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ORIGINAL ARTICLE

A three-gene phylogeny supports taxonomic rearrangements in the family *Didymiaceae* (*Myxomycetes*)

Ilya S. Prikhodko[1] · Oleg N. Shchepin[1,2] · Nadezhda A. Bortnikova[1] · Yuri K. Novozhilov[1] · Vladimir I. Gmoshinskiy[3,4] · Gabriel Moreno[5] · Ángela López-Villalba[2,5] · Steven L. Stephenson[6] · Martin Schnittler[2]

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Abstract

Myxomycetes, or plasmodial slime molds, are a monophyletic group of amoeboid protists whose classification is based mainly on morphological features of fruiting bodies. Although published phylogenies based on one or two genetic markers have clarified the boundaries of the main order-level systematic groups, the position and composition of some families and genera of myxomycetes are still a topic for discussion. In this study, we reconstructed the phylogeny of the family *Didymiaceae* based on three independent genetic markers: the 18S rDNA gene, the translation elongation factor 1-alpha, and the cytochrome c oxidase subunit 1 gene. Maximum likelihood and Bayesian inference phylogenetic analyses produced congruent topologies and showed that of the five major genera of the family, only species of the genus *Diachea* form a monophyletic clade, while the other four genera are clearly para- or polyphyletic. Species of the genus *Didymium* form a monophyletic clade with the only species of the genus *Mucilago*. The polymorphic species *Lepidoderma tigrinum* is clearly placed among 13 species of *Diderma*, including the type species of the genus. All other studied species of *Lepidoderma* form a separate clade together with *Diderma fallax*. We thus extend the latest nomenclatural revisions by disbanding the genera *Mucilago* and *Lepidoderma*, whereby the single species of *Mucilago* is transferred to the genus *Didymium* and *L. tigrinum* to *Diderma*. Extended taxon sampling allows the transfer of more nivicolous species of the former genus *Lepidoderma* to *Polyschismium*.

Keywords 7 new combinations · *Diderma* · Nomenclature · *Lepidoderma* · *Mucilago* · *Myxomycetes*

Introduction

Myxomycetes (= *Myxogastria*), the true or plasmodial slime molds, form a monophyletic clade within the Amoebozoa supergroup (Adl et al. 2012, 2019) and are characterized by a distinctive life cycle involving multinucleate unicellular plasmodia and complex fruiting bodies. According to the nomenclatural database of Lado (2005–2022), more than 1100 valid species are recognized, and these have been traditionally divided into five orders, distinguished on the basis of macro- and microscopic features of the fruiting bodies and spores. The foundations of this classification were laid as early as the nineteenth century in the first monograph on the group (Rostafiński 1874).

Although the classification of myxomycetes has remained relatively stable for more than a century, the advent of

Section Editor: Tanay Bose

✉ Ilya S. Prikhodko
Hypnothotep@gmail.com

[1] Laboratory of Systematics and Geography of Fungi, The Komarov Botanical Institute of the Russian Academy of Sciences, Prof. Popov Street 2, St. Petersburg 197376, Russia

[2] Institute of Botany and Landscape Ecology, Ernst Moritz Arndt University Greifswald, Soldmannstr. 15, D-17487 Greifswald, Germany

[3] Department of Mycology and Algology, Faculty of Biology, Moscow State University, Leninskie Gory 1/12, Moscow 119992, Russia

[4] Polistovsky National Nature Reserve, Sovetskaya st., 9B, Bezhanitzy 182840, Russia

[5] Departamento Ciencias de la Vida (Botanica), Universidad de Alcala, Alcala de Henares, 28805 Madrid, Spain

[6] Department of Biological Sciences, University of Arkansas, Fayetteville, AR 72701, USA

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molecular phylogenetics has forced it to change rapidly. The application of molecular phylogenetic methods confirmed some of the major systematic groups of myxomycetes (Fiore-Donno et al. 2005) and showed a basic bifurcation into the dark- and bright-spored myxomycetes (Fiore-Donno et al. 2012, 2013). However, the expansion of species sampling and the improvement of phylogenetic analyses also revealed the artificial nature of many traditional taxa, especially families and genera. Sequencing of the 18S rRNA (SSU) gene has made it possible to substantiate the separation of several species of the genus *Lamproderma* Rostaf. into the independent genus *Meriderma* Mar. Mey. & Poulain (Fiore-Donno et al. 2008; Poulain et al. 2011), now placed in the order *Meridermatales* (Leontyev et al. 2019). Further work in this direction pointed to the poly- or paraphyly of the species-rich genera *Lamproderma* (Fiore-Donno et al. 2012; Novozhilov et al. 2022b) and *Physarum* Pers. (Nandipati et al. 2012; Cainelli et al. 2020). Similarly, a two-gene (Fiore-Donno et al. 2013; Ronikier et al. 2020) and a three-gene phylogeny (García-Cunchillos et al. 2022) using SSU, the nuclear translation elongation factor 1-alpha gene (*EF1A*) and the mitochondrial small subunit ribosomal RNA (mtSSU) elucidated the complex structure of families and genera in the orders *Liceales* and *Trichiales*. Although the last major SSU-based phylogeny and systematic revision (Leontyev et al. 2019) made it possible to identify monophyletic groups at the order level, the question of delimitation of some genera and families of myxomycetes remains open.

The *Didymiaceae* is the second largest family of the dark-spored myxomycetes after the *Physaraceae*. The core of the family is constituted by two large genera. *Diderma* Pers. was first described at the end of the 18th century (Persoon 1794). Currently, this genus comprises 85 valid species (Lado 2005–2022; Novozhilov et al. 2022a) united by a one-, two- or three-layered peridium with granular lime. Another large genus, *Didymium* Schrad., includes ca. 100 species with single-layered peridium covered by crystalline lime (Shrader 1797). All other genera of the family are less diverse. *Lepidoderma* de Bary ex Rostaf (1873) includes 15 valid species with a one- or two-layered peridium abundantly covered with crystalline scales, *Diachea* Fr. includes 13 species (Fries 1825; Lado 2005–2022; Lado et al. 2022) with a stalk and columella filled with lime granules, and *Mucilago* P. Micheli ex Adans. is a monotypic genus with aethalia covered with crystalline lime (Adanson 1763) as is the case in *Didymium*.

Although the species of *Didymiaceae* have been addressed in different phylogenies (Fiore-Donno et al. 2008, 2010a, 2012; Cainelli et al. 2020; Novozhilov et al. 2022a, b), only one systematic revision based on molecular data has been published, thus far, and this transferred 9 of the 15 recognized species of *Lepidoderma* into the monophyletic genus *Polyschismium* Corda (Ronikier et al. 2022). At the same time, a number of published papers have pointed to a non-monophyletic nature of genera within the *Didymiaceae*. Wikmark et al. (2007a, b) showed trees based on SSU and 28S rDNA (LSU) in which *Didymium anellus* Morgan formed a supported clade with species of *Diderma* but not with the "Didymium" clade. SSU-based (Fiore-Donno et al. 2008, 2010a) and two-gene phylogenies (including as well *EF1A*; Fiore-Donno et al. 2010b, 2019; Cainelli et al. 2020; Ronikier et al. 2022) showed that *Lepidoderma tigrinum* (Schrad.) Rostaf., the type species of the genus *Lepidoderma*, formed a monophyletic clade with species of *Diderma*. Finally, it was shown in SSU-based (Fiore-Donno et al. 2010a; Wrigley de Basanta et al. 2015) and two-gene phylogenies (Zhao et al. 2021) that the monotypic genus *Mucilago* forms a well-supported clade within the genus *Didymium*. All these facts motivated us to carry out a molecular phylogenetic study aimed at clarifying the taxonomic status of the main genera making up the family *Didymiaceae* and their position in the order *Physarales*, with a special focus on *Lepidoderma* and *Mucilago*.

Materials and methods

Sampling and morphological studies

The studied specimens were collected in different regions of Europe, Asia, Australia, New Zealand, and North America and preserved in the Herbarium of the Komarov Botanical Institute RAS (LE), Collection of Myxomycetes at the Department of Mycology and Algology (Faculty of Biology, Lomonosov Moscow State University) (MYX), Herbarium of the Department of Plant Biology (University of Alcalá) (AH) and the private collections of M. Meyer (MM), M. Schnittler (Sc), and S.L. Stephenson (SLS). In addition, seven herbarium specimens, including holotypes of *Lepidoderma alpestroides* Mar. Mey. & Poulain and *L. perforatum* Mar. Mey. & Poulain and the paratype of *L. crustaceum* Kowalski, were requested from the Meise Botanic Garden (BR).

The identification of the air-dried fruiting bodies was confirmed based on macro- and micromorphology or by using DNA sequencing. Light microscopy was carried out with a Zeiss Axio Imager A1 light microscope (LM) with differential interference contrast (DIC), a Zeiss Stemi 2000 dissecting microscope (DM), and a Zeiss Axio Zoom.V16 (Carl Zeiss Microscopy Deutschland GmbH, Oberkochen, Germany) motorized stereomicroscope. For microscopy, sporocarps were preserved as permanent slides in polyvinyl-lactophenol. Microscopic measurements were made using Zeiss Zen 3.2 software. Scanning electron microscopy (SEM) was performed with a JSM-6390 LA (Jeol Ltd., Akishima, Japan) at the Core Facility Center of the Komarov Botanical Institute of the Russian Academy of Sciences and with Quattro S (Thermo Fisher Scientific, Waltham, USA) and JSM-6380LA (Jeol

Ltd., Akishima, Japan) at the Interdepartmental Laboratory of Electron Microscopy at the Faculty of Biology of Moscow State University. Specimens for SEM were mounted on copper stubs with a double-sided tape or a thin coat of varnish and coated with gold or gold-palladium, respectively.

DNA extraction, amplification, and sequencing

Extraction of genomic DNA was performed from matured air-dried fruiting bodies without a trace of fungal contamination. Approximately 2–5 sporocarps or small fragments of plasmodiocarps were placed in 2 ml safe-lock tubes with addition of steel balls 3 mm in diameter and frozen at –20 °C for at least 30 min. Afterwards, the samples were crushed in a TissueLyser LT homogenizer (QIAGEN, Hilden, Germany) for 1 min at 30 Hz. DNA was extracted either with PhytoSorb (Sintol, Moscow, Russia) according to the manufacturer's protocol with minor modifications (the spore homogenate was eluted with 450 µl of extraction buffer; lysis buffer was added without a preliminary precipitation step and supernatant transfer into a new sterile tube; final elution volume was 80–100 µl) or with the Mag-Bind Plant DNA DS 96 Kit (Omega Bio-tek, Norcross, USA) according to the manufacturer's protocol.

To reconstruct the phylogeny, three unlinked genetic markers were sequenced. A fragment of approximately 550 base pairs from the 5' end of the 18S rDNA gene (SSU) that is free of introns was obtained with forward primers S1 or S2 (Fiore-Donno et al. 2008) and reverse primers SU19R (Fiore-Donno et al. 2011) or SSU_rev (designed for this study by the first author). Overlapping fragments of the protein-coding gene for the translation elongation factor 1-alpha (*EF1A*) were amplified with primer pairs PB1F/PB1R (Novozhilov et al. 2013a; exon fragment ca. 800 bp) and/or a set of primers for a semi-nested PCR EF03(EF04)/KEF_R3 (Wrigley de Basanta et al. 2017; Ronikier et al. 2020; exon fragment ca. 1075 bp). In addition, partial sequences of the cytochrome c oxidase subunit 1 gene (*COI*) were obtained with primer pairs COMF/COMRs (Liu et al. 2015; Novozhilov et al. 2019; gene fragment ca. 850 bp) and/or COIF1/COIR1 (Feng and Schnittler 2015; gene fragment ca. 650 bp) when the first primer pair could not produce a PCR product with some species. A list of primers, their sequences and amplification protocols for different primer combinations are provided in Table 1.

PCR reactions were prepared with 10 µl of 2 × BioMaster HS-Taq PCR-Color reaction mix (Biolabmix, Novosibirsk, Russia) containing 100 mM KCl, 0.4 mM dNTPs, 4 mM $MgCl_2$, 0.06 U/µl TaqDNA polymerase, 0.2% Tween20, and several dyes (xylene cyanol, bromphenol blue, OrangeG, and tartrazine) with addition of 3 nmol of each primer, 1–3 µl of template DNA and diH_2O up to a total volume of 20 µl. The amplification was carried out with the C1000 Touch (Bio-Rad, Hercules, USA) thermal cycler. Products of amplification were stained with GelRed (Biotium, San Francisco, USA), separated by 1.2% agarose gel electrophoresis, observed in Gel Doc XR+ System (Bio-Rad, Hercules, USA), and then purified using the CleanMag DNA (Evrogen, Moscow, Russia) purification kit before semi-nested PCR or sequencing with the BrilliantDye Terminator v3.1 Cycle Sequencing Kit (NimaGen, Nijmegen, the Netherlands) using the primers mentioned earlier. Sequencing products were purified with the NimaGen D-Pure DyeTerminator Cleanup kit and analyzed on ABI 3500 automated DNA sequencer (Applied Biosystems, Foster City, USA) equipped with a standard 50 cm capillary array. Alternatively, purified PCR products were sent to Macrogen Europe B.V. (Amsterdam, the Netherlands) for commercial sequencing.

Alignments, model selection, and phylogenetic analyses

SSU, *EF1A*, and *COI* sequences were compiled into three single-gene alignments in Unipro UGENE (Okonechnikov et al. 2012) and aligned using MAFFT v7.496 online service (Katoh and Standley 2013; Katoh et al. 2019) with E-INS-I option for SSU and G-INS-i for *EF1A* and *COI* with default gap penalties. Since mitochondrial transcripts in myxomycetes are subject to insertional RNA editing and RNA editing patterns in described RNA transcripts are different (GenBank L14769, GU182127; Gott et al. 1993; Traphagen et al. 2010), we did not perform codon repair in *COI* sequences. Exon parts of *EF1A* sequences were determined according to the known protein and nucleotide *EF1A* sequences of *Meriderma carestiae* (GenBank MH460968; Fiore-Donno et al. 2019) and *Lepidoderma tigrinum* (GenBank EF513195; Fiore-Donno et al. 2010b).

After manual editing, trimming of the primer sequences, and removal of introns, three sets of nucleotide sequences were merged into a single alignment with three partitions using SequenceMatrix 1.8. (Vaidya et al. 2011). 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+I+G4 evolutionary model was selected for SSU partition and GTR+F+I+G4 for *COI* and *EF1A* partitions according to the ModelFinder tool implemented in IQ-TREE (Kalyaanamoorthy et al. 2017). One thousand ultrafast bootstrap (UBS) replicates (Hoang et al. 2018) were performed to obtain confidence values for the branches. Bayesian inference (BI) 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 phylogenetic analysis was run four times as four separate chains for 20×10^6 generations (sampling every 1000). The convergence of MCMC chains was estimated

Table 1 Primer pairs and amplification protocols used in this study

Name	F/R	Sequence (5'-3')	Amplification protocol
S1	F	AACCTGGTTGATCCTGCC	5 min at 95 °C, 36 cycles (30 s at 95 °C, 20 sec at 56 °C, 50 sec at 72 °C) and 5 min at 72 °C
S2	F	TGGTTGATCCTGCCAGTAGTGT	
SU19R	R	GACTTGTCCTCTAATTGTTACTCG	
SSU_rev	R	AGACTTGTCCTCYAATTGTTAC	
COIF1	F	CTGCWTTAATTGGTGGBTTTGG	5 min at 95 °C, 36 cycles (30 s at 95 °C, 20 s at 50.7 °C, 1 min at 72 °C) and 10 min at 72 °C
COIR1	R	ACGTCCATTCCKACWGTRTAC	
COMF	F	GCTCCTGATATGGCWTTTC	5 min at 95 °C, 36 cycles (30 s at 95 °C, 20 sec at 52 °C, 1 min at 72 °C) and 10 min at 72 °C
COMRs	R	CATGRAAWGCATATCWARACC	
PB1F	F	ACCCGTGAGCACGCTCTCCT	5 min at 95 °C, 36 cycles (30 s at 95 °C, 30 s 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 s at 95 °C, 30 s at 60 °C, 90 sec at 72 °C) and 10 min at 72 °C
EF04	F	TGGGTGTTGGACAAACTC	
KEF_R3	R	CCGTTCTTGATGTTCTTGG	

using Tracer 1.7.2 (Rambaut et al. 2018) and by the average standard deviation of split frequencies; based on the estimates, the first 25% generations were discarded as burn-in. Posterior probabilities (PP) for clades were exported to the ML-tree. Phylogenetic tree with combined supports was visualized using FigTree 1.4.4 and edited using CorelDRAW 24.0.

Results

Phylogeny

A total of 381 new nucleotide sequences were generated for this study, including sequences from four holotype and three paratype specimens of three species and one variety. Of the nine herbarium specimens that were more than 25 years old (seven from BR and two specimens of the type material of *Lepidoderma cristatosporum* G. Moreno, López-Vill., S.L. Stephenson & A. Castillo from AH), we were able to obtain only one SSU fragment from the holotype of *L. alpestroides*. Even in this case, the PCR product had to be sequenced several times to obtain a readable sequence assembled from several overlapping reads. Similarly, all attempts to amplify gene fragments from 20 years old specimens of *Lepidoderma trevelyanii* (Grev.) Poulain & Mar. Mey. stored in the Komarov Botanical Institute (LE) failed.

The final dataset consisted of 241 specimens belonging to 61 morphological species from 11 genera. The concatenated alignment for ML and BI analyses included 241 SSU sequences, 157 *COI* sequences, and 223 *EF1A* sequences with 2,636 sites, 1,603 distinct patterns, 144 singleton sites, and 1,370 constant (non-informative) sites in total. The topologies of ML and BI phylogenies were congruent and the clades that received high statistical support with UBS and PP were mostly identical. The list of successfully sequenced specimens, the concatenated alignment, partition file, and phylogenetic tree in the Newick format can be found in Online Resources 1–4 and FigShare (https://doi.org/10.6084/m9.figshare.21342834).

Figure 1 shows the resulting three-gene phylogeny. The tree is rooted with species of *Meriderma* (*Meridermataceae*, *Meridermatales*), being a sister clade relative to the order *Physarales*.

Three species of the genus *Lamproderma* (*Lamprodermataceae*), in turn, occupy a sister position to the clade uniting *Didymiaceae* and *Physaraceae*. *L. columbinum* (Pers.) Rostaf., *L. ovoideum* Meyl., and *L. ovoideoechinulatum* Mar. Mey. & Poulain form a monophyletic group, but the first species (which is the type species of the genus) is divided into two clades and the latter species shows prominent genetic diversity.

Diachea, with five species investigated, is the only large genus that forms a monophyletic clade in the family *Didymiaceae*. All five studied species of the genus appear monophyletic, despite long branches occurring in *D. subsessilis* and *D. leucopodia*. The most common species *D. leucopodia* (Fig. 2a) is as well very diverse genetically (see Online Resource 2); specimens collected in Russia and mainland China form two sister clades. This lends evidence that the type species of the genus *Diachea*, *D. leucopodia* (Fig. 2a), may represent a complex of several species. It should also be noted that of all studied species within the *Didymiaceae*, *D. silvaepluvialis* M.L. Farr (Fig. 2i) was the only one where *COI* could not be amplified with the COMF/COMRs primer combination, but it was amplified with COIF1/COIR1 instead.

The clade corresponding to the genus *Didymium* has maximum support, but immediately splits into two subclades. Clade 1 (UBS/PP supports = 86/0.99) combines three *Didymium* species (*D. reticulosporum* Novozh. & Zemly., *D. clavus* (Alb. & Schwein.) Rabenh., and *D. crustaceum*

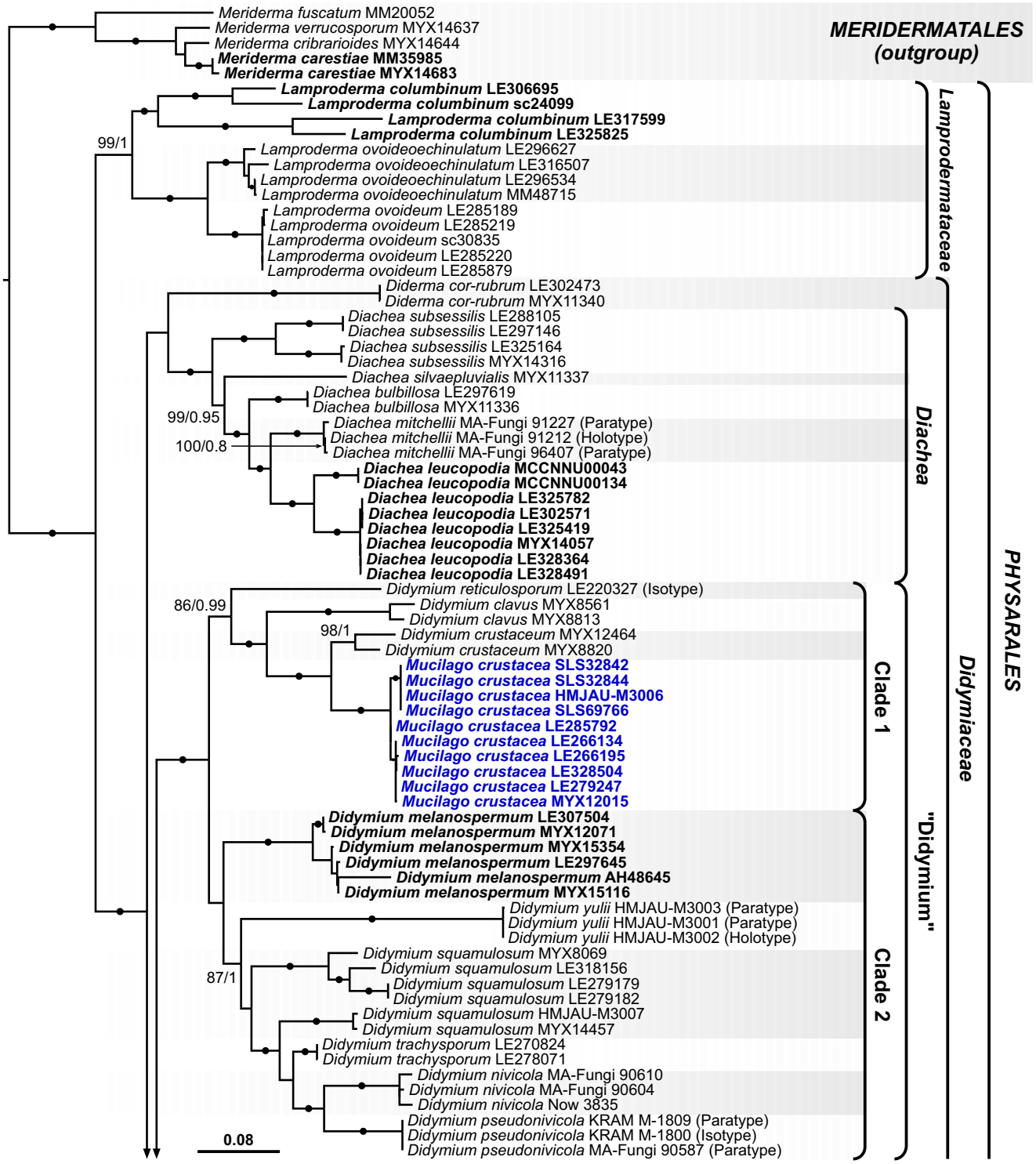


Fig. 1 Phylogenetic tree of the *Didymiaceae* family, obtained from the combined SSU, *COI*, and *EF1A* sequences. Bold font indicates the type species of a genus; species subjected to nomenclatural revision in the current article are highlighted in blue font. Branch supports are shown only for UBS/PP ≥ 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. The nomenclature used in the tree is checked against the most current version of the nomenclature database of Lado (2005–2022)

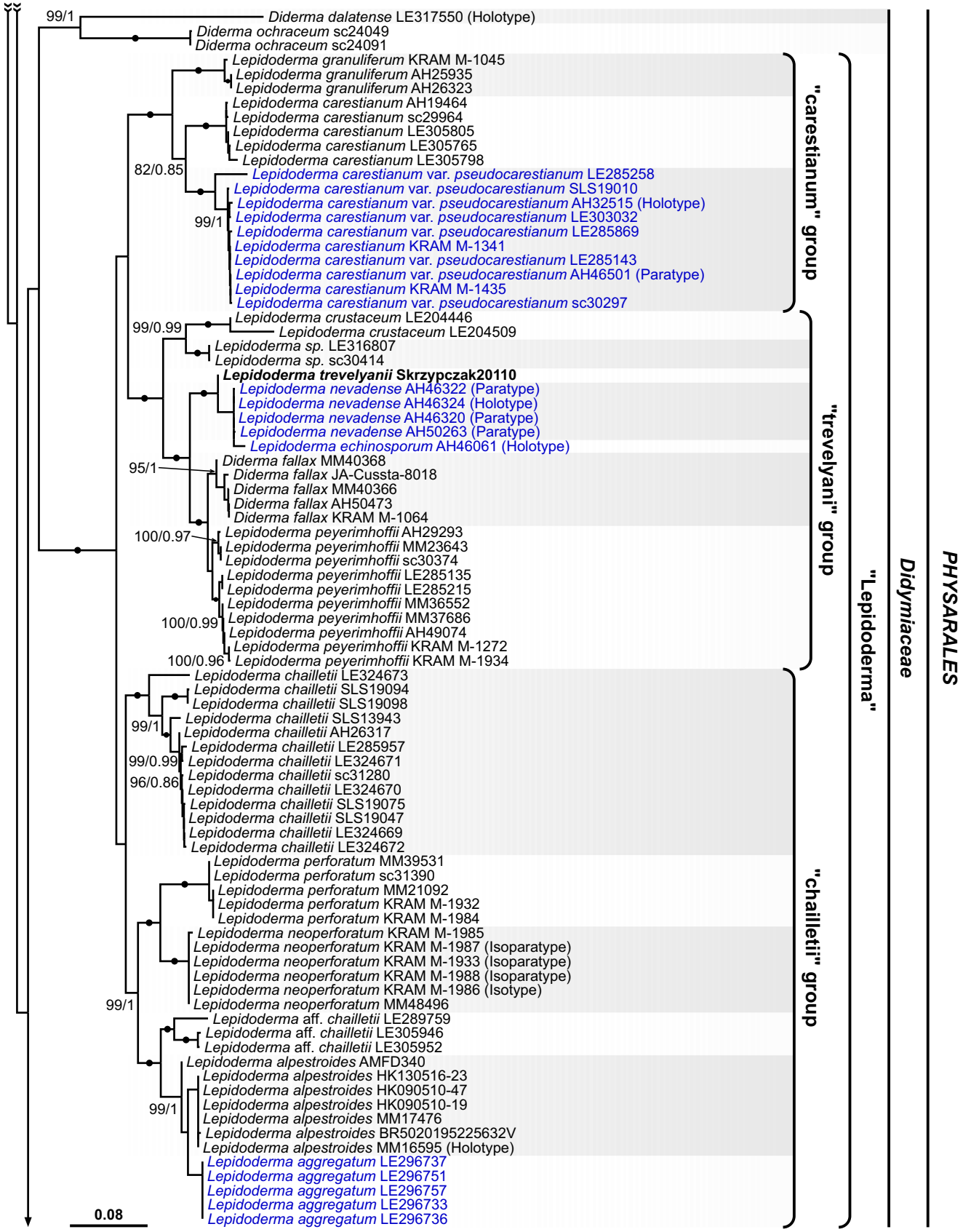


Fig. 1 (continued)

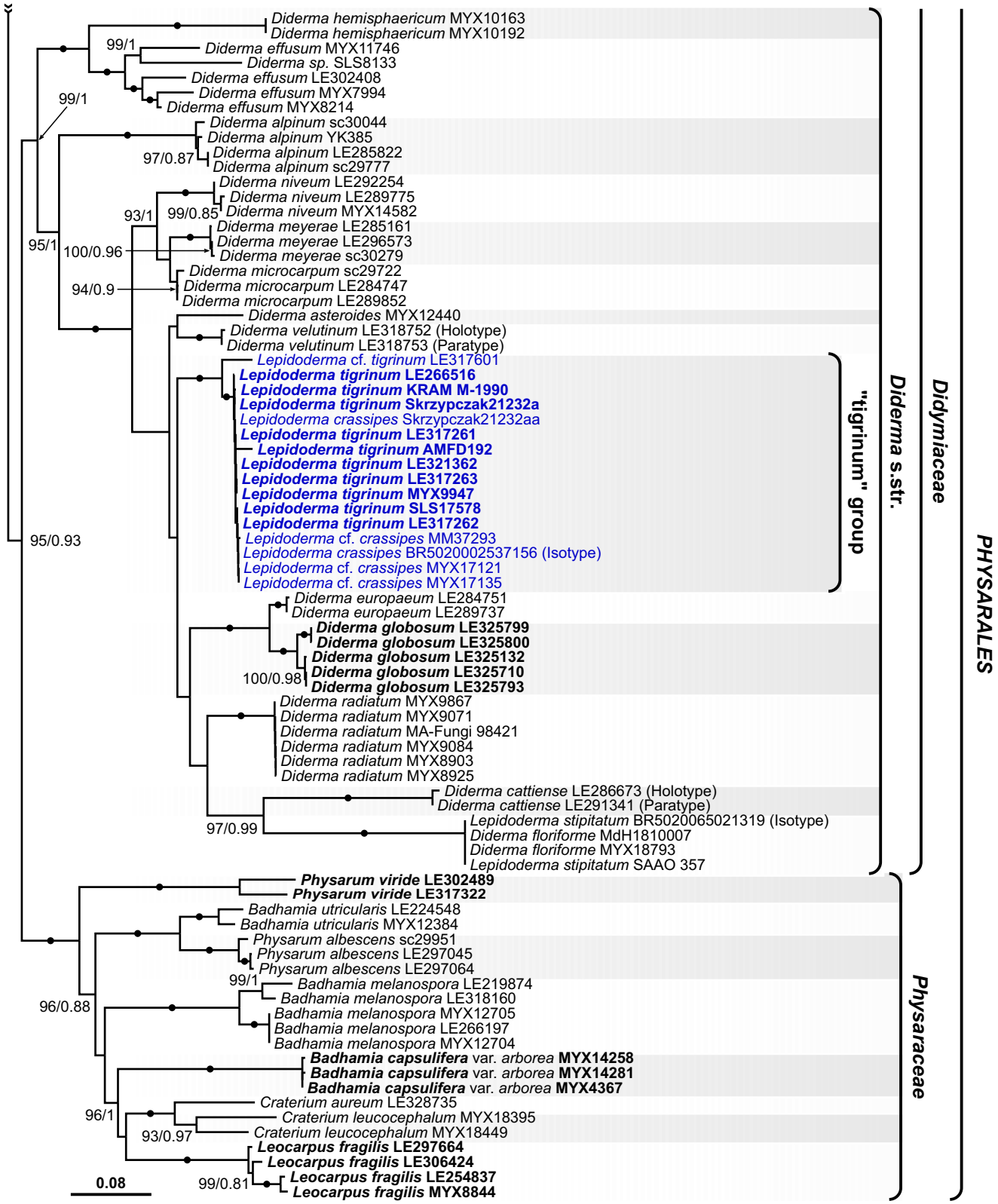
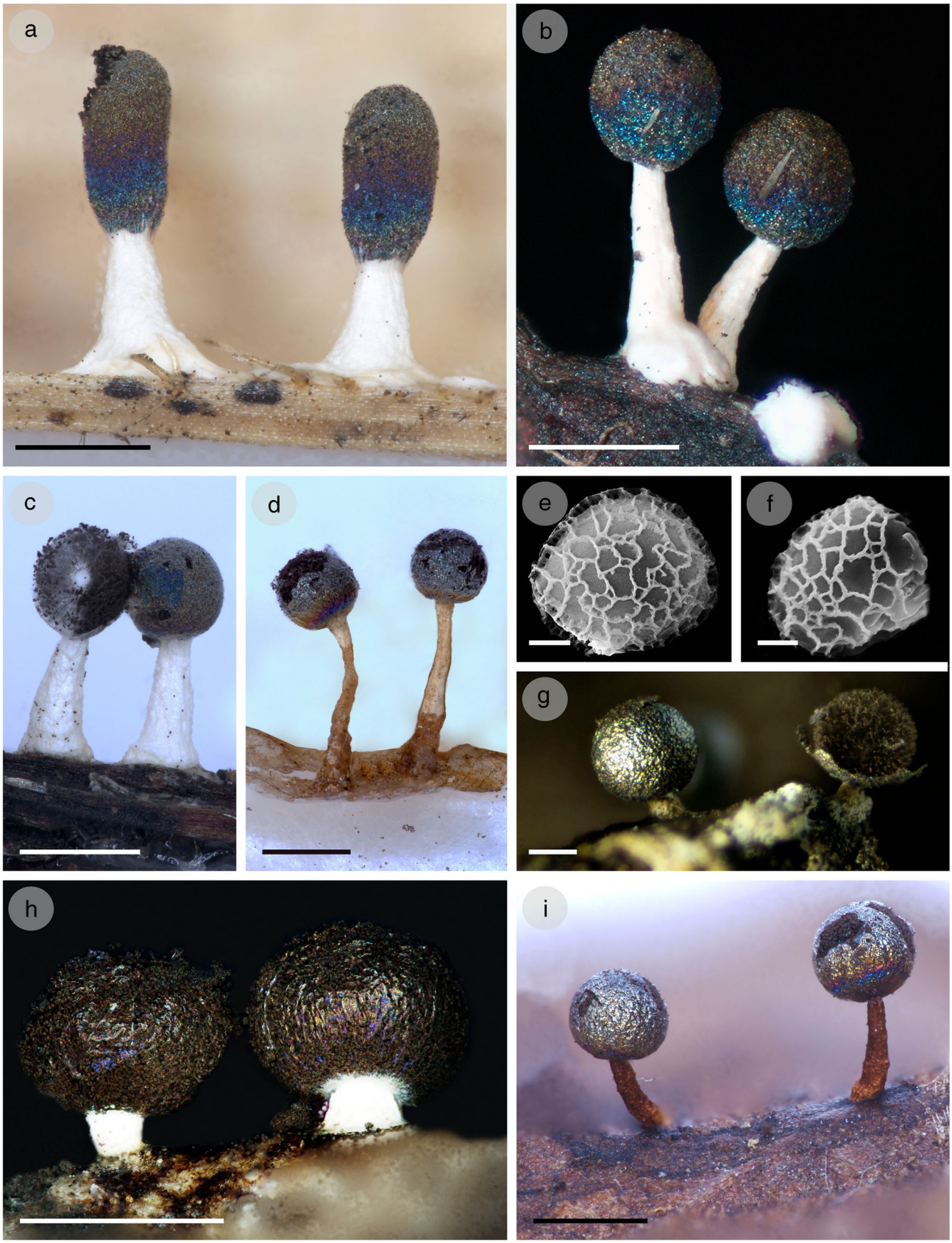


Fig. 1 (continued)


a
b
c
d
e
f
g
h
i

◀ **Fig. 2** Morphological characters of species of the genus *Diachea*. **a** sporocarps of *D. leucopodia* (LE328364). **b**, **c** – sporocarps of *D. bulbillosa*: **b** (LE297619), **c** (MYX11336). **d**–**h** – *D. subsessilis*: **d** sporocarps (LE288105), **e** spore (LE288105), **f** spore (MYX14316), **g** sporocarps (MYX14316), **h** sporocarps (LE325164). **i** sporocarps of *D. silvaepluvialis* (MYX11337). Scale bars: a–d, h–i = 500 μm; g = 200 μm; e, f = 2 μm

Fr.) together with *Mucilago crustacea* P. Micheli ex F.H. Wigg, the only species of the genus *Mucilago* P. Micheli ex Adans. Therefore, the genus *Didymium* appears to be paraphyletic. Clade 2 (UBS/PP supports = 77/0.94), combines the type species of the genus, *D. melanospermum* (Pers.) T. Macbr., *D. yulii* S.-Y. Liu & F.-Y. Zhao, *D. squamulosum* (Alb. & Schwein.) Fr. & Palmquist, *D. trachysporum* G. Lister, *D. nivicola* Meyl., and *D. pseudonivicola* Janik, A. Ronikier & Lado. The species *D. squamulosum* appears polyphyletic in this phylogeny, being represented by two separate clades.

All studied species of *Lepidoderma* except for *L. tigrinum* form a separate fully supported clade together with *Diderma fallax*. Three species groups corresponding to subclades can be distinguished. The "carestianum" group combines the strictly plasmodiocarpic *L. granuliferum* (W. Phillips) R.E. Fr. and two varieties of *L. carestianum* (Rabenh.) Rostaf., whereas the "chailletii" group includes species capable of forming both plasmodiocarps and sporocarps with varying degrees of aggregation. *Lepidoderma chailletii* Rostaf. (which appears to be polyphyletic), *L. perforatum* Mar. Mey. & Poulain, and *L. neoperforatum* A. Kuhnt are well separated from each other. Specimens identified morphologically as *L. aggregatum* Kowalski cluster together with *L. alpestroides*, but separately from other species of *Lepidoderma*. All studied specimens of *L. aggregatum* show prominent morphological differences from both *L. chailletii* and *L. alpestroides.*

The monophyletic "trevelyanii" group includes morphologically similar species with sessile or short-stalked sporocarps: *Lepidoderma crustaceum* Kowalski, *L. echinosporum* G. Moreno, López-Vill. & S.L. Stephenson, *L. nevadense* G. Moreno, A. Sánchez, Mar. Mey., López-Vill. & A. Castillo, *L. peyerimhoffii* Maire & Pinoy, *L. trevelyanii* (Grev.) Poulain & Mar.Mey. and *Diderma fallax* (Rostaf.) Lado. All these species are well separated into monophyletic subclades. However, two aberrant specimens morphologically similar to *L. peyerimhoffii* (Fig. 3e, f), but with unique nucleotide substitutions, group together with *L. crustaceum* (Fig. 3a, b, c and d).

Species of the last large genus in the *Didymiaceae*, *Diderma*, are scattered throughout the tree and appear in four clades. The most diverse *Diderma* clade includes the minimal monophyletic clade (UBS/PP = 100/1) with several species of *Diderma* and *Lepidoderma*, including the type taxa *D. globosum* Pers. and *L. tigrinum*. Three species (or species complexes) occupy a more basal position. These are *D. alpinum* (Meyl.) Meyl., *D. hemisphaericum* (Bull.) Hornem, and *D. effusum* (Schwein.) Morgan with the specimen SLS8133 with a lime deposition pattern different from that common in the genus *Diderma* (see Discussion).

Two more *Diderma* clades are small and occupy an unresolved position within the *Didymiaceae* in our phylogeny (Fig. 1). *Diderma cor-rubrum* T. Macbr. assumes a sister position to *Diachea* (UBS/PP support = 88/0.52); *D. dalatense* Novozh., Prikhodko & Shchepin and *D. ochraceum* Hoffm. occur in a sister position to the "Lepidoderma" clade (UBS/PP support = 76/0.34). The fourth clade is represented by *D. fallax*, which is nested in the "Lepidoderma" clade.

Finally, the family *Physaraceae* is placed inside the *Didymiaceae* with a high support (UBS/PP = 95/0.93) in a sister position to the clade uniting the majority of species of *Diderma*, thus making the *Didymiaceae* a paraphyletic taxon.

The aforementioned facts provide the justification to carry out a nomenclatural revision of several taxa in the family *Didymiaceae*.

Taxonomy

Diderma

This genus appears poly- and paraphyletic even for the 17 studied species (including the specimen SLS8133 not clearly assignable to a described species). However, there is a monophyletic group with 10–14 taxa (Fig. 1), including the type species *D. globosum*. In addition, this group includes *Lepidoderma tigrinum* and specimens primarily determined as *L. crassipes* and *L. stipitatum*. If the scales on the peridium are not considered, stalked species of the genus *Lepidoderma* are morphologically more similar to the stalked species of the genus *Diderma* than to other species of the genus *Lepidoderma*. Taking morphological and genetic relationships into account, we do not maintain *L. tigrinum* (including *L. crassipes*) in the genus *Lepidoderma* as in Ronikier et al. (2022) but transfer it to the genus *Diderma*.

Diderma tigrinum (Schrad.) Prikhodko, Shchepin, Novozh., López-Vill., G. Moreno & Schnittler, comb. nov. (Fig. 4)

MycoBank: 846132

Basionym: *Didymium tigrinum* Schrad., gen. nov. pl. 22 (1797).

≡ *Lepidoderma tigrinum* (Schrad.) Rostaf., in Fuckel, Jahrb. Nassauischen Vereins Naturk. 27-28:73 (1873).

= *Lepidoderma crassipes* Flatau, Massner & Schirmer, Z. Mykol. 53(1):146 (1987).

Note: The basis for the synonymization of *D. tigrinum* and *L. crassipes* is that specimens showing the typical morphology of *L. crassipes* (Fig. 4g, h, i, j and k) are mixed with *L. tigrinum* in the three-gene phylogeny (Fig. 1) and are nearly identical in sequences of three unlinked genetic markers (Online Resource 2) to specimens of *L. tigrinum*, which have a different character of lime deposition.

Fig. 3 **a, b** sporocarps of *Lepidoderma crustaceum* (Kowalski 6533; paratype). **c, d** *L. crustaceum* (LE204509): **c** sporocarps with club-shaped columella, **d** capillitium and spores (LM). **e, f** sporocarps of *Lepidoderma* sp. (LE316807). Scale bars: a, b, c, f = 500 µm; d = 20 µm; e = 1 mm

Didymium

At least nine species of this genus form a fully supported clade (UBS/PP = 100/1; Fig. 1), which includes both species with typical sporocarps and recently described species with aethalioid fructifications (*D. yulii*; see Online Resource 5e). In addition, this group comprises specimens of the monotypic genus *Mucilago* which form a clade sister to *D. crustaceum* (also fully resolved; UBS/PP = 100/1). Therefore, we transfer *M. crustacea* to the genus *Didymium* based on their genetic, macro- (aethaliod fructifications) and micromorphological similarity.

Didymium mucilago Prikhodko, Shchepin, Novozh., Schnittler & Stephenson, nom. nov. (Fig. 5a, b, c and d)

MycoBank: 846131

Etymology: The epithet *mucilago* refers to the generic name of the replaced synonym.

Fig. 4 Morphological diversity within the "tigrinum" group. **a**–**f** – *Lepidoderma tigrinum*: **a** – MYX9947, **b** – MYX18162, **c** – MYX18241, **d** – MYX18252, **e** – LE317601, **f** – BR5020063968456. *Lepidoderma crassipes*: **g** – MYX15367, **h**, **i** – MYX17121; **j**, **k** – MYX17135. Scale bars: a = 1000 μm; b–k = 500 μm

Replaced synonym: *Mucilago crustacea* P. Micheli ex F.H. Wigg., Prim. fl. holsat. 112 (1780).

Lepidoderma

After the nomenclatural revision by Ronikier et al. (2022), one *Diderma* species and nine valid species of the genus *Lepidoderma* were transferred to the resurrected genus *Polyschismium* Corda, namely, *D. fallax* (Rostaf.) Lado, *L. trevelyanii* (Grev.) Poulain & Mar.Mey., *L. alpestroides* Mar. Mey. & Poulain, *L. carestianum* (Rabenh.) Rostaf., *L. chailletii* Rostaf., *L. crustaceum* Kowalski, *L. granuliferum* (W. Phillips) R.E. Fr., *L. neoperforatum* A. Kuhnt, *L. perforatum* Mar. Mey. & Poulain, *L. peyerimhoffii* Maire & Pinoy. *L. stipitatum* Flatau has been synonymized with *Diderma floriforme* (Bull.) Pers., *L. crassipes* Flatau was

Fig. 5 **a**–**d** – *Mucilago crustacea*: **a** sporophores in field conditions (MYX12015), **b** peridium (MYX12015), **c** capillitium, spores, and stellate lime crystals (LE285792; SEM), **d** spore ornamentation (LE285792; SEM). **e**–**g** – *Didymium crustaceum*: **e** sporocarps (MYX8820), **f, g** sporocarps (MYX12464), **h** capillitium (MYX12464; SEM), **i** spore ornamentation (MYX12464; SEM), **j** lime crystal (MYX12464; SEM). Scale bars: a – 2 cm; b, e–g = 500 μm; h = 50 μm; c, i, j = 10 μm; d = 5 μm

reduced to a synonym of *L. tigrinum* (Schrad.) Rostaf. Thus, the revised genus *Lepidoderma* de Bary ex Rostaf. included the type species *L. tigrinum* and three valid species remained unstudied (*L. cristatosporum* G. Moreno, López-Vill., S.L.Stephenson & A. Castillo, *L. echinosporum* G. Moreno, López-Vill. & S.L. Stephenson and *L. nevadense* G. Moreno, A. Sánchez, Mar. Mey.). Another species, *L. aggregatum* Kowalski, was also not affected by the revision by Ronikier et al. (2022), since Lado (2005–2022) recognized it as a synonym of *L. chailletii*.

Having studied the type material of *Lepidoderma echinosporum* and *L. nevadense*, we were able to reliably attribute these two species to a monophyletic clade including *L. trevelyanii* (Grev.) Poulain & Mar.Mey. (≡ *Polyschismium trevelyanii* (Grev.) Corda), the type species of the resurrected genus *Polyschismium* (Ronikier et al. 2022). In addition, the reconstruction of the three-gene phylogeny (Fig. 1) made it possible to reliably separate species and varieties in the *carestianum-granuliferum* species complex, as well as to elevate *L. aggregatum* to species rank.

Polyschismium aggregatum (Kowalski) Prikhodko, Shchepin, Novozh., G. Moreno, López-Vill. & Schnittler, comb. nov.

MycoBank: 846133

Basionym: *Lepidoderma aggregatum* Kowalski, Mycologia 63(3):511 (1971).

Note: See discussion.

Polyschismium carestianum* var. *pseudocarestianum (G. Moreno, A. Sánchez, Mar. Mey., López-Villaba & A. Castillo) Prikhodko, Shchepin, Novozh., G. Moreno, López-Vill. & Schnittler, comb. nov.

MycoBank: 846137

Basionym: *Lepidoderma carestianum* var. *pseudocarestianum* G. Moreno, A. Sánchez, Mar. Mey., López-Villaba & A. Castillo, Bol. Soc. Micol. Madrid 42:51 (2018).

Note: See discussion.

Polyschismium cristatosporum (G. Moreno, López-Vill., S.L. Stephenson & A. Castillo) Prikhodko, Shchepin, Novozh., G. Moreno, López-Vill. & Schnittler, comb. nov.

MycoBank: 846134

Basionym: *Lepidoderma cristatosporum* G. Moreno, López-Vill., S.L. Stephenson & A. Castillo, Mycoscience 59(5):387 (2018).

Note: Only two type specimens collected in the same location were available for study, but amplification and sequencing failed due to fungal contamination of the specimens. The morphological similarity of this species with *L. crustaceum* gives us reasons for transferring the species to the monophyletic genus *Polyschismium*. Considering the differences in the ornamentation of spores (Moreno et al. 2018a), we treat it as independent taxon until proven otherwise.

Polyschismium echinosporum (G. Moreno, López-Vill. & S.L. Stephenson) Prikhodko, Shchepin, Novozh., G. Moreno, López-Vill. & Schnittler, comb. nov.

MycoBank: 846135

Basionym: *Lepidoderma echinosporum* G. Moreno, López-Vill. & S.L. Stephenson, in Crous et al., Persoonia 37:231 (2016).

Polyschismium nevadense (G. Moreno, A. Sánchez, Mar. Mey., López-Vill. & A. Castillo) Prikhodko, Shchepin, Novozh., G. Moreno, López-Vill. & Schnittler, comb. nov.

MycoBank: 846136

Basionym: *Lepidoderma nevadense* G. Moreno, A. Sánchez, Mar. Mey., López-Vill. & A. Castillo, Bol. Soc. Micol. Madrid 42:67 (2018).

Discussion

The phylogeny presented herein (Fig. 1) is based on three independent genetic markers and confirms some results obtained in previous studies, especially that of Ronikier et al. (2022) (discussed below). The high statistical support for many of the deeper nodes in the presented three-gene phylogeny and extensive species sampling allow us to define monophyletic clades that can be seen as core groups of the four species-rich genera in the *Didymiaceae*. In addition, the genus *Lepidoderma* was exhaustively sampled and can thus be revised entirely.

Didymiaceae and *Physaraceae*

As already reported by Nandipati et al. (2012), Leontyev et al. (2019) and Cainelli et al. (2020), the family *Physaraceae* forms a monophyletic clade sister to *Diderma*. This means that there is no monophyletic unit corresponding to the family *Didymiaceae*, except that it will be united with the *Physaraceae* (Fig. 1). However, since not all the deeper nodes of our phylogeny are resolved, we will discuss only our conclusions concerning the generic level.

Our results on the eight studied species of the *Physaraceae* (including type species of three genera) indicate as well that the genera *Physarum* Pers. and *Badhamia* Berk. are polyphyletic, which confirms previous reported results (Nandipati et al. 2012; Cainelli et al. 2020, Ronikier et al. 2022).

Delimitation of the four genera within the *Didymiaceae*

In our phylogeny, there are four clades within the *Didymiaceae* with three or more species which are nearly completely resolved (UBS/PP = 99/1 or better), as shown in detail in Fig. 1. The first one contains five species of the genus

Diachea Fr. It was classified as a taxon *incertae sedis* by Leontyev et al. (2019), since the single complete SSU sequence from *D. subsessilis* (GenBank JQ031964; Fiore-Donno et al. 2012) formed one of the most poorly supported branches within the *Physarales*. In the phylogenies of Fiore-Donno et al. (2012) and Cainelli et al. (2020), this sequence formed a weakly supported sister branch to the species of *Didymium*. In the most relevant molecular phylogenetic work on the genus *Diachea*, Lado et al. (2022) used a different set of genes, but their data set is fragmentary and at least six species of *Diachea* (including the newly described *D. mitchellii* Lado & Treviño) do not form a reliably supported clade in the ML analysis. In our analysis, the five studied species form a monophyletic clade, but the position of this clade still remains unresolved.

The second large clade unites species of the genus *Didymium* Schrad., including the type species *D. melanospermum* (Online Resource 5a–d) and *Mucilago crustacea* (Fig. 5a, b, c and d), the latter appearing as a sister taxon to *D. crustaceum* (Fig. 5e, f, g, h, i and j) which has densely crowded fructifications but with discernible sporocarps. This confirms the results of previous studies based on one or two genes (Fiore-Donno et al. 2010a; Wrigley de Basanta et al. 2015; Zhao et al. 2021). Thus, we propose to reject the monotypic genus *Mucilago* and propose a new name *Didymium mucilago* for its single species. The description of the species *Didymium yulii* (Zhao et al. 2021) has already widened the range of fructification forms in the genus *Didymium*, so *M. crustacea* is just another aethalioid species. The transition from sporocarpic to aethalioid fructifications apparently occurred independently in two evolutionary lines of the genus *Didymium*. A tendency to form compound fructifications can be observed in nearly all major branches of the myxomycetes (Clark & Haskins 2014; Leontyev et al. 2019).

With ca. 100 validly described species, *Didymium* represents approximately one tenth of the total species diversity of myxomycetes (Lado 2005–2022). Moreover, some species are likely to represent complexes of several species, such as *D. squamulosum* (Online Resource 6c–g), which appears paraphyletic in our phylogeny (Fig. 1). With only nine studied species, we cannot make a final taxonomical revision of this genus. However, in contrast to previous studies (Fiore-Donno et al. 2010a; Wrigley de Basanta et al. 2017, Ronikier et al. 2022), we were able to resolve a core clade of the genus around the type species *D. melanospermum*.

The third large clade, mainly represented by species of *Lepidoderma*, includes 11 of the 15 currently accepted species (Lado 2005–2022). One species in this clade, *L. aggregatum* Kowalski, is considered to be a synonym of *L. chailletii* (Lado 2005–2022). Three other species, *L. crassipes*, *L. tigrinum*, and *L. stipitatum* are placed in a clade formed mostly by species of *Diderma* (see below). For *L. cristatosporum* we were not able to obtain molecular data. *Lepidoderma trevelyanii*, *L. crassipes*, and *L. stipitatum* were treated in Ronikier et al. (2022); the first is the only non-nivicolous species in the resurrected genus *Polyschismium*, the second and third turned out to be heterotypical synonyms of *L. tigrinum* and *Diderma floriforme*, respectively. Our taxon sampling completes that of Ronikier et al. (2022) and allows us to recombine the remaining species of the former genus *Lepidoderma*.

The fourth clade, named *Diderma* s. str., unites 12 species of *Diderma* clustered around the type species *D. globosum* and the "tigrinum" group (with two synonymous species, see above). Thus, unlike Ronikier et al. (2022), we have reasons to disband the genus *Lepidoderma* and transfer *L. tigrinum* to the genus *Diderma*.

The phylogenetic group *Diderma* s. str. includes species from different ecological groups with variable peridium morphology. The support of ML and BI analyses does not allow us to reliably determine the branching order of species within this clade (Fig. 1), but we can distinguish some major branches. The most basal branch (UBS/PP = 100/1) includes *D. hemisphaericum* (Fig. 6a, b), *D. effusum* (Fig. 6e, f), and an unidentified specimen covered with relatively large lime scales (SLS8133; Fig. 6c, d). This branch is followed by *D. alpinum* and the nivicolous species complex (*D. niveum*, *D. meyerae*, and *D. microcarpum*) (UBS/PP = 93/1), which corresponds to the general topology of the two-gene tree obtained earlier (Novozhilov et al. 2022a). Other monophyletic branches include the type species *D. globosum* paired with *D. europaeum* (≡ *Diderma globosum* var. *europaeum* Buyck) (UBS/PP = 100/1), and D. cattiense paired with *D. floriforme* (UBS/PP = 97/0.99; including the isotype of *Lepidoderma stipitatum*). *D. floriforme* was the first species mentioned in the protologue of the genus *Diderma* (Persoon 1794), but it was later transferred to a monotypic genus *Leangium* Link or designated as the type species of the subgenus *Leangium* within *Chondrioderma* Rostaf. In addition to *D. floriforme*, *Leangium* included D. fallax (Rostaf.) E. Sheld. (≡ *Polyschismium fallax* (Rostaf.) A. Ronikier, J.M. García-Martín, A. Kuhnt, J.C. Zamora, M. de Haan, Janik & Lado; = *Leangium lyallii* (Massee) E. Sheld.) and *D. radiatum* (L.) Morgan (≡ *Leangium radiatum* (L.) E. Sheld.). The current position of these three species in the three-gene phylogeny (Fig. 1) clearly demonstrates that *Leangium* is an artificial taxonomic group.

Three other studied species of *Diderma* form two separate "Diderma" s.l. clades. The first is formed by *D. dalatense* (see Fig. 1 in Novozhilov et al. 2019) and *D. ochraceum* (Fig. 7a, b), and the second is formed by *D. cor-rubrum* (Fig. 7c, d). All these species have a three-layered peridium, consisting of an inner membranous layer, a middle layer of granular lime and an outer cartilaginous layer, but differ considerably in stalk and sporotheca characters. In the three-gene phylogeny these taxa appear in two long branches close to the genera *Lepidoderma* and *Diachea*, respectively (Fig. 1). The low support values, variations in topology depending on species sampling and different patterns of RNA editing in *COI*

Fig. 6 **a**, **b** – *Diderma hemisphaericum*: **a** MYX10163, **b** MYX10192. **c, d** *Diderma sp.* (SLS8113). **e**, **f** – *D. effusum*: **e** MYX7994, **f** MYX8214. Scale bars: a, b, e, f = 500 μm; c, d = 200 μm

sequences point more towards a long branch attraction artifact than towards valid relationships between the branch of *D. dalatense* and *D. ochraceum* with the "Lepidoderma" clade or the *D. cor-rubrum* branch with the genus *Diachea*. A final solution of this problem requires a broader taxon and/or genetic loci sampling or phylogenomic approach as the best option.

Species of the former genus *Lepidoderma*

The genus *Lepidoderma* was described in the second half of the 19th century (Rostafiński 1873) as a monotypic genus when the morphological species *L. tigrinum* (Schrad.) Rostaf. was distinguished from the genus *Didymium* based on the peridium structure. This species and two more species,

Fig. 7 **a**, **b** *Diderma ochraceum* Sc24049. **c**, **d** *D. cor-rubrum* LE302473. Scale bars: a, b = 1000 μm; c, d = 500 μm

L. carestianum and *L. chailletii*, assigned to the genus a year later (Rostafiński 1874) were characterized by a thin dark single-layered peridium, which was covered with abundant light lime scales, underlying the etymology of the genus name. All SSU-based and two-gene phylogenies (Fiore-Donno et al. 2008, 2010a, 2010b; Cainelli et al. 2020; Furulund et al. 2021; Zhao et al. 2021) published to date indicated that the type species *L. tigrinum* and one or several *Diderma* specimens formed a monophyletic clade, whereas *Diderma fallax* clustered with other species of *Lepidoderma*, close to *L. peyerimhoffii* (Shchepin et al. 2016). This is shown as well in the two-gene phylogeny presented in Ronikier et al. (2022). We confirm these results using additional SSU, *COI*, and *EF1A* sequences obtained from 12 nivicolous species of *Lepidoderma, D. fallax*, 17 species of *Diderma* (including the type species *D. globosum*), and two species of the *tigrinum* complex (Online Resources 1, 2).

Lepidoderma tigrinum is widespread in coniferous forests of Europe and North America and can also be found in other regions, although less frequently (Martin and Alexopoulos 1969). Usually, fruiting bodies of this species are formed on the large wet coarse wood debris of coniferous trees covered by mosses, liverworts, and algae in moist habitats (Martin and Alexopoulos 1969; Kowalski 1971; Schnittler and Novozhilov 1996; Ing 1999). Most authors have pointed out that the morphology of this species is quite distinct. Kowalski (1971) noted that "when the large, furrowed, stout stipe is well-developed, *L. tigrinum* is clearly separated from other members of *Lepidoderma* which are predominantly sessile". However, both stalk length and sporotheca size can vary considerably. Meylan described two forms – *L. tigrinum* f. *gracile* (Meylan 1910), which forms stalks up to 2 mm long, and *L. tigrinum* f. *microcarpon* (Meylan 1931), which has sporangia 0.5 mm in diameter. Kowalski (1971) studied these forms and concluded that this variation was still within the range of *L. tigrinum*. The color of peridium scales is variable as well. On the basis of the yellow-colored lime scales *L. fulvum* Massee (1892) was described. Later, Kowalski (1971) proposed that this species be regarded as a synonym of *L. tigrinum,* although the specimen he studied did not have the type designation and did not have the herbarium voucher listed in the protologue. Finally, Schnittler et al. (2010) provided evidence that at low pH values the lime scales may be either partially or completely absent. Under such conditions the sporocarps of *L. tigrinum* become almost black, which makes it very difficult to find them in nature.

Flatau (1984) and Flatau et al. (1987) described two species morphologically similar to *L. tigrinum*; these were *L. stipitatum* and *L. crassipes*, respectively. Ronikier et al. (2022) demonstrated the first species to be synonymous with *Diderma floriforme* and the latter with *Lepidoderma tigrinum* (these authors maintain *Lepidoderma* as a separate genus).

Herein, we complete the transfer of all other species of *Lepidoderma* to *Polyschismium*. All these species are nivicolous or at least late-autumn (*L. trevelyanii*) and have no or a weakly developed stalk. At least 13 species belong to a monophyletic clade (Fig. 1), but many species are quite polymorphic and may represent species complexes.

The clade with other species of *Lepidoderma* consists of three groups. The "carestianum" group (Fig. 1) includes *Lepidoderma carestianum* (Rabenh.) Rostaf. and *L. granuliferum* (W. Phillips) R.E. Fr., which is consistent with the SSU-based phylogeny of Shchepin et al. (2016) and the two-gene phylogeny of Ronikier et al. (2022). Kowalski (1971) and Moreno et al. (2018b) studied the type material of both species and clearly separated them based on capillitium structure. "A filamentous capillitium with threads creating an intricate net with abundant globular nodules covered by an asteriform membrane" (Moreno et al., 2018b) was recorded for *L. granuliferum*, whereas the capillitium of *L. carestianum* is "reticulate, with plentiful to scanty lime nodules onto the threads", smooth (under a LM) and the nodules are not covered by an asteriform membrane. In addition, Moreno et al. (2018b) recognized *L. carestianum* var. *pseudocarestianum*, with the capillitial threads rarely anastomosing and lacking lime nodules but having funnel-shaped tips. We consider the absence of nodules on weakly branched capillitial filaments to be the most stable character of this variety, whereas funnel-shaped tips of capillitium threads are absent in some specimens. Considering these diagnostic features, we included in the analysis specimens attributed to both *L. granuliferum* and two varieties of *L. carestianum* (Fig. 8; Online Resource 1), which are the three taxa that we currently accept. More material needs to be studied to decide whether (1) *Lepidoderma granuliferum* should be retained or considered as a synonym or a variety of *L. carestianum*, as it was done more than once in the 20th century (Lister 1911, 1925, Hagelstein 1944); (2) *L. carestianum* var. *pseudocarestianum* should be elevated to the species rank or kept as it is. Regardless the subtle morphological differences, rather long branches in our molecular phylogeny underpin the genetic diversity within the "carestianum" complex.

The core of the second *Lepidoderma* branch, designated as the "trevelyanii" group (Fig. 1), is formed by two widely distributed nivicolous species. The sporocarps of *Lepidoderma peyerimhoffii* Maire & Pinoy (Fig. 9a, b and c) and *Diderma fallax* (Rostaf.) E. Sheld (Fig. 9d, e and f) are similar, since both species have a peridium which breaks into polygonal plates along the lines and a well-developed pseudocolumella that can attach to the inner surface of the peridium in the former species. However, *L. peyerimhoffii* has a three-layered peridium with a crystalline middle layer, while *D. fallax* has a two-layered peridium and the outer layer consists of globular calcium deposits. Ronikier et al. (2022) showed that *L. trevelyanii* (Grev.) Poulain & Mar. Mey. (Fig. 9g, h and i) belongs as well to this group.

Lepidoderma alpestroides (Fig. 10a, b) and *L. aggregatum* (Fig. 10c, d) share the last *Lepidoderma* clade together with *L. chailletii* in the three-gene phylogeny ("chailletii" group). While the plasmodiocarpic *L. alpestroides* has been perceived as an independent species since its description, the sporocarpic *L. aggregatum* was considered to be close to *L. crustaceum* (Kowalski 1971; Schnittler & Novozhilov 1999) or *L. chailletii* (Moreno et al. 2004, 2018a, b) and was also synonymized with the latter (Lado 2005–2022). This study provides evidence that at least five specimens of *L. aggregatum* collected in the Caucasus (Novozhilov et al. 2013b) form a clade sister to *L. alpestroides* and separately from *L. chailletii* and *L. crustaceum* (Fig. 1). The peridium in *L. aggregatum* and *L. alpestroides* is covered with a lime crust and spore ornamentation consists of spines with coralloid apices. However, *L. aggregatum* is characterized by aggregated sporocarps, which occur in dense and scattered groups, and a peridium covered with densely arranged scales of lime, whereas *L. alpestroides* has flattened plasmodiocarps and a peridium covered with a solid lime crust. At the moment, we consider them as closely related but independent species within the genus *Polyschismium*.

In contrast to Ronikier et al. (2022), the broader specimen sampling for *Lepidoderma chailletii* (Fig. 10e, f, g and h) shows that this is clearly a polyphyletic taxon, which was suggested earlier (Shchepin et al. 2016). For the current work, we studied specimens collected in both the Northern and Southern Hemispheres (Online Resource 1), which allowed us to confirm the previously reported results using a different set of specimens and a different outgroup. Although most specimens of *L. chailletii* were fairly conservative in their SSU sequences, three specimens from the Khibiny mountains (Murmansk Oblast, Russia) share unique indels in SSU and substitutions in *EF1A* sequences (see Online Resource 2). As a result, the morphospecies *L. chailletii* appears polyphyletic as it forms two fully supported clades (UBS/PP supports = 100/1, Fig. 1) that are separated by other species.

Taxonomic value of morphological structures

Our phylogeny proved that but not all morphological traits used to delimit the genera of *Didymiaceae* are wrong. The absence of lime in the sporotheca, together with a partially to completely lime-incrusted stalk, still seem to delimit the genus *Diachea* (Fig. 1). In turn, fruiting body structure (aethalioid *vs.* solitary) has little weight for the delimitation of genera, as shown for *Mucilago* (aethalioid) and *Didymium* (solitary). A similar case is *Fuligo vs. Physarum* (Stephenson et al. 2020; Shchepin et al. 2021).

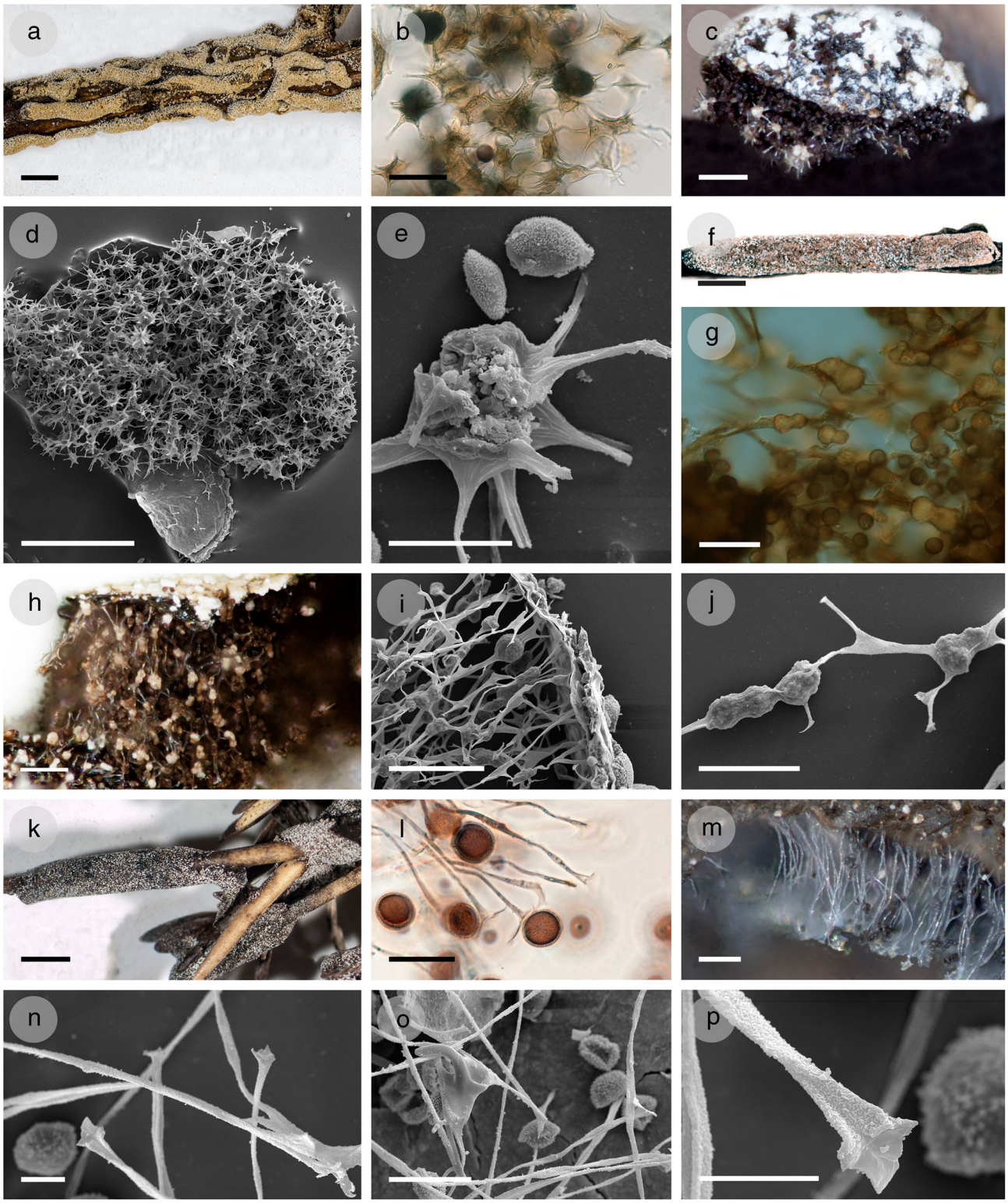


Fig. 8 **a**, **b** – *Lepidoderma granuliferum* (AH25935): **a** sporocarps, **b** capillitium with stellate lime nodes (LM). **c**–**e** – *L. granuliferum* (LE285870): **c** sporocarp fragment under high magnification (LM), **d** capillitium (SEM), **e** asteriform lime nodule of the capillitium (SEM). **f**–**j** – *L. carestianum* (LE305798): **f** sporocarp, **g** capillitium with rounded nodes (LM), **h** capillitium under high magnification, **i** fragment of the capillitium, attached to peridium (SEM), **j** nodules of the capillitium (SEM). **k**, **l** – *L. carestianum* var. *pseudocarestianum* (AH32515): **k** sporocarp, **l** capillitium and spores (LM). **m**–**p** – *L. carestianum* var. *pseudocarestianum* (LE285258): **m** capillitium under high magnification, **n**–**p** – fragments of the capillitium (SEM). Scale bars: a = 2000 μm; b, e, g, j = 50 μm; c, i, m, n = 100 μm; d = 250 μm; f, h, k = 1000 μm; l, o = 30 μm; p = 10 μm

Fig. 9 **a**–**c** – *Lepidoderma peyerimhoffii* (LE285135): **a, b** sporocarps with pseudocolumella-like structures, **c** spores (LM). **d**–**f** – *Diderma fallax* (MM40366): **d, e** sporocarps with pseudocolumella-like structures, **f** spores (LM). **g**–**i** – *L. trevelyanii* (LE47463): **g**, **h** sporocarps, **i** spores (LM). Scale bars: a = 1000 µm; b, d, e, g, h = 500 µm; c, f, i = 10 µm

It is unfortunate that the structure of lime deposits (solid and crystalline = *Didymium*, solid and globular = *Diderma*, scaly and globular = *Lepidoderma*) seems not to be a useful character to separate the three genera. To some extent, the difference of crystalline *vs.* globular lime granules is still useful, since the species investigated for the "Didymium" clade have crystalline, and those of the *Diderma* and "Lepidoderma" clades have globular lime. In contrast, scaly *vs.* solid lime crust appears to be a species-specific character. *Lepidoderma tigrinum* has scaly lime, whereas almost all other species in *Diderma* s. str. clade (Fig. 1) form solid lime crusts (another exception occurs in the unidentified specimen SLS8133; Fig. 6c, d). Moreover, as


a
b
c
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◀ **Fig. 10** **a, b** *Lepidoderma alpestroides* (MM16595; holotype). **c, d** *L. aggregatum*: **c** – LE285260, **d** LE296733. **e–h** *L. chailletii*: **e** LE305946, **f** LE305952, **g** LE285957. Scale bars: a, d = 1000 μm; b, c, e–h = 500 μm

shown by the great variation within this species (Fig. 4), the process of lime formation likely depends as well on environmental factors (humidity, pH, and temperature) and does not seem to be strictly determined by the genotype. Ronikier et al. (2022) demonstrated this convincingly and thus synonymized *L. crassipes* with *L. tigrinum*.

Similarly, the presence or absence of an outer cartilaginous peridial layer is a character with low "phylogenetic" value (e.g., the subgenus *Leangium* defined by a three-layered peridium turned out to be an artificial group). Moreover, two phylogenetically similar species, *Diderma fallax* and *Lepidoderma peyerimhoffii*, have two and three layers of peridium, respectively.

Value of SSU sequences for species delimitation

The fragment of ca. 550 base pairs from the 5' end of the SSU (18S rDNA) which is free of introns is now widely used as a barcoding marker in myxomycetes (Schnittler et al. 2017). This marker can usually distinguish between cryptic species (*Trichia varia*, Feng and Schnittler 2015; *Hemitrichia serpula*, Dagamac et al. 2017; *Physarum albescens*, Shchepin et al. 2021) and the barcoding gap is usually sufficient to separate all species in the group (Borg Dahl et al. 2018).

However, in this study we found two cases that challenge these statements. First, barcodes obtained from the type (MM16595 = BR5020150510636) and authentic (MM17476) specimens of *L. alpestroides*, as well as a sequences taken from Fiore-Donno et al. (2012) and Ronikier et al. (2022), are nearly or completely identical to sequences obtained from collections of *L. aggregatum* stored in the Komarov Botanical Institute (LE) (see Online Resources 1, 2). A similar high level of SSU conservation occurs between *L. peyerimhoffii* and *Diderma fallax*, which differ in only four positions in their 567-nucleotide-long barcodes but show different RNA editing patterns in *COI* and 92 distinct positions in a 762-nucleotide-long *EF1A* gene fragment. In Shchepin et al. (2021), two pairs of the discovered cryptic species also shared one SSU variant. Thus, we conclude that the beginning of the 18S rRNA gene cannot always be used as a universal DNA barcode in *Myxomycetes*; additional unrelated genetic markers such as *EF1A*, *COI*, and/or mtSSU are needed to resolve these cases.

Prospects for COI application in taxa delimitation

The mitochondrial cytochrome c oxidase subunit 1 (*COI*) gene has been used as the main DNA barcode for *Metazoa* for three decades (Folmer et al. 1994; Hebert et al. 2003; Huang et al. 2008; Che et al., 2011; Yang et al. 2020; Ahmed et al., 2022) and has also proven effective in distinguishing species-level taxa in the *Amoebozoa* supergroup (Nassonova et al. 2010; Kosakyan et al. 2012; Tekle 2014). Meanwhile, *COI* and other mitochondrial genetic markers have been difficult to apply to *Myxomycetes* since their mitochondrial RNA transcripts undergo editing, due to which mtDNA does not encode open reading frames (Gott et al. 1993; Horton and Landweber 2002; Traphagen et al. 2010). Each RNA transcript is subject to modification by RNA polymerase, which adds nucleotides or dinucleotides to the growing chain and can also non-synonymously remove or replace nucleotides. As the original mtDNA matrix is poorly subjected to stabilizing selection, the number and position of the editing sites vary from taxon to taxon (Horton and Landweber 2000; Chen et al. 2012). This yields additional phylogenetic signals but complicates the design of universal primers.

In 2015, two papers were published aimed at resolving the phylogeny of *Myxomycetes* (Liu et al. 2015) and at identifying hidden biodiversity within the *Trichia varia* morphospecies (Feng and Schnittler 2015) using *COI* gene fragments. In both publications, primers were proposed for the amplification of two different gene fragments (see methods), which have not been widely used and have been applied only to describe four new species (Bortnikov et al. 2018; Novozhilov et al. 2019, 2022b; Stephenson et al. 2020) and reveal the extent of hidden biodiversity within *Lepidoderma chailletii* (Shchepin et al. 2016) and *Physarum albescens* (Shchepin et al. 2021).

Since RNA-editing patterns have been described for only a small number of taxa, and all previous studies have been based on a small sample of specimens or a small sample of taxa, it is difficult to say whether applying *COI* would result in a significant change in tree topologies with respect to single- and two-gene trees. By obtaining *COI* sequences from about 50 species, we were able to show that *COI* increases phylogenetic resolution at all taxonomic levels from order to species. *COI* shows notable variability in those taxa where SSU sequences are extremely conservative (e.g., *Badhamia capsulifera var. arborea*, *Lepidoderma peyerimhoffii*, and *Diderma fallax*) and at the same time supports phylogenies obtained with more traditional SSU and *EF1A* markers (Fiore-Donno et al. 2012; Leontyev et al. 2019; Novozhilov et al. 2022a). Despite the fact that sequences were obtained with different primer pairs and differed in both length and RNA editing patterns, consistent topologies and high resolution make *COI* a promising genetic marker for reconstructing evolutionary relationships in *Myxomycetes*.

Conclusion

This study demonstrates that a three-gene approach can reliably delimit genera in dark-spored myxomycetes. We could at least define the core groups of four species-rich genera in the family *Didymiaceae* by rearranging some species and thus resolving the

known cases of paraphyly for *Didymium*, *Diderma*, and *Lepidoderma*. The majority of species of *Lepidoderma* together with *Diderma fallax* fall into the genus *Polyschismium*, while *Lepidoderma tigrinum* and two allied species were transferred to the genus *Diderma*, thus invalidating the genus *Lepidoderma*. *Mucilago crustacea* received the new name *Didymium mucilago*. Several morphospecies in the *Didymiaceae* (e.g., *D. melanospermum*, *D. squamulosum*, *Diachea leucopodia*, *Diderma globosum*) seem to be complexes of cryptic species (Fig. 1); resolving these complexes requires a multilocus analysis with a broad specimen sampling. The family *Didymiaceae* proved to be a non-natural taxon due to the position of the family *Physaraceae* sister to *Diderma*. We found that deeper phylogenetic relationships of myxomycetes and taxa with long branches remain difficult to resolve even by a three-gene phylogeny and require phylogenomic approaches, like the one used in Kang et al. (2017) for cultivable members of the Amoebozoa.

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Author contribution Ilya S. Prikhodko, Yuri K. Novozhilov, and Oleg N. Shchepin contributed to the study conception and design. All authors participated in the collection of herbarium specimens for morphological and molecular phylogenetic analyses. Light and scanning electron microscopy was performed by Yuri K. Novozhilov, Vladimir I. Gmoshinskiy, Gabriel Moreno, and Ángela López-Villalba. Ilya S. Prikhodko, Oleg N. Shchepin, and Nadezhda A. Bortnikova were responsible for DNA extraction, PCR analysis, and sequencing. Ilya S. Prikhodko and Oleg N. Shchepin performed phylogenetic analyses. Ilya S. Prikhodko and Vladimir I. Gmoshinskiy prepared illustrations on the phylogeny and morphology of the studied material, respectively. The first draft of the manuscript was written by Ilya S. Prikhodko, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The DNA sequences generated during the current study are available in NCBI Genbank. The list of material used in the phylogenetic reconstruction, the concatenated alignment, partition file, and phylogenetic tree in the Newick format have been submitted as supplementary material and can also be found in FigShare (https://doi.org/10.6084/m9.figshare.21342834).

Declarations

Competing interests The authors declare no competing interests.

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