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Protistology

Original article

Reassessing the phylogenetic position of the genus *Kelleromyxa* (Myxomycetes = Myxogastrea) using genome skimming data

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Summary

The order Physarales is the largest and the best studied group of myxomycetes, or plasmodial slime molds, distinguished on the basis of the morphology of fruiting bodies (sporophores) and molecular data. However, the structure of the order, as well as the boundaries separating its families, becomes a hot issue in the context of current phylogeny reconstructions. To solve the issue of the systematic position of the genus *Kelleromyxa* Eliasson, we carried out low-pass genome sequencing («genome skimming»), which allowed us to obtain contigs suitable for two-gene phylogeny reconstruction. Different methods of phylogeny reconstruction showed the same result: the genus *Kelleromyxa* cannot be clearly assigned to one of the families in the order Physarales, but assumes an intermediate position between Didymiaceae and Physaraceae. In turn, the topologies and statistical support of Maximum likelihood and Bayesian inference phylogenetic analyses clearly indicate the paraphyletic nature of both families, which only reinforces the conflict of morphological and molecular genetic concepts of taxa in myxomycetes.

Key words: Amoebozoa, Physarales, genome skimming, molecular phylogeny, small-subunit ribosomal RNA, taxonomy, translation elongation factor 1-alpha

Introduction

In the present work, we examine the phylogeny of the monospecific genus *Kelleromyxa* Eliasson, which comprises a single species *K. fimicola* (Dearn. and Bisby) Eliasson, first described in 1929 as *Licea fimicola* Dearn. and Bisby (Bisby et al., 1929). This species is one of the few obligate coprophilous species of myxomycetes and inhabits weathered dung of herbivores (Eliasson and Lundqvist, 1979; Eliasson and Keller, 1999; Eliasson, 2013; Calaça et al., 2020). The idea of *Licea fimicola* belonging to the genus *Licea* was originally questioned by Eliasson et al. (1991). Characters distinguishing this species from

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others in the genus *Licea* are the phaneroplasmodium (in contrast to protoplasmodium occurring in most *Licea* species), the presence of capillitial threads (absent in *Licea*) and the presence of calcium crystals in the peridium detected by X-ray analysis (absent in most *Licea*), although a crystalline substance, which may contain calcium was found in *L. alexopouli* (Mock and Kowalski, 1976). Spore color was the most significant difference: spores are dark in *L. fimicola*, whereas the other *Licea* spp. show brightly colored spores, as typical for the group of bright-spored myxomycetes (Leontyev et al., 2019).

Our previous study, based on the analysis of one complete and three partial sequences of the nuclear 18S rDNA (Erastova et al., 2013), confirmed the position of the genus *Kelleromyxa* within the order Physarales and its association with the dark-spored evolutionary lineage. However, the tree topology derived from a single marker did not allow us to assign *Kelleromyxa* reliably to one of the described families. A later phylogeny of myxomycetes by Leontyev et al. (2019) did not clarify the position of the genus in the myxomycete system, since the monophyletic clade "*Kelleromyxa* + *Physaraceae*" had low statistical support. This is a case where a monospecific genus disrupts the monophyly of a larger taxon, creating a taxonomic problem.

Thus, we decided to resolve this taxonomic conflict by reconstructing the two-gene phylogeny of the order Physarales using sequences of *Kelleromyxa fimicola* obtained by low-pass genome sequencing ("genome skimming").

Material and methods

Material studied

Sporophores of *Kelleromyxa fimicola* were obtained from moist chamber cultures set up with weathered samples of horse dung collected in August 2008 in the central Altay Mts. (Novozhilov et al., 2010). The samples were broken into pieces, air-dried *in situ* and transported back to the laboratory in sealed paper bags. The locality was geo-referenced using a portable GPS device (model Garmin 12, using WGS 84). In the laboratory, cultures were prepared by placing pieces on paper towels in Petri dishes (9 cm in diameter) lined with filter paper as described by Erastova et al. (2013). Voucher specimens were deposited in the mycological herbarium of the Komarov Botanical Institute, Laboratory of Systematics and Geography of Fungi (LE).

Specimen examined: LE255175; 18.01.2008, Russia, Central Altai Mts., Kurayskaya steppe, shallow valley of the Chichke River (branch of the Akturu River), 87°47'54"N, 50°33'32"E, 1530 m a.s.l.; on weathered dung of horse in moist chamber culture (collectors Y.K. Novozhilov, M. Schnittler, A.V. Vlasenko).

Morphological analyses

Air-dried sporophores were studied with a Zeiss Axio Imager A1 light microscope (LM) with differential interference contrast (DIC), a Stemi 2000 dissecting microscope (DM), a Zeiss motorized stereomicroscope ZEISS Axio Zoom.V16, and a JSM-6390 LA scanning electron microscope (SEM) at the Core Facility Center of the Komarov Botanical Institute of the Russian Academy of Sciences. For light microscopy, sporophores were preserved as permanent slides in polyvinyl-lactophenol. For SEM micrographs specimens were mounted on copper stubs using double-sided tape and sputter-coated with gold. Microscopic measurements were made with the program Axio Vision 4.8.0.0 (Carl Zeiss Imaging Solutions GmbH, free license). Color notations in parentheses are from the ISCC-NBS color-name charts illustrated with centroid colors (Centore, 2016).

DNA extraction

Extraction of genomic DNA for Sanger sequencing was performed from matured air-dried sporophores without a trace of fungal contamination. Approximately 2-5 sporophores were placed in 2 ml plastic tubes with screw caps. Ceramic balls 3 mm in diameter were added, tubes were frozen at -20 °C for at least 30 min and samples were finally crushed in a Bioprep-24 homogenizer (Hangzhou Allsheng Instruments, Hangzhou, China) with three cycles of 10 seconds at a speed of 6 m/sec, with intervals of 5 sec. DNA was extracted either with a PhytoSorb kit (Sintol, Moscow, Russia) according to the manufacturer's protocol with minor modifications (spore homogenate was eluted with 450 µl of extraction buffer; lysis buffer was added without preliminary precipitation step and supernatant transfer into a new sterile tube; final elution volume was 80-100 µl) or with a MagPure Plant DNA Kit (Magen Biotechnology, Guangzhou, China) according to the manufacturer's protocol using an automated

Table 1. Primer pairs and amplification protocols used in this study.

Name	F/R	Sequence (5' - 3')	Amplification protocol
S2	F	TGGTTGATCCTGCCAGTAGTGT	5 min at 95 °C, 36 cycles (30 sec at 95 °C, 20 sec at 56 °C, 50 sec at 72 °C) and 5 min at 72 °C
SSU_rev	R	AGACTTGTCCTCYAATTGTTAC	
PB1F	F	ACCCGTGAGCACGCTCTCCT	5 min at 95 °C, 36 cycles (30 sec at 95 °C, 30 sec at 65.4 °C, 1 min at 72 °C) and 10 min at 72 °C
PB1R	R	CGCACATGGGCTTGGAGGGG	
EF03	F	TGATCTACAAGTGCGGTG	5 min at 95 °C, 35 cycles (30 sec at 95 °C, 30 sec at 60 °C, 120 sec at 72 °C) and 10 min at 72 °C
KEF_R3	R	CCGTTCTTGATGTTCTTGG	
EF04	F	TGATCTACAAGTGCGGTG	5 min at 95 °C, 30 cycles (30 sec at 95 °C, 30 sec at 60 °C, 120 sec at 72 °C) and 10 min at 72 °C
KEF_R3	R	CCGTTCTTGATGTTCTTGG	

DNA extraction station Auto-Pure 96 (Hangzhou Allsheng Instruments, Hangzhou, China).

Since magnetic methods of purification cause uncontrolled fragmentation of high molecular weight genomic DNA, DNA extraction from a *Kelleromyxa fimicola* specimen was carried out using the ExtractDNA Blood and Cells (Evrogen, Moscow, Russia) spin column extraction kit. For this kit, sporophores containing about 1×10^5 spores were resuspended in 100 µl PBS solution (pH 7.4) and the mixture was left to incubate at room temperature for one hour. Cell lysis and DNA extraction was performed according to the manufacturer's protocol, with a final elution volume of 60 µl.

DNA amplification and Sanger sequencing

To expand taxon sampling for a phylogenetic tree, two unlinked genetic markers were studied for different species of Physarales with Sanger sequencing. A fragment of approximately 550 base pairs from the 5' end of the nuclear 18S rDNA gene (nrSSU), which is free of introns, was obtained with forward primer S2 (Fiore-Donno et al., 2008) and reverse primer SSU_rev (Prikhodko et al., 2023). Fragments of the protein-coding gene for the translation elongation factor 1-alpha (EF1α) was either obtained using the PB1F/PB1R primer pair alone (Novozhilov et al., 2013) or assembled from reads obtained using PB1F/PB1R and a set of primers for a semi-nested PCR EF03(EF04)/KEF_R3 (Wrigley de Basanta et al., 2017; Ronikier et al., 2020). The list of primers, their sequences, and amplification protocols for different primer combinations are provided in Table 1.

PCR reactions were prepared with 2 × BioMaster HS-Taq PCR-Color reaction mix (Biolabmix, Novosibirsk, Russia) with 50 mM KCl, 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.06 U/µl TaqDNA polymerase, 0.2% Tween20, several dyes (xylene cyanol, bromphenol blue, OrangeG, tartrazine) with addition of 3 nmol of each primer, 2 µl of template DNA and diluted with diH_2O to obtain a total volume of 20 µl. The amplification was carried out via thermal cycler C1000 Touch (Bio-Rad, Hercules, CA, USA). Products of amplification were stained with GelRed (Biotium, San Francisco, CA, USA), separated by 1.2% agarose gel electrophoresis, observed in Gel Doc XR+ System (Bio-Rad, Hercules, CA, USA), and then purified using the CleanMag DNA (Evrogen, Moscow, Russia) purification kit before being sequenced with the BrilliantDye Terminator v3.1 Cycle Sequencing Kit (NimaGen, Nijmegen, the Netherlands). Sequencing products were purified with the Nimagen D-Pure DyeTerminator Cleanup kit, and then analyzed on ABI 3500 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

It is worth noting that attempts to amplify and sequence mitochondrial cytochrome c oxidase I (COI) using the previously described protocol (Prikhodko et al., 2023) were also carried out, but failed for key

Physaraceae species such as *Badhamia capsulifera* and *Physarum viride*. Thus, we decided to exclude this gene from further analysis and discussion.

Low-pass genome sequencing

The spectral characteristics of the *Kelleromyxa fimicola* DNA extract and its DNA content were measured with an Implen P300 nanophotometer (Implen, USA). Further DNA quality control, library preparation and shotgun whole-genome sequencing were performed by a third-party organization (Institute of Genomic Analysis, Moscow, Russia). DNA was fragmented using Covaris ME220 (Covaris, USA) to achieve fragment length distribution peak in range 250-320 bp, then DNA library was prepared using MGIEasy Universal DNA Library Prep (MGITech, China). Sequencing with target coverage of the nuclear genome 2X was performed on DNBSeq 400 (MGITech, China) using DNBSEQ-G400RS High-throughput Sequencing Set (FCL PE150) according to manufacturer's instructions. The resulting paired-end reads of 150 bp length were assembled in contigs using SPAdes 3.15.4 (Prjibelski et al., 2020) with a --careful flag for a more thorough error correction. Summary statistics were calculated using QUAST 5.2.0 (Mikheenko et al., 2018). Contigs containing orthologs of the genes of interest were identified using the --usearch_global command in VSEARCH 2.18.0 (Rognes et al., 2016) based on similarity to available sequences.

Sequence alignment and phylogenetic analyses

18S rDNA and EF1α sequences were combined in two multiple alignments in Unipro UGENE (Okonechnikov et al., 2012) and aligned using MAFFT online service (Katoh and Standley, 2013; Katoh et al., 2019) with E-INS-I or G-INS-i options, respectively, and default gap penalties. After manual editing, primer trimming, and shortening of excessively long contigs derived from *Kelleromyxa fimicola* and *Echinostelium bisporum*, two sets of nucleotide sequences were merged into a single alignment using SequenceMatrix 1.9 (Vaidya et al., 2011). The nrSSU sequences were analyzed as a single partition, while two separate partitions were defined for the EF1α sequences: the first and second positions of each codon were analyzed separately from the third positions. The exon parts of the EF1α sequences were determined according to the sequence from *Echinostelium bisporum* (GenBank MH814572) obtained from transcriptome data (Fiore-Donno et al., 2019).

The final alignment consists of 113 sequences with 2257 sites, 1272 distinct patterns, 265 singleton sites and 1184 non-informative (constant) sites. Maximum likelihood (ML) analyses were performed using IQ-TREE 1.6.12 (the last stable release; Nguyen et al., 2015) launched on the local machine. The TIM2e+R4 model was selected for the nrSSU partition according to the ModelFinder tool implemented in the program (Kalyaanamoorthy et al., 2017). TPM3+F+I+G4 and TVM+F+G4 models were selected for the first two and third positions of each codon in EF1α partitions, respectively. Ultrafast bootstrap analysis with one thousand replicates (Hoang et al., 2018) was performed to obtain confidence values for the branches. Bayesian analysis was performed with the same dataset using mrBayes 3.2.7a (Huelsenbeck and Ronquist, 2001) run on CIPRES Science Gateway (Miller et al., 2010); the GTR+G+I model was applied. The phylogenetic analysis was run four times as four separate chains for 10 million generations (sampling every 1000). The convergence of MCMCMC was estimated using TRACER 1.7.2 (Rambaut et al., 2018); based on the estimates by TRACER, the first 2.5 million generations were discarded as burn-in. Posterior probabilities (PP) of splits were exported to the best-scoring ML-tree. Phylogenetic tree with combined supports was visualized using FigTree 1.4.4 and edited using CorelDRAW 24.0.

To test for the influence of nrSSU alignment uncertainty and bias of the tree reconstruction methods, two additional analyses were performed. First, nrSSU sequences were aligned using PRANK v.170427 (Löytynoja, 2014), which tends to introduce more gaps compared to MAFFT and is thus more conservative in finding homologous sites. The resulting alignment 1418 bp long was concatenated with EF1α exon alignment and analyzed using RAxML-NG 1.2.0 (Kozlov et al., 2019). The analysis was performed with 100 distinct starting trees (50 random + 50 maximum parsimony) and the same partitions and models as for IQ-Tree, with a slight change in the model for nrSSU (TIM2ef+I+G4) suggested by ModelTest-NG 0.1.7 (Darriba et al., 2019). For the best-scoring tree, non-parametric bootstrap support values were calculated based on 1000 replicates. Second, sequences were analyzed using BAli-Phy 4.0-beta4 (Redelings, 2021). BAli-Phy is a Markov chain Monte Carlo program

that jointly estimates phylogeny and alignment from unaligned sequence data and treats indels as evolutionary events. Two partitions were defined: unaligned nrSSU sequences under TN93+G+I model and a fixed EF1α exon alignment under GTR+G+I model. The analysis was run in three independent chains until approximately 17600 generations were sampled in each chain, with 10% burn-in. A greedy consensus tree with split posterior probabilities was computed based on the sampled trees. The two resulting trees were visualized using FigTree 1.4.4 and compared with the best-scoring IQ-Tree ML phylogeny.

Results

Morphology of *Kelleromyxa fimicola*

In moist chamber cultures, we observed phaneroplasmodia up to 6 mm in size with a vivid yellowish pink (25) color, leaving numerous conspicuous black sclerotia and plasmodial tracks on the surface of filter paper (Fig. 1, A). Immature sporophores (Fig. 1, B) are deep pink (3). During the maturation, the color turns to shiny black (267; Fig. 1, C). The peridium consists of two closely adhering layers: the inner layer is membranous with a papillose ornamentation; the outer layer is darker and has a leathery appearance (Fig. 1, D). Capillitial threads are well developed mostly as short, narrow (0.5–1.0 μm) simple peridial outgrowths (Fig. 1, E). The spore ornamentation consists of long (0.9–1.2 μm) and scattered large blunt spinules (Fig. 1, F) and ridges that are visible even by LM (Fig. 1, G). Thus, the morphological features of specimen LE255175 studied here agree with previous descriptions (Erastova et al., 2013).

Low-pass genome sequencing data analysis

4.7 million paired-end reads were assembled into 33851 contigs, with N50=71, L50=10641, GC-content 40.37% and largest contig 110432 bp. Read coverage was 1038 for nrSSU, 4 for EF1α and 491–550 for mitochondrial genes.

Phylogenetic analysis

A total of 38 new nucleotide sequences were generated for this study, including close to complete sequences of nuclear 18S rDNA (nrSSU), nuclear translation elongation factor 1-alpha (EF1α), mitochondrial cytochrome c oxidase I (COI), and mitochondrial 16S rDNA gene (mtSSU) of *Kelleromyxa fimicola*. The list of newly obtained sequences, the concatenated alignment, partition file, and phylogenetic tree in the Newick format can be found in Supplementary materials and FigShare (DOI:10.6084/m9.figshare.23460152).

Figure 2 shows the resulting two-gene phylogeny. The tree is rooted with *Barbeyella minutissima* and *Echinostelium bisporum* (Echinosteliales), followed by two sister clades consisting of species of the genus *Meriderma* (Meridermatales) and representatives of the order Physarales, respectively. Within Physarales, we can clearly distinguish two monophyletic clades: three species of genus *Lamproderma* (Lamprodermataceae = LAM), including type species *L. columbinum*, form a well-supported clade (UBS/PP = 98/1), occupying a sister position to the complex clade uniting Didymiaceae and Physaraceae (UBS/PP = 99/0.95). The genus *Kelleromyxa* is positioned between Didymiaceae and Physaraceae, but cannot be clearly assigned to one of the two families because of the tree topology and insufficient support for the clade uniting *Kelleromyxa* and Physaraceae (UBS/PP = 82/0.82).

Branches that received high statistical support in Figure 2 were reproduced in the two additional analyses that relied on different alignment and tree reconstruction methods (PRANK+RAxML-NG and BAli-Phy). The position of the clades with short branches and low support, in contrast, varied between the analyses. However, *Kelleromyxa fimicola* was always placed within Physarales with low statistical support (Supplementary File S4).

Discussion

The phylogeny presented herein (Fig. 2) is based on two independent genetic markers and confirms main results obtained in previous studies (discussed below). In addition, the use of a wide list of taxa and high statistical support for a number of clades allows us to draw new conclusions regarding taxonomy of Physarales.

The phylogenetic position of the genus *Kelleromyxa*

As stated by Erastova et al. (2013), the main limitation hampering a reliable assignment of the genus *Kelleromyxa* to one of the families of dark-

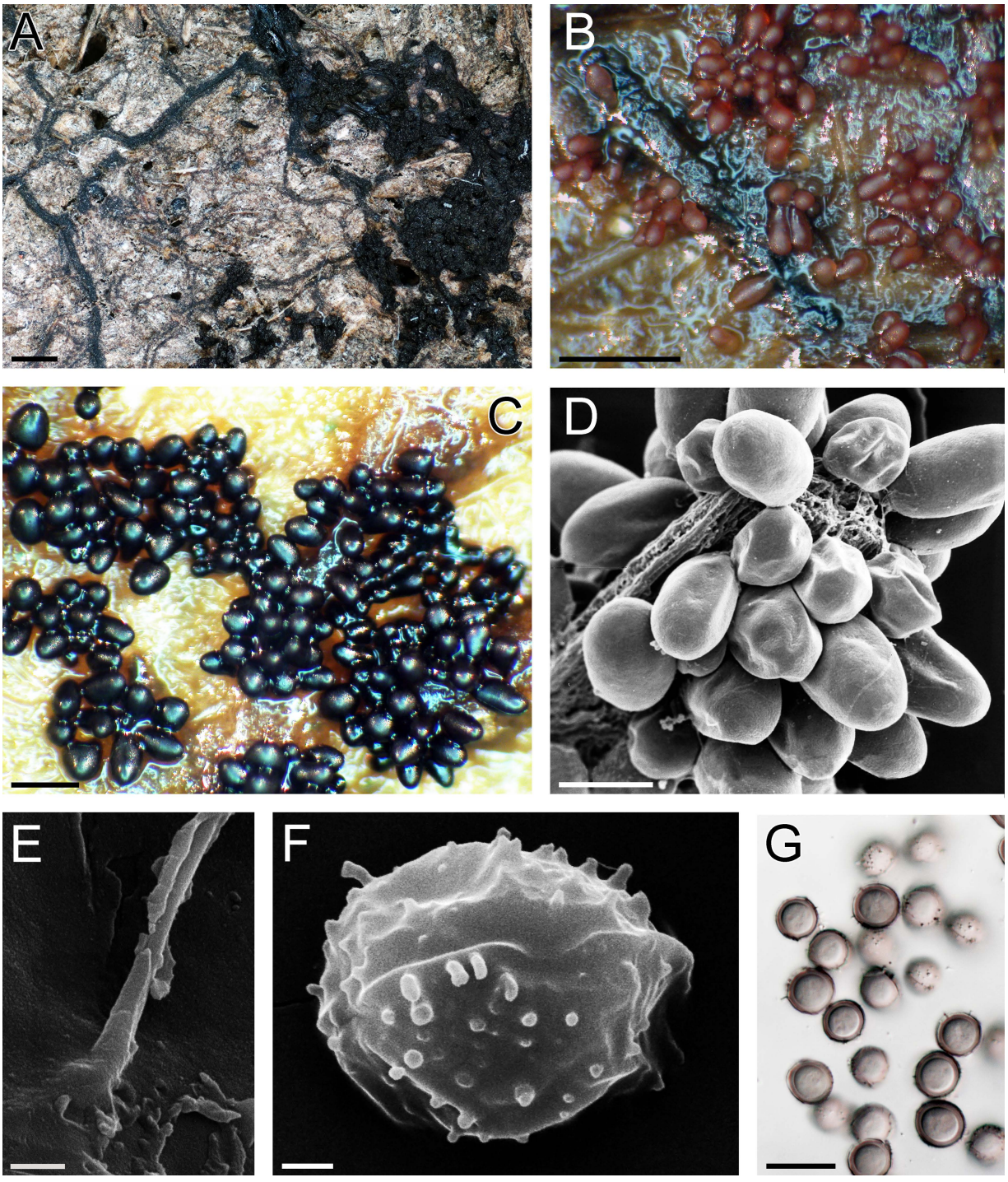

Fig. 1. Morphological characters of *Kelleromyxa fimicola* (LE255175). A – Traces of a phaneroplasmodium and sclerotia on filter paper in moist chamber culture; B – group of immature sporophores on weathered horse dung in moist chamber culture; C – mature sporophores in moist chamber culture; D – mature sporophores under SEM; E – a capillitial thread connected to the inner side of the peridium under SEM; F – spore ornamentation under SEM; G – spores under light microscope (oil immersion, ×100, top and median view). Scale bars: A–D = 100 μm; E, F = 1 μm; G = 10 μm.

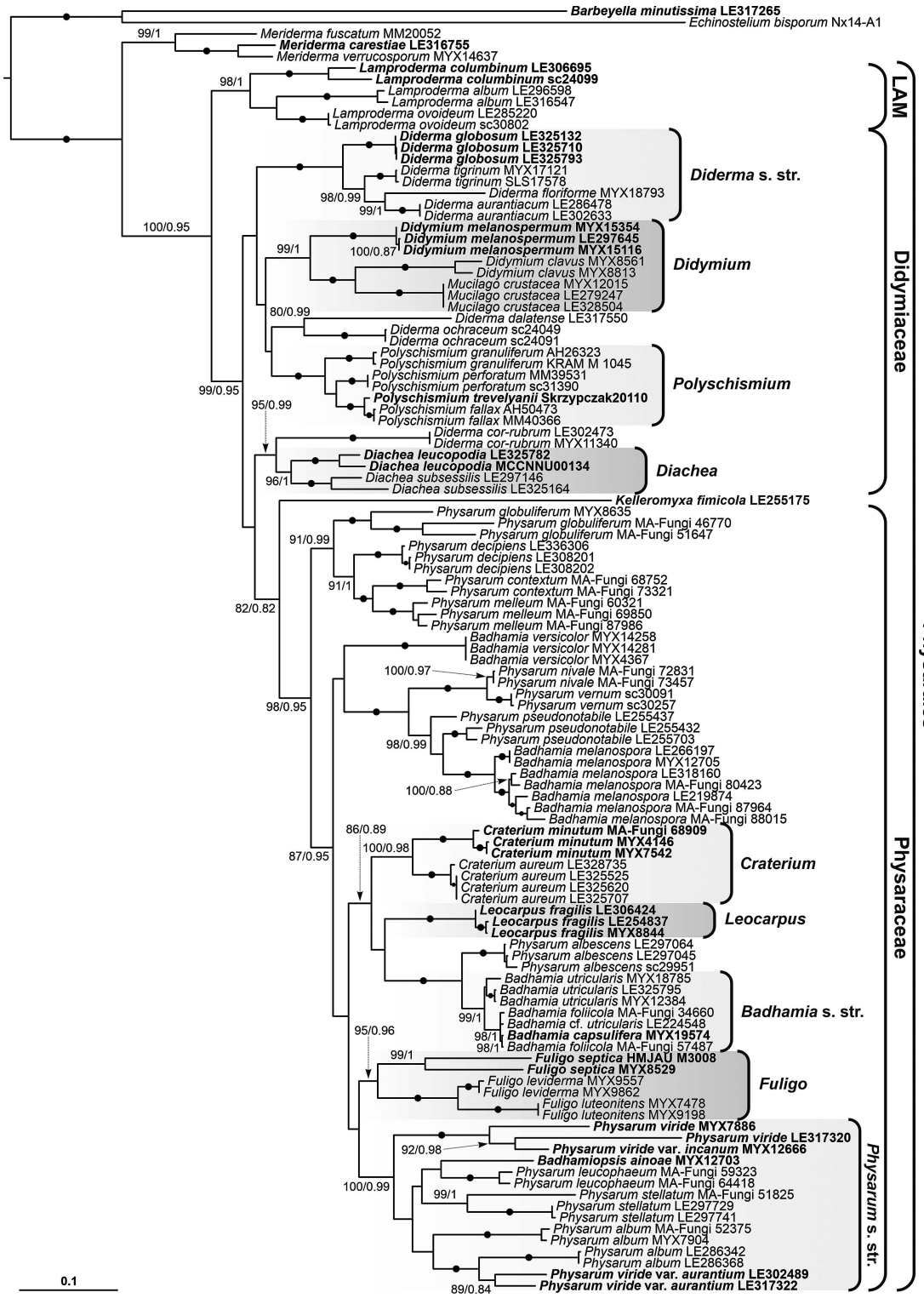

Fig. 2. Maximum-likelihood phylogenetic tree of the order Physarales obtained from concatenated nrSSU and EF1α sequences using IQ-Tree. Bold font indicates the type species of a genus. Branch supports are shown only for UBS/PP ≥ 80/0.8; black dots indicate maximum supports in both analyses (UBS/PP = 100/1); scale bars represent the mean number of nucleotide substitutions per site. LAM = Lamprodermataceae family. The nomenclature used in the tree is checked against the current version of the nomenclature database of Lado (2005–2023).

-spored myxomycetes was «limited extent of taxon sampling». Specifically, the previous phylogeny reconstruction was performed without including representatives of the genera *Craterium, Leocarpus,* and *Willkommlangea* (Physarales) in the analysis. By expanding the list of taxa included in the phylogenetic analysis and adjusting the list of specimens so that each specimen on the tree is represented by a pair of nrSSU and EF1α sequences, we attempted to avoid these limitations. However, all our analyses resulted in the same problematic topology that occurred earlier in the single-gene (Cainelli et al., 2020), two-gene (Ronikier et al., 2022), and three-gene (Prikhodko et al., 2023) phylogenies. Specifically, our results confirm that there are no monophyletic units corresponding to the morphologically circumscribed families Didymiaceae and Physaraceae, but there is a large monophyletic clade, sister to the family Lamprodermataceae, in which species traditionally assigned to the families Didymiaceae and Physaraceae are consistently grouped.

There are several possible explanations for this contradiction:

1) currently, nucleotide sequences have been obtained from only about one-tenth of the entire described diversity of the families Didymiaceae and Physaraceae, which does not provide sufficient resolution for phylogenetic analyses;

2) the two-gene DNA sequence data that we use may lack phylogenetic signal to resolve the relationships between families; García-Cunchillos et al. (2022) stated that the analysis of the EF1α gene «only supported phylogenetic affinities among specimens of the same species or closely related taxa»;

3) the phylogenetic reconstruction strategy we chose may be incorrect if there is a gene tree – species tree discordance due to rapid speciation and large ancestral population of the common ancestor of Physaraceae and Didymiaceae; in this case, a species tree inference under multi-species coalescent model with more loci should be applied;

4) the traditionally distinguished families Didymiaceae and Physaraceae may indeed turn out to be artificial taxa, since key morphological features may not correlate with specific gene trees but result from convergent evolution.

At this stage, it is impossible to prove or disprove the latter statement; therefore, the separation of the genus *Kelleromyxa* into an independent family or its assignment to one of the described families of Physarales seems impossible without expanding the sampling of taxa and genome loci. In particular, molecular data from the monotypic genera *Erionema* Penz., *Physarella* Peck, and *Willkommlangea* Kuntze are lacking for a reliable reconstruction of the Physaraceae phylogeny.

Conflicts between morphological characters and phylogenetic data

Our attempt to clarify the phylogeny of the genus *Kelleromyxa* not only showed the need to introduce additional markers into the analysis, but also highlighted a number of unresolved questions in the phylogeny of the entire family Physaraceae.

In our current phylogeny, the «Physaraceae» clade is represented by species of six main genera of the family Physaraceae. Taking into account the current sampling of species, tree topology and statistical supports, the genera *Craterium, Leocarpus,* and *Fuligo* appear to be monophyletic. Meanwhile, *Badhamia* and *Physarum* are clearly para- or polyphyletic taxa, which is in accordance with Nandipati et al. (2012).

With ca. 160 validly described species (Lado, 2005-2023), *Physarum* Pers. is the most diverse genus of myxomycetes. It includes species with different morphologies of sporophores and ecological affinities united by one common feature – a capillitium of the «physaroid» type with hyaline tubules connecting calcareous nodes (Martin and Alexopoulos, 1969). The structure of the phylogenetic tree shows a disagreement between the molecular data and the taxonomy of the group based on the morphological similarity of the sporophores. Stalked vs. sessile sporophores are a good example. Traditionally, the presence of a stalk in fruiting bodies is a trait that was given a rather high taxonomic weight (Martin and Alexopoulos, 1969; Farr, 1976). In contrast, the most basal position within Physaraceae is occupied by a monophyletic clade (UBS/PP = 91/0.99), which includes sessile or plasmodiocarpic species (*Physarum decipiens* and *Ph. contextum*) and species with sporangia on calcified stalks (*Ph. globuliferum* and *Ph. melleum*). At the same time, the type variety of a type species *Physarum viride* var. *aurantium*, together with *Ph. viride* var. *incanum* and three other species with stalked sporangia (*Ph. album, Ph. leucophaeum*, and *Ph. stellatum*) forms a well-supported terminal clade, designated by us as «*Physarum* s. str.» (Fig. 2; UBS/PP = 100/0.99). However, the monophyletic nature of this clade is disrupted by the presence of *Badhamiopsis ainoae*, the

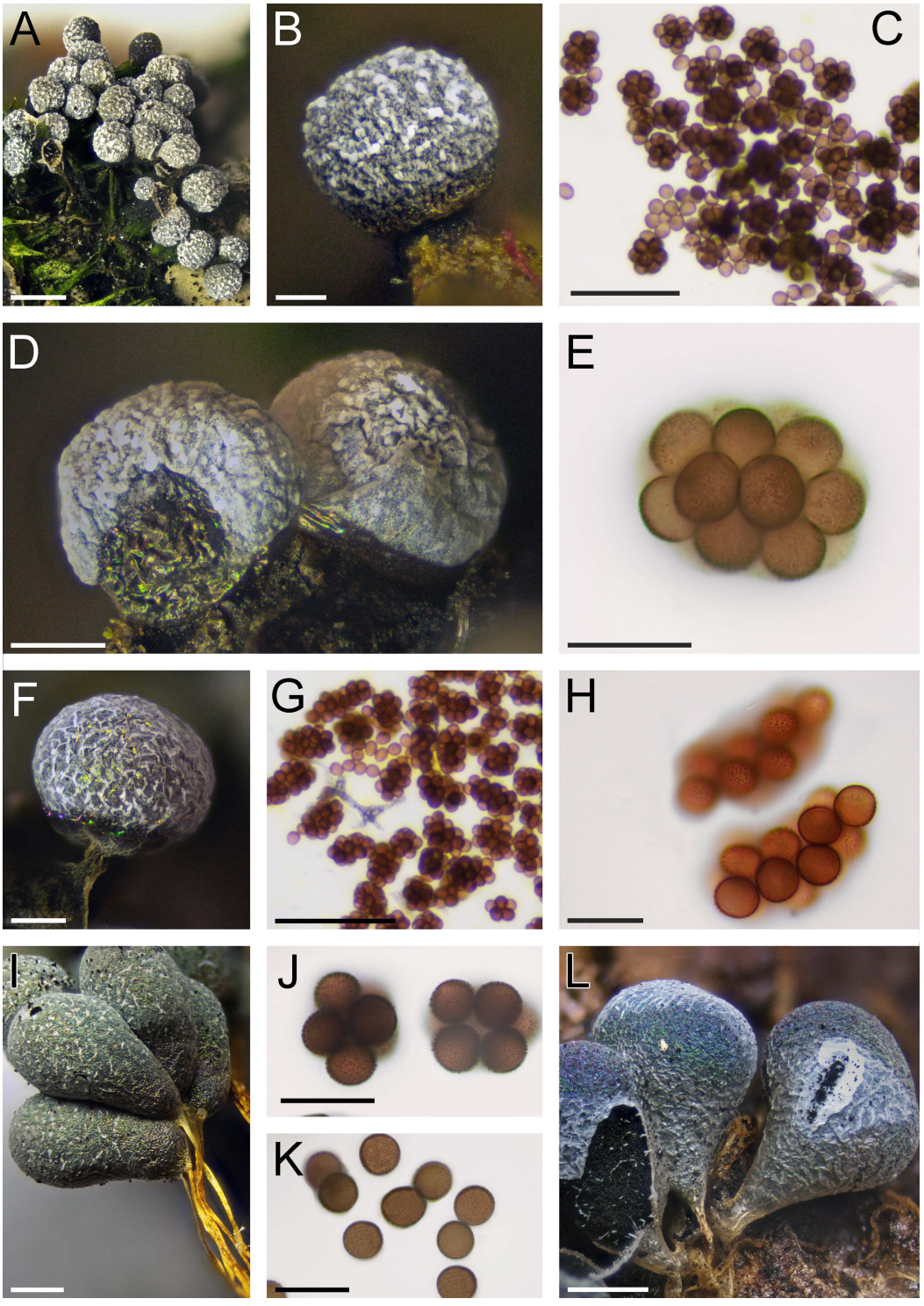

Fig. 3. Morphological characters of studied *Badhamia* species. A–E – *Badhamia versicolor* (A–C from MYX14281, D–E from MYX4367); F–H – *Badhamia capsulifera* (MYX19574); I–L – *Badhamia utricularis* (I–J from MYX18785, K–L from MYX12384). A, B, D, F, I, L – mature sporangia; C, G – spores under light microscopy (×10, without cover glass); E, H, J, K – spores under light microscopy (×100, oil immersion, top and median view). Scale bars: A, I, L = 500 μm; B, C, G = 100 μm; D = 200 μm; E, H, J, K = 20 μm; F = 200 μm.

type species of the genus *Badhamiopsis* T.E. Brooks and H.W. Keller, with unique capillitium represented by tubular invaginations from the upper peridium, simple or occasionally bifurcate, more or less spike-like, usually enclosing dense deposits of white lime granules (Keller and Brooks, 1976).

A similar conflict between morphology and molecular phylogeny can be observed among the species of the genus *Badhamia* Berk., a genus traditionally characterized by uniform calcification of capillitium rarely turning into a «physaroid» type. Specimens of *Badhamia versicolor* (Fig. 3, A–E), which were initially wrongly attributed to *B. capsulifera* var. *arborea* (Prikhodko et al., 2023), are characterized by spherical or somewhat flattened sporangia, and constantly differ from type species *B. capsulifera* (Fig. 3, F–H) only by a smaller diameter of sporophores (0.2–0.5 mm vs. 0.5–1.5 mm) and a smaller number of spores in clusters (6–20 vs. 10–40 pcs.). However, *B. versicolor* and *B. capsulifera* do not appear closely related in any of our phylogenies obtained with different methods. At the same time, *Badhamia utricularis* (Fig. 3, I–L), which is characterized by drooping obpyriform sporangia with stalks up to several centimeters long and spores dispersing both in small clusters (Fig. 3, J) and solitary (Fig. 3, K), forms a monophyletic clade with the sessile or short-stalked species *B. capsulifera*, regardless of the phylogeny reconstruction strategy.

In light of these results, we favor molecular topology, considering that the traditionally accounted morphological characters of the genera *Badhamia, Badhamiopsis,* and *Physarum* do not adequately reflect phylogenetic relationships. These results have implications for understanding the distribution of homoplasy in data sets of morphological characters of myxomycete species, as well as the utility of morphology as a test of molecular hypotheses (Pisani et al., 2007; Oyston et al., 2022). As mentioned for stalked vs. sessile fructifications, we must assume frequent convergent evolution, especially in conspicuous characters like solitary vs. compound fructifications, spinulose vs. reticulated spores, or colored vs. colorless lime.

Acknowledgments

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Supplementary material

File S1. Concatenated alignment.

File S2. Partition file in RAxML format.

File S3. Raw phylogenetic tree in the Newick format.

File S4. Additional trees obtained by BAli-Phy and RAxML-NG.

File S5. nrSSU alignment obtained with PRANK

File S6. Alignment obtained with BAli-Phy

Table S1. List of sequences used in this study.