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Primers are designed for amplification and direct sequencing of ITS region of rDNA from Myxomycetes

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Abstract: Four new primers were designed, based on comparison of *Physarum polycephalum* sequences retrieved from Genbank (primers PHYS-5 and PHYS-4) and our own sequences (primers PHYS-3 and PHYS-2), to amplify the ITS regions of rDNA, including the 5.8S gene segment from *Lamproderma* species. Sequencing analysis shows that *Lamproderma* contains ITS1-5.8S-ITS2 regions of approximately 900 bp, which is similar in size to most eukaryotes. However, the corresponding region in another common myxomycete, *Fuligo septica*, is more than 2000 bp due to the presence of large direct-repeat motifs in ITS1. Myxomycete rDNA ITS regions are interesting both as phylogenetic markers in taxonomic studies and as model sequences for molecular evolution.

Key words: DNA amplification, *Lamproderma*, ribosomal DNA, sequencing

INTRODUCTION

Myxomycetes, or plasmodial slime molds, are eukaryotic organisms characterized by two morphologically distinct assimilative stages. One of the stages consists of uninucleate amoebae with or without flagella, and the other consists of a distinctive multinucleate structure, the plasmodium, and a static reproductive phase, the sporophore, which is minute (0.1–2 mm) (Lado and Pando 1997). Relationships among members of the myxomycetes, as well as the other two groups of slime molds (dictyostelids and protostelids), are not well defined, and morphological and biochemical data have not provided conclusive evidence to support one phylogenetic tree (Rusk et al 1995). Based on small-subunit ribosomal-DNA (SSU rDNA) sequence phylogeny, myxomycetes are included in the Protozoa (Cavalier-Smith 1993). However, only one myxomycete species (*Physarum polycephalum* Schwein.) was included in the analysis. Based on gene analysis of the elongation factor EF-1α, Baldauf (1999) concluded that the clade formed by *Physarum*, *Dictyostelium* and *Planoprotostelium* is the sister group of Animalia and Fungi. Furthermore, it was stressed that analyses based on any single molecule appear to be inaccurate in reconstructing all higher-order taxonomic relationships. In general, literature on the origin and evolution of myxomycetes, based on molecular methods, is scarce. The primary problem is the difficulty of isolating DNA from a single sporophore in sufficient quantity and quality to amplify and obtain sequences from the target regions. DNA extractions from myxomycetes have been done from plasmodia in culture (Rusk et al 1995, Baldauf and Doolittle 1997). However, the plasmodium of many species remains unknown, or the plasmodia do not grow well and form no sporophores, which makes it impossible to establish the identity of the species. The two objectives of this study were to develop an easy and reliable method to isolate DNA from, a single sporophore for amplification and sequencing and to develop primers by using published sequence data from conserved rDNA genes of related species. By this approach, it would be possible to create a sequence database from single sporophore to identify the myxomycetes and to establish the relationship with its plasmodial phase. Three species from the genus *Lamproderma* (Stemonitales) were chosen for this study. *Lamproderma* species previously have not been included in DNA sequencing studies, despite the fact that they represent a significant taxonomic problem among myxomycetes (Kowalski 1970, 1975). Significant morphological variability is observed among *Lamproderma* species in extreme climatic habitats, such as those near melting snow in alpine areas (nivicolous species). Such climate conditions might include strong, rough and fast changes in temperature, humidity or UV radiation during fructification.

MATERIALS AND METHODS

Myxomycete isolates.—Specimens were obtained from wood or plant material as denoted in TABLE I. The specimens are:

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TABLE I. Specimens studied

Species	Collection	Locality	Date	Substrate	Morphological Characters	Sequence
Lamproderma atrosporum	MA-Fungi 47898	Spain: Lérida, Alto Aneu, 1600 m	29 Apr 1999	Wood of *Betula pendula*	Sporocarps stalked.	AJ302667
	MA-Fungi 47897	Spain: Lérida, Alto Aneu, 1600 m	29 Apr 1999	Wood of *Betula pendula*	Capillitium dark brown, the tips expanded into a funnel-shape and attached to the peridium (Fig. 2A).	AJ302666
	MA-Fungi 47896	France: Albertville, foret de Esserts-Blay, 1300 m	13 Jun 1999	Wood	Spores subreticulate with some dispersed warts, 13–14 μm diam (Fig. 2B).	AJ302665
Lamproderma atrosporum cf.	MA-Fungi 47899	Spain: Lérida, Alto Aneu, 1800 m	30 Apr 1999	Herbaceous stems	Sporocarps stalked. Capillitium dark brown to black, the tips expanded into a funnel-shape and attached to the peridium (Fig. 2C). Spores reticulate, 10.5–13.5 μm diam, showing a border of 1–1.5 μm thickness (Fig. 2D).	AJ302668
Lamproderma ovoideum	MA-Fungi 47900	Spain: Huesca, Benasque, 1800 m	30 Apr 1999	Wood of *Pinus nigra*	Sporocarps sessile to short stalked. Capillitium dark brown, the tips acute and not attached to the peridium (Fig. 3E). Spores densely spinulose, 14–16 μm diam, (Fig. 3F).	AJ302669
Lamproderma sauteri	MA-Fungi 47894	Spain: Lérida, Alto Aneu, 1560 m	30 Apr 1999	Herbaceous stems and bryophytes	Sporocarps stalked, sometimes sessile.	AJ302663
	MA-Fungi 47895	Spain: Lérida, Alto Aneu, 1560 m	30 Apr 1999	Wood of *Rosa* sp	Capillitium pale brown, the tips scanty, flexuose, acute, colourless and not attached to the peridium (Fig. 2G). Spores densely spinulose, 11–13 μm diam, (Fig. 2H).	AJ302664

TABLE II. Primers designed in this study

Primer	Position[1]	Sequence
PHYS-5	SSU-1932	5′…gga agc aga agt cgt aac aag g…3′
PHYS-2	5.8S-30	5′…ctg cgc tct tca tcg aag c…3′
PHYS-3	5.8S-48	5′…gca tcg atg aag aac gca g…3′
PHYS-4	LSU-39	5′…ttc ctc cgc tga cta ata tgc…3′

[1] Position in *Physarum polycephalum* SSU, 5.8S and LSU rRNA coding sequences corresponding to 3′ primer.

Lamproderma atrosporum Meyl. (MA-Fungi 47896; MA-Fungi 47897; MA-Fungi 47898; MA-Fungi 47899), *L. ovoideum* Meyl. (MA-Fungi 47900) and *L. sauteri* Rostaf. (MA-Fungi 47894; MA-Fungi 47895).

SEM technique.—The critical-point dried technique was used in scanning electron microscopy (SEM) preparations, and the specimens were examined by a Jeol T 330 A scanning electron microscope at 10–15 kV.

DNA extractions.—DNA was extracted using the methods of Whiting et al (1997), modified according to Martín and Winka (1997), and a E.Z.N.A Fungi DNA miniprep kit (Omega Biotech), following the instructions of the manufacturer but without adding RNase and β-mercaptoethanol to the lysis buffer. In both protocols, an overnight incubation was done in the lysis buffer. DNA was resuspended in 100 μL prewarmed sterile water (FLUKA, Ref. 95305).

DNA amplification and sequencing.—DNA amplification was performed with two protocols: a) standard procedure described in White et al (1990) in a total reaction volume of 20 μL and b) Ready-To-Go® PCR Beads (Amersham-Pharmacia Biotech) in individual reactions to a final volume of 25 μL, as described in Winka et al (1998). The primers ITS1F, ITS5, ITS1, ITS4, ITS3 and ITS2 (Gardes and Bruns 1993, White et al 1990) were tested to use in PCR amplifications of the ITS regions, including the 5.8S rDNA. However, no PCR products were obtained due to differences between the primers and the target regions. Four new primers were designed, based on comparison of *P. polycephalum* sequences retrieved from GenBank (primers PHYS-5 and PHYS-4) and our own sequences (primers PHYS-3 and PHYS-2) to amplify ITS regions of rDNA included the 5.8S (TABLE II). Thirty-five cycles were conducted in a PE-9700 thermocycler: 94 C for 1 min, 52 C for 2 min, 72 C for 3 min, with a final extension at 72 C for 10 min. PCR products were separated on 2.0% agarose gels, stained with ethidium bromide and viewed under UV light. Amplification products were cleaned with the E.Z.N.A. Clean kit (Omega Biotech), and both strands were sequenced separately with primers PHYS-5, PHYS-4, PHYS-3 and PHYS-2 at the Automatic Sequencing Service (CIB-CSIC, Madrid).

Sequence alignment and phylogenetic analysis.—Sequence Navigator™ Sequence Comparison for pairwise comparisons and SEQAPP software for multiple sequences were used to search for the best alignment. Where ambiguities in the alignment occurred, the alignment chosen was the one generating the fewest potentially informative characters. Alignment gaps were marked ''–'' and unresolved nucleotides or unknown sequences were indicated with ''N''. Parsimony and maximum-likelihood analyses were performed with the computer program PAUP 4.0b* (Phylogenetic Program Using Parsimony) of Swofford (1996). Branch robustness was estimated by bootstrap analysis (Felsenstein 1985) of 10 000 heuristic replicates using the fast stepwise-addition option.

RESULTS

The capillitium of *L. atrosporum*, as seen by SEM (FIG. 2A, C), has slightly expanded tips and is attached to fragments of the peridium in a funnel shape. In *L. ovoideum* (FIG. 2E) and *L. sauteri* (FIG. 2G), the peridium does not remain attached to the capillitium, the tips are acute, sometimes bifurcate and without expansions. The spores of *L. atrosporum* are subreticulate with some dispersed warts or reticulate when observed by SEM (FIGS. 2A, B), but in some collections (MA-Fungi 47899) the reticulum is very well developed (FIG. 2D) with a more open mesh and prominent muri with some holes; this ornamentation is defined by Rammeloo (1975) as ''reticulum formed by muri and muri perforati''. In *L. ovoideum* and *L. sauteri*, the spores are densely baculate (FIGS. 2F, H), many bacula then become slightly pilate, and occasionally in *L. ovoideum* some bacula can be fused.

The best PCR products were obtained from DNA isolated with E.Z.N.A. Fungal DNA miniprep kit (Omega Biotech, Doraville, USA) (Martín and García-Figueres 1999), which yielded a DNA total concentration of 2–20 ng/μL, and using Ready-To-Go® PCR Beads (Amersham-Pharmacia Biotech) (Winka et al 1998).

The new primers were used to sequence both strands from species of the genus *Lamproderma*. ITS-1 and ITS-2 were found to be approximately 325 bp and 400 bp in size, respectively (http://www.fagmed.uit.no/info/imb/amb/), which is similar to most other eukaryotes analyzed.

Sequences have been lodged in the EMBL database with accession numbers: *L. atrosporum* (AJ302665, MA-Fungi 47896; AJ302666, MA-Fungi 47897; AJ302667, MA-Fungi 47898; AJ302668, MA-Fungi 47899), *L. ovoideum* (AJ302669, MA-Fungi 47900) *L.*

TABLE III. Uncorrected ("p") distance matrix (PAUP* 4.0) between *Lamproderma* collections (MA-Fungi) mentioned in this study

Species	Uncorrected ("p") value						
L. sauteri 47895	—						
L. sauteri 47894	0.00768	—					
L. atrosporum 47896	0.27437	0.27974	—				
L. atrosporum 47897	0.29140	0.29717	0.00368	—			
L. atrosporum 47898	0.28963	0.29516	0.00599	0.00695	—		
L. atrosporum 47899	0.30172	0.30692	0.08690	0.08153	0.08122	—	
L. ovoideum 47890	0.33066	0.33713	0.10119	0.09846	0.10313	0.08484	—

sauteri (AJ302663, MA-Fungi 47894; AJ302664, MA-Fungi 47895); *Fuligo septica* (AJ312113).

The alignment of ITS rDNA sequences of *Lamproderma* species produces no ambiguous regions and 1005 characters were included in the analyses. The alignment is available in TreeBASE http://herbaria.harvard.edu/treebase/. TABLE III shows the uncorrected "p" distance matrix. From the characters, 158 were parsimony informative. Three most-parsimonious trees (MPT) were obtained by an exhaustive search (tree length = 271; consistency index CI = 0.9779; retention index RI = 0.9653; rescaled consistency index = 0.9439). The strict consensus tree is shown in FIG. 1. Similar tree topology was generated from maximum-likelihood analysis (not shown) under Felsenstein (1985) settings. Both analyses agree, placing *L. sauteri* in different clades from *L. atrosporum* and *L. ovoideum*.

DISCUSSION

The reported primers also are compatible to rDNA ITS regions of more distantly related myxomycetes.

Lamproderma ITS sequences did not contain any obvious repetitive features. The 5.8S segment, which is an integrated region of the large subunit (LSU) rDNA, was found to be almost identical among the different *Lamproderma* species and 86%, 83% and 81% similar to 5.8S rDNA of the Physarales myxomycetes *Didymium iridis* (Johansen et al 1992), *P. polycephalum* (Otsuka et al 1983), and *Fuligo septica*, respectively. Some myxomycetes belonging to the order

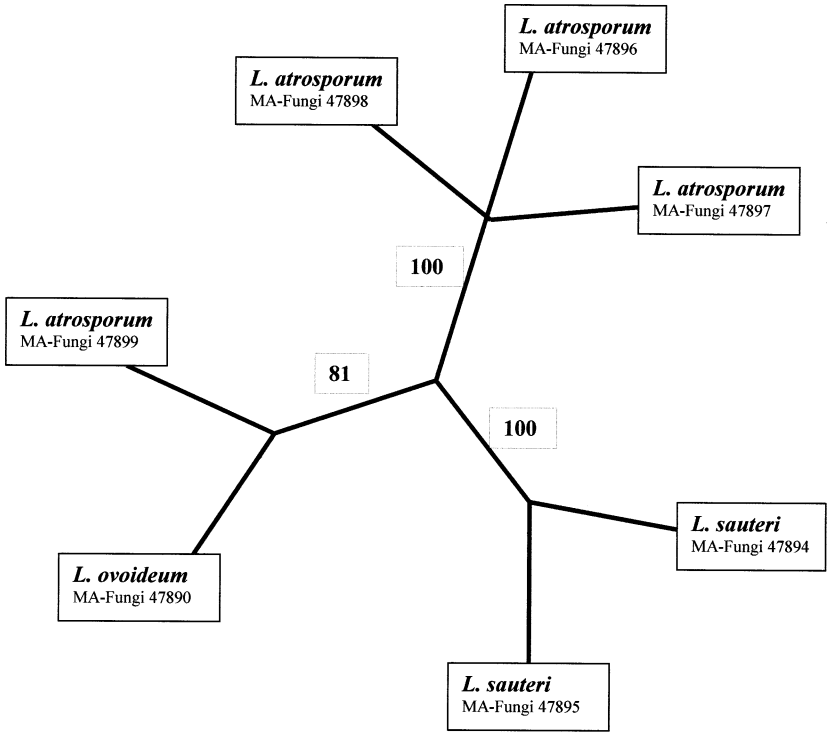

FIG. 1. Strict-consensus tree of three MPTs using PAUP 4.b* under exhaustive search based on 158 informative characters. Bootstrap values are indicated above branches. *Lamproderma atrosporum*, *L. ovoideum* and *L. sauteri*.

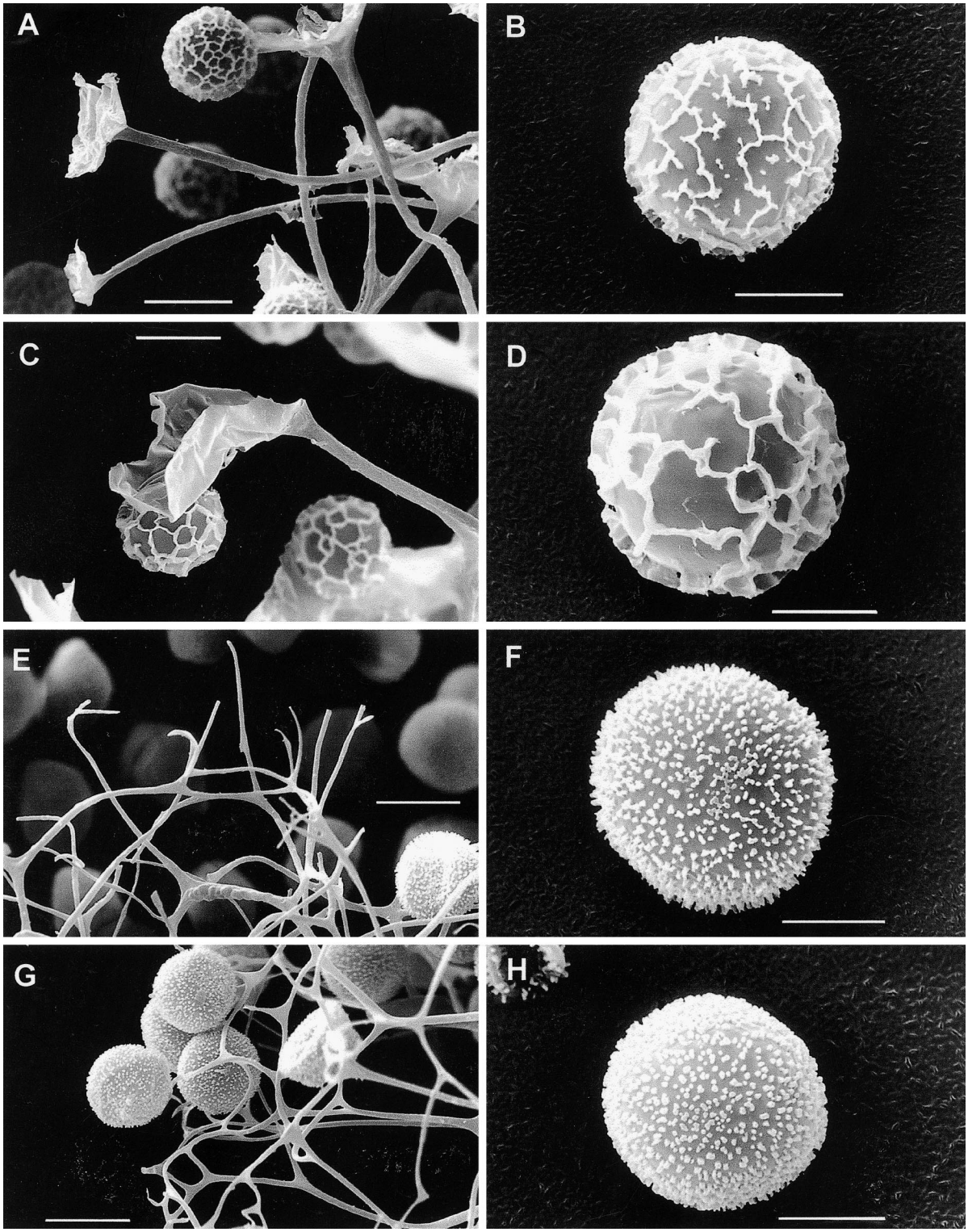

FIG. 2. Scanning-electron micrograph of *Lamproderma* species. A–B: *Lamproderma atrosporum* (MA-Fungi 47897). A. Capillitium, the tips expanded into a funnel-shape and attached to the peridium. B. Spore subreticulate with some dispersed warts. C–D: *L. atrosporum* cf. (MA-Fungi 47899). C. Capillitium, the tips expanded into a funnel-shape and attached to the peridium. D. Spore reticulate. E–F: *L. ovoideum* (MA-Fungi 47900). E. Capillitium, the tips acute and not attached to the peridium. F. Spore densely baculate, some bacula fused. G–H: *L. sauteri* (MA-Fungi 47894). G. Capillitium, the tips are scanty, flexuose, acute and not attached to the peridium. H. Spore densely baculate or slightly pilate. Scale bars: A, C, E, G = 10 μm, B, D, F, H = 5 μm.

Physarales appear to contain unusually large ITS regions. ITS-1 in both *F. septica* and *D. iridis* (Johansen et al 1997) are approximately 1.5 kb in size. Both ITS-1 regions consist of a complex, but unrelated, pattern of direct repeat motifs. Sequence and repeat organization of *F. septica* ITS-1 is shown at http://www.fagmed.uit.no/info/imb/amb/ and contains three different direct-repeat motifs, named A (26 bp), B (ca 95 bp) and C (ca 235 bp), with copy numbers of 3, 5 and 2, respectively. Individual repeats are not 100% identical to each other. In *D. iridis*, we found that ITS-1 repeat-copy numbers were fixed within a particular natural isolate or strain (Johansen et al 1997), somewhat similar to nuclear microsatellites, and probably informative in evaluating relationships among closely related species or strains.

The four *L. atrosporum* collections do not form a monophyletic clade because one of the collections (MA-Fungi 47899) forms a group with *L. ovoideum*, with a high bootstrap value. As shown in FIG. 2 and mentioned in TABLE I, *L. atrosporum* collections that form a clade have subreticulate spores, whereas the collection not included in the clade has reticulate spores. According to Clark and Haskins (1998), the observed morphological differences in other myxomycetes are related to the reproductive systems expressed. Here, a biological species appears to have larger spores than those from an apomitic isolate. Clark (2000) considers myxomycete morphospecies, in general, to be a complex of heterothallic sexual isolates and apomitic independent lines, allowing independent evolution, which thus could result in the accumulation of morphological variations. Because *Lamproderma* species cannot be grown in culture, we do not have data concerning the reproductive systems of collections in this study. However, the differences, observed both microscopically and in comparing the sequence of ITS rDNA, suggest that *L. atrosporum* MA-Fungi 47899 is an independent taxon from *L. atrosporum* s.e. Based on DNA isolate from a single sporophore, sequences of the ITS regions, including the 5.8S rDNA, have been obtained from a number of myxomycetes to create a sequence database.

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