respectively (Fiore-Donno et al. 2010b). Within the dark-spored clade, in need of revision because of the 'rampant' non-monophyly of many taxa (Adl et al. 2019), the order *Physarales* stands out by presenting conspicuous calcareous deposits in different parts of their fruiting bodies (Keller et al. (2022),

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with the exception of the monospecific genera *Kelleromyxa*, *Protophysarum* and *Trabrooksia*, in which calcium is only detectable by high-resolution analyses (Blackwell 1974, Eliasson et al. 1991). So defined, *Physarales* has been recovered as monophyletic in numerous phylogenetic studies (Fiore-Donno et al. 2008, Kamono et al. 2013, Cainelli et al. 2020, Strelow et al. 2020), although the taxonomic sampling in these publications has been scarce (less than 10 % of the currently accepted species included, and frequently less than 5 %).

The order *Physarales* is the largest group of *Myxomycetes* and currently comprises more than 450 accepted species (Lado 2005–2023). This includes several recently described taxa (García-Martín et al. 2018, Kuhnt 2019, 2021, Novozhilov et al. 2019, Stephenson et al. 2020), as well as two model spe-

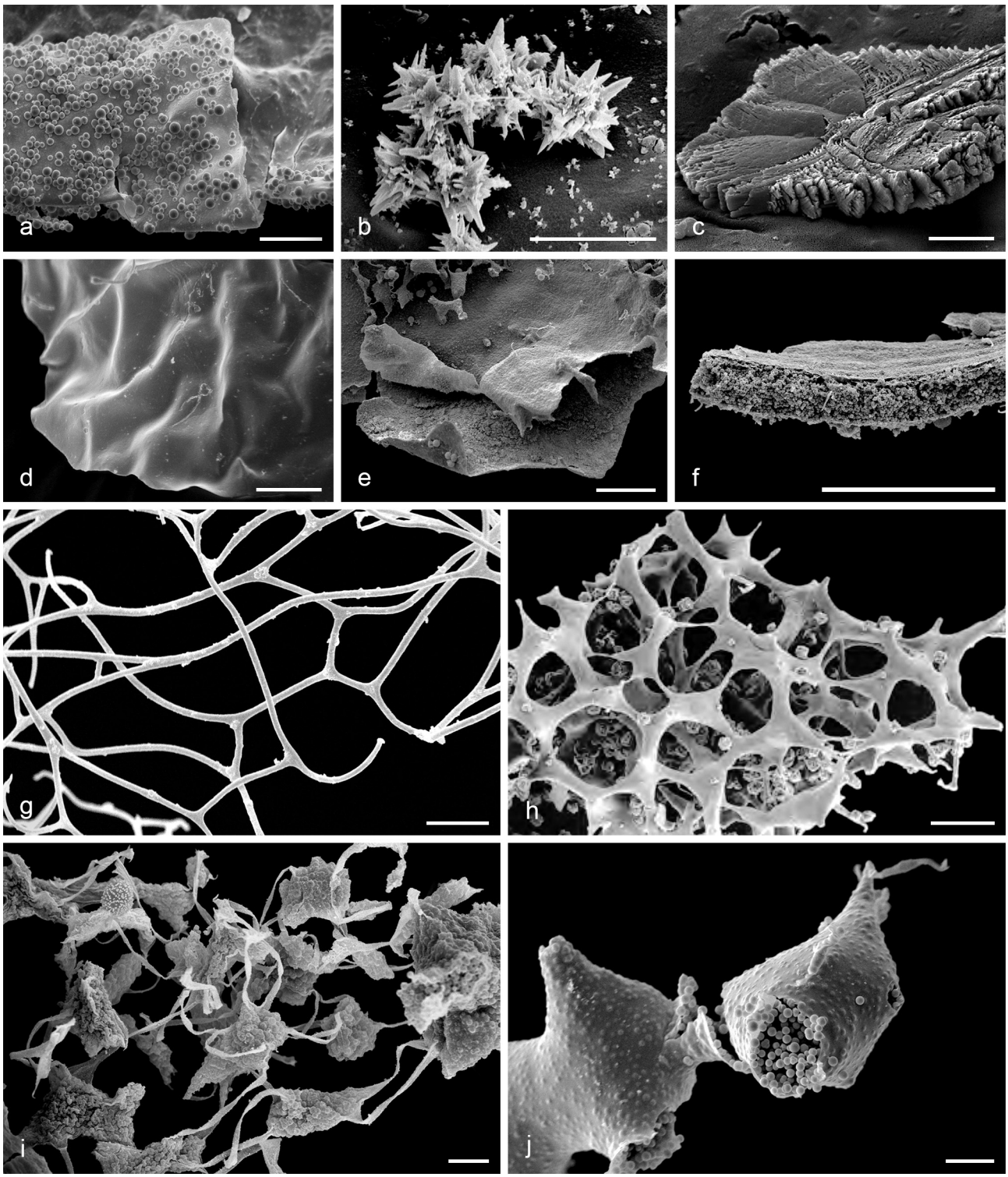

Fig. 1 Different types of calcareous deposits, peridia and capillitia in *Physarales* by SEM. a. Lime granules on the peridium of *Physarum polygonosporum* (MA-Fungi 90742); b. lime crystals covering the peridium of *Didymium dubium* (9276 aet); c. crystalline lime scale on the peridium of *Polyschismium carestianum* s.lat. (Lado 821); d. smooth single peridium of *Diachea mitchellii* (MA-Fungi 91227); e. double peridium of *Physarum andinum* (MA-Fungi 80936); f. triple peridium with a thick middle layer of *Diderma rufostriatum* (Lado 21040); g. capillitium netted, tubules non-calcareous, typical of the family *Didymiaceae* (Lado 19200); h. capillitium netted, tubules entirely calcareous ('badhamioid'), typical of the genus *Badhamia*; i. capillitium netted, formed by large calcareous nodes connected by non-calcareous tubules ('physaroid'), characteristic of most members of the family *Physaraceae* (MA-Fungi 17294); j. detail of capillitial nodes filled with numerous small calcareous granules (aet 12094). — Scale bars: a–d, g, i–j = 10 µm; e–f, h = 100 µm. Image 1h used with permission of Dr. Harold W. Keller, adapted from Hatano & Keller (2008).

cies, *Physarum polycephalum* and *Didymium iridis*, used in a plethora of studies on mitochondrial editing (Hendrickson & Silliker 2010a, b, Traphagen et al. 2010, Houtz et al. 2018), spatial memory (Reid et al. 2012), computing (Adamatzky 2016), chemical sensors (Whiting et al. 2014), plasmodial biological activities (Nguyen et al. 2017), and many other topics.

Many *Physarales* are worldwide distributed in terrestrial ecosystems (Lado & Rojas 2018), even in extreme environments (Ronikier & Lado 2013, Wrigley de Basanta et al. 2015, 2018, Novozhilov et al. 2022b), where they are likely more widespread than currently acknowledged (Fiore-Donno et al. 2016). Their fructifications are often found on rotten wood and other vegetal remnants (Novozhilov et al. 2022a) and, exceptionally, from aquatic environments (Kappel & Anken 1992, Lindley et al. 2007) or on living substrates (Townsend et al. 2005, Zhang et al. 2007), while vegetative phases have been isolated as endocommensals (Dyková et al. 2007). *Physarales*, as other *Myxomycetes*, prey on soil bacteria and other unicellular organisms (Keller et al. 2022), probably playing a significant role in nutrient cycling in terrestrial ecosystems, given that nutrients

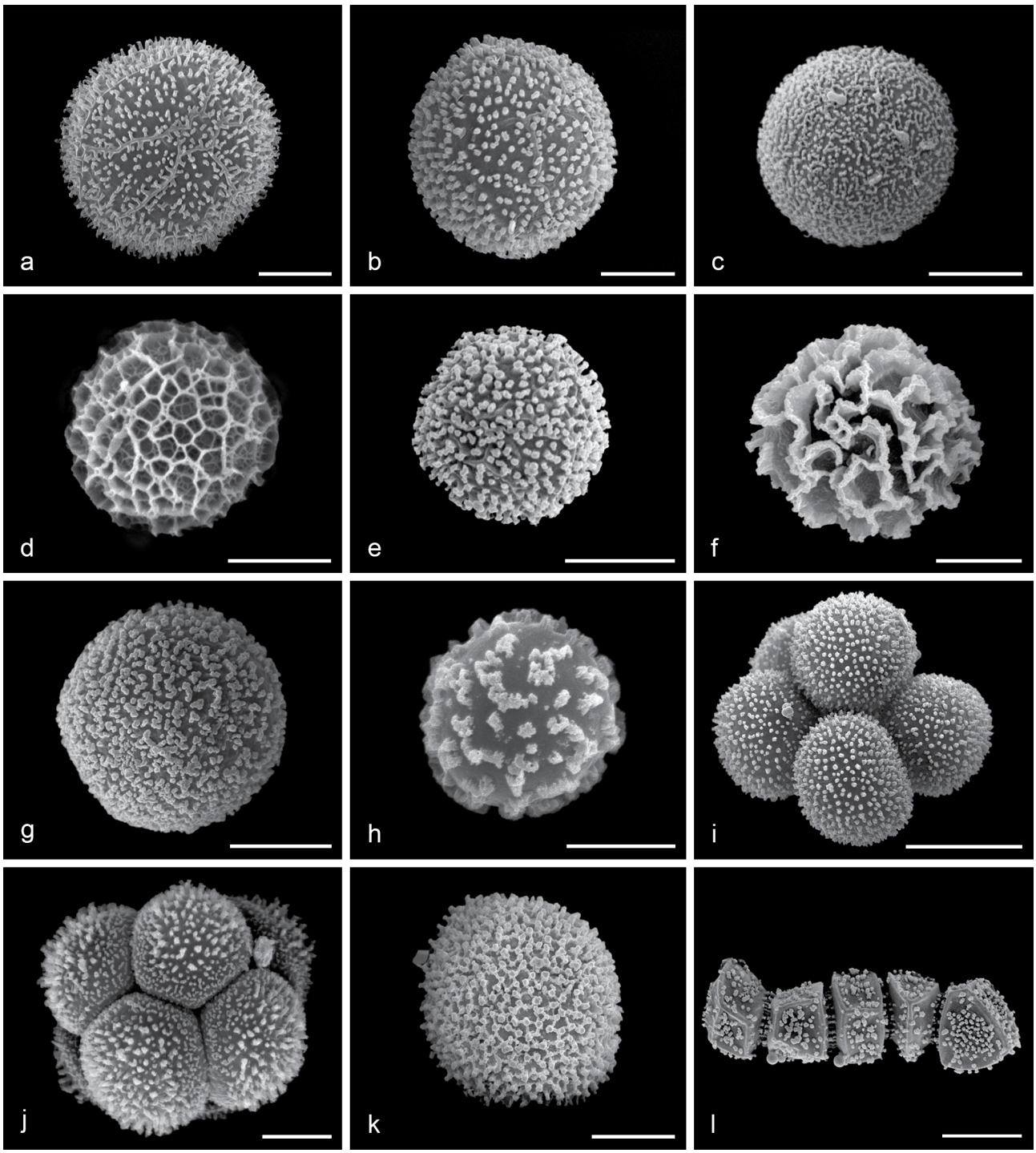

Fig. 2 Spore ornamentation by SEM. a. Distinctly spinulose spore of *Polyschismium carestianum* s.lat. (MA-Fungi 63016); b. spore with evenly distributed bacula of *Didymium xerophilum* (MA-Fungi 86885); c. minutely warted spore of *Protophysarum phloiogenum* (egc211); d. reticulate spore of *Didymium operculatum* (MA-Fungi 74050). Note the second banded reticulum beneath the outer one; e. pilate spore of *Didymium squamulosum* (MA-Fungi 80633); f. spore ornamented with incomplete ridges (cristae) about 1 µm high of *Craterium muscorum* (MA-Fungi 91170); g. spore densely and uniformly ornamented with warts (inconspicuous pila) of *Diachea mitchellii* (MA-Fungi 91212); h. spore ornamented with short, branched, low crests in *Diderma gracile* (MA-Fungi 78767); i. loose cluster of 4–6 subglobose warted spores of *Physarum lakhanpalii* (MA-Fungi 81609); j. compact cluster of 4–20 spores, coarsely spinulose on the exposed area, minutely warted elsewhere, of *Badhamia nitens* (MA-Fungi 85881); k. subreticulate and warted-spinulose spore of *Fuligo cinerea* (MA-Fungi 88376); l. chain formed by five polyhedral spores in *Physarum polygonosporum* (MA-Fungi 90750). — Scale bars: a–h, j–k = 5 µm; i, l = 10 µm.

are released through the feeding activities of bacterivores (White et al. 2020).

Most knowledge on the systematics of the *Physarales* comes from morphological studies of their fruiting bodies. A detailed review and the terminology used to describe their structural elements have been extensively treated in different monographs (Martin & Alexopoulos 1969, Nannenga-Bremekamp 1991, Neubert et al. 1995, Poulain et al. 2011). The traditional classification of the order *Physarales* recognises two families: *Didymiaceae* and *Physaraceae*. Members of both families present calcareous deposits in different parts of their fruiting bodies, but they differ in: 1) the type of capillitium; 2) the shape of the peridial calcareous deposits; 3) the chemical composition of calcium compounds; and 4) the way these deposits are excreted when fructification begins (Gustafson & Thurston 1974, Schoknecht 1975, Schoknecht & Keller 1989). Castillo et al. (1998) erected the family *Protophysaraceae* to accommodate *Protophysarum phloiogenum*, a species that does not present visible lime in the capillitium nor the peridium, lately proved to be phylogenetically related to the family *Didymiaceae* (Fiore-Donno et al. 2010a). Additionally, the intra-ordinal classification of *Physarales* has recently undergone a substantial change with the inclusion of a fourth family, i.e., *Kelleromyxaceae*, since *Kelleromyxa fimicola*, the only species of the genus and the family, did not seem to show clear morphological or molecular affinities to the other families (Erastova et al. 2013).

Within the order *Physarales*, between 16 and 18 genera have been recognised according to a combination of phenotypic traits, such as: 1) fructification type (sporocarps, plasmodiocarps, aethalia or pseudoaethalia); 2) habit (e.g., crowded, heaped, scattered, solitary, closely packed); 3) structure and location of the calcareous deposits (Fig. 1a–c); 4) number and nature of peridial layers (membranous, cartilaginous, coriaceous or calcareous) (Fig. 1d–f); 5) dehiscence of the peridium (e.g., circumscissile, irregular); 6) capillitial morphology (homogeneous, with two distinct morphologies within the same fruiting body – the so-called 'duplex capillitium' – , degree of calcification, etc.; Fig. 1g–j); 7) presence and nature of a stalk and columella; and 8) colour, shape, size and ornamentation of the spores (Fig. 2). However, overemphasizing the importance of some of these traits can lead to artificially circumscribed genera, especially if classifications are based on unreliable characters that may be affected by environmental conditions, e.g., the development and morphology, but also the calcification process of the fruiting bodies (Farr 1961, Alexopoulos 1982).

During the last years, numerous molecular studies based on nearly complete sequences of the nuclear small subunit rRNA gene (hereafter nSSU) have been conducted for different taxa of *Myxomycetes* (Nandipati et al. 2012, Erastova et al. 2013, Kretzschmar et al. 2016, Cainelli et al. 2020, Strelow et al. 2020), offering deep insights into the phylogenetic relationships among and within these groups. Leontyev et al. (2019) presented a new high-rank classification of *Myxomycetes*, based on a single-gene phylogeny (often with partial sequences) and 'assuming that each taxon should correspond to a monophyletic clade'. They proposed an 'emended' circumscription of the order *Physarales*, however represented by only 18 species of traditional *Physarales* (less than 5 % of all currently recognised species) and without a clear inclusion of the genus *Diachea* in *Physarales* based on their tree. Despite this, they transferred five genera previously attributed to the order *Stemonitidales* to *Physarales* justifying their decision on the basis of some unifying characters (e.g., a persistent but separating peridium, weakly melanized capillitial tips, iridescence of the peridium) that are not free of considerable variation among the included species, so that the circumscription of *Physarales* proposed by Leontyev et al. (2019) is, at best, hardly diagnosable. Moreover, they did not consider the type of fruiting body development as a distinguishing trait between *Stemonitidales* and *Physarales*. Therefore, in this study we use the traditional interpretation of *Physarales* (s.str.), as an order distinguished from *Stemonitidales* (s.lat.) by three main characters: 1) the type of plasmodium (phaneroplasmodium and aphanoplasmodium, respectively); 2) its subhypothallic development (epihypothallic in *Stemonitidales*); and 3) the generalised presence of calcareous deposits in some parts of their sporophores (generally absent not only in *Stemonitidales*, but also in other orders of *Myxomycetes*).

Comparatively few phylogenetic studies have been dedicated to *Physarales*, especially through multigene analytic approaches, the most complete being published by Prikhodko et al. (2023), who focused on *Didymiaceae* and analysed three unlinked DNA regions (nSSU, *EF-1α* and COI). There are only five specimens of this order for which complete nSSU sequences and the elongation factor-1 alpha (*EF-1α*) genes are available (Fiore-Donno et al. 2005, 2019), most genera have hitherto remained understudied from a molecular perspective, and some genera have never been molecularly characterised. More importantly, some species representing generic types have never been included in preceding molecular studies. These circumstances, coupled with its enormous diversity, have prevented both a detailed understanding of its internal evolutionary interrelationships and the recognition of monophyletic genera. Consequently, the systematics of *Physarales* remains controversial (Walker & Stephenson 2016, Lado & Eliasson 2022).

Therefore, the objective of the study is to propose a revised and updated, more natural classification of the order *Physarales*. Under this general aim, we also address the following specific goals: 1) to confirm its monophyly; 2) to test whether the traditional families and genera are statistically supported; 3) to assess whether morphological taxonomic characters give any information about its evolutionary relationships; and 4) to identify stable morphological and molecular traits defining each supported clade.

MATERIAL AND METHODS

Taxon sampling

In our main analyses, we included 384 specimens representing around 150 species from 16 out of the 16–18 genera currently recognised in *Physarales* (Fig. 3, S1–S4). Of these, 284 samples (Table S1–S2), collected from distant locations to cover the geographic range of the species analysed, were identified by the authors using a compound Nikon Eclipse 80i compound microscope, and a Nikon SMZ1500 dissecting microscope equipped with a Leica DFC 550 digital camera for light micrographs. To examine specific morphological characters, a scanning electron microscopy (SEM) from the Scanning Electron Microscopy Department of the Real Jardín Botánico of Madrid, model Jeol T 330, was used at 10–15 kV. We included, whenever possible, material of species whose types are also the types of different generic names, as well as some rare taxa not molecularly characterised before, such as *Badhamiopsis ainoae* and *Willkommlangea reticulata*. Seventeen samples corresponding to *Stemonitidales*, the closest relatives to *Physarales* (Fiore-Donno et al. 2010b, 2012, Kretzschmar et al. 2016), were included as outgroup. Most voucher specimens are deposited in the *Myxomycetes* collection of the MA-Fungi herbarium housed at the Real Jardín Botánico, CSIC (Madrid, Spain), although a few samples from other herbaria were also used (Table S1). Institutional herbarium codes follow Index Herbariorum (Thiers 2023).

Besides newly generated data for the present study, we used 366 GenBank sequences (Table S2). Among these, 157 sequences had been previously obtained by us from some of

the MA-Fungi specimens selected for this study, and four sequences (i.e., JX312801, JX312803, JX312804 and AY230189) corresponded to loaned specimens from which we generated additional data. Other 195 GenBank sequences corresponded to vouchered specimens of morphospecies absent in our sampling, or represented by a single individual, for which two DNA regions were publicly available. Finally, sequences corresponding to the four genes analysed here were bioinformatically extracted from the transcriptomes of *Physarum polycephalum* (Glöckner & Marwan 2017, accessible through http://www.physarum-blast.ovgu.de) and *Didymium iridis* (Jiang et al. 2018, GenBank accession number SRR6706112), using as queries public sequences of these genes from other specimens of the same species.

DNA isolation

DNA was extracted from mature fruiting bodies as described elsewhere (Wrigley de Basanta et al. 2015, García-Martín et al. 2018, 2019). The concentration of DNA extractions was estimated using a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, USA) and further assessed by electrophoresis in 1 % agarose gels. Most of the extractions showed either no signal, as previously noted (Fiore-Donno et al. 2012), or a smear. Some samples were subjected to the method for nucleic acid purification (phenol/chloroform) by Sambrook & Russell (2006).

Gene sampling

We amplified partial sequences of three unlinked nuclear regions, nSSU, *EF-1α* and *α-Tub*, and one mitochondrial gene, mtSSU, using the primers shown in Table S3. In the case of the nSSU, we firstly attempted to obtain its full-length by amplifying four overlapping segments, using four primer pairs designed by Fiore-Donno et al. (2008). Despite the fragment amplified with S4/S900R is only interrupted by two introns, i.e., S516 and S529 (Fig. S5), we consistently failed to get it from many samples so that we decided to focus our efforts on: 1) the 5' end fragment (~ 600 bp, referred to as nSSU-5'), which comprises four hypervariable helices and is the most easily amplifiable due to the lack of introns; and 2) a fragment of around 800 bp, excluding introns, close to the 3' end (nSSU-3'), using a semi-nested PCR. This approach targeted c. 70–75 % of the exonic region of the gene nSSU.

PCR cycling parameters and purification

PCR reactions were conducted with newly obtained DNA extractions, except for *Protophysarum phloiogenum*, for which we used the DNA obtained by Walker et al. (2003). PCR mixtures (final volume of 25 µL) contained 3 µL of DNA template, 1 µL of each primer (10 mM), 12.5 µL of MyTaq™ Red Mix 2× (BioLine, United Kingdom), 1 µL of DMSO and 7.5 µL of Milli-Q water. PCR cycling, carried out in an Eppendorf Mastercycler® ep gradient S Thermocycler (Eppendorf, Germany), included an initial denaturation step at 94 °C for 1 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and extension at 72 °C for 3 min, with a final extension stage at 72 °C for 10 min. After amplification, PCR products were examined in 1 % agarose gels in 1× TA buffer.

When the concentration of the target fragment was below the level of detection (no bands observed), 1 µL of the PCR product was subjected to an additional round of amplification, substituting one of the primers used in the first PCR for an internal primer (Table S3). The same reaction conditions and cycling parameters, but with an increased annealing temperature (54 °C), were used for these semi-nested PCRs.

When multiple bands were obtained, the one with the expected size was excised from the gel and purified by using the QIAquick® Gel Extraction kit (QIAGEN, Germany). Alternatively, instead of using a commercial kit, we followed the purification method used by Dentinger et al. (2010). In short, individual bands were excised from the gels, and each cut piece of gel placed in the top of a filter tip previously cut to fit within a microcentrifuge tube. We spun up the tubes at 13 000 rpm for 10 min and, subsequently, we removed the tip leaving the extracted DNA within the flow-through. Single bands were purified with Illustra ExoProStar 1-Step™ (GE Healthcare Life Sciences, United Kingdom). Cleaned amplicons were sequenced in both directions by Macrogen (Spain) with the same primers used for amplification.

The total number of sequences obtained varied among the four loci. To avoid excessive missing data, as a general rule, only those specimens with available data for a minimum of three regions were selected for our analyses. Exceptionally, a few specimens of some key taxa, such as *Willkommlangea reticulata*, *Kelleromyxa fimicola* or *Mucilago crustacea*, from which only one or two genes could be sequenced, were also included in our analyses (Table S1). Besides, to expand the taxon sampling with some other species analysed in previous studies, we also added selected specimens with at least two unlinked DNA regions available in GenBank, typically, partial nSSU and *EF-1α* (Table S2). In the case of clearly monophyletic species with numerous available sequenced samples and little intraclade variability, such as *Didymium yulii* or *Physarum polygonosporum*, a maximum of four specimens were included.

Sequences edition and alignment

Sequence edition and consensus assembly were performed in Geneious v. 7.1.9. (www.geneious.com; Kearse et al. 2012). The resulting sequences were trimmed to exclude primer regions and blasted against the NCBI GenBank database to verify that they were not contaminants. A total of 956 partial sequences newly generated for this study were submitted to the mentioned repository under accession numbers shown in Table S1.

Single-gene datasets were individually compiled and aligned. In the case of the nSSU gene, we used a preexistent dataset that takes into account the secondary structure of this gene (Fiore-Donno et al. 2012) as seed alignment, using the add-option in the MAFFT online server (Katoh et al. 2019). Obvious misplacements, especially at both ends, were manually corrected prior to creating a mask that excluded parts that could not be confidently aligned. Thus, our final nSSU alignment comprised 346 sequences and 1252 aligned positions. For the mtSSU, we automatically aligned our sequences with the E-INS-i strategy in MAFFT v. 7.017 (Katoh & Standley 2013), as implemented in Geneious, and manually removed a central fragment (~ 260 bp) that could not be unambiguously aligned. For the *EF-1α* and *α-Tub* sequences, we translated them into amino acid sequences to detect sequencing errors, prior to be aligned with MAFFT. Manual adjustments were made when alignment errors, due to particularly long or divergent intron sequences, were detected. Spliceosomal (protein-coding genes) and group I introns (nSSU-3') were visually detected and removed prior to phylogenetic analyses. All alignments used in this study are available in Figshare (https://doi.org/10.6084/m9.figshare.21502335.v1; Alignment S1–S7).

Single-gene phylogenetic analyses

For each locus, both Maximum Likelihood and Bayesian Inference analyses (henceforth ML and BI, respectively) were conducted using resources available in CIPRES Science Gateway portal (Miller et al. 2010). Specifically, the individual ML phylogenetic trees were estimated using IQ-TREE v. 2.1.2 (Minh et al. 2020). The optimal partitioning scheme and the

corresponding best-fit model of nucleotide substitution were selected by the integrated version of ModelFinder (Kalyaanamoorthy et al. 2017). Both nSSU and mtSSU genes were not partitioned, while *EF-1α* and *α-Tub* were originally partitioned by codon position (see Table S4). Branch support (BS) was assessed by using the 'complete bootstrap' option with 1 000 non-parametric bootstrap replicates. Besides, considering that rogue taxa can decrease BS values, branch supports for ML trees were also calculated with the transfer bootstrap expectation (TBE) method (Lemoine et al. 2018), using BOOSTER (available at https://booster.pasteur.fr/). To run the analyses, the best replicate obtained with IQ-TREE was used as reference tree, along with the bootstrap trees inferred using the same method.

Single-gene Bayesian analyses were carried out using the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) method as implemented in MrBayes v. 3.2.6 (Huelsenbeck & Ronquist 2001, Ronquist et al. 2012). For each of the protein-coding genes, the partitioning scheme used in the Bayesian analysis was that previously obtained with IQ-TREE, unlinking model parameters across different partitions. We used the reversible jumping model choice (lset nst = mixed) to estimate the best-fit substitution models for each partition (Huelsenbeck et al. 2004), allowing a gamma distributed rate heterogeneity across sites, with four rate categories, and a proportion of invariant sites. Two independent runs, each with eight chains, were executed for a maximum of 1×10^8 generations, sampling every 1 000, with the first 50 % discarded as burn-in and the posterior probabilities (PP) being calculated from the remaining ones. The analyses were automatically stopped before completing the maximum number of generations if the average standard deviation of split frequencies (σ) fell below 0.01. In addition, the convergence to the stationary distribution of all parameters was assessed in Tracer v. 1.7.1 (Rambaut et al. 2018) by checking the value of the effective sample size for each parameter (ESS > 200). Single-gene trees were visualized with FigTree v. 1.4.3 (Rambaut 2018), and compared to each other. For each locus, both ML and BI analyses yielded very similar topologies so that only those phylogenies obtained from the Bayesian analyses, showing PP, BS and TBE support values, can be found in Fig. S1–S4. Significant support was assumed for those nodes with PP ≥ 0.95, BS ≥ 70 % and TBE ≥ 75 %. Certain topological differences existed among our four single-gene trees, involving closely related species, but these were not supported by all approaches and did not affect their backbones.

Multigene phylogenetic analyses

Two approaches were used for building a multilocus phylogenetic tree: 1) concatenation of the four datasets into a single matrix; and 2) pseudocoalescence-based species tree. For the concatenated analysis, the choice of the best partition scheme (testing eight potential partitions: nSSU, mtSSU and each codon position of the *EF-1α* and *α-Tub*) and substitution models was again performed with IQ-TREE. It was also used to estimate the concatenated ML tree using the 'complete bootstrap' option and 1 000 non-parametric bootstrap replicates to assess the robustness of the analysis. The concatenated Bayesian analysis followed the same settings used for each gene dataset. Once again, only the Bayesian concatenated tree, showing node supports from both phylogenetic methods is presented (Fig. 3).

We also estimated a pseudocoalescent species tree with ASTRAL III v. 5.6.3 (Zhang et al. 2018), using the four independent ML phylogenetic trees previously obtained with IQ-TREE. For each tree, those branches receiving BS support values ≤ 20 % were collapsed using Newick Utilities v. 1.6 (Junier & Zdobnov 2010), as recommended by Zhang et al. (2018). Branch support was calculated as local posterior probabilities based on quartet support (QS) and was considered significant when QS ≥ 0.95 (Sayyari & Mirarab 2016). Specimens were assigned to candidate species on the basis of: 1) morphological criteria; and 2) the information provided by both our single-gene and concatenated trees. For example, individuals originally identified as one species but that formed independent, well-supported, non-monophyletic groups (e.g., *Fuligo candida*) were treated as putative different species. This pseudocoalescence-based species tree is shown in Fig. S6.

Morphological character evaluation

Different morphological traits traditionally used to distinguish among families, genera and species (Table S5) were illustrated into our multigene tree (Fig. 3). Specifically, we have evaluated: 1) the type of fructification (sporocarps, plasmodiocarps and aethalia); 2) the presence/absence of a stalk; 3) the number of peridial layers (single, double or triple peridium); 4) the type of peridial lime (if present, globular or crystalline); 5) presence/absence of a totally calcareous capillitium; 6) presence/absence of a columella; 7) presence/absence of a pseudocolumella; and 8) presence/absence of clustered spores (Fig. 3).

Molecular signatures

To better characterise the clades obtained, we looked for specific nSSU signatures, following Sheikh et al. (2018). We also identified molecular signatures by comparing our own sequences of the other three genes analysed in this study.

RESULTS

Sequence data

We have newly obtained 956 partial sequences corresponding to four independent genes from 285 dark-spored myxomycete

Table 1 Characteristics of the *EF-1α* and *α-Tub* introns found in sequences included in this study.

EF-1α				*α-Tub*			
Intron	Corresp.[1]	Length (bp)*	No. sequences with introns (taxa)	Intron	Corresp.[2]	Length (bp)*	No. sequences with introns (taxa)
1st	Intron 4	61–553	All (obligatory intron)	1st	–	59	1 (*D. infundibuliforme* 78327)
2nd	–	86	1 (*W. reticulata* 79031)	2nd	–	93–108	7 (Subclade 6-VII)
3rd	Intron 10	88	1 (*K. fimicola* LE274210)	3rd	Intron 6	66–200	41 (*Physarales* + *Stemonitidales*)
4th	Intron 15	36–99	10 (Subclades 6-IV + 6-VIII)	4th	–	98	1 (*D. leptotrichum* 83181)
5th	–	75–89	7 (Subclade 6-VII)	5th	–	78–109	6 (Subclade 2-IV)
–	–	–	–	6th	–	54–237	89 (*Physaraceae* + *K. fimicola*)

[1] Correspondence among the *EF-1α* introns found here and those reported by Fiore-Donno et al. (2010b), see Alignment S6 in FigShare. Note that the second *EF-1α* intron does not correspond to any previously known.

[2] Correspondence among the *α-Tub* introns observed in our sequences and those interrupting an almost complete *α-Tub* sequence of *Ph. polycephalum* (Walden et al. 1989). Four of the six *α-Tub* introns found in our sequences are inserted in sites previously unseen in sequences of *Myxomycetes* and *Dictyostelids* available in GenBank (see Alignment S7 in FigShare).

* Length considering complete introns only. Taxa are named according to Fig. 3.

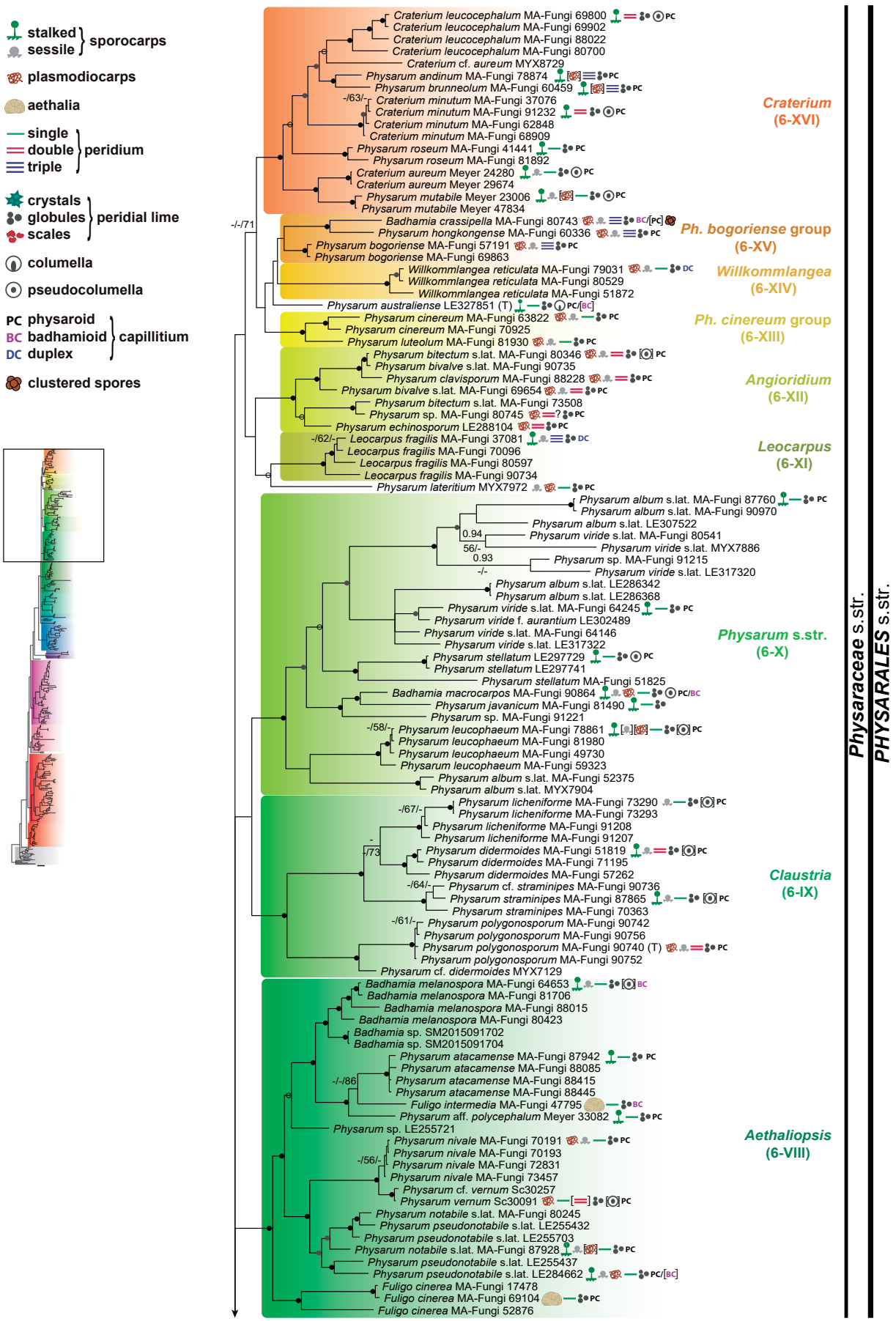

Fig. 3 Fifty percent majority-rule Bayesian tree of the order *Physarales* based on 3 367 nucleotide positions from 384 specimens. Species names are followed by herbaria codes. Type specimens are indicated by '(T)'. Black solid dots indicate nodes highly supported by all three analyses (PP ≥ 0.95, BS ≥ 70 %, and TBE ≥ 75 %). Grey solid dots represent nodes that are supported by two analyses. Those nodes only supported by one analysis are represented by white empty circles. Other values are included only when PP ≥ 0.90, BS ≥ 50 %, and TBE BS ≥ 70 %. The scale bar represents the average number of substitutions per site. Continuous and discontinuous vertical lines represent mono- and paraphyletic groups, respectively. An interrupted branch (//) indicates its length has been reduced. Morphological traits have been illustrated only once when the species is represented by several specimens. Traits occurring only occasionally for a given species appear between square brackets.

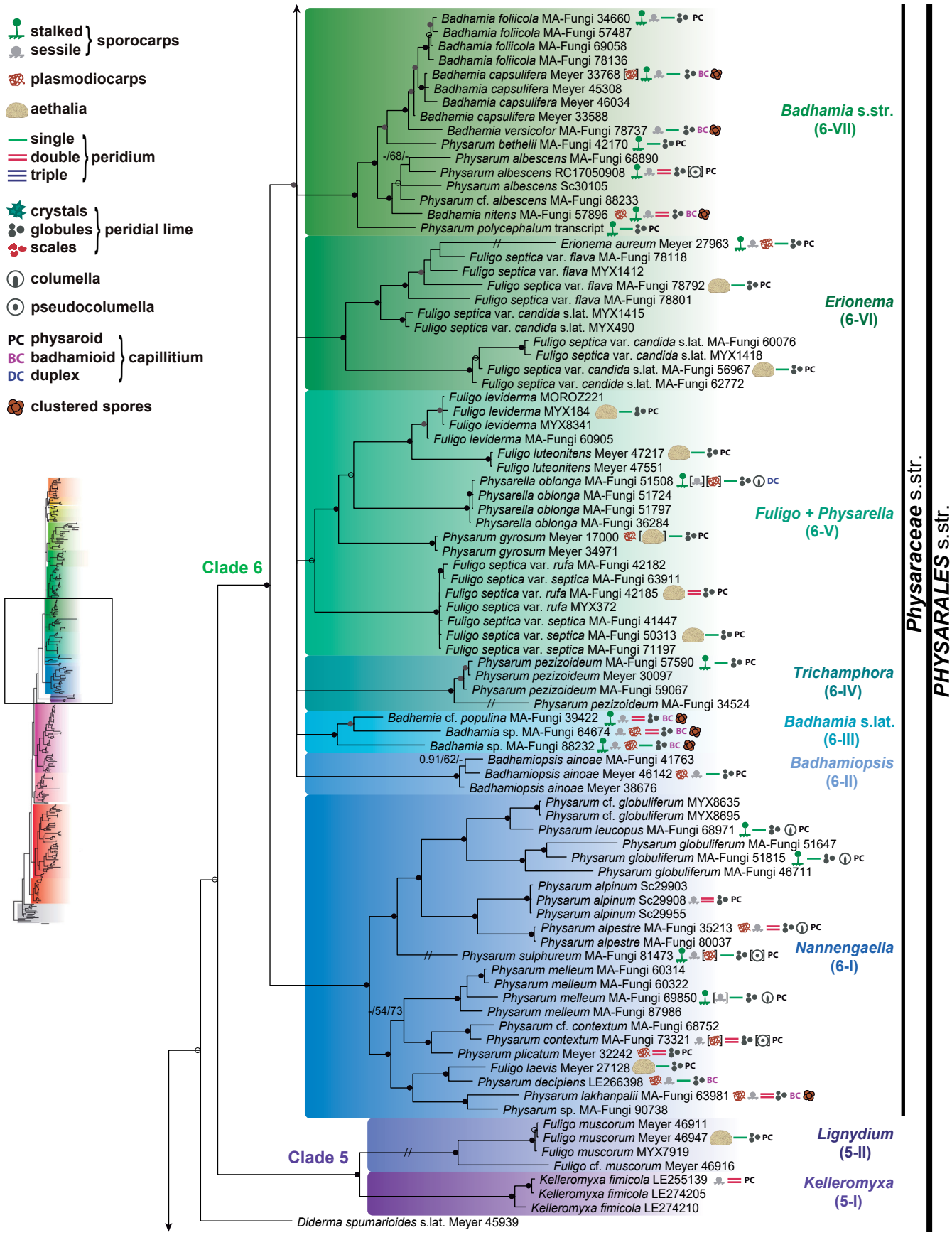
stalked
sessile
sporocarps
plasmodiocarps
aethalia
single
double
triple
peridium
crystals
globules
scales
peridial lime
columella
pseudocolumella
PC physaroid
BC badhamioid
DC duplex
capillitium
clustered spores
Clade 6
Clade 5
Badhamia foliicola MA-Fungi 34660
Badhamia foliicola MA-Fungi 57487
Badhamia foliicola MA-Fungi 69058
Badhamia foliicola MA-Fungi 78136
Badhamia capsulifera Meyer 33768
Badhamia capsulifera Meyer 45308
Badhamia capsulifera Meyer 46034
Badhamia capsulifera Meyer 33588
Badhamia versicolor MA-Fungi 78737
Physarum bethelii MA-Fungi 42170
-/68/-
Physarum albescens MA-Fungi 68890
Physarum albescens RC17050908
Physarum albescens Sc30105
Physarum cf. albescens MA-Fungi 88233
Badhamia nitens MA-Fungi 57896
Physarum polycephalum transcript
Badhamia s.str. (6-VII)
Erionema aureum Meyer 27963
Fuligo septica var. flava MA-Fungi 78118
Fuligo septica var. flava MYX1412
Fuligo septica var. flava MA-Fungi 78792
Fuligo septica var. flava MA-Fungi 78801
Fuligo septica var. candida s.lat. MYX1415
Fuligo septica var. candida s.lat. MYX490
Fuligo septica var. candida s.lat. MA-Fungi 60076
Fuligo septica var. candida s.lat. MYX1418
Fuligo septica var. candida s.lat. MA-Fungi 56967
Fuligo septica var. candida s.lat. MA-Fungi 62772
Erionema (6-VI)
Fuligo leviderma MOROZ221
Fuligo leviderma MYX184
Fuligo leviderma MYX8341
Fuligo leviderma MA-Fungi 60905
Fuligo luteonitens Meyer 47217
Fuligo luteonitens Meyer 47551
Physarella oblonga MA-Fungi 51508
Physarella oblonga MA-Fungi 51724
Physarella oblonga MA-Fungi 51797
Physarella oblonga MA-Fungi 36284
Physarum gyrosum Meyer 17000
Physarum gyrosum Meyer 34971
Fuligo septica var. rufa MA-Fungi 42182
Fuligo septica var. septica MA-Fungi 63911
Fuligo septica var. rufa MA-Fungi 42185
Fuligo septica var. rufa MYX372
Fuligo septica var. septica MA-Fungi 41447
Fuligo septica var. septica MA-Fungi 50313
Fuligo septica var. septica MA-Fungi 71197
Fuligo + Physarella (6-V)
Physarum pezizoideum MA-Fungi 57590
Physarum pezizoideum Meyer 30097
Physarum pezizoideum MA-Fungi 59067
Physarum pezizoideum MA-Fungi 34524
Trichamphora (6-IV)
Badhamia cf. populina MA-Fungi 39422
Badhamia sp. MA-Fungi 64674
Badhamia sp. MA-Fungi 88232
Badhamia s.lat. (6-III)
0.91/62/-
Badhamiopsis ainoae MA-Fungi 41763
Badhamiopsis ainoae Meyer 46142
Badhamiopsis ainoae Meyer 38676
Badhamiopsis (6-II)
Physarum cf. globuliferum MYX8635
Physarum cf. globuliferum MYX8695
Physarum leucopus MA-Fungi 68971
Physarum globuliferum MA-Fungi 51647
Physarum globuliferum MA-Fungi 51815
Physarum globuliferum MA-Fungi 46711
Physarum alpinum Sc29903
Physarum alpinum Sc29908
Physarum alpinum Sc29955
Physarum alpestre MA-Fungi 35213
Physarum alpestre MA-Fungi 80037
Physarum sulphureum MA-Fungi 81473
Physarum melleum MA-Fungi 60314
Physarum melleum MA-Fungi 60322
Physarum melleum MA-Fungi 69850
Physarum melleum MA-Fungi 87986
-/54/73
Physarum cf. contextum MA-Fungi 68752
Physarum contextum MA-Fungi 73321
Physarum plicatum Meyer 32242
Fuligo laevis Meyer 27128
Physarum decipiens LE266398
Physarum lakhanpalii MA-Fungi 63981
Physarum sp. MA-Fungi 90738
Nannengaella (6-I)
Fuligo muscorum Meyer 46911
Fuligo muscorum Meyer 46947
Fuligo muscorum MYX7919
Fuligo cf. muscorum Meyer 46916
Lignydium (5-II)
Kelleromyxa fimicola LE255139
Kelleromyxa fimicola LE274205
Kelleromyxa fimicola LE274210
Kelleromyxa (5-I)
Diderma spumarioides s.lat. Meyer 45939
Physaraceae s.str.
PHYSARALES s.str.

Fig. 3 (cont.)

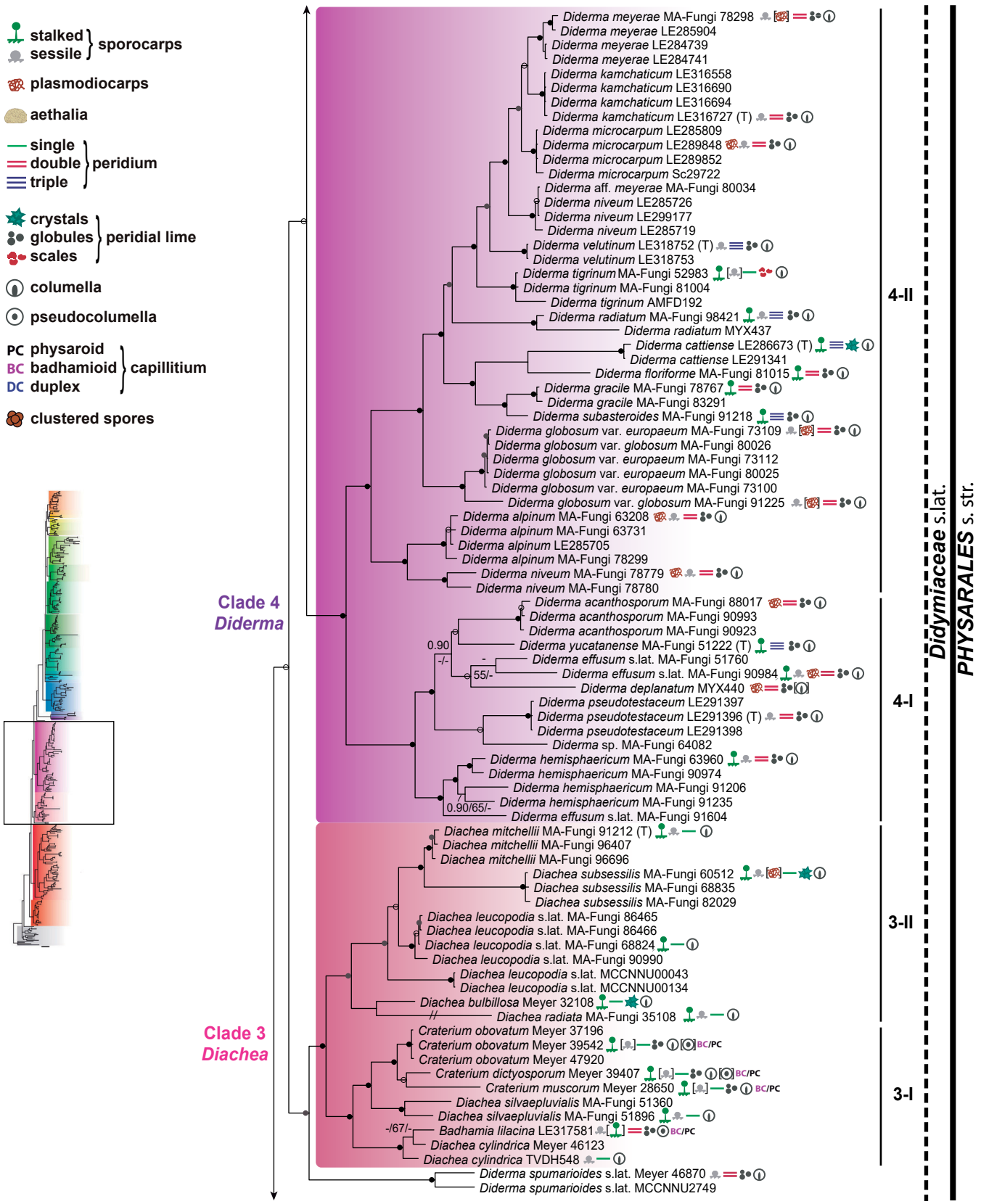
stalked
sessile
sporocarps
plasmodiocarps
aethalia
single
double
triple
peridium
crystals
globules
scales
peridial lime
columella
pseudocolumella
PC physaroid
BC badhamioid
DC duplex
capillitium
clustered spores
Clade 4
Diderma
Clade 3
Diachea
Diderma meyerae MA-Fungi 78298
Diderma meyerae LE285904
Diderma meyerae LE284739
Diderma meyerae LE284741
Diderma kamchaticum LE316558
Diderma kamchaticum LE316690
Diderma kamchaticum LE316694
Diderma kamchaticum LE316727 (T)
Diderma microcarpum LE285809
Diderma microcarpum LE289848
Diderma microcarpum LE289852
Diderma microcarpum Sc29722
Diderma aff. meyerae MA-Fungi 80034
Diderma niveum LE285726
Diderma niveum LE299177
Diderma niveum LE285719
Diderma velutinum LE318752 (T)
Diderma velutinum LE318753
Diderma tigrinum MA-Fungi 52983
Diderma tigrinum MA-Fungi 81004
Diderma tigrinum AMFD192
Diderma radiatum MA-Fungi 98421
Diderma radiatum MYX437
Diderma cattiense LE286673 (T)
Diderma cattiense LE291341
Diderma floriforme MA-Fungi 81015
Diderma gracile MA-Fungi 78767
Diderma gracile MA-Fungi 83291
Diderma subasteroides MA-Fungi 91218
Diderma globosum var. europaeum MA-Fungi 73109
Diderma globosum var. globosum MA-Fungi 80026
Diderma globosum var. europaeum MA-Fungi 73112
Diderma globosum var. europaeum MA-Fungi 80025
Diderma globosum var. europaeum MA-Fungi 73100
Diderma globosum var. globosum MA-Fungi 91225
Diderma alpinum MA-Fungi 63208
Diderma alpinum MA-Fungi 63731
Diderma alpinum LE285705
Diderma alpinum MA-Fungi 78299
Diderma niveum MA-Fungi 78779
Diderma niveum MA-Fungi 78780
Diderma acanthosporum MA-Fungi 88017
Diderma acanthosporum MA-Fungi 90993
Diderma acanthosporum MA-Fungi 90923
Diderma yucatanense MA-Fungi 51222 (T)
Diderma effusum s.lat. MA-Fungi 51760
Diderma effusum s.lat. MA-Fungi 90984
Diderma deplanatum MYX440
Diderma pseudotestaceum LE291397
Diderma pseudotestaceum LE291396 (T)
Diderma pseudotestaceum LE291398
Diderma sp. MA-Fungi 64082
Diderma hemisphaericum MA-Fungi 63960
Diderma hemisphaericum MA-Fungi 90974
Diderma hemisphaericum MA-Fungi 91206
Diderma hemisphaericum MA-Fungi 91235
Diderma effusum s.lat. MA-Fungi 91604
Diachea mitchellii MA-Fungi 91212 (T)
Diachea mitchellii MA-Fungi 96407
Diachea mitchellii MA-Fungi 96696
Diachea subsessilis MA-Fungi 60512
Diachea subsessilis MA-Fungi 68835
Diachea subsessilis MA-Fungi 82029
Diachea leucopodia s.lat. MA-Fungi 86465
Diachea leucopodia s.lat. MA-Fungi 86466
Diachea leucopodia s.lat. MA-Fungi 68824
Diachea leucopodia s.lat. MA-Fungi 90990
Diachea leucopodia s.lat. MCCNNU00043
Diachea leucopodia s.lat. MCCNNU00134
Diachea bulbillosa Meyer 32108
Diachea radiata MA-Fungi 35108
Craterium obovatum Meyer 37196
Craterium obovatum Meyer 39542
Craterium obovatum Meyer 47920
Craterium dictyosporum Meyer 39407
Craterium muscorum Meyer 28650
Diachea silvaepluvialis MA-Fungi 51360
Diachea silvaepluvialis MA-Fungi 51896
Badhamia lilacina LE317581
Diachea cylindrica Meyer 46123
Diachea cylindrica TVDH548
Diderma spumarioides s.lat. Meyer 46870
Diderma spumarioides s.lat. MCCNNU2749
0.90
-/-
55/-
0.90/65/-
-/67/-
BC/PC
4-II
4-I
3-II
3-I
Didymiaceae s.lat.
PHYSARALES s. str.

Fig. 3 (cont.)

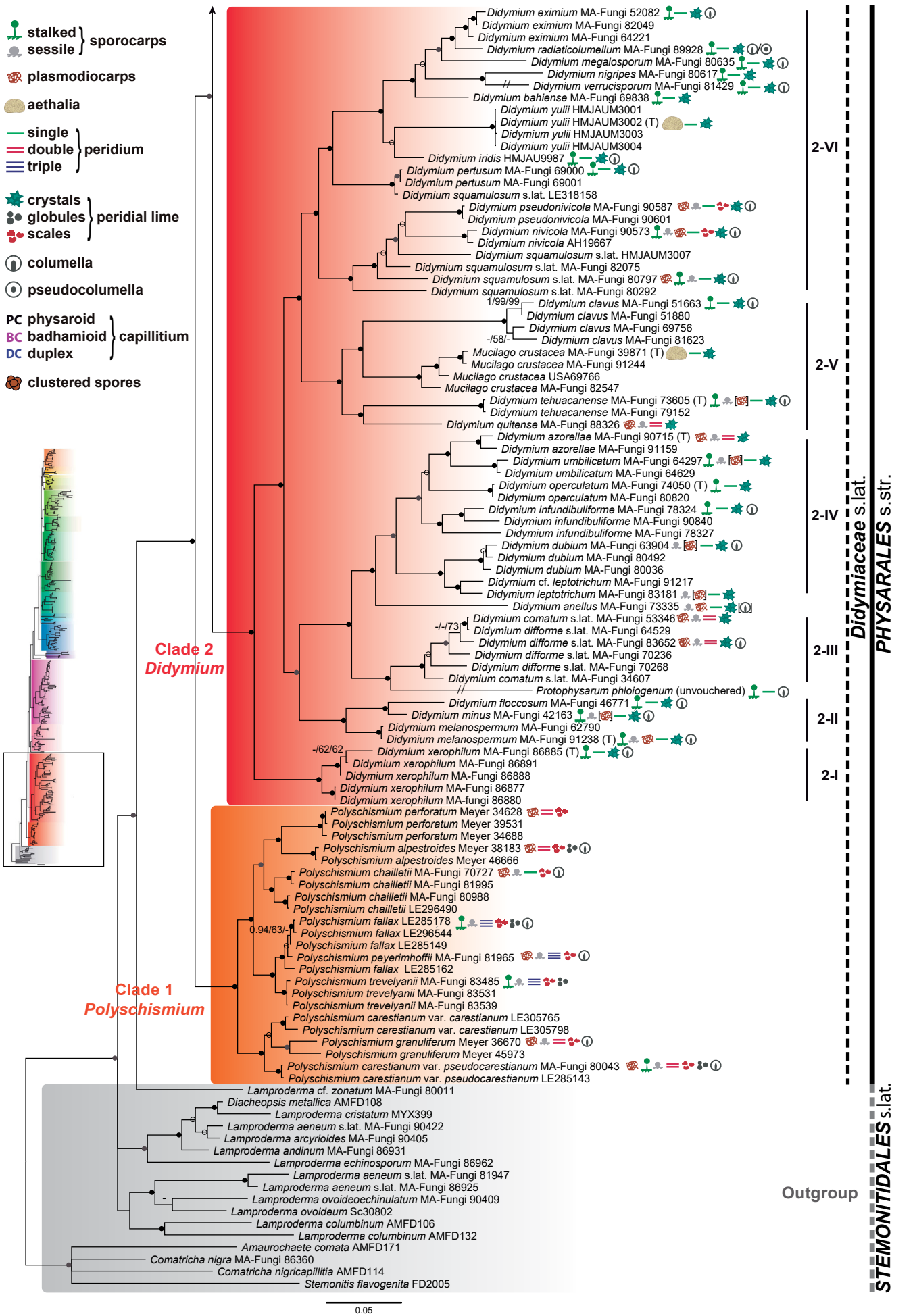
stalked
sessile
sporocarps
plasmodiocarps
aethalia
single
double
triple
peridium
crystals
globules
scales
peridial lime
columella
pseudocolumella
PC physaroid
BC badhamioid
DC duplex
capillitium
clustered spores
Didymium eximium MA-Fungi 52082
Didymium eximium MA-Fungi 82049
Didymium eximium MA-Fungi 64221
Didymium radiaticolumellum MA-Fungi 89928
Didymium megalosporum MA-Fungi 80635
Didymium nigripes MA-Fungi 80617
Didymium verrucisporum MA-Fungi 81429
Didymium bahiense MA-Fungi 69838
Didymium yulii HMJAUM3001
Didymium yulii HMJAUM3002 (T)
Didymium yulii HMJAUM3003
Didymium yulii HMJAUM3004
Didymium iridis HMJAU9987
Didymium pertusum MA-Fungi 69000
Didymium pertusum MA-Fungi 69001
Didymium squamulosum s.lat. LE318158
Didymium pseudonivicola MA-Fungi 90587
Didymium pseudonivicola MA-Fungi 90601
Didymium nivicola MA-Fungi 90573
Didymium nivicola AH19667
Didymium squamulosum s.lat. HMJAUM3007
Didymium squamulosum s.lat. MA-Fungi 82075
Didymium squamulosum s.lat. MA-Fungi 80797
Didymium squamulosum s.lat. MA-Fungi 80292
1/99/99
Didymium clavus MA-Fungi 51663
Didymium clavus MA-Fungi 51880
Didymium clavus MA-Fungi 69756
-/58/-
Didymium clavus MA-Fungi 81623
Mucilago crustacea MA-Fungi 39871 (T)
Mucilago crustacea MA-Fungi 91244
Mucilago crustacea USA69766
Mucilago crustacea MA-Fungi 82547
Didymium tehuacanense MA-Fungi 73605 (T)
Didymium tehuacanense MA-Fungi 79152
Didymium quitense MA-Fungi 88326
Didymium azorellae MA-Fungi 90715 (T)
Didymium azorellae MA-Fungi 91159
Didymium umbilicatum MA-Fungi 64297
Didymium umbilicatum MA-Fungi 64629
Didymium operculatum MA-Fungi 74050 (T)
Didymium operculatum MA-Fungi 80820
Didymium infundibuliforme MA-Fungi 78324
Didymium infundibuliforme MA-Fungi 90840
Didymium infundibuliforme MA-Fungi 78327
Didymium dubium MA-Fungi 63904
Didymium dubium MA-Fungi 80492
Didymium dubium MA-Fungi 80036
Didymium cf. leptotrichum MA-Fungi 91217
Didymium leptotrichum MA-Fungi 83181
Didymium anellus MA-Fungi 73335
-/-/73
Didymium comatum s.lat. MA-Fungi 53346
Didymium difforme s.lat. MA-Fungi 64529
Didymium difforme s.lat. MA-Fungi 83652
Didymium difforme MA-Fungi 70236
Didymium difforme s.lat. MA-Fungi 70268
Didymium comatum s.lat. MA-Fungi 34607
Protophysarum phloiogenum (unvouchered)
Didymium floccosum MA-Fungi 46771
Didymium minus MA-Fungi 42163
Didymium melanospermum MA-Fungi 62790
Didymium melanospermum MA-Fungi 91238 (T)
-/62/62
Didymium xerophilum MA-Fungi 86885 (T)
Didymium xerophilum MA-Fungi 86891
Didymium xerophilum MA-Fungi 86888
Didymium xerophilum MA-Fungi 86877
Didymium xerophilum MA-fungi 86880
Clade 2
Didymium
2-VI
2-V
2-IV
2-III
2-II
2-I
Didymiaceae s.lat.
PHYSARALES s.str.
Polyschismium perforatum Meyer 34628
Polyschismium perforatum Meyer 39531
Polyschismium perforatum Meyer 34688
Polyschismium alpestroides Meyer 38183
Polyschismium alpestroides Meyer 46666
Polyschismium chailletii MA-Fungi 70727
Polyschismium chailletii MA-Fungi 81995
Polyschismium chailletii MA-Fungi 80988
Polyschismium chailletii LE296490
0.94/63/-
Polyschismium fallax LE285178
Polyschismium fallax LE296544
Polyschismium fallax LE285149
Polyschismium peyerimhoffii MA-Fungi 81965
Polyschismium fallax LE285162
Polyschismium trevelyanii MA-Fungi 83485
Polyschismium trevelyanii MA-Fungi 83531
Polyschismium trevelyanii MA-Fungi 83539
Polyschismium carestianum var. carestianum LE305765
Polyschismium carestianum var. carestianum LE305798
Polyschismium granuliferum Meyer 36670
Polyschismium granuliferum Meyer 45973
Polyschismium carestianum var. pseudocarestianum MA-Fungi 80043
Polyschismium carestianum var. pseudocarestianum LE285143
Clade 1
Polyschismium
Lamproderma cf. zonatum MA-Fungi 80011
Diacheopsis metallica AMFD108
Lamproderma cristatum MYX399
Lamproderma aeneum s.lat. MA-Fungi 90422
Lamproderma arcyrioides MA-Fungi 90405
Lamproderma andinum MA-Fungi 86931
Lamproderma echinosporum MA-Fungi 86962
Lamproderma aeneum s.lat. MA-Fungi 81947
Lamproderma aeneum s.lat. MA-Fungi 86925
Lamproderma ovoideoechinulatum MA-Fungi 90409
Lamproderma ovoideum Sc30802
Lamproderma columbinum AMFD106
Lamproderma columbinum AMFD132
Amaurochaete comata AMFD171
Comatricha nigra MA-Fungi 86360
Comatricha nigricapillitia AMFD114
Stemonitis flavogenita FD2005
Outgroup
STEMONITIDALES s.lat.
0.05

Fig. 3 (cont.)

specimens (276 of the order *Physarales* plus nine of the order *Stemonitidales*). Specifically, 153 nSSU-5', 140 nSSU-3', 189 *EF-1α*, 250 mtSSU, and 224 *α-Tub* gene sequences have been generated and deposited in GenBank (Table S1). The combined dataset (Alignment S5, 384 specimens) consisted of 3 367 characters (1 252 nSSU, 941 *EF-1α*, 375 mtSSU and 799 *α-Tub*) of which 1 837 were variable (711 SSU, 531 *EF-1α*, 246 mtSSU and 349 *α-Tub*) and 1 478 were parsimony informative (563 nSSU, 423 *EF-1α*, 217 mtSSU and 275 *α-Tub*).

Spliceosomal introns in the EF-1α and α-Tub genes

Some sequences of the *EF-1α* and *α-Tub* genes were interrupted by several introns, flanked by GT-AG borders, distributed throughout their coding regions (Table 1). Most of them were extremely variable in sequence and length, which precluded their unambiguous alignment. Specifically, *EF-1α* contained five insertion sites (Alignment S6), of which one was obligatory (that located most close to the 5' end of the gene), being the only intron present in sequences corresponding to the family *Didymiaceae*. As for *α-Tub*, we identified a total of six introns, varying in length between 54 and 237 bp (Alignment S7). Three *α-Tub* introns (1st, 4th and 5th, see Table 1) appeared unique to some species of the genus *Didymium* within 'Subclade 2-IV'. The 2nd was unique to the 'Subclade 6-VII' (*Aethaliopsis*). The 3rd was present in various species dispersed throughout the phylogeny (*Didymiaceae*, *Physaraceae* and some *Stemonitidales*). The 6th was found in several *Physaraceae* and *Kelleromyxa*. *Diderma*, *Polyschismium* and *Mucilago* lacked *α-Tub* introns.

Phylogenetic analyses

The optimal partitioning scheme and the corresponding best-fit model of nucleotide substitution for the final matrices analysed in this study are shown in Table S4. Individually, none of the four regions analysed was able to confidently resolve all relationships within this order, as evidenced by the low support values recovered for most internal branches in our single-gene trees (Fig. S1–S4). Certain topological differences, not supported in all analyses, and generally affecting short branches, were observed among closely related samples in the four single-gene trees. This is exemplified by some members of the 'Subclade 4-I' (see Fig. 3): *Diderma acanthosporum* and *D. effusum* appeared as sister species with moderate support in the nSSU tree (Fig. S1) but, in contrast, *D. acanthosporum* is phylogenetically closer to *D. hemisphaericum* and *D. pseudotestaceum* than to *D. effusum* in the *EF-1α* tree (Fig. S2), also with moderate support.

The topology of the multilocus pseudocoalescent species tree inferred with ASTRAL (Fig. S6) largely agreed with that obtained from the concatenated analysis (Fig. 3). The final normalized quartet score was 0.99, indicating very low discordance among the four single-locus trees. As indicated in Fig. S6, branch support values were generally very low, but the six main clades observed in Fig. 3 were also recovered here.

The phylogeny obtained from the combined dataset showed improved but not full resolution. Considering that the topologies of the concatenated Bayesian and ML analyses were similar, only the Bayesian tree, showing support values from all three phylogenetic methods, is presented (Fig. 3). As this figure illustrates, the order *Physarales* is recovered as monophyletic with high support (PP = 1, BS = 96 %, TBE = 99 %), the family *Didymiaceae* is paraphyletic with respect to all other members of the order, and the family *Physaraceae* s.str. is highly supported as monophyletic (PP = 1, BS = 97 %, TBE = 100 %). Six major imbricated clades are recovered: 1) 'Clade 1, *Polyschismium*'; 2) 'Clade 2, *Didymium*'; 3) 'Clade 3, *Diachea*'; 4) 'Clade 4, *Diderma*'; 5) 'Clade 5, *Kelleromyxa* + *Fuligo muscorum*'; and 6) 'Clade 6, *Physaraceae* s.str.'.

The highly supported '*Polyschismium* clade' (PP = 1, BS = 83 %, TBE = 99 %) includes *Polyschismium fallax*, formerly treated in the genus *Diderma*, and all the species traditionally ascribed to *Lepidoderma* (except for the type, i.e., *L. tigrinum*) that were analysed in this study, which now also belong to *Polyschismium.*

The 'Clade 2, *Didymium*' is also highly supported (PP = 1, BS = 99 %, TBE = 100 %), and is subdivided into six well-supported monophyletic groups: 1) 'Subclade 2-I', formed by all samples of *Didymium xerophilum* (PP = 1, BS = 100 %, TBE = 100 %); 2) 'Subclade 2-II', constituted by three *Didymium* species, including the type of the genus *Didymium* (PP = 1, BS = 100 %, TBE = 100 %); 3) 'Subclade 2-III' with *D. comatum* and *D. difforme* (PP = 1, BS = 89 %, TBE = 98 %); 4) 'Subclade 2-IV' formed by six *Didymium* species (PP = 1, BS = 66 %, TBE = 97 %); 5) 'Subclade 2-V' consisting of three species of *Didymium* plus *Mucilago crustacea*, (PP = 1, BS = 99 %, TBE = 100 %); and 6) 'Subclade 2-VI', formed by at least 11 *Didymium* species (PP = 1, BS = 98 %, TBE = 99 %), one of them aethalioid (*D. yulii*). In addition, two morphologically divergent taxa, i.e., *Protophysarum phloiogenum* and *D. anellus*, were found within the '*Didymium* clade' in separated positions.

The 'Clade 3, *Diachea*' is significantly supported by standard bootstrapping (BS = 70 %), and highly supported by both transfer bootstrap (TBE = 98 %) and Bayesian posterior probabilities (PP = 1). This clade is constituted by all isolates of the genus *Diachea* included in this study, one *Badhamia* and three species of *Craterium*.

The 'Clade 4, *Diderma*' received maximum support (PP = 1, BS = 100 %, TBE = 100 %) and is further divided into two highly supported monophyletic groups: 1) 'Subclade 4-I' (PP = 1, BS = 100 %, TBE = 100 %), with at least six species; and 2) 'Subclade 4-II' (PP = 1, BS = 87 %, TBE = 98 %), formed by at least 12 species, including the types of *Diderma* (*D. globosum*) and *Lepidoderma* (*L. tigrinum*).

The 'Clade 5, *Kelleromyxa* + *Fuligo muscorum*' received maximum support (PP = 1, BS = 100 %, TBE = 100 %) and is constituted by three specimens of *K. fimicola* and four of *F. muscorum*, each species forming a well-defined and highly supported clade (PP = 1, BS = 100 %, TBE = 100 %).

The 'Clade 6, *Physaraceae* s.str.' is highly supported (PP = 1, BS = 97 %, TBE = 100 %), and formed by all members of the family *Physaraceae* analysed in this study. Within this group, there is one early diverging, well differentiated and fully supported group ('Subclade 6-I') and a large polytomy constituted by all other members of this family, distinguished here in 15 monophyletic groups (subclades '6-II' to '6-XVI'), plus two unassigned species (*Ph. lateritium* and *Ph. australiense*). Most subclades arising from this polytomy comprise representatives from different genera. See Discussion and Taxonomy for additional details on the members of each clade and subclade.

There is one additional species whose analysed specimens showed two distant taxonomic placements: *Diderma spumarioides*. Two specimens (MCCNNU2749 and Meyer 46870) formed a clade with maximum support (PP = 1, BS = 100 %, TBE = 100 %) closely related to *Diachea*, a relationship supported by the Bayesian analysis (PP = 0.95 %) and transfer bootstrap (TBE = 97 %), but not by standard bootstrap (BS = 51 %). One isolated specimen (Meyer 45939) was placed as sister to clades (5) + (6), but the relationships at this level are only supported by transfer bootstrap (PP = 0.94, BS = 30 %, TBE = 99 %).

Morphological character evaluation

All morphological traits evaluated here are homoplastic to some extent, since they are present in different clades distributed along the tree (Fig. 3). Specifically, the badhamioid capillitium

(Fig. 1h) is found in the subclades '3-I', '6-I', 6-III', '6-VII', '6-VIII' and '6-XV'. Species with a capillitium with two morphologies, interpreted in the literature as a 'duplex capillitium', appear in the subclades '6-V' (*Physarella oblonga*), '6-XI' (*Leocarpus fragilis*), and also in *Badhamiopsis* and *Willkommlangea* ('6-II' and '6-XIV', respectively). Strictly sporocarpic species are distributed throughout the tree (see Fig. 3; Table S5), species developing as aethalia appear in seven different subclades ('2-V', '2-VI', '5-II', '6-I', '6-V', '6-VI' and '6-VIII'), and those that frequently form sporocarps or plasmodiocarps are distributed in multiple monophyletic groups, i.e., subclades '2-II' to '2-VI', '4-I', 4-II', '6-I' to '6-III' and '6-VI' to '6-XV', along with the '*Polyschismium* clade' and '*Didymium anellus*'. Some species rarely forming plasmodiocarps are found in the following subclades: '2-II', '2-IV', '2-V', '3-II', '4-II', '6-I', '6-V', '6-VIII', '6-X' and '6-XVI'. Most species analysed here are either sessile or can be both stalked or sessile, with the absence of the stipe occurring many times ('*Polyschismium* clade' plus subclades '2-II' to '2-VI', '3-I', '3-II','4-I', '4-II', '5-I', '6-I' to '6-III' and '6-V' to '6-XVI'. Species with a single peridium (Fig. 1d) are spread across the tree, those with a double peridium also appear in all six main clades, while a few species with a three-layered peridium (Fig. 1e) are found in '*Polyschismium* clade', '*Diderma* clade' and three subclades within the '*Physaraceae* clade', i.e., '6-XI', '6-XV' and '6-XVI'.

The columella is or can be present in some species belonging to five of the six major monophyletic groups in Fig. 3, with the exception of the '*Kelleromyxa* + *Fuligo muscorum* clade'. Within the '*Physaraceae* clade', the columella is present only in some species of the subclades '6-I' and '6-V', and *Ph. australiense*. Finally, species with clustered spores not easily dissociating form part of the subclades '6-I', '6-III', '6-VII' and '6-XV'.

Molecular signatures

A summary of the different molecular signatures found for most monophyletic groups recovered in our multigene phylogeny is presented in Table S6. As this table indicates, most clades and subclades presented one or more molecular signatures but no unique molecular synapomorphies were found for a minority of monophyletic groups, even after inspecting all four genes analysed in this study. Full details of the molecular signature(s) found for each clade or subclade are given in the Taxonomy section.

DISCUSSION

Extensive morphological homoplasy

From the mapping of different phenotypic traits on our multilocus tree of *Physarales*, we can now confirm that the macroscopic characters defining the genera of this order may have undergone frequent reversal and/or parallelism (homoplasy) during evolution. Consequently, the order *Physarales*, and especially the family *Physaraceae*, is a paradigm of taxonomic confusion given that earlier authors may have overemphasized on macromorphology as the main diagnostic criterion. For example, the aethalioid fruiting body used to define the polyphyletic genus *Fuligo* seems to be a derived character, which may have been acquired, at least, six independent times (*Mucilago crustacea*, *Didymium yulii*, *Fuligo muscorum*, *F. laevis*, and at least twice in the subclades '6-V', '6-VI' and '6-VIII' considered altogether). While, the stalk has been lost/acquired several times, exemplified by the occurrence of both stalked and sessile species in the same monophyletic groups. Likewise, although the vast majority of *Physarales* have free spores, these are arranged in clusters in some species distributed across different parts of the tree, and the same happens with the calcification of the capillitium (i.e., species with a badhamioid capillitium do not form a monophyletic group). In addition to homoplasy, polymorphism is also related to some of the mentioned characters. For example, the degree of calcification of the capillitium has been confirmed to be variable (Eliasson & Adamonyte 2009), and certain species can develop different sporophore types depending on the substrate (Ronikier & Lado 2013). Thus, the genus *Badhamia* is a prime example illustrating how the strong systematic importance placed on both the clustered spores and the badhamioid capillitium resulted in the circumscription of a highly polyphyletic taxon.

In our judgment, the high degree of morphological homoplasy and polymorphism calls for caution when establishing generic circumscriptions and intrafamilial relationships, so that further research is warranted to explore unnoticed non-homoplastic synapomorphies, possibly ultrastructural ones. Nonetheless, certain observed phenotypic characters or combinations of characters may be informative and even diagnostic within particular groups. For instance, this is the case of the combination 'capillitium type + type of fructification', valid to recognise and distinguish the genera *Willkommlangea* and *Leocarpus*.

It is also important to stress that the phylogenetic relationships recovered in this study do not correlate with the ecogeographic features of the species analysed. In this sense, species representing an ecological guild, e.g., nivicolous, succulenticolous, foliicolous or lignicolous taxa, are dispersed across the tree. Similarly, individuals of the same species collected in distant locations grouped together without a clear geographical pattern, although the present study is not focused on species delimitation and other patterns may be observed with additional sampling.

First steps towards a new classification of the order Physarales

Generic delimitations in *Physarales* have been much debated in the literature (Lado & Eliasson 2022), and based on the monophyletic groups found in our multi locus phylogeny (Fig. 3), a deep revision of the taxonomy of the order is necessary. Nonetheless, considering that all studied phenotypic characters are affected by homoplasy, at least to some extent, that the backbone of the phylogeny is still not fully solved as noted above, and that many species and some genera, e.g., *Trabrooksia* or *Physarina*, are still lacking, adopting a totally new classification would be imprudent and futile, as it may undergo major changes in the close future. Instead, we present an updated classification for the order *Physarales*, which incorporates only those nomenclatural changes we believe well justified by our findings, in this way contributing to nomenclatural stability.

The division of *Physarales* into families seems challenging. While *Physaraceae* s.str. is rather well-circumscribed, at least in terms of phylogenetic relationships, *Didymiaceae* is repeatedly recovered as paraphyletic to *Physaraceae*, both in previous studies (Fiore-Donno et al. 2012, 2019, Erastova et al. 2013, Kamono et al. 2013, Kretzschmar et al. 2016, Prikhodko et al. 2023), and in our analyses. One possible solution would be to recognise each major clade in *Didymiaceae* s.lat. (i.e., 1–4) as an independent family. However, that would imply that each of those putative families would contain a single genus, which would not provide much additional information or practical usefulness. Besides, the branches connecting these four main clades are considerably short and with low to moderate support (i.e., there are comparatively few synapomorphies supporting them), so alternative topologies cannot be discarded. For these reasons, and because *Didymiaceae* can still be morphologically diagnosed (capillitium generally not calcareous and calcareous columella frequently present; in the very few species with calcareous capillitium the lime is, at least, partially crystalline), we argue for temporarily maintaining *Didymiaceae* in its traditional

sense (Nannenga-Bremekamp 1991, Poulain et al. 2011, Lado & Eliasson 2022). From a morphological point of view and showing an enhanced consistency with phylogenetic relationships, another option would be recognising two families, i.e., *Didymiaceae* s.lat., formed by species with crystalline calcareous deposits (at least, partially), and *Didermaceae*, constituted by species presenting exclusively globular lime deposits, as pointed out by Krzemieniewska (1960). *Diderma tigrinum*, however, would be an exception within a putative *Didermaceae* by having crystalline plates.

The family *Kelleromyxaceae* could be recognised either as traditionally, i.e., including only the morphologically highly divergent genus *Kelleromyxa*, or it could include *Fuligo muscorum*, according to our results. The relationship between these two taxa does not seem to be spurious judging from the considerably long branch and high support obtained in our analyses, but the group *Kelleromyxa*/*F. muscorum* cannot be diagnosed based on phenotypic characters. If *F. muscorum* is excluded, then the problem would be similar to the one already mentioned for *Didymiaceae*: a new family name for only the *F. muscorum* clade would be needed, and then both *Kelleromyxaceae* and this putative new family would contain a single genus (and perhaps a single species), adding little new meaning to the names at the rank of family.

Finally, the family *Physaraceae* is considered rather well-defined and well-supported in a strict sense, when excluding two species groups formerly included in it: species of *Craterium* p.p. (+ *Badhamia lilacina*) with a true calcareous columella (with a somewhat crystalline lime), which should be included in *Diachea* and *Fuligo muscorum* s.lat., commented above. Therefore, the presence of lime in the capillitium is not anymore exclusive of *Physaraceae*, although the mentioned exceptions are truly very few when considering the number of species known in *Physarales*. More details on the morphology of these species are provided next in the taxonomic part. Alternatively, the family *Kelleromyxaceae* could be merged within *Physaraceae* in agreement with some other taxonomic treatments (e.g., Poulain et al. 2011, Leontyev et al. 2019, Lado & Eliasson 2022), in which case the sole exception of species with calcareous capillitium occurring outside *Physaraceae* would be the group of *Craterium* p.p. (+ *Badhamia lilacina*) with true columella. The drawback of this last approach is that the major clade including both clades 5 and 6 is only supported by transfer bootstrap in the ML analysis.

At the generic level, only some of the several clades distinguished in Fig. 3 correspond to currently accepted genera. When the groups are well-supported, the relationships among nearby clades are reasonably certain, and it is possible to diagnose them in terms of molecular and morphological characters, then we performed any required nomenclatural changes, e.g., we resurrected some validly published old genera or proposed new genera, with their corresponding combinations. Other clades with more uncertain relationships, or lacking statistical support or diagnosability, may also contain species representing the types of validly published genera that have been synonymised in previous studies, but formal combinations in these available generic names are avoided and discouraged until additional data (providing further resolution) are gathered; these generic names are used here only tentatively. The genera *Physarum*, *Fuligo* and *Badhamia* are thus maintained even if they are polyphyletic since, eventually, some of their respective members could cluster together when additional data are analysed, resulting in more easily diagnosable groups based on phenotypic characters. For simplicity, the accepted genera, those newly proposed here, and the different informal or tentative groups are listed in the next section, 'Taxonomy', following the order in which they appear in Fig. 3.

TAXONOMY

For each genus, the following information is provided: i) name of the taxon with reference to the protologue and indication of the clade in our phylogeny; ii) MycoBank number (only for new nomenclatural proposals); iii) etymology (only for new taxa); iv) species name which type corresponds to the type of the generic name (indicated as 'type species' by editorial command), and current species name if different; v) synonymous generic names with their types and current names; vi) morphological diagnosis; vii) molecular diagnosis following the example of Sheikh et al. (2018) (the positions of the signatures are indicated between brackets, followed by the name of the corresponding gene); viii) the accepted species (or taxa) included (only those assessed in this study); ix) new combinations at species level (if applicable) with reference to their basionyms; and x) notes. For further information regarding homo- and heterotypic synonyms, see Lado (2005–2023). We followed the rules of the ICN (Turland et al. 2018) for nomenclatural purposes, since *Myxomycetes* are explicitly mentioned as affected by the provisions of this Code in the Preamble (Pre. 8, as 'slime molds').

Polyschismium Corda, Icon. Fungorum 5: 20. 1842 [**Clade 1**]

Type species. *Leangium trevelyanii* Grev., Scott. Crypt. Fl. 3 (27): pl. 132. 1824.

Obligate synonym. *Polyschismium trevelyanii* (Grev.) Corda, Anleit. Stud. Mykol. 81: 202. 1842.

Doubtful synonym. *Lepidodermopsis* R. Wilczek & Meyl., Bull. Soc. Vaud. Sci. Nat. 58: 179. 1934 (nom. illeg., Art. 53.1, non *Lepidodermopsis* Höhn., 1909).

Type species. *Lepidodermopsis vermicularis* R. Wilczek & Meyl., Bull. Soc. Vaud. Sci. Nat. 58: 179. 1934 (according to Kowalski (1971), a sclerotial state of a myxomycete with crystalline scales on the peridium, perhaps a synonym of *Polyschismium granuliferum* (W. Phillips) Ronikier et al., see below).

Morphological diagnosis — Sporophores plasmodiocarpic or sporocarpic, sessile or very shortly stipitate. Peridium single to triple, cartilaginous or membranous, with large prismatic calcareous plates, dehiscing irregularly. Occasionally stellate dehiscence. Columella present or absent, calcareous. Capillitium limeless, formed by slender tubules, occasionally with calcareous nodes and reticulate. Spores, generally, densely spinulose (Fig. 2a), but variation occurs (e.g., *P. nevadense* and *P. cristatosporum*).

Molecular diagnosis — TAANAT (128–133, nSSU), ATATT (469–473, nSSU); YYGAAATAG (28–36, mtSSU), KACCRAC (78–84 mtSSU), GTCCTTAAGA (273–282, mtSSU); W (137, *α-Tub*) combined to C (167, *α-Tub*), A (206, *α-Tub*) and T (*α-Tub*, 221).

Accepted species. *Polyschismium alpestroides* (Mar. Mey. & Poulain) A. Ronikier, J.M. García-Martín, A. Kuhnt, J.C. Zamora, M. de Haan, Janik & Lado, *P. carestianum* (Rabenh.) A. Ronikier, J.M. García-Martín, A. Kuhnt, J.C. Zamora, M. de Haan, Janik & Lado, var. *carestianum*, *Polyschismium carestianum* var. *pseudocarestianum* (G. Moreno, A. Sánchez, Mar. Mey., López-Vill. & A. Castillo) Prikhodko, Shchepin, Novozh., G. Moreno, López-Vill. & Schnittler, *P. chailletii* (Rostaf.) A. Ronikier, J.M. García-Martín, A. Kuhnt, J.C. Zamora, M. de Haan, Janik & Lado, *P. fallax* (Rostaf.) A. Ronikier, J.M. García-Martín, A. Kuhnt, J.C. Zamora, M. de Haan, Janik & Lado, *P. granuliferum* (W. Phillips) A. Ronikier, J.M. García-Martín, A. Kuhnt, J.C. Zamora, M. de Haan, Janik & Lado, *P. peyerimhoffii* (Maire & Pinoy) A. Ronikier, J.M. García-Martín, A. Kuhnt, J.C. Zamora, M. de Haan, Janik & Lado, *P. perforatum* (Mar. Mey. & Poulain) A. Ronikier, J.M. García-Martín, A. Kuhnt, J.C. Zamora, M. de Haan, Janik & Lado, *P. trevelyanii* (Grev.) Corda.

Notes — The 'Clade 1, *Polyschismium*' of our multigene phylogeny includes all the species traditionally placed in the genus *Lepidoderma* analysed here, except for the type, *L. tigrinum* (see observations under the genus *Diderma*). This clade also

Fig. 4 Macromorphology of some members of the '*Polyschismium* clade' and '*Didymium* clade'. a. *Polyschismium trevelyanii* (MA-Fungi 14311); b. *Polyschismium chailletii* (MA-Fungi 90017); c. *Didymium xerophilum* (MA-Fungi 86877); d. *Didymium melanospermum* (MA-Fungi 91238); e. *Didymium phloiogenum* (211egc, formerly *Protophysarum phloiogenum*); f. *Didymium difforme* (MA-Fungi 89922); g. *Didymium anellus* (MA-Fungi 87954); h. *Didymium dubium* (MA-Fungi 89924). — Scale bars: a, c = 0.5 mm; b, f–h = 1 mm; d = 0.2 mm; e= 0.1 mm.

comprises *P. fallax*, formerly included in the genus *Diderma.* The sporophores of this species can bear a more or less densely covering of lime squamules, giving it the appearance of other *Polyschismium* species, with which it shares large spines as sporal ornamentation (Meylan 1927, Martin & Alexopoulos 1969, Lado et al. 2005, Poulain et al. 2011, Moreno et al. 2018).

The genus *Polyschismium* has just been resurrected by Ronikier et al. (2022) to accommodate 10 predominantly nivicolous species previously ascribed to *Diderma*. All of them, including *Polyschismium trevelyanii* (Fig. 4a), the type of this genus, are stemless to very shortly stalked, and have a non-calcareous capillitium, a limy columella, and a multi-layered peridium covered by lime squamules (with the exception of *P. chailletii* that has a single peridium, Fig. 4b). Both our study and that of Ronikier et al. (2022) were simultaneously under review, hence the shared authorship of the new combinations.

We have only considered here the species analysed in this study, although *Polyschismium* also includes *P. crustaceum*, *P. neoperforatum* (Ronikier et al. 2022), *P. aggregatum*, *P. echinosporum*, *P. nevadense* and, most likely, *P. cristatosporum* (Prikhodko et al. 2023). In this way most of the remaining *Lepidoderma* s.lat. also belong to *Polyschismium*, with the exception of the prominently stipitate *L. crassipes* and *L. stipitatum*, synonymised with *L. tigrinum* and *D. floriforme*, respectively (Ronikier et al. 2022).

Didymium Schrad., Nov. Gen. Pl.: 20 (1797) **[Clade 2]**

Type species. Didymium farinaceum Schrad., Nov. Gen. Pl.: 22.1797. (Nom. illeg., Art. 52.1, *Physarum melanospermum* Pers., Neues Mag. Bot. 1: 88. 1794 was cited as a synonym.)

Obligate synonym. Didymium melanospermum (Pers.) T. Macbr., N. Amer. Slime-moulds, ed. 1: 88. 1899.

Typus. Neotype designated here for *Ph. melanospermum* Pers., Spain, Cáceres, Jarandilla de la Vera, on *Pinus pinaster*, 28 Dec. 2014, *J.C. Zamora* s.n. (MA-Fungi 91238, MBT 10009957).

Synonyms. Cionium Link, Ges. Naturf. Freunde Berlin Mag. Neuesten Entdeck. Gesammten Naturk. 3: 28. 1809.
Type species. Not designated in the protologue. Although the name *Cionium farinaceum* (Schrad.) Nees, Syst. Pilze: 114. 1816. (or authorship variants) is listed as the type in some databases (e.g., Index Fungorum, MycoBank), we are not aware of any publications where such typification is effectively published.
Mucilago P. Micheli ex F.H. Wigg., Prim. Fl. Holsat.: 112. 1780.
Type species (designated here): *Mucilago crustacea* P. Micheli ex F.H. Wigg., Prim. Fl. Holsat.: 112. 1780. MBT 10009956.
Mucilago Haller, Hist. Stirp. Helv. 3: 110. 1768. Nom. illeg. (Art. 53.1).
Spumaria Pers., in Gmelin, Syst. Nat., ed. 13 (Leipzig), 2: 1466. 1792.
Type species. *Spumaria mucilago* Pers., in Gmelin, Syst. Nat., ed. 13 (Leipzig), 2: 1466. 1792. (Nom. illeg., Art. 52.1, *Mucor spongiosus* Leyss., Fl. Halens., editio altera: 305 (1783) was cited as a synonym.)
Amphisporium Link, Ges. Naturf. Freunde Berlin Mag. Neuesten Entdeck. Gesammten Naturk. 7: 41. 1815.
Type species. *Amphisporium versicolor* Link, Ges. Naturf. Freunde Berlin Mag. Neuesten Entdeck. Gesammten Naturk. 7: 41. 1815.
Disporium Léman, Dict. Sci. Nat. 13: 351. 1819. (Nom. illeg., Art. 52.1, published as a replacement name of *Amphisporium* Link, 1815.)
Type species. *Disporium versicolor* (Link) Léman, Dict. Sci. Nat. 13: 351. 1819.
Lepidodermopsis Höhn., Sitzungsber. Kaiserl. Akad. Wiss., Math.-Naturwiss. Cl. 118: 438. 1909. (Non *Lepidodermopsis* R. Wilczek & Meyl., 1934).
Type species. *Lepidodermopsis leonina* (Berk. & Broome) Höhn., Sitzungsber. Kaiserl. Akad. Wiss., Math.-Naturwiss. Cl. 118: 439. 1909.
Squamuloderma Kowalski, Mycologia 64: 1282. 1973 '1972'.
Type species. Squamuloderma nullifilum Kowalski, Mycologia 64: 1284. 1973 '1972' as '*nullifila*'.
Pseudodidymium R. Michel, Walochnik & Aspöck, Acta Protozool. 42: 342. 2003.
Type species. Pseudodidymium cryptomastigophorum R. Michel, Walochnik & Aspöck, Acta Protozool. 42: 342. 2003.
Protophysarum M. Blackw. & Alexop., Mycologia 67 (1): 33. 1975.
Type species. Protophysarum phloiogenum M. Blackw. & Alexop., Mycologia 67: 33. 1975.

Morphological diagnosis — Sporophores sporocarpic, plasmodiocarpic or only rarely aethalioid, stalked or sessile. Peridium calcareous, lime in the form of stellate crystals, sometimes compacted and forming a crust, only exceptionally, peridium without conspicuous lime deposits. Stalk often present, limeless or calcareous, usually continuing into a columella, which may be sometimes absent or replaced by a pseudocolumella. Capillitium present, rarely absent, consisting of slender limeless tubules, only occasionally containing lime crystals.

Molecular diagnosis — This genus differs from all others by having CTC in positions 584–586 of the *EF-1α* gene combined with AA in positions 593–594, G in 618 and C in 663. Within it, molecular signatures have been found for the different groups of related species (subclades).

a. Subclade 2-I: AAGGCTTC (252–259, nSSU); ACAACCCCGTTTT (295–307, *EF-1α*), TCCCATCTCC (379–388, *EF-1α*), TTTCGCCCCCGGA (607–619, *EF-1α*), CTCTTGAG (893–900, *EF-1α*); GAGGCGAAATT (99–107, mtSSU), GTCCTTAG (273–280 mtSSU); AAGACTCTCCGTT (50–62, *α-Tub*), AGCTGTGGTGGAGCCT (122–137, *α-Tub*).
b. Subclade 2-II: GCCTTGY (107–113, nSSU); ATCCCCCAACCTTG (418–431, *EF-1α*); CTAT-TTGAGTTCTAAAGGCGA (84–105, mtSSU), GGCGGAAGCGG (164–175, mtSSU); AGACCCACCTACACCAACTTG (246–266, *α-Tub*).
c. Subclade 2-III: TCTGTCCTTCTCGACTT (642–658, nSSU); AACCCTCAAGCCTAA (580–594, *EF-1α*), ATTCCC (677–682, *EF-1α*); AAATCTATG (67–75, mtSSU), GCCGAAG (158–164, mtSSU); TACCCACTCTCTCCTC (155–170, *α-Tub*).
d. Subclade 2-IV: ATTGRACATCGT (424–435, nSSU); GTGACCATC (65–73, *EF-1α*); TGGGCGA (99–105, mtSSU); CTTATGTACCGTGGA (534–548, *α-Tub*).
e. Subclade 2-V: TWMGAATC (110–117, mtSSU); TTGGATAAC (195–203, *α-Tub*).
f. Subclade 2-VI: TGCAATGCAT (428–437, nSSU); AGGAGACRTTCGAAR (150–164, mtSSU), GATCGAC (177–183, mtSSU).

Accepted species. Didymium anellus Morgan, *D. azorellae* D. Wrigley, Lado & Estrada, *D. bahiense* Gottsb., *D. clavus* (Alb. & Schwein.) Rabenh., *D. comatum* (Lister) Nann.-Bremek., *D. difforme* (Pers.) Gray, *D. dubium* Rostaf., *D. eximium* Peck, *D. floccosum* G.W. Martin, K.S. Thind & Rehill, *D. iridis* (Ditmar) Fr., *D. infundibuliforme* D. Wrigley, Lado & Estrada, *D. leptotrichum* (Racib.) Massee, *D. megalosporum* Berk. & M.A. Curtis, *D. melanospermum* (Pers.) T. Macbr., *D. minus* (Lister) Morgan, *D. nigripes* (Link) Fr., *D. nivicola* Meyl., *D. operculatum* D. Wrigley, Lado & Estrada, *D. pertusum* Berk., *D. phloiogenum* (M. Blackw. & Alexop.) J.M. García-Martín & Lado, *D. pseudonivicola* Janik, A. Ronikier & Lado, *D. quitense* (Pat.) Torrend, *D. radiaticolumellum* Bellido, G. Moreno, Mar. Mey. & J.F. Moreno, *D. spongiosum* (Leyss.) J.M. García-Martín, J.C. Zamora & Lado, *D. squamulosum* (Alb. & Schwein.) Fr. & Palmquist, *D. tehuacanense* Estrada, D. Wrigley & Lado, *D. umbilicatum* D. Wrigley, Lado & Estrada, *D. verrucisporum* A.L. Welden, *D. xerophilum* Lado, Estrada & D. Wrigley, *D. yulii* S.-Y. Liu & F.-Y. Zhao.

Didymium spongiosum (Leyss.) J.M. García-Martín, J.C. Zamora & Lado, *comb. nov.* — MycoBank MB 846253

Basionym. *Mucor spongiosus* Leyss. Fl. Halens., Ed. Altera: 305. 1783.

Typus. Lectotype designated here, f. 2 of plate 96 in Micheli (1729, Nov. Pl. Gen. (Florentiae), MBT 10009954). Epitype designated here, to support the lectotype cited above, Spain, Valencia, Fontanars dels Alforins, N38°48'02" W00°46'39", 623 m, on *Brachypodium retusum*, 08 Jan. 1997, *M. Oltra* 1904 (MA-Fungi 39871, MBT 10009953).

Synonyms. Mucilago crustacea P. Micheli ex F.H. Wigg., Prim. Fl. Holsat. 112. 1780. (Non *D. crustaceum* Fr., 1829).
Typus. Neotype designated here (MA-Fungi 39871, see specimen data above, MBT 10009956).
Spumaria mucilago Pers., in Gmelin, Syst. Nat., ed. 13 (Leipzig), 2: 1466. 1792. Nom. illeg., Art. 52.1 (*Mucor spongiosus* Leyss. cited in synonymy).
Didymium mucilago Prikhodko, Shchepin, Novozh., Schnittler & Stephenson. Mycol. Progress 22, 2023. (Published as a replacement name of *Mucilago crustacea* but lacking priority, Art. 11.4 final sentence).

Didymium phloiogenum (M. Blackw. & Alexop.) J.M. García-Martín & Lado, *comb. nov.* — MycoBank MB 846255

Basionym. Protophysarum phloiogenum M. Blackw. & Alexop., Mycologia 67: 33. 1975.

Typus. USA, Colorado, Boulder, on bark of living *Ulmus americana* in moist chamber, 21–27 May 1965, *C.J. Alexopoulos* UTMC-519 (BPI 737584).

Notes — Our concept of *Didymium* includes all *Didymium* species analysed plus *Mucilago crustacea* and *Protophysarum phloiogenum*, the genus becoming monophyletic in this way. This large group is characterised by the crystalline peridial lime loosely scattered or forming a crust, or eggshell-like, but never in the form of scales (Alexopoulos 1982), with the exception of *P. phloiogenum*, in which the lime deposits are not conspicuous (see comments below). In our analyses, *Mucilago crustacea* is deeply nested in *Didymium* (Fig. 3), as several previous molecular studies have shown (Fiore-Donno et al. 2008, 2010a, Prikhodko et al. 2023), but against others (Nandipati et al. 2012). Despite that the extreme morphological parallelism between *Didymium* and *Mucilago* has been debated since the 20th century, when Lister (1925) noted that 'the sporangia (of *Mucilago*) are confluent to form an aethalium, otherwise the characters are those of the genus *Didymium*', early authors defended the idea that species with such distinct fruiting bodies could not belong to the same genus. However, our analyses with a wide sampling of *Didymium* species leave little doubts about the phylogenetic position of *M. crustacea* within *Didymium*. Moreover, one aethalioid species of *Didymium* has been recently described (Zhao et

al. 2021), so the erection of a genus exclusively based on the type of fructification is not justified.

From a nomenclatural point of view, the genus *Mucilago* (1780) was coined before *Didymium* (1797) that, currently, with more than 90 species, is the second largest myxomycete genus after *Physarum* (Lado 2005–2023). Following the principle of priority of the ICN (Turland et al. 2018), a large number of new combinations would be required to provide nomenclatural consistency. However, to avoid losing the use of this well-known generic name and for the sake of simplicity, a proposal to conserve *Didymium* against *Mucilago* and its synonym *Spumaria* has already been published (Zamora et al. 2023). According to Rec. 14A.1, we currently use the name *Didymium* for all included species. The combination of *Mucilago crustacea* under *Didymium* is precluded by the existence of the validly published *D. crustaceum*, a different species. Thus, we have combined the next available synonym, *Mucor spongiosus*, under *Didymium*, following Art. 11.4 (last sentence).

No original material (either cited in the protologue or not) is known to exist for *M. crustacea*, and consequently we designate the strain MA-Fungi 39871 as neotype as it is well-preserved, agrees with the concept of the species, and it has data for all four genes analysed. We have also lectotypified the name *Mucor spongiosus* (basionym of *D. spongiosum*) with Micheli's (1729) pl. 96, f. 2, the only original material known to exist. Since the taxonomic identity of this illustration is ambiguous (it could potentially represent a species of the genus *Fuligo* (*Physaraceae*), or even *Brefeldia* (*Stemonitidales*)), we have also designated a recently collected, sequenced and unambiguous specimen as epitype for *Mucor spongiosus* (the same designated as neotype for *Mucilago crustacea*).

The typification of *D. melanospermum*, the type of the genus *Didymium*, was also investigated. No original material seems to exist, as no illustrations or specimens were cited in the protologue, and no material under the name *Ph. melanospermum* is kept in Persoon's herbarium (L). Therefore, we have designated an abundant and recently collected specimen agreeing with the protologue and with its current concept, of which we have molecular data for all four DNA regions. These actions clarify the application and preserve the current usage of all mentioned names.

We can distinguish six well-supported subclades plus two rather isolated species that could potentially represent different lineages worth of taxonomic recognition. Clark & Haskins (2018) recognised six groupings of stipitate species within *Didymium*, but we only recovered what they called 'empty tube non-calcareous long stipe species group' (see below), while members of other assemblages (e.g., *D. xerophilum*, *D. tehuacanense* and *D. pertusum*, from their 'refuse matter or lime filled tube group' or *D. operculatum* from the 'empty tube externally calcareous striated stipe group') are scattered across the genus. Based on complete nSSU sequences Fiore-Donno et al. (2010a) considered two groups, i.e., '*Didymium* 1' (paraphyletic), and '*Didymium* 2' (monophyletic), the former including *M. crustacea*. Using partial nSSU sequences, Kamono et al. (2013) observed the same two groups, but '*Didymium* 1' also included *Protophysarum phloiogenum* and *Diachea subsessilis*, apart from *M. crustacea*. Considering that *Didymium* is rather well defined, that not all species have been here included (which could result in a different branching pattern), and that this genus has appeared as monophyletic in most previous molecular studies, we do not recommend splitting it into several smaller genera until convincing evidence to do so is produced.

a. Subclade 2-I: Five specimens of *D. xerophilum* (Fig. 2b, 4c) appear clearly separated as the earliest diverging species of the 'Clade 2, *Didymium*', forming the fully supported 'Subclade 2-I' (Fig. 3). It can be distinguished from all its congeners because of the exclusive character combination 'funnel-shaped invagination of the peridium + calcareous stalk + columella' (Wrigley de Basanta et al. 2015), and differs from its morphologically closest species, *D. infundibuliforme* in that the latter does not have a columella (Wrigley de Basanta et al. 2009).
b. Subclade 2-II: This subclade harbours the type of the genus *Didymium*, *D. farinaceum* (≡ *D. melanospermum*), Fig. 4d, and also *D. minus*, and *D. floccosum*. They share a subglobose or slightly flattened sporotheca deeply but narrowly umbilicate below, covered by an areolate peridium frosted with stellate crystals, a dark columella and an ochraceous to dark-brown, stout stalk, which give them a very similar general appearance. However, no apomorphies could be found for this subclade, as the mentioned traits are present in many other species of *Didymium*, such as *D. nigripes*.
c. Subclade 2-III: Sister to the species *Protophysarum phloiogenum* (Fig. 4e, see below), it is found a group formed by *D. comatum* and *D. difforme* (Fig. 4f), both developing as small, sessile, sporocarpic to plasmodiocarpic fruiting bodies strongly calcified, with abundant lime crystals densely compacted forming an eggshell-like covering. The taxonomy of these species is still intricate, and some authors have considered them as varieties of a single species (Lister 1901, Lyon 1977, Clark & Haskins 2014), or as different species (Nannenga-Bremekamp 1966). This would explain the intermingled pattern observed in Fig. 3.
d. Subclade 2-IV: This subclade is sister to *D. anellus* (Fig. 4g, see below) and unites plasmodiocarpic and sporocarpic species, sessile or stalked, having either a single (e.g., *D. dubium*, Fig. 4h–SEM, and *D. umbilicatum*, Fig. 5a), or double peridium (*D. azorellae*) with irregular or circumscissile dehiscence. At molecular level, three of these species (*D. azorellae*, *D. operculatum* (Fig. 2d) and *D. infundibuliforme* (Fig. 5b)) have an exclusive *α-Tub* intron (Table 1).
e. Subclade 2-V: This clade exhibits high morphological diversity as it merges species with sporocarpic (e.g., *D. clavus*, Fig. 5c), plasmodiocarpic (e.g., *D. quitense*, Fig. 5d) and aethalioid sporophores, with non-calcareous stalks (when present), and the columella can be present or absent. Since *Mucilago crustacea* (Fig. 5e) is included here, there are two possible options to ensure nomenclatural consistency: either subdivide *Didymium* into several genera or consider both genera *Mucilago* and *Didymium* as synonyms. As indicated above, we advocate for the latter option, as the alternative would require extensive nomenclatural changes and a better taxon sampling to firmly establish those putative new genera.
f. Subclade 2-VI: This group partially corresponds to the 'empty tube non-calcareous long stipe group' proposed by Clark & Haskins (2018). In particular, it accommodates *D. bahiense* (Fig. 5f), *D. eximium*, *D. megalosporum*, *D. nigripes* (Fig. 5g), and *D. verrucisporum*, recognisable by their long fibrous-membranous stalks, longitudinally striated, and their limy columella or pseudocolumella. All these five species were also included in the so-called 'long-stalked *Didymium* group' of Nannenga-Bremekamp (1972). However, this subclade also contains *D. squamulosum* (Fig. 2e, 5h), a species very variable in morphology (from stalked sporocarps to short plasmodiocarps) probably composed of distinct biological entities (ElHage et al. 2000), *D. pertusum*, which has a grooved stipe filled with calcareous nodules and no columella, and *D. yulii*, an aethalioid species. Thus, it seems difficult to find a morphological trait common to all these taxa.

Protophysarum and *Didymium anellus*: The sole species of the genus *Protophysarum*, viz., *P. phloiogenum* (Fig. 2c, 4e), is set apart by a very long branch (Fig. 3). Calcareous deposits

Fig. 5 Macromorphology of some members of the '*Didymium* clade'. a. *Didymium umbilicatum* (MA-Fungi 73566); b. *Didymium infundibuliforme* (MA-Fungi 78320); c. *Didymium clavus* (MA-Fungi 80283); d. *Didymium quitense* (MA-Fungi 95031); e. *Didymium spongiosum* (MA-Fungi 39871, formerly *Mucilago crustacea*); f. *Didymium bahiense* (MA-Fungi 89915); g. *Didymium nigripes* (MA-Fungi 80617); h. *Didymium squamulosum* (1igc). — Scale bars: a–c, f, h = 0.5 mm; d = 1 mm; e = 1 cm; g = 0.2 mm.

cannot be detected under a light microscope, but they are associated with the peridium and the capillitium at ultrastructural level (Blackwell 1974, Blackwell & Alexopoulos 1975). Besides, *P. phloiogenum* presents calcareous mitochondrial granules deposited in a highly similar way to the process reported in *D. squamulosum* (Blackwell 1974). *Protophysarum* was firstly placed in the family *Physaraceae* (Blackwell & Alexopoulos 1975) and later, given the absence of visible lime in the capillitium and peridium, Castillo et al. (1998) erected the family *Protophysaraceae* to accommodate it. However, our results (Fig. 3), which confirm Fiore-Donno et al. (2010a) and Cainelli et al. (2020) previous findings, show that *Protophysarum* is deeply nested within *Didymium*, or at the very least within the family *Didymiaceae*, so the recognition of the family *Protophysaraceae* is unjustified. *Protophysarum* could be considered either as a synonym of *Didymium* or as an independent genus. The latter possibility, however, would complicate the taxonomy of *Didymium* for little gain (several hardly diagnosable genera would need to be erected for the subclades discussed above), so that we have preferred to transfer *P. phloiogenum* to *Didymium*. On the other hand, *D. anellus* (Fig. 4g) is also set apart by a long branch. This species differs for all other members of the genus by a combination of features such as the formation of flattened plasmodiocarps with abundant lime crystals covering the peridium, but not as an eggshell, with circumscissile dehiscence, and an expanding capillitium.

Diachea Fr., Syst. Orb. Veg.: 143. 1825 [**Clade 3**]

Type species. Stemonitis elegans Trentep., in Roth, Catal. Bot. 1: 220. 1797.

Synonyms. Scyphium Rostaf., Sluzowce Monogr.: 148. 1874.
Type species: *Scyphium rubiginosum* Rostaf., Sluzowce Monogr.: 148. 1874.
Diachaeella Höhn., Sitzungsber. Kaiserl. Akad. Wiss., Math.-Naturwiss. Cl. 118: 436. 1909.
Type species: *Didymium bulbillosum* Berk. & Broome, J. Linn. Soc., Bot. 14: 84. 1873.

Morphological diagnosis — Sporophores sporocarpic, typically stalked, rarely sessile. Peridium persistent, membranous, with or without lime, often iridescent. Stalk usually calcareous, containing amorphous lime granules and/or crystals. True columella always present, continuous with the stalk, calcareous, also with lime granules or crystals. Capillitium varying from limeless to almost entirely calcareous, filiform, branched and anastomosed, originating from the columella and forming a net of tubules. Spores pilate to cristate-reticulate, with elements coronated by small warts or spine-like projections, sometimes inconspicuous (Fig. 2f, g, see also Keller et al. (2004)).

Molecular diagnosis — The genus *Diachea* differs from all others by having ATCCGCGGCCTACGGGTCG in positions 1106–1124 of the nSSU gene, TRKRG in positions 99–101 combined with TGA in 103–105 of mtSSU, and YGGTTTCGGWTCYCTYCTCCTYGAGAGACTT in positions 26–56 of the *α-Tub* gene. There are also several signatures for each of both subclades recognised within *Diachea*.

a. Subclade 3-I: ATCCCCCCAAGTGTCCACTGCTGTG (104–128, *α-Tub*), TGCCCCTGTC (401–410, *α-Tub*).
b. Subclade 3-II: CTCCAACACCACCGCCATYGCYGAA (719–743, *α-Tub*).

Accepted species. Badhamia lilacina (Fr.) Rostaf., *Diachea bulbillosa* (Berk. & Broome) Lister, *D. cylindrica* Bilgram, *D. dictyospora* (Rostaf.) J.M. García-Martín, J.C. Zamora & Lado, *D. leucopodia* (Bull.) Rostaf., *D. mitchellii* Lado & Treviño (Fig. 2g), *D. muscorum* (Ing) J.M. García-Martín, J.C. Zamora & Lado, *D. obovata* (Peck) J.M. García-Martín, J.C. Zamora & Lado, *D. radiata* G. Lister & Petch, *D. silvaepluvialis* M.L. Farr, *D. subsessilis* Peck.

Diachea dictyospora (Rostaf.) J.M. García-Martín, J.C. Zamora & Lado, *comb. nov.* — MycoBank MB 846256

Basionym. Badhamia dictyospora Rostaf., in Sluzowce Monogr. suppl.: 4. 1876.

Diachea obovata (Peck) J.M. García-Martín, J.C. Zamora & Lado, *comb. nov.* — MycoBank MB 846257.

Basionym. Craterium obovatum Peck, in Bull. Buffalo Soc. Nat. Sci. 1: 64. 1873.

Diachea muscorum (Ing) J.M. García-Martín, J.C. Zamora & Lado, *comb. nov.* — MycoBank MB 846258.

Basionym. Craterium muscorum Ing, in Trans. Brit. Mycol. Soc. 78: 443. 1982.

Notes — The 'Clade 3' is a well-defined, monophyletic and well-supported natural group, whose members exhibit several molecular signatures in different genes, and that we recognise here as an amended genus *Diachea*. *Diachea leucopodia* (Fig. 6a), and *D. radiata* have also been previously analysed in a phylogenetic context by Fiore-Donno et al. (2010a), but they did not form a clade and their relationships remained uncertain. The genus *Diachea* was first described by Fries (1825) placed in the 'subord. IV. Trichiacei', although it was later transferred to the 'subord. II. Myxogastres', 'sect. III. Stemonitei', due to its iridescent peridium and non-calcareous capillitial system (Fries 1829). Rostafiński (1874) emphasized its similarities to some *Physarales* (calcareous stalk and presence of a calcareous columella). Since then, it has been shifted back and forth between *Physarales* and *Stemonitidales* (Alexopoulos 1982, Kalyanasundaram & Mubarak Ali 1989, Lado 2005–2023). Within *Physarales*, *Diachea* has been placed either in *Physaraceae* (Lister 1925) or *Didymiaceae* (Keller & Braun 1999).

The somewhat unexpected inclusion of three species with cupulate sporophores, formally placed in *Craterium*, i.e., *C. dictyosporum*, *C. muscorum* (Fig. 2f) and *C. obovatum* (Fig. 6b), challenges its definition, as *Diachea* now includes some species with a decidedly calcareous capillitium. Nevertheless, these three deviant *Craterium* species have been treated as borderline with other genera, such as *Badhamia*, by other authors (e.g., Poulain et al. (2011)), and even an independent genus, *Scyphium*, was created to accommodate *C. obovatum* (as *S. rubiginosum*, Rostafiński (1874)). Moreover, the lime present in the stalk and columella is more or less crystalline, forming irregular aggregates to somewhat prismatic or rhombohedral crystals, which is a character previously recorded in other species of *Diachea* (Keller et al. 2004, Cavalcanti et al. 2009), but not in *Craterium* s.str. What certainly does not characterise the genus *Diachea* anymore is the absence of lime in the sporotheca, one of the characters emphasized in Prikhodko et al. (2023). The species *Badhamia lilacina*, formerly placed in *Craterium* as *Craterium lilacinum*, should also be transferred to *Diachea* on account of its phylogenetic relationships and morphological resemblance to the mentioned *Craterium* p.p. species (Massee 1892). However, for the time being, we prefer not to combine this species name because we have not seen the specimen included in the analyses (*B. lilacina* LE 317581).

Diachea, as currently circumscribed here, can be morphologically characterised by the combination of sporocarpic sporophores, with a conspicuous, true calcareous columella, and free spores (forming very loose clusters only in *D. mitchellii*, Lado et al. (2022)), with a complex pilate to cristate ornamentation, in which the main elements are warty or spiny at the apex. It can be separated in two well-supported groups according to our phylogenetic reconstructions, with clear molecular signatures and partially overlapping morphological traits:

a. Subclade 3-I: This group comprises subsessile to stipitate species with dark stalks and capillitium and peridium with varying degrees of calcification, from limeless to almost entirely calcareous. If considered useful, it could be recognised as an infrageneric taxon (e.g., subgenus or section) for which *Scyphium* could be used as basionym.
b. Subclade 3-II: *Diachea* species often with white or pale stalks (except *D. mitchellii*) and limeless or almost limeless capillitium and iridescent peridium. This group contains the types of both genera *Diachea* and *Diachaeella* (*D. leucopodia* and *D. bulbillosa*, respectively).

Diderma Pers., Neues Mag. Bot. 1: 89. 1794 [**Clade 4**]

Type species. Diderma globosum Pers., Neues Mag. Bot. 1: 89. 1794.

Synonyms. Leangium Link, Ges. Naturf. Freunde Berlin Mag. Neuesten Entdeck. Gesammten Naturk. 3: 26. 1809.
Type species. Diderma floriforme (Bull.) Pers., Neues Mag. Bot. 1: 89. 1794.
Chondrioderma Rostaf., Vers. Syst. Mycetozoen: 13. 1873.
Type species. Diderma testaceum (Schrad.) Pers., Syn. Meth. Fung. 1: 167. 1801.
Lepidoderma de Bary ex Rostaf., Vers. Syst. Mycetozoen: 13. 1873.
Type species. Didymium tigrinum Schrad., Nov. Gen. Pl.: 22. 1797.
Wilczekia Meyl., Bull. Soc. Vaud. Sci. Nat. 56: 68. 1925.
Type species. Wilczekia evelinae Meyl., Bull. Soc. Vaud. Sci. Nat. 56: 69. 1925.

Morphological diagnosis — Sporophores sporocarpic or plasmodiocarpic, sessile to prominently stalked. Peridium single

Fig. 6 Macromorphology of some members of the '*Diachea* clade' and '*Diderma* clade'. a. *Diachea leucopodia* (MA-Fungi 78316); b. *Diachea obovata* (MM39542, formerly *Craterium obovatum*); c. *Diderma acanthosporum* (MA-Fungi 90993); d. *Diderma hemisphaericum* (MA-Fungi 90961); e. *Diderma niveum* (L20749); f. *Diderma globosum* (MA-Fungi 87098); g. *Diderma floriforme* (MA-Fungi 36755); h. *Diderma tigrinum* (MA-Fungi 52983). — Scale bars: a = 0.2 mm; b, d–e, g–h = 0.5 mm; c, f = 1 mm.

to triple, membranous to calcareous, the outer layer often eggshell-like, containing globular lime granules, rarely with angular crystals or scales. Columella usually present, calcareous. Capillitium consisting of slender, branched and anastomosed, non-calcareous tubules.

Molecular diagnosis — No signatures are common to all members of this clade, but several ones have been found for each of the two different subclades distinguished in Fig. 3.

a. Subclade 4-I: ACGCTTCTCCTAAAGACTAAGCCATGCATGCY (1–32, nSSU); MTYGAGAAGKSMGACAAG (410–427, *EF-1α*); TGTGGKKATCA (210–220, mtSSU); CTTGGAYATTGATA (233–246, *α-Tub*).
b. Subclade 4-II: T (565, *EF-1α*) combined with A (595, *EF-1α*) and G (627, *EF-1α*); WTCYCCYCARGTCTCWACNGCYGTG (104–128, *α-Tub*).

Accepted species. *Diderma acanthosporum* Estrada & Lado, *D. alpinum* (Meyl.) Meyl., *D. cattiense* Novozh. & D.W. Mitch., *D. deplanatum* Fr., *D. effusum* (Schwein.) Morgan, *D. floriforme* (Bull.) Pers., *D. globosum* Pers. var. *globosum* Pers., *D. globosum* var. *europaeum* Buyck, *D. gracile* Aramb. (Fig. 2h), *D. hemisphaericum* (Bull.) Hornem., *D. kamchaticum* Novozh., Schnittler, Shchepin, Prikhodko, *D. meyerae* H. Singer, G. Moreno, Illana & A. Sánchez, *D. microcarpum* Meyl., *D. niveum* (Rostaf.) E. Sheld., *D. pseudotestaceum* Novozh. & D.W. Mitch., *D. radiatum* (L.) Morgan, *D. subasteroides* M.L. Farr, *D. tigrinum* (Schrad.) Prikhodko, Shchepin, Novozh., López-Vill., G. Moreno & Schnittler, *D. yucatanense* Estrada, Lado & S.L. Stephenson, *D. velutinum* Bornikov.

Notes — The '*Diderma* clade' of our multigene phylogeny largely corresponds to the previous concept of the genus *Diderma*, comprising most *Diderma* species, except for *D. fallax* and *D. spumarioides*, but also including *Diderma tigrinum*, placed in the genus *Lepidoderma* until very recently (Prikhodko et al. 2023). Given that this distant position from other former *Lepidoderma* (now *Polyschismium*) was repeatedly observed regardless the gene analysed (Fig. S1–S4), and that this has been previously and independently found in other studies (Fiore-Donno et al. 2010a, Erastova et al. 2013, Kamono et al. 2013, Novozhilov et al. 2013, Prikhodko et al. 2023), we are confident that this intergeneric relationship is not spurious. Until very recently, *Lepidoderma tigrinum* (Fig. 6h) has been considered one of the few non-nivicolous members of the traditional genus *Lepidoderma* (most species now included in *Polyschismium*), but it has just been transferred to *Diderma* (Prikhodko et al. 2023). It differs from the others by presenting a single membranous peridium, sparsely covered with large lime scales, and a distinctive stout furrowed stalk.Both morphological features separate it from other members of *Lepidoderma*, predominantly stemless and having a single to triple peridium densely covered with crystalline lime scales, lose or laterally united to form a crust-like outer wall. Indeed, Kowalski (1971) stated that '*L. tigrinum* does not appear to be closely related to any known species of *Lepidoderma*'. This evidence along with the fact that *Diderma* was erected before *Lepidoderma* lead to the need of transferring *L. tigrinum* to *Diderma*. Two other clearly stipitate *Lepidoderma* s.lat. species, *L. crassipes* and *L. stipitatum*, have been recently synonymised with *L. tigrinum* and *D. floriforme*, respectively (Ronikier et al. 2022), a conclusion accepted here.

Based on distinct morphological characteristics, Link (1809) recognised two genera, *Diderma* (peridium double) and *Leangium* (peridium simple). Taking into account the information provided by our own multigene dataset and, in agreement with Poulain et al. (2011), rather than supporting the subgeneric division of the genus into *D.* subg. *Diderma* and *D.* subg. *Leangium*, we distinguish the following two well-supported subclades:

a. Subclade 4-I: In this group we find mostly species with a double peridium, the outer porcelain-like, a stalk that, when present, is calcareous, and frequently a columella that appears as a coloured thickened base (often pinkish to brown), among others, *D. acanthosporum* (Fig. 6c) and *D. hemisphaericum* (Fig. 6d). The columella is, however, absent in *D. deplanatum*, which, contrary to the rest of members of this subclade, mainly fructifies as extensive plasmodiocarps. The sporotheca is often more or less flattened.
b. Subclade 4-II: This monophyletic subclade combines species with double and triple peridium. It includes the species of the so-called '*Diderma niveum-alpinum* complex' (Poulain et al. 2011), i.e., *D. alpinum*, *D. niveum* (Fig. 6e), *D. microcarpum*, *D. meyerae* and the type of *Diderma*, *D. globosum* (Fig. 6f). All of them have pale sporocarps with globose or subglobose sporothecae and larger spores than the species of the 'Subclade 4-I' (9–13 µm diam vs 6–9 µm diam). Furthermore, most of them have a prominent globose to hemispheric columella, and are predominantly nivicolous, with some exceptions, such as *D. floriforme* (Fig. 6g), *D. gracile*, *D. velutinum*, and *D. tigrinum* (Fig. 6h), or the colourful neotropical species *D. subasteroides* and the paleotropical *D. cattiense*. Many authors have discussed the similarities and differences of the species of this subclade and proposed several segregations and synonyms (Neubert et al. 1995, Ing 1998, Moreno et al. 2003, Stephenson et al. 2007, Lado & Ronikier 2008, Poulain et al. 2011, Novozhilov et al. 2013). The clustering pattern seems to indicate that neither the type of peridium nor the number of peridial layers is a reliable taxonomic trait for grouping the species of *Diderma*. For example, *D. subasteroides* and *D. gracile*, which are sister species, exhibit a three- and two-layered peridium, respectively. Similarly, the type of dehiscence does not seem to be a decisive character either as both species just mentioned show clearly different mechanisms, three layers sticking together during dehiscence vs two separating layers, respectively (Arambarri 1973).

Kelleromyxa Eliasson, in Eliasson, Keller & Schoknecht, Mycol. Res. 95: 1205. 1991 [**Subclade 5-I**]

Type species. *Kelleromyxa fimicola* (Dearn. & Bisby) Eliasson, Mycol. Res. 95: 1206. 1991.

Morphological diagnosis — Sporophores sporocarpic, fusiform, black, sometimes laterally compressed, erect on a constricted base. Peridium double, both layers closely adhered. Columella absent. Capillitium arising from the inner peridial layer as a sparse system of branching threads, sometimes unbranched and anastomosing, with dark nodules.

Molecular diagnosis — ACCCCCCGGGT (111–121, nSSU), CACCCCAAGGT (182–192, nSSU), GTACGGCAGT (300–309, nSSU), GGGACGTGCCG (314–324, nSSU); TTATTRACTAC (63–83, mtSSU), AACGTCCTTAAAAA (270–283, mtSSU). Moreover, this species has an *EF-1α* intron that does not interrupt the sequences of other *Physarales*, although it is present in *Ceratiomyxa fruticulosa* (Alignment S6).

Accepted species. *Kelleromyxa fimicola* (Dearn. & Bisby) Eliasson.

Notes — The genus *Kelleromyxa* poses a special taxonomic problem since *K. fimicola* (Fig. 7a) was initially placed in the order *Cribrariales* (Bisby et al. 1929). The reasons adduced were the seemingly lacking capillitium and the absence of conspicuous lime concretions in its shiny, black, spindle-shaped sporocarps. However, the presence of a true capillitium has been proved, consisting of short unbranched threads, while detectable amounts of calcium have been found on its cartilaginous peridium by using X-ray spectroscopy (Eliasson et al. 1991). Thus, based on these SEM and spectroscopic studies, *K. fimicola* was tentatively assigned to the order *Physarales*. This placement was later confirmed thanks to DNA analyses (Erastova et al. 2013), and it was then postulated as a separate monogeneric family representing a link between *Didymiaceae* and *Physaraceae*. We confirm the phylogenetic position of *Kelleromyxa* in our analyses, although the newly found strongly supported relationship with *Fuligo muscorum* raises doubts about the convenience of retaining *Kelleromyxaceae* as a different family (see Discussion).

Lignydium Link, Ges. Naturf. Freunde Berlin Mag. Neuesten Entdeck. Gesammten Naturk. 3: 24. 1809 [**Subclade 5-II**]

Type species. Lignydium griseoflavum Link, Ges. Naturf. Freunde Berlin Mag. Neuesten Entdeck. Gesammten Naturk. 3: 24. 1809.

Morphological diagnosis — Sporophores aethalioid, pulvinate, small, on a pallid or dull orange hypothallus. Peridium very thin, orange yellow to greyish yellow, bearing scattered

Fig. 7 Macromorphology of some members of the '*Kelleromyxa* clade' and '*Physaraceae* clade'. a. *Kelleromyxa fimicola* (LE274210); b. *Lignydium muscorum* (MM46947, formerly *Fuligo muscorum*); c. *Nannengaella mellea* (MA-Fungi 90062, formerly *Physarum melleum*); d. *Nannengaella globulifera* (MA-Fungi 46711, formerly *Physarum globuliferum*); e. *Nannengaella alpestris* (MA-Fungi 35213, formerly *Physarum alpestre*); f. *Nannengaella laevis* (MM27128, formerly *Fuligo laevis*); g. *Badhamiopsis ainoae* (MA-Fungi 64480). — Scale bars: a, c = 0.2 mm; b, e–f = 1 mm; d, g = 0.5 mm.

deposits of lime. Internal walls poorly developed, forming a pale orange-yellow or yellowish pseudocapillitium. Capillitium consisting of ochraceous to dull orange fusiform or branching lime nodes, connected by short hyaline tubules. Pseudocapillitium pale orange-yellow or yellowish. Spores yellowish brown, irregularly and rather sparsely warted.

Molecular diagnosis — CGACAAGGC (128–136, nSSU), GYACAGCAGT (300–309, nSSU), GGGACGTGCTG (314–324, nSSU); ACC (659–661, *EF-1α*), CAGAAGGTT (899–907, *EF-1α*); TTATCYACTAC (63–83, mtSSU), AACGTCCTTATTAA (270–283, mtSSU); ATTCTCT (746–752, *α-Tub*).

Accepted species. Lignydium muscorum (Alb. & Schwein.) Kuntze.

Notes — The genus *Lignydium* is here resurrected for the species widely known as *Fuligo muscorum* (Fig. 7b), which is not closely related to any other species previously treated as *Fuligo* s.lat.

Nannengaella J.M. García-Martín, J.C. Zamora & Lado, *gen. nov*. — MycoBank MB 846267 [**Subclade 6-I**]

Etymology. Named after Neeltje Elizabeth ('Elly') Nannenga-Bremekamp, a talented scientist and illustrator, specialists on *Myxomycetes*, being the author of many species, including some *Physarales*.

Type species. Nannengaella globulifera (Bull.) J.M. García-Martín, J.C. Zamora & Lado (see below).

Morphological diagnosis — Sporophores sporocarpic, generally stalked, or sessile plasmodiocarps. Hypothallus frequently calcareous. Stalk calcareous, entirely filled with lime. Peridium covered with very abundant lime, especially when plasmodiocarpic. Columella calcareous, usually dome-shaped or as a conical extension of the stalk. In some cases, with a calcareous pseudocolumella. Capillitium netted, with limeless tubules connecting calcareous nodes.

Molecular diagnosis — GGT (924–926, nSSU), CAM (989–991, nSSU); TGTTCR (156–161, mtSSU), GGTGTTMAAT (263–272, mtSSU), CAAACAYC (305–312, mtSSU), TGRCTC (362–367, mtSSU).

Accepted species. Nannengaella alpestris (Mitchel, S.W. Chapm. & M.L. Farr) J.M. García-Martín, J.C. Zamora & Lado, *N. alpina* (Lister & G. Lister) J.M. García-Martín, J.C. Zamora & Lado, *N. contexta* (Pers.) J.M. García-Martín, J.C. Zamora & Lado, *N. globulifera* (Bull.) J.M. García-Martín, J.C. Zamora & Lado, *N. laevis* (Pers.) J.M. García-Martín, J.C. Zamora & Lado, *N. lakhanpalii* (Nann.-Bremek. & Y. Yamam.) J.M. García-Martín, J.C. Zamora & Lado, *N. leucopus* (Link) J.M. García-Martín, J.C. Zamora & Lado, *N. mellea* (Berk. & Broome) J.M. García-Martín, J.C. Zamora & Lado, *N. plicata* (Nann.-Bremek. & Y. Yamam.) J.M. García-Martín, J.C. Zamora & Lado, *N. sulphurea* (Alb. & Schwein.) J.M. García-Martín, J.C. Zamora & Lado, *Physarum decipiens* M.A. Curtis.

Nannengaella alpestris (Mitchel, S.W. Chapm. & M.L. Farr) J.M. García-Martín, J.C. Zamora & Lado, *comb. nov.* — MycoBank MB 846268

Basionym. Physarum alpestre Mitchel, S.W. Chapm. & M.L. Farr, Mycologia 78: 68. 1986.

Nannengaella alpina (Lister & G. Lister) J.M. García-Martín, J.C. Zamora & Lado, *comb. nov.* — MycoBank MB 846269

Basionym. Physarum virescens var. *alpinum* in Lister & G. Lister, J. Bot. 46: 216. 1908.

Nannengaella contexta (Pers.) J.M. García-Martín, J.C. Zamora & Lado, *comb. nov.* — MycoBank MB 846270

Basionym. Diderma contextum Pers., Observ. Mycol. 1: 89. 1796.

Nannengaella globulifera (Bull.) J.M. García-Martín, J.C. Zamora & Lado, *comb. nov.* — MycoBank MB 846271

Basionym. Sphaerocarpus globulifer Bull., Hist. Champ. France 134 (1791) (we treat the specific epithet as a masculine adjective instead of a name in apposition since Bulliard (1791) also used the unambiguous adjectival form '*globuliferus*' in the corresponding plate. Persoon (1801), the first author who combined the specific epithet in a non-masculine genus, also treated it as adjective in the combination *Physarum globuliferum* (Bull.) Pers.).

Nannengaella lakhanpalii (Nann.-Bremek. & Y. Yamam.) J.M. García-Martín, J.C. Zamora & Lado, *comb. nov.* — MycoBank MB 846272

Basionym. Physarum lakhanpalii Nann.-Bremek. & Y. Yamam., Proc. Kon. Ned. Akad. Wetensch. C 90: 335. 1987.

Nannengaella laevis (Pers.) J.M. García-Martín, J.C. Zamora & Lado, *comb. nov.* — MycoBank MB 846273

Basionym. Fuligo laevis Pers., Syn. Meth. Fung. 1: 160. 1801.

Nannengaella leucopus (Link) J.M. García-Martín, J.C. Zamora & Lado, *comb. nov.* — MycoBank MB 846274

Basionym. Physarum leucopus Link, Ges. Naturf. Freunde Berlin Mag. Neuesten Entdeck. Gesammten Naturk. 3: 27. 1809.

Nannengaella mellea (Berk. & Broome) J.M. García-Martín, J.C. Zamora & Lado, *comb. nov.* — MycoBank MB 846275

Basionym. Didymium melleum Berk. & Broome, J. Linn. Soc., Bot. 14: 83. 1873.

Nannengaella plicata (Nann.-Bremek. & Y. Yamam.) J.M. García-Martín, J.C. Zamora & Lado, *comb. nov.* — MycoBank MB 846276

Basionym. Physarum plicatum Nann.-Bremek. & Y. Yamam., Proc. Kon. Ned. Akad. Wetensch. C 93: 284. 1990.

Nannengaella sulphurea (Alb. & Schwein.) J.M. García-Martín, J.C. Zamora & Lado, *comb. nov.* — MycoBank MB 846277

Basionym. Physarum sulphureum Alb. & Schwein., Consp. Fungorum Lusat.: 93. 1805.

Notes — The 'Subclade 6-I' is the most distinct group within the large '*Physaraceae* s.str. clade', being consistently recovered in all analyses. All its members are characterised by their strongly calcified stalk (when present), columella (or sporophore base) and peridium, and present several molecular motifs, which, in our opinion, grant its recognition as a distinct genus. Thus, here we proposed to segregate it from the rest of *Physaraceae* as an independent entity for which the name *Nannengaella* is proposed. The marginal support (PP = 0.94) obtained in the Bayesian analysis for its sister group (the large polytomy within *Physaraceae* s.str.) is more likely an indicator of the heterogeneity of such group and not indicative of 'Subclade 6-I' being perhaps nested within any of the other subclades of *Physaraceae* s.str. An ample variety of sporophore morphologies occurs in this genus, being sporocarpic and stipitate (*N. leucopus*, *N. mellea* (Fig. 7c), *N. sulphurea*, and *N. globulifera* (Fig. 7d)), to plasmodiocarpic and sessile (*N. alpestris* (Fig. 7e), *N. alpina*, *N. plicata*), including connecting morphologies with sessile sporocarps and vermicular plasmodiocarps (*N. contexta* and *N. lakhanpalii*), and even one aethalioid species (*N. laevis*, Fig. 7f). Therefore, the type of fructification is a character of little taxonomic value that does not seem appropriate to use to delimitate this genus. This subclade includes one specimen of the species *Ph. decipiens* (LE266398), but we have not transferred it to *Nannengaella* because the specimen was not studied.

Badhamiopsis T.E. Brooks & H.W. Keller, Mycologia 68: 835. 1976. [**Subclade 6-II**]

Type species. Badhamiopsis ainoae (Yamash.) T.E. Brooks & H.W. Keller, Mycologia 68: 836. 1976.

Morphological diagnosis — Sporophores plasmodiocarpic, flattened and effused, sessile. Columella absent. Capillitium consisting of simple, or occasionally forked, calcareous spike-like invaginations of the upper peridium, filled with granular lime.

Molecular diagnosis — TGTGTGCAATCTCCA (201–215, nSSU), ATCTTTCTGGGT (256–267, nSSU); GGGTCGTGTC (532–571, *EF-1α*), TCGCCGC (910–916, *EF-1α*); GTTAACTGAA (75–84, mtSSU), ATCTGT (281–286, mtSSU); CGCNATCGCTGAA (731–743, *α-Tub*).

Accepted species. Badhamiopsis ainoae (Yamash.) T.E. Brooks & H.W. Keller.

Notes — *Badhamiopsis* is a genus of corticolous myxomycetes with a unique capillitium (Fig. 7g), consisting of calcareous peridial invaginations that form spike-like vertical columns within its plasmodiocarps. Thus, the sporophores acquire a segmented appearance. *Badhamiopsis* was removed from

Badhamia by Keller & Brooks (1976) to bring coherence to the latter, and it seems to be a distinct genus based on our results (Fig. 3), although it should be taken into account that the relationships among *Badhamiopsis*, *Badhamia* s.lat. and *Badhamia* s.str. clades are still uncertain. Several new species have been recently proposed based on morphological studies (Kuhnt 2021).

'*Badhamia* s.lat. group' [Subclade 6-III]

Morphological diagnosis — Sporophores plasmodiocarpic or sporocarpic, mostly sessile. Peridium single or double, membranous but frosted with calcareous deposits, or entirely calcareous. Columella absent. Capillitium netted, rigid, entirely calcareous or with calcareous nodes connected by short limeless tubules. Spores conglobate.

Molecular diagnosis — CTCG (1095–1098, nSSU); TCTCGACGCT (463–472, *EF-1α*); GATGTATCGTGGTGACGTTGTT (536–557, *α-Tub*).

Accepted species. B. cf. *populina* Lister & G. Lister and *Badhamia* sp.

Notes — This small clade is constituted by species sharing firmly conglobate-adnate spores, verrucose on the exposed surface and smooth or subsmooth on the inner part, plus sporocarpic to short plasmodiocarpic sporophores with single to double layered, calcareous peridium (see Fig. 8a). The existence

Fig. 8 Macromorphology of some members of the '*Physaraceae* clade'. a. *Badhamia* sp. (MA-Fungi 88232); b. *Trichamphora pezizoidea* (MM30097, formerly *Physarum pezizoideum*); c. *Fuligo septica* var. *septica* (MA-Fungi 14371); d. *Physarella oblonga* (MA-Fungi 51797); e. *Fuligo gyrosa* (MM17000, formerly *Physarum gyrosum*); f. *Erionema aureum* (TNSMR1366); g. *Fuligo candida* (MA-Fungi 87151). — Scale bars: a, d = 0.5 mm; b, e–f = 1 mm; c = 1 cm; g = 2 mm.

of clustered spores has been reported in different species of *Physarales*, including *Ph. lakhanpalii* (Fig. 2i, see *Nannengaella* above) and numerous species of the genus *Badhamia*, so this character alone is clearly insufficient to diagnose the 'Subclade 6-III'. What is more, even the character combination 'clustered spores + sessile sporophores with one- or two-layered calcareous peridium' is exhibited by other species, which in our sampling comprise *Ph. lakhanpalii* ('Subclade 6-I', see above), *Badhamia versicolor* and *B. nitens* (Fig. 2j, both in 'Subclade 6-VII' (see below). Since there is not a unique combination of morphological synapomorphies for this subclade, it is exclusively supported here by the existence of molecular signatures in the *EF-1α* and *α-Tub* genes (see Taxonomy section). Given the uncertain phylogenetic relationships in the large polytomy within *Physaraceae* s.str., we cannot discard alternative topologies placing the 'Subclade 6-III' together with other subclades, even with *Badhamia* s.str.

Trichamphora Jungh., Praem. Fl. Crypt. Java: 12. 1838 [**Subclade 6-IV**]

Type species. *Trichamphora pezizoidea* Jungh., Praem. Fl. Crypt. Java: 12. 1838.

Morphological diagnosis — Sporocarps sporocarpic, stalked. Sporotheca discoid. Peridium single. Stalk long, red brown and translucent. Capillitium usually dense, netted, branched, hyaline, limeless or weakly limy, connecting the lower and upper peridium.

Molecular diagnosis — AAACCAGGGATAGGACT (626–642, nSSU), CGGGGGGAGTATGGTT (695–710, nSSU); TCTCGACARCATCACYGAGCCT (463–484, *EF-1α*); AAACCGATTCAA (176–187, mtSSU).

Accepted species. Trichamphora pezizoidea Jungh.

Notes — The only species of this subclade, *Ph. pezizoideum* (Fig. 8b), was originally described as *Trichamphora pezizoidea* (Junghuhn 1838), and later transferred to *Physarum* (Pavillard & Lagarde 1903). Farr (1964) considered two varieties, which were later separated as species by Ukkola & Härkönen (1996), erecting *Badhamia gigantospora* for the large-spored taxon. In addition to the spore size, *B. gigantospora* has stout and almost fully calcified capillitial columns, very different to the netted and almost limeless capillitum of *T. pezizoidea*, so the taxonomic position of the former remains uncertain for the time being. Still, *T. pezizoidea* is well characterised by its unique lenticular sporotheca ('*Peziza*-like') combined to a netted capillitium, hair-like and tortuous through the spore mass, that connects the upper and lower parts of the sporotheca. All four specimens of *T. pezizoidea* analysed have an *EF-1α* intron that is only shared with members of the 'Subclade 6-VIII' (Table 1).

'*Fuligo* + *Physarella* group' [**Subclade 6-V**]

Morphological diagnosis — Sporophores aethalioid, rarely sporocarpic or plasmodiocarpic, stalked or sessile. Stalk, when present, long, fibrous, non-calcareous. Peridium single, calcareous, lime forming an almost continuous crust, or membranous and frosted with lime. When sporocarpic, the sporotheca is thimble-shaped and presents spine-like capillitial processes. When aethalioid or plasmodiocarpic, capillitium rigid, forming a net with spindle-shaped lime nodes and sometimes spike-like projections from the peridium.

Molecular diagnosis — T (69, nSSU); TAAGTTCWCYGAGATYWTSTCCAAGGTCGACCGT (880–913, *EF-1α*).

Fuligo Haller, Hist. Stirp. Helv. 3: 110. 1768

Type species. *Mucor septicus* L., Sp. Pl., ed. 2, 2: 1656. 1763.

Synonym. *Aethalium* Link, Ges. Naturf. Freunde Berlin Mag. Neuesten Entdeck. Gesammten Naturk. 3: 24. 1809.
Type species. *Fuligo septica* (L.) F.H. Wigg., Prim. Fl. Holsat.: 112. 1780.

Accepted species. Fuligo gyrosa (Rostaf.) E. Jahn, *F. leviderma* H. Neubert, Nowotny & K. Baumann, *F. luteonitens* L.G. Krieglst. & Nowotny, *F. septica* var. *rufa* (Pers.) Lázaro Ibiza, *F. septica* (L.) F.H. Wigg. var. *septica*.

Physarella Peck, Bull. Torrey Bot. Club 9: 61. 1882

Type species. *Physarella mirabilis* (Peck) Peck, Bull. Torrey Bot. Club 9: 61. 1882.

Accepted species. Physarella oblonga (Berk. & M.A. Curtis) Morgan.

Notes — The 'Subclade 6-V' consists of six taxa, including one representative of *Physarum* (*Ph. gyrosum* ≡ *F. gyrosa*) and the types of the genera *Fuligo* (*F. septica* var. *septica*, Fig. 8c) and *Physarella* (*Ph. oblonga*, Fig. 8d). While *Ph. oblonga* has radically different fructifications, stipitate sporocarps, rarely plasmodiocarps (Alexopoulos 1982), *Fuligo* species are always aethalioid. *Physarum gyrosum* (Fig. 8e) precisely bridges the morphological gap between these two sporophore morphologies, by forming aggregate plasmodiocarps or pseudoaethalia. The relationship between *Fuligo* and *Ph. gyrosum* (≡ *F. gyrosa*) has been earlier suggested (Jahn 1902, Martin & Alexopoulos 1969). Interestingly, the highly distinctive capillitium of *Ph. oblonga*, with both spike-like projections borne from the peridium and a network of threads with some calcareous nodes, is very similar to that of *F. gyrosa*, and together with the occasional occurrence of plasmodiocarps in *Ph. oblonga*, it is not surprising that both species may belong to the same genus. In our tree, *Ph. oblonga* is closely related to *F. leviderma*, *F. luteonitens*, and *F. gyrosa*, with high support, and the relationship between *Ph. oblonga* and *F. leviderma* has been previously observed by Kamono et al. (2013) and Strelow et al. (2020). However, a different relationship was recovered when studying other specimens (Erastova et al. 2013).

Besides *F. muscorum* and *F. laevis*, which are here recognised in the genera *Lignydium* and *Nannengaella*, respectively, different *Fuligo* species analysed in this study are interspersed along the '*Physaraceae* clade' (subclades '6-V', '6-VI', and '6-VIII'), rendering the genus polyphyletic as currently defined. The transfer of *F. laevis* ('Subclade 6-I') to *Nannengaella* and *F. muscorum* ('Subclade 5-II') to *Lignydium* is granted based on the molecular results, but other possible combinations should only be done when additional data are available. The combination of *F. cinerea* and *F. intermedia* ('Subclade 6-VIII') to the genus *Aethaliopsis* would remediate the most important part of the polyphyly, but this change would be well-justified only if additional data confirmed that the 'Subclade 6-VI' constitutes an independent entity, not closely related to '*Fuligo* + *Physarella*', something impossible to state with the current data, since the relationships among these clades are unsupported. Unfortunately, the entire '*Fuligo* + *Physarella*' group is also only supported by transfer bootstrap in the ML analysis, so we prefer to avoid nomenclatural changes for the time being. Besides, a coherent morphological diagnosis of *Fuligo* including also *Ph. oblonga* is, at the moment, challenging.

Erionema Penz., Myxomyc. Fl. Buitenzorg: 36. 1898. (Non *Erionema* Maire, 1906) [**Subclade 6-VI**]

Type. *Erionema aureum* Penz., Myxomyc. Fl. Buitenzorg: 37. 1898.

Morphological diagnosis — Sporophores aethalioid or massed plasmodiocarps. Peridium strongly calcareous, the lime

almost forming a crust. Stalk absent. Columella absent. Capillitium netted, protruding elastically to some extent, lime nodes sparse or absent.

Molecular diagnosis — C (694, *EF-1α*) combined with A (718, *EF-1α*), R (751, *EF-1α*), A (764 *EF-1α*) and A (778, *EF-1α*); A (149, mtSSU) combined with ACTT (155–158, mtSSU); C (602, *α-Tub*) combined with T (626, *α-Tub*), T (632 *α-Tub*) and A (647, *α-Tub*).

Accepted species. Erionema aureum Penz., *Fuligo candida* Pers. and *F. flava* Pers.

Notes — This well-supported subclade includes the species *E. aureum* (Fig. 8f), type of the genus *Erionema*, plus two former varieties of *F. septica* (*F. septica* var. *candida* (Fig. 8g) and *F. septica* var. *flava*) that can be distinguished by the colour of the plasmodium and the peridial cortex (Hoppe 2017). Being not closely related to *F. septica* var. *septica*, or in any case clearly independent as distinct clades, these taxa are therefore reinstated at species level by resurrecting *F. flava* and *F. candida*. However, since the relationships with most other subclades within the 'Clade 6', and especially with '*Fuligo* + *Physarella*', are unresolved, we refrain from directly accepting *Erionema* as an independent genus, and instead we left uncombined the *Fuligo* species included in the 'Subclade 6-VI'. For simplicity, '*Fuligo* + *Physarella*' and *Erionema* can be treated as '*Fuligo* s.lat.' for the time being.

Badhamia Berk., Proc. Linn. Soc. London 2: 199. 1852. s.str. [**Subclade 6-VII**]

Type species. Sphaerocarpus capsulifer Bull., Hist. Chap. France: 139. 1791. We consider the specific epithet in the basionym a masculine adjective as in *Sphaerocarpus globulifer*, see above).

Morphological diagnosis — Sporophores sporocarpic to subplasmodiocarpic, sessile to stalked, then typically with weak stalks, sometimes thread-like to more or less membranous. Sporotheca subglobose to ovoid or slightly flattened, sometimes lobulated or multiple. Peridium single or double, papery-membranous, often iridescent and somewhat translucent, frosted with lime or, when double, the external layer entirely calcareous. Stalk, when present, non-calcareous or only the wall presents some lime deposits. Columella absent. Capillitium netted, the limeless tubules connected by lime nodes, sometimes entirely calcareous. Spores free or clustered.

Molecular diagnosis — GTGGCCA (219–225, nSSU), ATTGGGCAAAGCT (424–436, nSSU), TCGCYNCCTK (969–978, nSSU), GAYAAGGCGTC (1096–1106, nSSU); GRWG (619–622, *EF-1α*); GAAAAKAY (29–36, mtSSU); GCGCGCCGTGTGT (701–713, *α-Tub*).

Accepted species. Badhamia albescens (Ellis ex T. Macbr) J.M. García-Martín, J.C. Zamora & Lado, *B. bethelii* (T. Macbr. ex G. Lister) J.M. García-Martín, J.C. Zamora & Lado, *B. capsulifera* (Bull.) Berk., *B. foliicola* Lister, *B. nitens* Berk., *B. polycephala* (Schwein.) J.M. García-Martín, J.C. Zamora & Lado, *B. versicolor* Lister.

Badhamia albescens (Ellis ex T. Macbr) J.M. García-Martín, J.C. Zamora & Lado, *comb. nov.* — MycoBank MB 846260

Basionym. Physarum albescens Ellis ex T. Macbr., N. Amer. Slime-moulds, ed. 2: 86. 1922.

Badhamia bethelii (T. Macbr. ex G. Lister) J.M. García-Martín, J.C. Zamora & Lado, *comb. nov.* — MycoBank MB 846261

Basionym. Physarum bethelii T. Macbr. ex G. Lister, in Lister, Monogr. Mycetozoa, ed. 2: 57. 1911.

Badhamia polycephala (Schwein.) J.M. García-Martín, J.C. Zamora & Lado, *comb. nov.* — MycoBank MB 846262

Basionym. Physarum polycephalum Schwein., Schriften Naturf. Ges. Leipzig 1: 63. 1822.

Notes — So far, the genus *Badhamia* has been thought to comprise 34 variable taxa (Lado 2005–2023), mainly based on the presence of an entirely calcareous capillitium, but being otherwise a very heterogeneous group. A tentative circumscription of *Badhamia* is here limited to those *Badhamia* species constituting the well-supported 'Subclade 6-VII' since it comprises the type of the generic name, *B. capsulifera* (Fig. 9b). It also includes one species with free spores, *B. foliicola*, along with *B. nitens* (Fig. 2j) and *B. versicolor*, both with distinguishable conglobate-adnate spores, like *B. capsulifera*. This feature was highlighted by Berkeley (1852) when he described the genus *Badhamia*, but it is not synapomorphic. Neither is the presence of a badhamioid capillitium, commented above, so that it is difficult to find unique morphological synapomorphies to define this group. However, the usual presence of peridial iridescence in most of the included species (except for *B. versicolor*) is a relatively uncommon character in the family *Physaraceae*, and together with the papery consistency of the peridium in several species, and the absence of stout stalks, may help to define *Badhamia* s.str. The species *Badhamia utricularis* also presents a papery, somewhat translucent, and iridescent peridium, and weak, thread-like to more or less membranous stalks. It surely belongs to this group, but it was not included in the current analyses due to the scarcity of molecular data (only nSSU available).

The strain of *Ph. polycephalum* with a sequenced transcriptome used in this study is strongly supported as closely related to *Badhamia* s.str. and not to *Physarum* s.str. in all analyses. Even if this result may be somewhat inconvenient, because *Ph. polycephalum* must be combined in *Badhamia* in order to avoid many other combinations and conservation proposals to change the types of *Badhamia* and *Physarum*, it is not particularly surprising from a morphological point of view, as the peridial and stalk features of *Ph. polycephalum* agree well with those of other species of the 'Subclade 6-VII', such as *Ph. albescens* (Fig. 9a).

Within *Physarales*, there are two introns that seem to be unique to the members of this subclade: the 2nd intron of the *α-Tub* gene, and 5th *EF-1α* intron. The latter, as just mentioned, does not seem to exist in other *Physarales*, but it is present in some species of other orders of *Myxomycetes*, such as *Semimorula liquescens* and *Cribraria violacea* (Alignment S6).

Aethaliopsis Zopf, in Schenk, Handb. Bot. 3 (2): 149. 1885. [**Subclade 6-VIII**]

Type species. Aethaliopsis stercoriformis Zopf, in Schenk, Handb. Bot. 3: 150. 1885.

Morphological diagnosis — Sporophores sporocarpic, plasmodiocarpic or aethalioid, stalked or sessile. Columella absent. Capillitium netted, rigid, net entirely or partially calcareous. Spores globose to ellipsoid, rounded or angular, ornamented with warts evenly distributed, groups of warts or reticula.

Molecular diagnosis — AKTC (221–224, nSSU) combined with G (230, nSSU), CAT (923–925, nSSU), TCCG (971–974, nSSU); AATAWCCGATTTG (174–186, mtSSU). Furthermore, most members of this subclade (i.e., *Ph. atacamense*, *F. cinerea*, *F. intermedia*, *B. melanospora*, and *Ph. nivale*) have an intron interrupting their *EF-1α* sequences (our 4th intron, corresponding to the 15th intron reported by Fiore-Donno et al. (2010b)), absent in all other groups, except for *Trichamphora* (Table 1; Alignment S6).

Fig. 9 Macromorphology of some members of the '*Physaraceae* clade'. a. *Badhamia albescens* (L24133, formerly *Physarum albescens*); b. *Badhamia capsulifera* (MM33588); c. *Physarum vernum* (MA-Fungi 87214); d. *Badhamia melanospora* (MA-Fungi 88043); e. *Physarum atacamense* (MA-Fungi 87936); f. *Fuligo cinerea* (MA-Fungi 87149). — Scale bars: a, e = 0.5 mm; b–c = 1 mm; d = 0.2 mm; f = 0.1 mm.

Accepted species. Badhamia melanospora Speg., *Fuligo cinerea* (Schwein.) Morgan, *F. intermedia* T. Macbr., *Physarum atacamense* D. Wrigley, Lado & Estrada, *Ph. notabile* T. Macbr., *Ph. nivale* (Meyl.) Mar. Mey. & Poulain, *Ph. pseudonotabile* Novozh., Schnittler & Okun, *Ph. vernum* Sommerf.

Notes — '*Aethaliopsis*' includes species well adapted to extreme environmental conditions (deserts and alpine zones), belonging to the genera *Physarum* (Fig. 9c, e), *Badhamia* (Fig. 9d), and *Fuligo* (Fig. 9f) in their classical senses. Within the 'Subclade 6-VIII' we recovered a species complex, formed by *Ph. notabile*, *Ph. pseudonotabile*, *Ph. nivale*, and *Ph. vernum*, that requires further studies to clarify its taxonomy. The 'Subclade 6-VIII' also includes a specimen currently referred to as *F. cinerea* (Fig. 2k, 9f), a synonym of *Aethaliopsis stercoriformis*, the type of its genus, which differs from other species formerly treated in *Fuligo* by its subreticulate and relatively large spores (10–15 µm diam vs 6–9 µm diam) and their white or pale grey aethalia, regarded to be rather similar to some densely massed forms of *Physarum* (Martin & Alexopoulos 1969). Consequently, if this group is recognised as a valid genus after the polytomy from which it emerges was resolved, the name *Aethaliopsis* should be used for it. However, until phylogenetic resolution is achieved at this level, we refrain from making formal combinations and call for caution since alternative

topologies, with nomenclatural relevance, are possible. Once again, our molecular data contradict the traditional segregation of genera based solely on the type of fruiting bodies, i.e., aethalia vs sporocarps or plasmodiocarps (see also *Mucilago* in the 'Clade 2, *Didymium*'), and the diagnosis of this group based on the studied phenotypic characters remains difficult.

Claustria Fr., Summa Veg. Scand.: 451. 1849 [**Subclade 6-IX**]

Type species. Claustria didermoides (Pers.) Fr., Summa Veg. Scand.: 451. 1849.

Doubtful synonym. Hyperamoeba Alexeieff, Arh. Russk. Protistol. Obsc. 2: 280. 1923.
Type species. Hyperamoeba flagellata Alexeieff, Arh. Russk. Protistol. Obsc. 2: 280. 1923.

Morphological diagnosis — Sporophores plasmodiocarpic or sporocarpic, sessile or short-stalked. Peridium single or double, membranous, heavily encrusted with lime. Columella absent, sometimes with a pseudocolumella. Spores angular, very dark in colour.

Molecular diagnosis — CAAAGCCT (430–437, nSSU), GTAGTTCGTGGATTGATTTGTCTGGTT (873–899, nSSU); TGAAAA ß(166–171, mtSSU), CCCCA (235–239, mtSSU).

Accepted species. Physarum didermoides (Pers.) Rostaf., *Ph. licheniforme* (Schwein.) Lado, *Ph. polygonosporum* Mosquera, García-Martín & Lado, *Ph. straminipes* Lister.

Notes — The 'Subclade 6-IX' forms part of a large polytomy within the '*Physaraceae* s.str. clade', since the sister relationship with *Physarum* s.str. is unsupported in the Bayesian analysis, and not even recovered in the ML analysis. In any case, this group is highly supported and it is diagnosable on the basis of very dark angular spores (see, for example, those of *Ph. polygonosporum*; Fig. 2l, 10a) and several molecular signatures. This could indicate that this subclade truly represents a separate genus. Another species included in this subclade, i.e., *Ph. didermoides* (Fig. 10b), contains *Spumaria didermoides*, the type of the genus *Claustria*, among its taxonomic synonyms. Therefore, this generic name could be resurrected for this subclade if it still constitutes an independent and well-defined entity when the polytomy within *Physaraceae* is resolved. We do not recommend performing nomenclatural combinations based on the current evidence since the relationships of this clade with others may be substantially altered with the inclusion of additional data. The type of *Hyperamoeba*, *H. flagellata*, has not been included in our analyses because the scarcity of molecular data (Cavalier-Smith et al. 2004), but the only sequence publicly available for this species (AF411289) shows high similarity to the nSSU sequences of members of this group, so we assume that *H. flagellata* may belong here.

Physarum Pers., Neues Mag. Bot. 1: 88. 1794. s.str. [**Subclade 6-X**]

Type species. Physarum aureum Pers., Neues Mag. Bot. 1: 88. 1794. (Non *Ph. aureum* Brândza, 1929).

Synonyms. Sphaerocarpus Bull., Hist. Champ. France: 123. 1791. (Nom. illeg., Art. 53.1, non *Sphaerocarpus* Ludwig, 1760, non *Sphaerocarpus* Adans, 1763).
Type species. Sphaerocarpus trichioides Bull., Hist. Champ. France: 124. 1791.
Tilmadoche Fr., Summa Veg. Scand.: 454. 1849.
Type species. Tilmadoche leucophaea (Fr. & Palmquist) Fr., Summa Veg. Scand.: 454. 1849.

Doubtful synonyms. Cytidium Morgan, J. Cincinnati Soc. Nat. Hist. 19: 8. 1896.
Type species. Cytidium pulcherrimum (Berk. & Ravenel) Morgan, J. Cincinnati Soc. Nat. Hist. 19: 8. 1896.

Morphological diagnosis — Sporophores sporocarpic, stipitate, only occasionally sessile, or plasmodiocarpic. Sporotheca lenticular, subglobose, obconic or turbinate, often nodding. Stalk dark, usually limeless, sometimes partially calcareous. Peridium membranous, encrusted with yellow, whitish or grey lime, sometimes nearly limeless. Columella absent. Capillitium netted, with limeless tubules connecting lime nodes, sometimes the nodes agglutinated and forming a pseudocolumella. Spores free.

Molecular diagnosis — GTG (873–875, nSSU) combined with TA (899–900, nSSU).

Accepted species. Physarum album (Bull.) Chevall., *Ph. javanicum* Racib., *Ph. leucophaeum* Fr. & Palmquist, *Ph. macrocarpon* Ces., *Ph. stellatum* (Massee) G.W. Martin, *Ph. viride* (Bull.) Pers.

Notes — This subclade comprises the type of the genus *Physarum*, *Ph. viride* (Fig. 10c), which is shown to be polyphyletic, along with four additional species of the same genus sharing stalked sporocarps and lenticular to subglobose-depressed sporothecae, usually nodding. The polyphyly of *Ph. viride* is not surprising given the morphological plasticity of this species regarding, e.g., the shape of the sporotheca and colour (Martin & Alexopoulos 1969), also reflected by the long list of currently accepted synonyms (Lado 2005–2023) needing an urgent revision. Moreover, there is another species currently not recognised in *Physarum*, *Badhamia macrocarpos*, that was originally described as *Ph. macrocarpon*, and has free spores and a capillitium often somewhat physaroid (Martin & Alexopoulos 1969). Thus, it seems appropriate to reassign it to the genus *Physarum*. The epithet '*macrocarpon*' is an adjective transcribed from the Greek 'μακροκαρπόν', and it was spelled as such in the protologue. Since it is correct, it should not be altered to '*macrocarpum*', and it is to be spelled as '*macrocarpos*' when combined in a feminine genus such as *Badhamia* (Art. 23.5, Exs. 6 and 9). This error seems to have remained unnoticed since 1874, when the combination in *Badhamia* was made by Rostafiński.

The genus *Cytidium* is only tentatively treated as a synonym given that no specimens of *Ph. pulcherrimum*, which type is the type of that generic name, have been analysed in our study. For comparison with *Trichamphora*, see observations under that generic name.

Leocarpus Link, Ges. Naturf. Freunde Berlin Mag. Neuesten Entdeck. Gesammten Naturk. 3: 25. 1809 [**Subclade 6-XI**]

Type species. Diderma vernicosum Pers., Ann. Bot. (Usteri) 15: 34. 1795.

Synonym. Tripotrichia Corda, Icon. Fungorum 1: 22. 1837.
Type species. Tripotrichia elegans Corda, Icon. Fungorum 1: 22. 1837.

Morphological diagnosis — Sporophores sporocarpic, sessile or stalked. Sporotheca ellipsoid or ovoid. Peridium triple, the three layers firmly appressed, the outer layer brittle, shiny, cartilaginous and limeless, the middle calcareous, the inner membranous. Columella absent. Capillitium consisting of a network of rigid, white, calcareous nodes connected with, but largely distinct from, a network of slender, colourless, flattened tubules.

Molecular diagnosis — ACAGTTGTAAACTATAGCAAGCAC (83–106, nSSU), CGGTGCACGC (221–230, nSSU), CACCTTAGAGAAATCAGAGTCTTTGG (665–690, nSSU), TTCAGCCCGGCTCGCAAGA (942–960, nSSU); ACTCGACCAG (463–472, *EF-1α*), TACTGAAGTCAAGTCTGTCGAA (628–649, *EF-1α*); AGCCCT (57–62, mtSSU); TAACGAAGCYATCTAT (200–215, *α-Tub*).

Accepted species. Leocarpus fragilis (Dicks.) Rostaf.

Notes — *Leocarpus* is recovered as a fully supported monospecific genus, with an unsupported sister relationship with

Fig. 10 Macromorphology of some members of the '*Physaraceae* clade'. a. *Physarum polygonosporum* (MA-Fungi 90740); b. *Physarum didermoides* (MA-Fungi 80353); c. *Physarum viride* (L23250); d. *Leocarpus fragilis* (MA-Fungi 87158); e. *Physarum bitectum* (MA-Fungi 83516); f. *Physarum bivalve* (MA-Fungi 90049). — Scale bars: a–b, d–f = 0.5 mm; c = 0.2 mm.

Ph. lateritium. The latter is one of the few sessile *Physarum* species with an orange red peridium (together with, for example, *Ph. rubiginosum*), a pigmentation that may be similar to that found in *L. fragilis* (Fig. 10d). However, the capillitium system and the peridium structure are decidedly different in *Ph. lateritium*, and since the observed relationship with *Leocarpus* is only supported by transfer bootstrap in the ML analysis, we prefer to keep *Ph. lateritium* as *incertae sedis*.

Angioridium Grev., Scott. Crypt. Fl. 6: pl. 310. 1827 [**Subclade 6-XII**]

Type species. *Angioridium sinuosum* (Bull.) Grev., Scott. Crypt. Fl. 6: pl. 310. 1827.

Morphological diagnosis — Sporophores plasmodiocarpic, sessile, terete or strongly laterally compressed. Peridium typically white to greyish, double, with both layers distant and often distinctly separating at dehiscence, calcareous. Columella absent. Capillitium netted, composed of hyaline tubules connecting numerous white lime nodes. Spores free.

Molecular diagnosis — CGGGRAGGYTGGGGGRCGTGC (187–207, nSSU), CGCGWKGY (274–281, nSSU); TAACATCTTTTG (269–280, mtSSU).

Accepted species. Physarum bitectum G. Lister, *Ph. bivalve* Pers., *Ph. clavisporum* G. Moreno, A. Sánchez, A. Castillo & Illana, *Ph. echinosporum* Lister.

Notes — This robust subclade encompasses four species very similar on morphological grounds: *Physarum bitectum* (Fig. 10e), *Ph. bivalve* (Fig. 10f), *Ph. echinosporum*, and *Ph. clavisporum*. We do not perform combinations for these species because this subclade forms part of the polytomy within the '*Physaraceae* clade', and further rearrangements may appear with additional data, compromising any nomenclatural changes. Nonetheless, its high statistical support and the existence of, at least, three molecular signatures unique to the species of this group in two different genes, together with a rather uniform morphology, likely indicate that it is truly an independent genus. The name *Angioridium* would be available for this group since one of the included species, *Ph. bivalve*, is a synonym of *Angioridium sinuosum*, type of the generic name.

'Physarum cinereum group' [Subclade 6-XIII]

Morphological diagnosis — Sporophores sporocarpic, sessile, subglobose or plasmodiocarpic, terete, not strongly laterally compressed. Peridium single, membranous but dusted or densely covered with yellow or white lime. Columella absent.

Molecular diagnosis — ATATTCACTGAA (73–84, mtSSU).

Accepted species. Physarum cinereum (Batsch) Pers., *Ph. luteolum* Peck.

Notes — Subclade formed by only two members in our analyses, but several species with similar morphologies to that of *Ph. cinereum* (Fig. 11a) and *Ph. luteolum*, such as *Ph. daamsii* or *Ph. nitens*, could potentially be members of this group.

Willkommlangea Kuntze, Revis. Gen. Pl., 2: 875. 1891 [Subclade 6-XIV]

Type species. Willkommlangea reticulata (Alb. & Schwein.) Kuntze, Revis. Gen. Pl., 2: 875. 1891.

Synonym. Cienkowskia Rostaf., Vers. Syst. Mycetozoen: 9. 1873. (Nom. illeg., Art. 53.1, non *Cienkowskia* Regel & Rach, 1858, nec *Cienkowskya* Solms, 1867).
Type species. Cienkowskia reticulata (Alb. & Schwein.) Rostaf., Sluzowce Monogr.: 91. 1874.

Morphological diagnosis — Sporophores plasmodiocarpic, sessile. Columella absent. Capillitium consisting of flat, angular, calcareous nodes massed into transverse plates, and a delicate network of yellowish tubules bearing a few rounded calcareous nodes. The transverse plates divide the interior of the sporotheca into distinct segments.

Molecular diagnosis — CTAGATCTA (184–192, mtSSU); CGGTCT (279–284, mtSSU).

Accepted species. Willkommlangea reticulata (Alb. & Schwein.) Kuntze.

Notes — *Willkommlangea* is considered here as a monospecific and easily recognisable genus. The 'duplex capillitium' of its type, *W. reticulata* (Fig. 11b), and the presence of a species-specific *EF-1α* intron (Table 1; Alignment S6), as well as various molecular signatures in the mtSSU gene, are strikingly different compared to other *Physaraceae*. Its phylogenetic affinities remain uncertain.

'Physarum bogoriense group' [Subclade 6-XV]

Morphological diagnosis — Sporophores plasmodiocarpic, elongated, sometimes branched. Peridium yellowish, triple. Columella absent. Spores free or in loose clusters.

Molecular diagnosis — CTTGTCTGGGT (257–267, nSSU); AGATCTT (683–689, *α-Tub*) combined with C (764, *α-Tub*).

Accepted species. Badhamia crassipella K.D. Whitney & H.W. Keller, *Physarum bogoriense* Racib., *Ph. hongkongense* Chao H. Chung (Fig. 11c).

Notes — This unsupported subclade is constituted by three species of two genera sharing a rather similar morphology, including an unusual three-layered peridium (Poulain et al. 2011). As Whitney & Keller (1982) stated, placing *B. crassipella* in *Badhamia* 'is largely a matter of opinion', and our analyses do not recover this species as closely related to *Badhamia* s.str. This group could represent an independent genus, but given its low support, we rather prefer to wait for additional data before describing it.

Craterium Trentep., in Roth, Catal. Bot. 1: 224. 1797 [Subclade 6-XVI]

Type species. *Craterium pedunculatum* Trentep., in Roth, Catal. Bot. 1: 224. 1797.

Synonyms. Cupularia Link, Handbuch 3: 421. 1833.
Type species. *Cupularia leucocephala* (Pers. ex J.F. Gmel.) Link, Handbuch 3: 421. 1833.
Crateriachea Rostaf., Vers. Syst. Mycetozoen: 11. 1873.
Type species. *Crateriachea mutabilis* Rostaf., Sluzowce Monogr.: 126. 1874.
Iocraterium E. Jahn, Hedwigia 43: 302. 1904.
Type species. *Iocraterium rubescens* (Rex) E. Jahn, Hedwigia 43: 302. 1904.

Morphological diagnosis — Sporophores sporocarpic, stalked. Sporotheca cyathiform or subglobose. Peridium double or triple, rarely single, calcareous or cartilaginous, the lower part tending to persist after dehiscence as a more or less regular cup. Capillitium netted, formed by slender tubules connecting calcareous nodes that often aggregate in the centre to form a pseudocolumella.

Molecular diagnosis — RCTTYCYGGGT (257–267, nSSU); AAGGTGTCC (1099–1107, nSSU).

Accepted species. Craterium andinum (A. Ronikier & Lado) J.M. García-Martín, J.C. Zamora & Lado, *C. aureum* (Schumach.) Rostaf., *C. brunneolum* (W. Phillips) J.M. García-Martín, J.C. Zamora & Lado, *C. leucocephalum* (Pers. ex J.F. Gmel.) Ditmar, *C. minutum* (Leers) Fr., *C. crateriachea* (Lister) J.M. García-Martín, J.C. Zamora & Lado, *C. roseum* (Berk. & Broome) J.M. García-Martín & Lado .

Craterium andinum (A. Ronikier & Lado) J.M. García-Martín, J.C. Zamora & Lado, *comb. nov.* — MycoBank MB 846263

Basionym. Physarum andinum A. Ronikier & Lado, Mycologia 105: 164. 2013.

Craterium brunneolum (W. Phillips) J.M. García-Martín, J.C. Zamora & Lado, *comb. nov.* — MycoBank MB 846264

Basionym. Diderma brunneolum W. Phillips, Grevillea 5: 114. 1877.

Craterium crateriachea (Lister) J.M. García-Martín, J.C. Zamora & Lado, *comb. nov.* — MycoBank MB 849742

Basionym. Physarum crateriachea Lister, Guide Brit. Mycetozoa (London): 20. 1894.

Craterium roseum (Berk. & Broome) J.M. García-Martín & Lado, *comb. nov.* — MycoBank MB 846266

Basionym. Physarum roseum Berk. & Broome, J. Linn. Soc., Bot. 14: 84. 1873.

Notes — Traditionally, *Craterium* comprised species, such as its type *C. minutum* (Fig. 11d), with a well-defined cupulate sporotheca that, in some cases, could be irregular. Our amended concept of this genus, now achieving monophyly, includes most former species of *Craterium* analysed in this

Fig. 11 Macromorphology of some members of the '*Physaraceae* clade'. a. *Physarum cinereum* (MA-Fungi 87190); b. *Willkommlangea reticulata* (MA-Fungi 82977); c. *Physarum hongkongense* (MA-Fungi 87196); d. *Craterium minutum* (MA-Fungi 34992); e. *Craterium brunneolum* (MA-Fungi 82870, formerly *Physarum brunneolum*); f. *Craterium crateriachea* (MM47834, formerly *Physarum mutabile*). — Scale bars: a, c = 1 mm; b, e–f = 0.5 mm; d = 0.2 mm.

study (excluding those with a true calcareous columella, see *Diachea*), plus four species formerly placed in *Physarum* with a thick calcareous or cartilaginous peridium, remaining as an irregular cup after its dehiscence. Our results support the hypothesis of Martin & Alexopoulos (1969), who noted that 'the genus [*Craterium*] is close to *Physarum* as some of the species in this genus have a persistent, cup-like base'. This is exactly the case of *Ph. brunneolum* (Fig. 11e) and, to a lesser extent, of *Ph. andinum* and *Ph. mutabile* (Fig. 11f), since the dehiscence of its peridium is irregular, but still leaves a cup in the lower part of the sporotheca (Ronikier & Lado 2013). Besides, this genus also comprises *Ph. roseum*, a species with peridium that only partially persists after dehiscence as a rudimentary cup. The definition of the genus *Craterium* is thus enlarged to accommodate these former *Physarum* species. The existence of the name *Craterium mutabile* (an accepted synonym of *C. aureum*) precludes the combination of *Crateriachea mutabilis* (≡ *Ph. mutabile*) in *Craterium*, so we used the next available synonym, *Ph. crateriachea*, as basionym for the new combination in *Craterium*.

GENERA INCERTAE SEDIS

Carcerina Fr., Summa Veg. Scand.: 451. 1849

Type species. *Carcerina spumarioides* (Fr. & Palmquist) Fr., Summa Veg. Scand.: 451. 1849.

Morphological diagnosis — Sporophores sporocarpic, sessile, deeply embedded in a membranous hypothallus, usually covered with lime. Peridium double, both layers closely appressed. Columella hemispheric or conical, white ochraceous. Capillitium consisting of yellowish or pale brown threads with scattered warts and small thickenings, branched and anastomosed.

Molecular diagnosis — Not applicable (polyphyletic species).

Accepted species. Diderma spumarioides (Fr. & Palmquist) Fr.

Notes — The specimens of *D. spumarioides* analysed in this study do not form a clade, but are located in distant positions in our tree (Fig. 3). The specimen Meyer 45939 is sister to the 'Clade 5' (*Kelleromyxa* plus *Lignydium*) and 'Clade 6' (*Physaraceae*), although this relationship is only supported by transfer bootstrap (TBE = 99 %). The other two (Meyer 46870 and MCCNNU2749) form a well-supported clade, which seems to be sister to the genus *Diachea* (TBE = 97 %). Given the apparent polyphyletic nature of this species, and the lack of support for its relationships, to determine its actual phylogenetic placement is necessary to analysed additional specimens covering both the geographical and morphological range of *D. spumarioides*.

Physarina Höhn., Sitzungsber. Kaiserl. Akad. Wiss., Math.-Naturwiss. Cl. 118: 431. 1909 (non *Physarina* Caval.-Sm., 2012)

Type species. *Physarina echinocephala* Höhn., Sitzungsber. Kaiserl. Akad. Wiss., Math.-Naturwiss. Cl. 118: 432. 1909.

Morphological diagnosis — Sporophores sporocarpic, stalked. Stalk calcareous. Peridium membranous and marked with peg-like protuberances, filled with amorphous calcareous granules. Columella present. Capillitium filiform, limeless, radiating from the columella.

Molecular diagnosis — Not applicable.

Notes — From a morphological point of view this genus, which currently comprises three species, is clearly different to all other *Physarales*, which makes us to consider it as an independent entity within the order. However, given the lack of molecular data for this taxon, its phylogenetic position cannot be determined yet.

Trabrooksia H.W. Keller, Mycologia 72: 396. 1980

Type species. *Trabrooksia applanata* H.W. Keller, Mycologia 72: 396. 1980.

Morphological diagnosis — Sporophores plasmodiocarpic, flattened to depressed and sessile sporocarps. Peridium single, thin, limeless. Columella absent. Capillitium consisting of tubular, simple or, occasionally, forked invaginations of the upper peridium. Lime deposits absent.

Molecular diagnosis — Not applicable.

Notes — Monospecific genus not analysed in the present study, with no DNA data available. As stated by Keller (1980), *Trabrooksia* poses a special taxonomic problem due to the absence of visible and routinely testable lime in its sporophores so that its ascription to the order *Physarales* remains questionable.

SPECIES INCERTAE SEDIS

Physarum australiense S.L. Stephenson, Novozh. & Prikhodko, Novosti Sist. Nizsh Rast. 54: 400. 2020.

Physarum lateritium (Berk. & Ravenel) Morgan, J. Cincinnati Soc. Nat. Hist. 19: 23. 1896.

Morphological and molecular diagnoses — Not applicable.

Notes — Two of the species of *Physarales* analysed do not show very clear morphological and/or molecular affinities with the remaining taxa. *Physarum australiense* (Stephenson et al. 2020) is characterised by having brownish red or orangish lime knobs or large squamae on its single peridium, a limeless, brittle and black stalk, a large clavate calcareous columella, and a capillitium consisting of large white angular or rod-like nodes. This character combination, except for the columella, was also noted and illustrated on *Ph. squamosum* (Flatau & Schirmer 2004), so it would not be surprising if both taxa are closely related, or even conspecific. A relationship among *Ph. australiense* and two species of the '*Ph. bogoriense* group' was indicated by Stephenson et al. (2020), but it seems to be only supported by the nSSU gene (Fig. S1). *Physarum lateritium* is another species with a peridium covered by brick-red to bright red, small, irregular lime scales, but it fructifies as sessile sporocarps. Its phylogenetic position is highly uncertain, our analyses showing a relationship with *Leocarpus fragilis*, but only supported by TBE. Moreover, their morphologies are very different, and we have not been able to find molecular signatures in common.

PROVISIONAL KEY TO THE GENERA OF THE ORDER PHYSARALES

Notes — Genera *incertae sedis* or not treated here are marked with an asterisk (*). Groups not distinguishable by means of morphological characters, and/or without available names in the literature, have not been split (then left as, e.g., *Badhamia* s.lat. and *Physarum* s.lat.). Caution is advised for species not analysed in this study.

1. Calcareous deposits not visible, only detectable at ultrastructural level 2
1. Calcareous deposits conspicuous 4
2. Sporophores plasmodiocarpic (extending up to 5 mm in length and 2 mm or more across) to, occasionally, sessile sporocarps (0.2–0.4 mm diam); capillitium columnar, as invaginations of the peridium, forming simple or bifurcate columns from the base to the apex (it can be considered as a pseudocapillitium) . . . . . **Trabrooksia*
2. Sporophores sporocarpic, stalked or sessile (sporotheca < 0.25 mm diam); true capillitium present (sometimes reduced), thread-like, sometimes united to the peridium, but never as invaginations of the peridium 3
3. Sporophores black, spindle-shaped, sessile; capillitium reduced, consisting of short unbranched threads; coprophilous . . . . . *Kelleromyxa*
3. Sporophores grey to greyish brown, with a globose sporotheca, stalked; capillitium usually well-developed, branching and anastomosing; not coprophilous . . . . . *Didymium* p.p. (*D. phloiogenum*)
4. Capillitium calcareous 5
4. Capillitium not calcareous, rarely with aggregated lime crystals; occasionally no capillitium 22
5. Capillitium, at least in part, consisting of spike-like or columnar invaginations of the peridium, filled with lime . . 6
5. Capillitium without spike-like or columnar invaginations of the peridium 8

6. Capillitium as vertical, calcareous spikes/columns that become short limeless threads towards the base of the sporotheca, where they are attached; sporophores plasmodiocarpic, broader than high, sessile, broadly attached to the substrate . . . . . *Badhamiopsis*
6. Capillitium with both spike-like calcareous invaginations of the peridium, not vertically arranged, and a network of slender threads with scattered calcareous nodes; sporophores sporocarpic, plasmodiocarpic or aethalioid, higher than broad, sessile to stipitate 7
7. Sporophores sporocarpic, distinctly stipitate, rarely plasmodiocarpic . . . . . *Physarella*
7. Sporophores sessile to very shortly stipitate, plasmodiocarpic or aethalioid. . . . . *Fuligo* p.p. (*F. gyrosa*)
8. Capillitium with a double system of clearly calcareous tubes or plates, and a network of slender, limeless or nearly limeless tubules 9
8. Capillitium with a single system of calcareous tubes or a meshed net made of non-calcareous tubules connecting calcareous nodes. 10
9. Sporophores plasmodiocarpic, divided into compartments by vertical calcareous tubes or plates; peridium single . . . . . *Willkommlangea*
9. Sporophores sporocarpic, not divided into compartments by calcareous tubes or plates; peridium three-layered . . . . . *Leocarpus*
10. Sporophores aethalioid 11
10. Sporophores sporocarpic or plasmodiocarpic, rarely pseudoaethalioid 13
11. Sporophores with a distinctly thickened base . . . . . *Nannengaella* p.p. (*N. laevis*)
11. Sporophores without a clearly thickened base 12
12. Spores ≤ 9 µm diam . . . *Fuligo* p.p. (including *Erionema*)
12. Spores ≥ 10 µm diam . . . . . '*Aethaliopsis*' p.p.
13. Columella calcareous, columnar, usually dome-shaped or as a conical extension of the stalk or, if sessile, as a thickened base; stalk calcareous, sometimes absent; hypothallus often calcareous. 14
13. Columella rarely present, not calcareous; sometimes with a pseudocolumella formed by aggregation of lime nodes of the capillitium; stalk, when present, normally non-calcareous, sometimes frosted with lime; hypothallus usually non-calcareous 15
14. Sporocarps cup-shaped; columella columnar or cylindrical; calcareous deposits in stalk and columella at least partially crystalline (rhomboedric to irregular) . . . . . *Diachea* p.p.
14. Sporocarps not cup-shaped; columella dome-shaped or as a conical extension of the stalk; globular calcareous deposits . . . . . *Nannengaella* p.p.
15. Sporocarps cup-shaped, sometimes subglobose; peridium thick and usually cartilaginous; often, but not always, with dehiscence circumscissile, sometimes with a conspicuous preformed lid; the lower part of the peridium tending to persist as a more or less regular cup. . . . . *Craterium*
15. Sporocarps not cup-shaped, sometimes mixed with plasmodiocarps, or sporophores entirely plasmodiocarpic; peridium not cartilaginous; with irregular or areolate dehiscence, or dehiscence by fissures. 16
16. Spores angular, dark brown to blackish. . . . . '*Claustria*'
16. Spores globose, subglobose or slightly angular, violaceous brown to pale brown 17
17. Sporophores always plasmodiocarpic, sessile; peridium double or triple, with at least two distant layers often separating distinctly at dehiscence 18
17. Sporophores plasmodiocarpic or sporocarpic, sessile or stalked; peridium often single, sometimes double, but if also plasmodiocarpic, then, both layers closely appressed and with simultaneous dehiscence 19
18. Peridium double, whitish to greyish . . . . . '*Angioridium*'
18. Peridium triple, yellowish to ochraceous . . . . . *Physarum bogoriense* group
19. Peridium papery-membranous, often iridescent and somewhat translucent; stalk, when present, weak and thread-like to more or less membranous . . . . . *Badhamia* s.str.
19. Peridium rarely papery-membranous and iridescent; stalks, when present, generally stout, at least basally 20
20. Spores firmly conglobate . . . . . *Badhamia* s.lat.
20. Spores free, sometimes forming loosely adhering clusters 21
21. Sporotheca distinctly discoid-pezizoid; capillitium hyaline, weakly limy, connecting the lower and upper parts of the peridium. . . . . *Trichamphora*
21. Sporotheca very variable in shape, sometimes lenticular but not conspicuously discoid-pezizoid; capillitium different . . . . . '*Aethaliopsis*' p.p. + *Physarum* s.lat.
22. Peridium membranous, non-calcareous, iridescent . . . . . *Diachea* p.p.
22. Peridium calcareous (immature sporophores may present a non-calcareous peridium but they tend to mix with others with limy peridia) 23
23. Peridial lime exclusively in the form of minute globules (exceptionally lime needles also present in the middle peridial layer) 24
23. Peridial lime in the form of stellate or polyhedral crystals or scales, rarely mainly globules but then lime squamules also present, at least, in some fruiting bodies 26
24. Peridium marked with calcareous peg-like protuberances . . . . . **Physarina*
24. Peridium without peg-like protuberances, usually the lime is frosted or packed into a more or less eggshell-like layer, or packed forming a middle layer 25
25. Sporophores always long-stalked and without columella; sporotheca distinctly discoid-pezizoid; capillitium hyaline, netted; peridium single . . . . . *Trichamphora*
25. Sporophores sessile to stalked, often with columella (always present when the stalk is well-developed); sporotheca variable in shape but not distinctly discoid-pezizoid; capillitium usually brown, thread-like; peridium double or triple. . . . . *Diderma* p.p.
26. Peridium covered by stellate or polyhedral crystals, never globular, the crystals sometimes united into a continuous crust, then eggshell-like. . . . . *Didymium* p.p.
26. Peridium with conspicuous prismatic calcareous scales, rarely globular lime, inconspicuous or absent in some fruiting bodies 27
27. Sporophores sporocarpic, usually prominently stipitate, rarely sessile; peridium single, with scales . . . . . *Diderma* p.p. (*D. tigrinum*)
27. Sporophores sporocarpic to plasmodiocarpic, sessile or very shortly stipitate; peridium single to triple, with scales or, rarely, lime globules . . . . . *Polyschismium*

Future directions

Despite the advances on the phylogeny of the order *Physarales* presented here, data gaps are still preventing a completely resolved phylogenetic scheme for this order of *Myxomycetes*, especially in the case of the family *Physaraceae*. Then, we propose potential future directions to fill knowledge gaps that

we did not cover here: 1) to further expand the present study to the few genera that have not been analysed and, most importantly, to a much higher number of species of this order that could prove important for the resolution of *Physarales* phylogeny; 2) to use additional molecular regions, ideally those most informative phylogenetically; and 3) to conduct detailed ultrastructural studies in order to identify non-homoplastic synapomorphies. Then, we could map well-documented micro- and macromorphological traits and reconstruct the morphological characters that defined the last common ancestor of this order, and those of the ancestors of the different clades, which will allow us to get a better understanding of trait evolution across the tree of *Physarales*.

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Supplementary material

Fig. S1 Bayesian nSSU gene tree derived from 1252 nucleotide positions of 330 representative *Physarales*, with *Stemonitidales* as outgroup. Bayesian posterior probabilities (PP) along with maximum likelihood bootstrap (BS) and transfer booster bootstrap (TBE) support values are shown above and below each branch, respectively, if PP > 0.90, BS > 50 % and TBE > 70 %. Filled circles denote branches with full support (PP = 1, BS = 100 % and TBE = 100 %). Species names are followed by voucher number. The scale bar represents the average number of substitutions per site.

Fig. S2 Bayesian *EF-1α* gene tree derived from 941 nucleotide positions of 318 representative *Physarales*, with *Stemonitidales* as outgroup. Bayesian posterior probabilities (PP) along with maximum likelihood bootstrap (BS) and transfer booster bootstrap (TBE) support values are shown above and below each branch, respectively, if PP > 0.90, BS > 50 % and TBE > 70 %. Filled circles denote branches with full support (PP = 1, BS = 100 % and TBE = 100 %). Species names are followed by voucher number. The scale bar represents the average number of substitutions per site.

Fig. S3 Bayesian mtSSU gene tree derived from 375 nucleotide positions of 259 representative *Physarales*, with *Stemonitidales* as outgroup. Bayesian posterior probabilities (PP) along with maximum likelihood bootstrap (BS) and transfer booster bootstrap (TBE) support values are shown above and below each branch, respectively, if PP > 0.90, BS > 50 % and TBE > 70 %. Filled circles denote branches with full support (PP = 1, BS = 100 % and TBE = 100 %). Species names are followed by voucher number. The scale bar represents the average number of substitutions per site.

Fig. S4 Bayesian *α-Tub* gene tree derived from 799 nucleotide positions of 221 representative *Physarales*, with *Stemonitidales* as outgroup. Bayesian posterior probabilities (PP) along with maximum likelihood bootstrap (BS) and transfer booster bootstrap (TBE) support values are shown above and below each branch, respectively, if PP > 0.90, BS > 50 % and TBE > 70 %. Filled circles denote branches with full support (PP = 1, BS = 100 % and TBE = 100 %). Species names are followed by voucher number. The scale bar represents the average number of substitutions per site.

Fig. S5 Schematic representation of the nSSU gene. The two fragments amplified (nSSU-5' and nSSU-3') and the primers used appear in black, while introns are in white. The fragment that could not be amplified and the corresponding primer pairs are shown in grey.

Fig. S6 ASTRAL species tree based on the four maximum likelihood gene trees previously obtained with IQ-TREE. Numbers at nodes indicate local posterior probability support values (shown if > 0.90). Branch lengths are measured in coalescent units. Continuous and discontinuous vertical lines represent monophyletic and non-monophyletic groups, respectively.

Table S1 Summarised data on the 280 collections used for DNA extraction in this study.

Table S2 Summarised data on the 195 collections with data available in GenBank used in this study.

Table S3 Primers used for amplifying the molecular regions analyzed in this study.

Table S4 Best partition scheme and substitution model(s) for the final datasets, chosen according to BIC.

Table S5 Details of the morphological traits illustrated on the phylogenetic tree of Physarales shown in Fig. 3.

Table S6 Summary of the molecular signatures found for each clade or subclade recovered in Fig. 3.