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Evidence of Intra-individual SSU Polymorphisms in Dark-spored Myxomycetes (Amoebozoa)

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The nuclear small subunit rRNA gene (SSU or 18S) is a marker frequently used in phylogenetic and barcoding studies in Amoebozoa, including Myxomycetes. Despite its common usage and the confirmed existence of divergent copies of ribosomal genes in other protists, the potential presence of intra-individual SSU variability in Myxomycetes has never been studied before. Here we investigated the pattern of nucleotide polymorphism in the 5' end fragment of SSU by cloning and sequencing a total of 238 variants from eight specimens, each representing a species of the dark-spored orders Stemonitidales and Physarales. After excluding singletons, a relatively low SSU intra-individual variability was found but our data indicate that this might be a widely distributed phenomenon in Myxomycetes as all samples analyzed possessed various ribotypes. To determine if the occurrence of multiple SSU variants within a single specimen has a negative effect on the circumscription of species boundaries, we conducted phylogenetic analyses that revealed that clone variation may be detrimental for inferring phylogenetic relationships among some of the specimens analyzed. Despite that intra-individual variability should be assessed in additional taxa, our results indicate that special care should be taken for species identification when working with closely related species.

Key words: Amoebozoa; intra-individual variability; Myxomycetes; small subunit rRNA gene.

Introduction

Amoebozoa, one of the major divisions of eukaryotes, is a large group of mostly amoeboid organisms with different life cycles, often including sexuality, virtually found in all kinds of environments. Within this supergroup, Myxomycetes (Myxogastria or plasmodial slime molds), characterized by developing spore-bearing fruiting bodies at some point of their life cycle (Supplementary Material Fig. S1), is the most diverse monophyletic group (Baldauf et al. 2000; Fiore-Donno et al. 2005; Shadwick et al. 2009), with more than 1,000 species currently accepted (Lado 2005-2019Lado, -, 2019Lado 2005-2019).

Since vegetative and dormant phases are generally not morphologically distinguishable, the combination of multiple morphological features of their fruiting bodies has been traditionally used for diagnose and taxonomic purposes (Lado and Pando 1997; Martin and Alexopoulos 1969; Poulain

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et al. 2011). Over the last years, this morphological classification has been further improved by molecular studies (Erastova et al. 2013; Fiore-Donno et al. 2005, 2008, 2010, 2012, 2013; Nandipati et al. 2012), most of them exclusively based on partial or complete sequences of the gene coding for the nuclear ribosomal small subunit (nSSU rRNA gene, or 18S, hereafter called SSU).

In different Myxomycetes, SSU is known to be interrupted by up to 10 self-splicing group I introns (Fiore-Donno et al. 2013; Hedberg and Johansen 2013), variable in length and sequence (Supplementary Material Fig. S2C), which make it an unusually long gene difficult to amplify through a single PCR reaction. Apart from this, studies on two model species, i.e., *Didymium iridis* (Ditmar) Fr. and *Physarum polycephalum* Schwein., confirmed that SSU is arranged on a number of extrachromosomal nucleolar transcription units (Supplementary Fig. S2A–B) at different life cycle stages (Ferris and Vogt 1982; Hall and Braun 1977; Johansen et al. 1992; Silliker and Collins 1988). Besides, in *Ph. polycephalum* it has been proved that extrachromosomal rDNA is transmitted in a non-Mendelian fashion (Ferris et al. 1983). More in detail, the plasmodium comprises a mixture of both parental rDNA types in a different proportion, but after meiosis each spore inherits a single parental type, the predominant one being inherited more frequently.

It is precisely because of this highly repetitive nature that SSU has become the marker of choice for plasmodial slime molds, as the presence of several hundred copies (Campbell et al. 1979; Künzler 1985; Vogt and Braun 1976) enables its amplification from reduced quantities of template material. Consequently, thousands of SSU sequences have been generated and are now freely available in public repositories such as GenBank, making it possible to design specific primers for the group, unlike other gene sequences, still clearly underrepresented in such databases. Moreover, the SSU gene seems to be well suited for phylogenetic analyses at different taxonomic levels since it evolves relatively fast, so it is sufficiently variable among closely allied species and, at the same time, it contains highly conserved regions which make it suitable for inferring both generic and higher-level relationships. For these reasons, this marker has been preferentially used, not only in molecular studies on Myxomycetes, but also in those on its sister groups, i. e., protostelids (Fiore-Donno et al. 2010; Shadwick et al. 2018) and dictyostelids (Romeralo et al. 2011, 2010; Schaap et al. 2006; Sheikh et al. 2018).

Like other ribosomal genes, SSU may present a major problem inherent to its multicopy nature, i.e., intra-individual variation, also referred to as intra-strain or intragenomic variability in unicellular organisms. To this regard, even if a single plasmodium might be considered as one individual, and thus all fructifications originated from it (Eliasson 1981), it may be impossible to distinguish whether different sporophores come from the same or different plasmodia. Therefore, we considered a single sporophore as an individual for analytical purposes. As each sporophore contains numerous non-identical spores resulting from meiosis during their formation (Supplementary Material Fig. S1) we cannot talk about intragenomic variability, which could only be properly assessed by extracting DNA from single spores, and will rather use the conservative term “intra-individual”.

Although infrequently, intra-individual/intragenomic variability has been described in both ribosomal genes and their spacers of such diverse organisms as animals (Nyaku et al. 2013), plants (Denduangboripant and Cronk 2000; Fehrer et al. 2009), fungi (Kovács et al. 2011; Lindner and Banik 2011; Smith et al. 2007), diatoms (Alverson and Kolnick 2005), foraminiferans (Holzmann et al. 1996; Pillet et al. 2012; Weber and Pawlowski 2014), ciliates (Gong et al. 2013; Wang et al. 2017), apicomplexans (Vrba et al. 2011), dinoflagellates (Gribble and Anderson 2007), and even closer relatives of Myxomycetes within Amoebozoa (Geisen et al. 2014; Kudryavtsev and Gladkikh 2017; Kudryavtsev et al. 2009; Nassonova et al. 2010; Smirnov et al. 2007; Zlatogursky et al. 2016). Specifically in Myxomycetes, heterogeneous SSU sequences derived from single-spore PCRs have been detected in only one species from the bright-spored clade, *Trichia varia* (Pers. ex J.F. Gmel.) Pers. (Feng and Schnittler 2015). However, the possible existence of intra-individual SSU polymorphism has not been formally assessed by cloning single molecules from the pool of variants so far.

Paradoxically, while the SSU has been only recently proposed as a barcode marker for Myxomycetes (Borg Dahl et al. 2018; Shchepin et al. 2019), other genes less likely to be affected by intra-individual polymorphism (i.e., single-copy genes) remained underused for both barcoding and phylogenetic purposes. In fact, as far as we are aware, exclusively four nuclear markers (EF-1α, LSU, ITS and SSU) and COI as a mitochondrial representative (Liu et al. 2015), have been used in molecular studies on this group.

Here, to assess the occurrence of intra-individual SSU variability in Myxomycetes and to ensure that the polymorphisms did not correspond to the mixture of DNA from different sporophores, a single-sporophore DNA extraction was obtained from each of the isolates investigated, all representing dark-spored species. Specifically, the aims of this study were: (1) to determine whether intra-individual SSU variation exists in Myxomycetes, (2) if so, to document its extent and pattern and (3) to examine its potential effect on molecular analyses.

Results

A similar number of sequences of the first fragment of the SSU gene (between 27 and 31) were obtained from each of the eight specimens examined in this study. The full dataset (before removing singletons) comprised 238 sequences, eight obtained by direct sequencing and 230 by bacterial cloning. Once edited, their length did not greatly vary among individuals (543–571 base pairs). The reduced dataset (after removing singletons) contained 150 sequences.

Probability of Obtaining PCR Artefacts

For all eight specimens, the probability of the change(s) observed in different non-singleton clones being due to PCR errors was below a critical value (Supplementary Material Table S3). Accordingly, these cloned sequences were considered to exhibit actual substitutions.

Sequence Polymorphisms and Genetic Distance

Polymorphic sites including both transitions and transversions were detected among cloned sequences from all individuals, with transitions accounting for most variation. Considering the whole dataset (Supplementary Material Table S2), the total number of variable sites ranged from 18 up to 32, with the specimen *Lepidoderma chailletii* (MA-Fungi 90011) showing the highest value, opposite to *Didymium nigripes* (MA-Fungi 83234). On average, approximately 23 polymorphic sites existed among different copies in the samples investigated. Most nucleotide changes were singletons randomly distributed, although the same base replacements were also observed in different molecular clones in all eight specimens (Supplementary Material Table S2, Fig. S3). For each of the eight specimens studied, taking the sequence obtained by direct sequencing of the PCR product as reference, the observed polymorphic sites defined several co-occurring SSU variants, with *Physarella oblonga* (MA-Fungi 51797) and *L. chailletii* harboring the highest number of ribotypes (18 and 17, respectively).

After excluding those ribotypes that appeared only once (Supplementary Material Table S4), *Ph. oblonga* and *L. chailletii* still presented the highest number of variants (seven), contrary to *Lepidoderma peyerimhoffii* (MA-Fungi 81965), *Leocarpus fragilis* (MA-Fungi 91243) and *Lamproderma aeneum* (MA-Fungi 90427), each with only two ribotypes. In general, one variant was consistently more abundant than the others, this being particularly evident in the three specimens just mentioned, each with more than 88% of their clones corresponding to the same dominant ribotype. In contrast, both *Ph. oblonga* and *L. chailletii* showed different ribotypes in a similar proportion, with some cloned sequences matching that obtained by direct sequencing, and six additional ribotypes represented by a varying number of identical sequences (Supplementary Material Table S4).

As for the genetic distances calculated among all cloned sequences, the mean value of intra-individual sequence divergence ranged from 0.25% to 0.64% (Supplementary Material Table S2). The maximum genetic distance was recovered for *L. chailletii* 90011 (1.50%), followed by *Physarum andinum* (MA-Fungi 80920) (1.17%) and *L. aeneum* 90427 (1.02%). Conversely, the lowest maximum intra-individual sequence variability values were detected for *Badhamia utricularis* (MA-Fungi 64443) and *Didymium nigripes* 83234, with 0.82% and 0.85%, respectively.

Taking into account only those ribotypes that appeared several times (Supplementary Material Table S4), mean genetic distances were smaller than considering all 238 sequences (0.07% to 0.62%). The same value of maximum genetic distance (1.50%), corresponding to *L. chailletii*, was obtained.

Polymorphism Location

Due to the short length of the sequences generated in this study, the exact location of the polymorphic sites found in different specimens could not be mapped on their corresponding hypothetical secondary structures. Nevertheless, the approximate position of these sites was inferred by aligning our partial cloned sequences with that of *Ph. polycephalum*, with a well-known secondary structure (Johansen et al. 1988). In general, a higher proportion of substitutions (56.4%) were found on

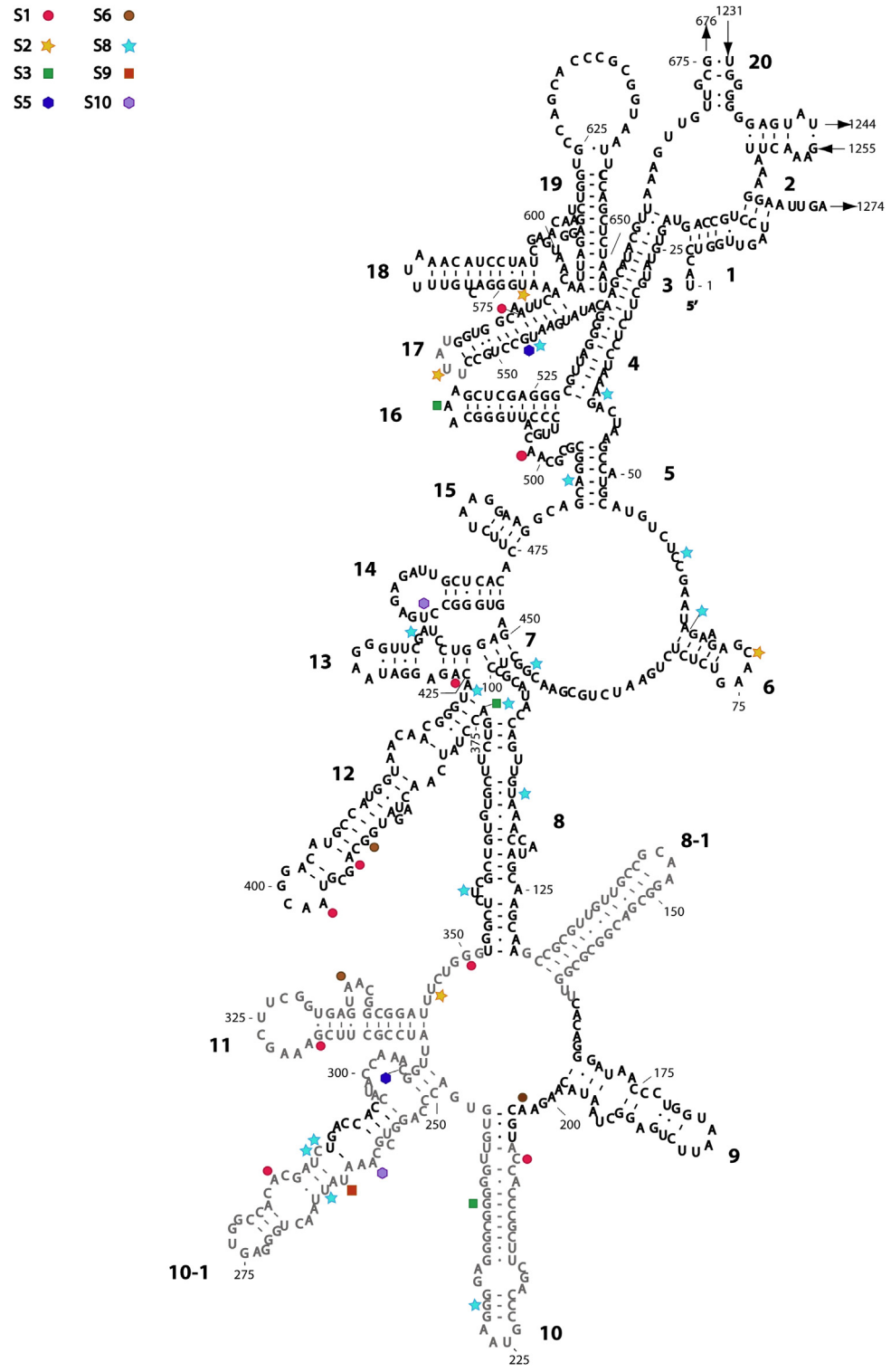

Figure 1. Schematic representation of the location of polymorphic sites in the SSU of the eight specimens examined, after excluding singleton sequences. The secondary structure of the SSU sequence of *Physarum polycephalum* X13160, published by Johansen et al. (1988), was used as reference model upon which we have mapped the substitution found in our cloned sequences. Nucleotide changes are represented by geometric forms, differently colored for each specimen. Double stranded helices found in this fragment of the SSU gene are named according to Wuyts et al. (2000). The four hypervariable helices E8-1, E10, E10-1 and E11 appear in grey.

Table 1. Nucleotide substitutions observed in different clones respect to the corresponding sequence obtained by direct sequencing of the PCR product.

Type of region	Specimen (MA-Fungi No.)	Variable nucleotide positions (150 non-singleton sequences)							Total No. (%)
Double-stranded helix	S1 (51797)	**319**/330 T/G → C/G	**392**/405 A/T → G/T	**426**/446 A/T → G/T	**566**/546 A/T → G/T				17 (43.6 %)
	S2 (64443)	**573**/594 T/A → C/A							
	S3 (80920)	**238**/212 G/C → A/C	**374**/423 A/T → G/T						
	S5 (81965)	**547**/564 G/C → A/C							
	S6 (83234)	**390**/407 A/C → G/C							
	S8 (90011)	**40**/529 A/G → G/G	**114**/367 T/G → C/G	**229**/222 G/C → A/C	**265**/286 C/C → T/C	**287**/264 T/A → C/A	**374**/423 A/T → G/T	**492**/51 A/T → G/T	**546**/566 T/A → C/A
Single-stranded loop	S1 (51797)	**208** C → T	**283** A → G	**350** A → G	**397** A → G	**501** A → G			22 (56.4 %)
	S2 (64443)	**73** C → T	**346** T → C	**555** T → A					
	S3 (80920)	**517** A → G							
	S5 (81965)	**305** A → G							
	S6 (83234)	**203** A → G	**335** T → C						
	S8 (90011)	**60** T → C	**66–67**** A → T	**96** G → A	**288** T → C	**358** T → A	**424** A → G	**441** G → A	
	S9 (91243)	**263** A → G							
	S10 (90427)	**260** A → G	**459** T → A						

Polymorphic positions, marked in bold, are numbered according to the reference sequence *Ph. polycephalum* X13160 (Johansen et al., 1988). Arrows indicate a change of a base or base pair observed in a given clone (after the arrow), respect to the sequence obtained by direct sequencing (before the arrow). For each polymorphic site found in a helical region, its complementary base is shown after the slash. Different types of resulting base pair are colored as follows: grey = canonical base pair (C/G); green = G/T wobble pair; blue = non-canonical C/A ; CT = non-canonical CT ; red = non-canonical G/G pair. **In the specimen S8, all sequences presented an insertion between positions 66 and 67, respect to *Ph. polycephalum,* which may represent a single nucleotide bulge loop (most S8 sequences presented an “A” in this bulge position, while three clones had a “T”).

single stranded regions (Fig. 1, Table 1), although these were also observed on base-paired helices (43.6%). The bulk of substitutions found on stem regions resulted on the formation of non-canonical, yet compatible, base pairs. Specifically, the pair GT (GU) was the most frequently found, followed by AC and CT. As an example, in the specimen *Ph. oblonga* 51797, five out of the nine non-singleton polymorphisms occurred in single stranded regions, while four base pairs (three GT and one GC) were located on stem (double-stranded) regions. Compensatory base changes, i. e., substitutions in both nucleotides to maintain the base pair itself, were not detected.

Phylogenetic Analyses

We conducted phylogenetic analyses (ML and BI) including all repeated cloned sequences and those generated by direct PCR. The clustering pattern was stable in both trees, identifying a distinct, and generally well-supported, sequence clade for each specimen analyzed (Supplementary Material Fig. S4).

More in detail, within the paraphyletic order Stemonitidales, a clade formed by all sequences obtained from the specimen *Lamproderma aeneum* 90427 was recovered with full support (PP = 1; aLRT = 94.2%; UFBS = 100%). Physarales appeared monophyletic with poor support (PP = 0.84; aLRT = 93.6%; UFBS = 83%), with the family Didymiaceae s. l. being paraphyletic with respect to Physaraceae.

Within Didymiaceae s. l., we highlight three main assemblages with varying support values: (1) a strongly supported clade comprising all clones of *Didymium nigripes* 83234 (PP = 1; aLRT = 92.9%; UFBS = 99%), forming part of a larger group, also receiving high support (PP = 0.99; aLRT = 95.0%; UFBS = 93%), that contained most *Didymium* species analyzed along with *Mucilago crustacea;* (2) a robust clade formed by all clones of *Lepidoderma chailletii* 90011 (PP = 1; aLRT = 95.8%; UFBS = 99%), sister to another monophyletic group constituted by three specimens of the same species; and (3) a weakly supported group (PP = 0.69; aLRT = 92.9%; UFBS = 73%) including all clones of *Lepidoderma peyerimhoffii* 81965, plus three GenBank sequences corresponding to *Diderma fallax* (MH930576 and MH930577) and *Lepidoderma crustaceum* (HE614619). Within it, two clones (i.e., 3 and 5) formed a strongly supported clade (PP = 1; aLRT = 92.8%; UFBS = 97%). Given the intermingled pattern of this "*L. peyerimhoffii + L. crustaceum + D. fallax* clade" an additional analysis was performed (see results below).

Within the family Physaraceae, which was recovered monophyletic receiving high support (PP = 1; aLRT = 99.8%; UFBS = 98%), we distinguish four main clusters of interest: (1) a fully supported clade formed by all clones of *Physarum andinum* 80920 (PP = 1; aLRT = 99.4%; UFBS = 100%), nested within a larger monophyletic group (PP = 1; aLRT = 95.4; UFBS = 96%) comprising three species of the genus *Craterium*; (2) a strongly supported monophyletic group (PP = 1; aLRT = 98.8%; UFBS = 100%) constituted by all clones of *Leocarpus fragilis* 91243 that forms part of a larger clade along with a number of GenBank sequences of the same species (PP = 1; aLRT = 94.6%; UFBS = 98%); (3) a moderately supported clade (PP = 0.92; aLRT = 91.7%; UFBS = 95%) comprising all sequences of *Physarella oblonga* 51797, sister, with high support, to a well-supported monophyletic group constituted by other isolates of *Ph. oblonga* (PP = 1; aLRT = 87.3%; UFBS = 95%); and (4) a highly-supported group (PP = 1; aLRT = 99.4%; UFBS = 100%) formed by all clones of *Badhamia utricularis* 64443 plus several GenBank sequences corresponding to *B. foliicola, B. utricularis* and *Ph. albescens*, the three last forming a well-defined and fully supported clade (PP = 1; aLRT = 100%; UFBS = 100%).

As for the tree built for the "*Lepidoderma peyerimhoffii + L. crustaceum + D. fallax* clade" (Fig. 2), the group formed by all clones of *L. peyerimhoffii* 81965 and the sequences of *D. fallax* and *L. crustaceum* received moderate support (PP = 0.92; aLRT = 84.4%; BS = 64%). Again, both clones 3 and 5 constituted a distinct well-supported clade (PP = 1; aLRT = 91.6%; BS = 87%). The group formed by most representatives of *D. fallax* plus *L. carestianum* HE614618 was recovered monophyletic with high support (PP = 1; aLRT = 85.8%; BS = 78%).

Discussion

Probably due to the difficulty of extracting enough DNA from single sporophores when working with Myxomycetes (especially, with species presenting minute fruiting bodies), to date, most molecular studies have been carried out using multiple-sporophore DNA extractions. However, in our experience, this routine could entail the unintentional use of a mixture of DNA from several individuals if those sporophores, while adjacent, originated from different plasmodia. Thus, we used

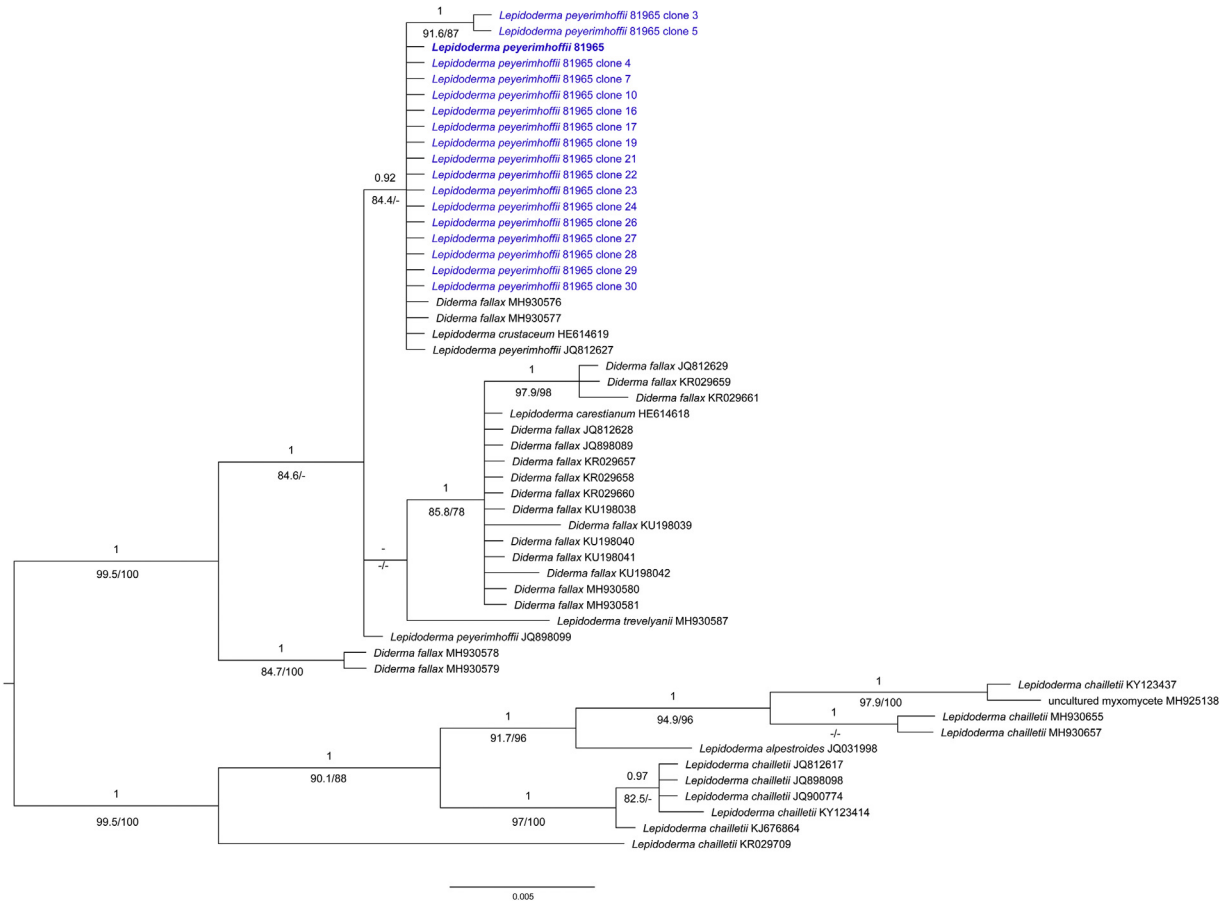

Figure 2. Fifty percent majority-rule Bayesian phylogenetic tree of the "*Lepidoderma peyerimhoffii* + *L. crustaceum* + *D. fallax* clade". Terminal names consist of species designation followed by voucher number and clone ID (the sequence obtained by direct PCR appears in bold). GenBank sequences are indicated by their corresponding accession numbers. Posterior probability (≥0.90) and SH-alrt (≥80%) plus bootstrap support values (≥ 70%) are shown above and below the branches, respectively.

single-sporophore DNA extractions to investigate the potential existence of intra-individual polymorphisms in the SSU of eight dark-spored species.

All clone libraries generated here usually comprised a highly represented copy and many singleton sequences or unique ribotypes (Supplementary Material Table S2), a common pattern considered unnatural. While some authors have stated that changes derived from experimental issues, i.e., base misincorporations during PCR, replication errors by competent cells or sequencing mistakes, are frequent (Lücking et al. 2014; Thielecke et al. 2017; Wang et al. 2017), others have confirmed that only a small number of variants can be attributed to technical artefacts (Alverson and Kolnick 2005; Thornhill et al. 2007). Moreover, it has been recently reported that unique substitutions are not necessarily a consequence of experimental errors (Borrelli et al. 2018; Thiéry et al. 2016). Taking this into account, we think that the polymorphisms found in the present study correspond to natural SSU variation because: 1) nucleotide misincorporation during PCR was circumvented by using a high-fidelity polymerase, which removes erroneous bases incorporated into the growing strand increasing the accuracy of DNA synthesis from the template (nonetheless, for the avoidance of doubt, future studies involving cloning from independent PCR reactions could be useful); 2) all PCR products were sequenced in both directions and no differences were observed when comparing the chromatograms; 3) assuming that occasional technical issues could have introduced some biases, singleton sequences were treated as artefacts and excluded from subsequent analyses, even if some authentic base substitutions might have been discarded (Supplementary Material Fig. S3). Still, different non-singleton ribotypes

were identified in all specimens examined (Supplementary Material Tables S2, S4). Considering the co-occurrence of the same ribotype in different bacterial colonies and that the probability of recovering our sequences due to PCR errors was statistically negligible (Supplementary Material Table S3), we think that these sequences harbor natural rather than artificial polymorphisms. Therefore, in the same way that the existence of different SSU copies has been corroborated in a variety of protists, including Amoebozoa (see Introduction), our data strongly suggest that this gene also shows genuine intra-individual variation in Myxomycetes. Moreover, as it has been detected in all specimens under study, intra-individual variation may be the rule rather than the exception in these amoeboid organisms, although more species should be studied to reach well-founded conclusions on this point.

Consistent with previous findings (Weber and Pawlowski 2014), the intra-individual variation found here is represented by SNPs, since no insertions or deletions were detected in our cloned sequences. Likewise, our results indicate that the level of intra-individual polymorphism varies among species, as demonstrated in other protists (Wang et al. 2017; Weber and Pawlowski 2014). Even so, there is something more remarkable about the variation depicted here: although a number of substitutions were located in the highly variable helices of the first fragment of SSU (i.e., E10, E10-1 and E11, shown in grey in Fig. 1), they were not restricted to these stretches. In other words, sequence variation reported here does not perfectly match the known variability of SSU, which is concentrated in the mentioned helical regions. A possible explanation for this finding is that the clones having these substitutions correspond to minor SSU variants from the pool existing within each individual, rarely obtained by direct sequencing of the PCR products (the signal of the dominant copy makes low-abundance variants to remain largely undetectable (Bik et al. 2013)). As a consequence, base changes not present in the dominant SSU copy but in minor ones, located outside of the mentioned helices, may have gone undetected so far.

Most variable sites were located in single-stranded segments although they also occurred in double-stranded regions, regardless if these were hypervariable or not (Fig. 1, Table 1). Among the latter, the largest proportion of changes resulted in the formation of the pairs GU (wobble pair) and CA (non-canonical), followed by CG (Watson-Crick canonical pair), CT and GG (both non-canonical). Interestingly, previous studies have confirmed that several non-canonical interactions, including those just cited, occur naturally in different RNAs and are crucial to their structural organization (see Davis et al. 2011; Halder and Bhattacharyya 2013; Nagaswamy et al. 2004, for more details).

In theory, changes in single-stranded regions or resulting in presumably stable interactions would not substantially affect the secondary structure of the SSU, so our clones could represent minor functional variants. But it has been proved that, even if the structure remains almost unaltered, the function of the ribosomal genes can be disabled by minor changes in binding or catalytic sites (Hershkovitz et al. 1999; Thornhill et al. 2007; Wuyts et al. 2001). Considering this, it would be necessary to conduct a rigorous empirical analysis to evaluate if the changes occurring in our clones, even if they mostly corresponded to stable pairs, have adverse effects on the structure and function of the SSU gene.

Alternatively, the existence of a few polymorphic sites in otherwise highly conserved regions allows us to hypothesize that some of our cloned sequences may be pseudogenes, as found in other protists (Santos et al. 2003; Thornhill et al. 2007). In functional genes, those sites with an important catalytic role have less freedom to mutate than the other bases, and so, substitutions are expected to occur in a non-random fashion (Wuyts et al. 2001). In contrast, given that pseudogenes may be non-functional elements (they do not need to preserve their structure) mutations can occur at any base, accumulating over their entire length, as observed in some of our clones. On the other hand, another interpretation is that not all genuine SSU variation has already been reported and so, the few polymorphic sites that we found in generally conserved positions would potentially represent additional variation.

The reasons for the observed pattern of intra-individual variation could be manifold. We suggest that it may be best explained by the combination of several factors, none of which are mutually exclusive. To begin with, intra-individual variability could be related to particularities inherent to the complex reproductive mechanism of Myxomycetes, which most probably encompass an intricate pattern of species with sexual and apogamic strains (Fiore-Donno et al. 2011). Although asexual reproduction cannot be rejected as a reproductive strategy, Myxomycetes are predominantly sexual organisms, with heterothallism (different mating types) being the most common scheme (Adler and Holt 1975; Clark and Haskins 2010; Feng and Schnittler 2015; Fiore-Donno et al. 2011; Keller et al. 2017; Stephenson and Schnittler 2017). This sophis-

ticated system, which impedes closely related individuals to mate avoiding inbreeding depression, includes the germination of the spores into uninucleate haploid amoebae (Supplementary Material Fig. S1). Nonetheless, the existence of amoebae and also plasmodia with different ploidy levels (Adler et al. 1975; Ferris et al. 1983) makes it more complicated and would explain certain variability based on allelic polymorphisms. In a similar manner, although myxomycete spores are typically uninucleate, several nuclei can occasionally survive per cell and, moreover, the number of nuclei per spore can vary within one sporophore (Novozhilov et al. 2013). Understandably, if coexisting nuclei were genetically different, this circumstance would be responsible for some variation. Additionally, even if the spores were uninucleate, the incomplete removal of one of the two parental rDNA types could result in heterogeneous SSU sequences in single spores, as reported for some specimens of *T. varia* (Feng and Schnittler 2015). Nevertheless, even under the simplest scenario (uninucleate haploid spores with complete deletion of one parental rDNA type), the genes found in different spores originated from the same plasmodium exhibit a mixture of alleles from both progenitors, as well as their recombination products (Fiore-Donno et al. 2011). That is, the multiple spores within a single sporophore are not expected to be identical, which probably accounts for an important fraction of the intra-individual SSU variability found here. Unfortunately, to date, neither the number of nuclei nor the spore ploidy has been documented for the species sampled in the present study, and so, further testing is needed to clarify their potential link to the variation found.

Another alternative or parallel explanation for the observed intra-individual SSU variability would be the existence of numerous divergent copies within each haploid nucleus. In most organisms different ribosomal gene copies are known to be homogeneous due to an extremely efficient molecular process, termed concerted evolution, that homogenizes them through the continual turnover of repeats by unequal recombination (Eickbush and Eickbush 2007; Ganley and Kobayashi 2007). However, in single-celled protists, it has been recently documented that the different copies not always evolve in a strictly concerted manner, with heterogeneity being positively correlated to the copy number (Gong et al. 2013; Kudryavtsev and Gladkikh 2017; Thornhill et al. 2007). Moreover, the extrachromosomal nature of ribosomal genes possibly contributes to intra-individual variation not only in protists (Gong et al. 2013; Kudryavtsev and Gladkikh 2017; Long and Dawid 1980), but also in distant organisms (Lindner and Banik 2011; Simon and Weiß 2008). Notably, both high copy number and extrachromosomal organization have been confirmed for the SSU of the model species (Campbell et al. 1979; Ferris et al. 1983; Torres-Machorro et al. 2010; Vogt and Braun 1976), and most likely are also characteristic in other myxomycete species. Considering this, the existence of a relaxed mechanism of concerted evolution (mutation rate exceeds the rate at which they are removed) in the species examined could be possible as the rate of evolution of the SSU gene in Myxomycetes is unusually high (Fiore-Donno et al. 2012; Fiore-Donno et al. 2019; Kretzschmar et al. 2016; Morin 2000). This high rate, combined with the repetitive nature of the SSU, may have driven intra-individual variation in this gene, as suggested in other groups (Gribble and Anderson 2007; Van de Peer et al. 2000).

Other explanations for our findings include hybridization and introgression as the source of genetic intra-individual variability (Alverson and Kolnick 2005; Pillet et al. 2012) but, to the best of our knowledge, no case of interspecific hybridization has been registered in Myxomycetes so far.

Surprisingly, perhaps because extensive cloning experiments have not been conducted in these amoeboid organisms, none of these hypotheses has been considered as a potential cause of intra-individual heterogeneity, even if intra-sporal variability has been proved to exist (Feng and Schnittler 2015). Indeed, all multiple SSU copies in a given specimen have been assumed to be homogeneous in some previous publications (Feng and Schnittler 2015, 2017; Schnittler et al. 2017), based on their interpretation of the study by Ferris et al. (1983) who, as initially mentioned, resolved that different rDNA types cannot occur simultaneously within a single spore. In our view, this does not necessarily imply that the whole spore population of a specimen (even when considered as a single sporophore) presents the same rDNA type.

Independently of the underlying causes, here we report for the first time the occurrence of intra-individual SSU variability in Myxomycetes. It has been shown earlier that most species possess several SSU ribotypes and that the number of variants varies among them (Schnittler et al. 2017). But, in light of our data, it seems clear now that the number of SSU ribotypes within each species must be much higher than previously thought. This intra-individual variation should be taken into account when assessing species richness in environmental studies. Despite that using only one locus (com-

mon practice in environmental barcoding studies) has important limitations when the goal is delimiting species accurately (discussed in Zamora et al. 2018), a single barcoding region can be used to get a rough estimate of the species richness. For the sake of this purpose, given that the occurrence of several ribotypes within the same specimen could inflate species richness estimates, it would be advisable to better characterize the extent of intra-individual, and thus intraspecific polymorphisms, in Myxomycetes. Then, perhaps, the 0.9% threshold for intraspecific variance identified by Borg Dahl et al. (2018) could be realistically adjusted, especially considering that the maximum intra-individual sequence divergence in our sample of *Lepidoderma chailletii* (MA-Fungi 90011) clearly exceeds this value (1.5%, Supplementary Material Table S4), even after singleton sequences were removed. In this regard, other authors have recently proved that applying the mentioned threshold value in metabarcoding studies results in an overestimation of diversity (Shchepin et al. 2019).

On the other hand, great care should be taken when dealing with very closely related species, as their boundaries could be blurred by the hitherto undetected intraspecific variability. This may be exemplified here by *L. peyerimhoffii* 81965 and two species represented by GenBank sequences, i.e. *L. crustaceum* and *D. fallax*, as they constitute a multispecies group within which two clones of *L. peyerimhoffii* 81965 form a distinct and well-differentiated clade (Fig. 2). In this particular case, if the sequences retrieved from GenBank correspond to well-identified specimens, it would indicate that SSU variation within an individual is larger than among different close species. However, this is something that needs to be confirmed as we have not studied the collections used to obtain the sequences of *D. fallax* and *L. crustaceum*. As for the remaining individuals included in this study, all clones, even the most divergent, formed part of well-supported monospecific clades, suggesting that intra-individual polymorphism might not have pernicious phylogenetic consequences when working with more distantly related species.

This study is the first step toward understanding genetic polymorphism in Myxomycetes and, while our data demonstrate that intra-individual (intra-sporophore) SSU variability exists in different species, some questions about the magnitude and origin of this heterogeneity remain unanswered. In fact, considering their complex life cycle, it would be of great interest to explore SSU variation from a multilevel perspective to determine whether 1) the numerous gene copies present in a single spore are identical; 2) the spores within the same sporophore are genetically different; and 3) the sporophores arising from the same plasmodium exhibit significant variation. Most importantly, it would be necessary to clearly establish the impact of this intra-individual SSU variability by generating data from numerous close species in order to test whether there is a gap between actual intraspecific (that accounting for intra-individual heterogeneity) and interspecific variability. It is only after such an ambitious assessment that we could have a complete image of SSU variation in Myxomycetes, and gain a better understanding of its underlying causes, its strengths and weaknesses for phylogenetic and barcoding studies.

While more research is warranted before broad generalizations can be made, the intra-individual variation observed here should not be ignored as it could be higher in other taxa, obscuring phylogenetic relationships and leading to diversity overestimation in barcoding analyses.

Methods

Taxa sampling: Eight mature specimens, each corresponding to a different morphospecies characterized by their large-sized sporophores, were specifically selected for this project (Supplementary Material Table S1). This includes six genera, i.e., *Badhamia*, *Didymium, Leocarpus, Lepidoderma, Physarella* and *Physarum* from the order Physarales s. str., and *Lamproderma* as representative of Stemonitidales. Voucher specimens were deposited in the MA-Fungi Herbarium of the Real Jardín Botánico, CSIC (Madrid, Spain).

DNA extraction: Genomic DNA was extracted from single sporophores following the CTAB protocol by Doyle and Doyle (1990), with minor modifications. First, each sporophore was placed in a 1.5 mL safe-lock Eppendorf tube containing one metallic bead. These tubes were frozen at −20 °C for 10 min before grinding the samples into powder by shaking the tubes at 12000 rpm for 2 minutes in a TissueLyser II (Qiagen, Germany). Then, 500 μL of CTAB buffer was added to each tube before being vortexed for 1 min and incubated at 55 °C overnight, with occasional shaking. The remaining steps of the extraction were performed according to the cited protocol.

nSSU rRNA gene amplification: The numerous introns often found in the SSU of Myxomycetes might hinder the amplification of the entire gene in a single PCR reaction (Fiore-Donno et al. 2012, 2013). Indeed, in previous studies, it was necessary to amplify four overlapping SSU fragments to obtain complete sequences (Aguilar et al. 2014; Fiore-Donno et al. 2008). In a pilot study prior to the present investigation, we aimed to amplify two large SSU fragments (second and two last parts of the gene, Supplementary Material Figure S2C) using the primer combinations S4/S900R and S11.5/RB2, respectively (Aguilar et al. 2014; Fiore-Donno et al. 2008). Unfortunately, the amplification of the second region failed in some specimens, while the existence of a large intron (>1000 bp) interrupting the other fragment compromised the quality of the sequences obtained from several samples. Thus, considering that these difficulties made the prospects of examining the full SSU gene

impossible to accomplish, we selected the 5' end fragment (ca. 600 bp), devoid of introns and recently proposed as barcode for Myxomycetes (Borg Dahl et al. 2018; Shchepin et al. 2019), to be amplified and subsequently cloned. The primers S2 (5'-TGGTTGATCCTGCCAGTAGTGT-3') and SR4Dark (5′-TGTCCTCTAATTGTTACTCGA-3′), designed by Fiore-Donno et al. (2008, 2012), were used for amplification of the target.

Each PCR reaction mixture contained 12.5 μL of a proofreading polymerase (MyFi™ Mix, Bioline, United Kingdom), 0.5 μL of each primer (10 nM), 5 μL of DNA template, and Milli-Q water to a final volume of 25 μL. PCR amplifications were carried out in a Mastercycler gradient S thermal cycler (Eppendorf, Germany) with the cycling parameters recommended by the manufacturers of the enzyme used: an initial denaturation step at 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min and polymerization at 72 °C for 1 min, with a final extension step at 72 °C for 10 min. After amplification, 3 μL of each PCR product were checked on 1% agarose gels. Reference bands were excised from the gels and purified with Wizard® SV Gel and PCR Clean-Up System (Promega, United States). To check the identity of the amplicons, half volume of each purified PCR product was sequenced by Macrogen (Spain) with the same primers used for amplification.

Cloning and sequencing: The remaining PCR products were cloned using a pGEM® T-Easy Vector System kit (Promega, United States), following the manufacturer's instructions. In short, amplicons were ligated into the pGEM® T-Easy vector which encodes the β-galactosidase gene. After transformation into Ready-to-Use JM109 competent cells (Promega, United States), the screening and selection of bacteria harboring recombinant plasmids were performed on LB agar plates containing ampicillin, X-gal and IPTG. Depending on their availability, we selected around 30 clearly isolated white colonies (recombinant clones) from each plate. Inserts were PCR amplified and Sanger-sequenced using the universal primers T7 (5′-TAATACGACTCACTATAGGG-3′) and SP6 (5′-GATTTAGGTGACACTATAG-3′). Raw data were assembled in Geneious 7.1.9 (https://www.geneious.com). After identifying and trimming the cloning vector, sequences obtained from each specimen were automatically aligned using MAFFT v7.017 (Katoh et al. 2002) with the E-INS-i algorithm and default settings, as implemented in Geneious. A total of 238 newly acquired sequences were submitted to GenBank under accession numbers MN333919 - MN334156 (Supplementary Material Table S1).

Descriptive statistics: Once aligned, all sequences generated from each specimen were analyzed with the software DnaSP v6.11.01 (Rozas et al. 2017) for characterizing intra-individual polymorphisms. Several statistics, such as the total number of polymorphic sites (random and parsimony informative substitutions) and the transition/transversion ratio, were measured along with the number and frequency of distinct ribotypes, i.e., unique sets of single nucleotide polymorphisms (SNPs) comprised in a given sequence. Singleton ribotypes, i.e., appearing only once within each specimen, were found in all eight samples (Supplementary Material Fig. S3, Table S2).

Probability of obtaining PCR artefacts: Singleton sequences are often considered to be methodological artefacts, i.e., sequences resulting from PCR, cloning or sequencing errors (e.g. Flynn et al. 2015; López-Escardó et al. 2018). Thus, in an attempt to circumvent what could be artificially induced variation, we deliberately reduced our initial dataset (from 238 to 150 sequences) by discarding all singleton cloned sequences, and retaining only those ribotypes that were repeatedly found within each individual. This conservative approach implied the exclusion of some point substitutions shared among different specimens (marked with an asterisk in Supplementary Material Fig. S3). Moreover, we further verified that the changes observed in the repeated sequences were not introduced during PCR by using the statistical method proposed by Cummings et al. (2010). Briefly, we used a binomial distribution function to calculate the probability of an amplicon to possess a specific number of substitutions due to PCR errors (see Supplementary Information for details).

Sequence polymorphisms and genetic distance: Assuming that the probability of recovering the same sequence several times due to technical errors was extremely low, both the number and frequency of retained ribotypes were estimated. Furthermore, genetic p-distances were calculated independently for each specimen in MEGA 7.0.26 (Kumar et al. 2016), with 1,000 bootstrapping replicates (Supplementary Material Table S4).

Polymorphism location: Predictive algorithms, commonly used to determine the secondary structure of ribosomal genes, may be unsuited for partial sequences. Thus, the location of the polymorphic sites found in our cloned sequences was indirectly deduced through a comparative analysis, assuming that homologous stem regions are maintained even if the loops may vary in length among taxa. To do so, we aligned the sequences obtained from each individual with that of the species *Ph. polycephalum* (accession number X13160), whose secondary structure is well-known (Johansen et al. 1988), using MAFFT. After minor manual modifications, we visually inspected the approximate position of the polymorphic sites with respect to the reference sequence (Fig. 1, Table 1).

Phylogenetic analyses: To place our data in a phylogenetic context, 182 sequences representing the closest allies to the eight morphospecies investigated here, obtained by BLAST searches against the NCBI database (identity >90%), were included in the analyses (Supplementary Material Table S5). Additionally, based on the phylogeny by Fiore-Donno et al. (2012), five sequences from the" *Comatricha* clade" and "*Stemonitis* clade" of Stemonitidales were selected as outgroup. The final alignment comprised 337 sequences spanning 17 myxomycete genera from two orders.

Two common phylogenetic approaches, i.e., Maximum Likelihood (ML) and Bayesian Inference (BI), were used to infer the relationships among these sequences. The ML phylogenetic analysis was performed with IQ-TREE 1.6.10 (Nguyen et al. 2015) using the substitution model selected by ModelFinder (Kalyaanamoorthy et al. 2017), according to the Bayesian Information Criterion. Because of the large size and complexity of the data matrix, the robustness of the inferred ML tree was assessed by the ultrafast bootstrap approximation using 1000 replicates (- bb 1000). To reduce the risk of overestimating branch supports due to severe model violations, we used the option - bnni (Hoang et al. 2017; Minh et al. 2013). Branch support was also assessed by using the non-parametric SH-approximate likelihood ratio test (SH-aLRT) (Anisimova and Gascuel 2006).

The Bayesian analysis was conducted in the CIPRES Science Gateway (Miller et al. 2010) using MrBayes v.3.2.6 (Huelsenbeck and Ronquist 2001; Ronquist et al. 2012). Since the best-fit substitution model suggested by ModelFinder (TIM3e + G4) cannot be implemented in MrBayes, it was replaced by the closest over-parameterized model (SYM + G). Four parallel runs, each one with six Metropolis-coupled Markov chains, were executed for 15 million generations, sampling every 1000 generations. The convergence of the runs was assessed through the standard deviation of split frequencies (<0.01) in MrBayes, and the effective sampling size crite-

rion (ESS > 200) in Tracer v1.6 (Rambaut et al. 2014). After discarding the first 25% generations as burn-in, the 50% majority-rule consensus tree with branch lengths and posterior probabilities was calculated from the remaining trees. Since, in general, there was topological congruence between the trees resulting from both methods, we only present the Bayesian consensus tree showing BI posterior probabilities (PP) and SH-aLRT/ultrafast bootstrap (UFBS) support values, above and below the branches, respectively (Supplementary Material Fig. S4). In parallel, given that our dataset spanned two orders and some variable regions were difficult to align, we also carried out these analyses using an alignment mask published by Fiore-Donno et al. (2012). Although the exclusion of part of the variation resulted in lower resolution within some minor clades of interest, the results concerning major clades were similar, reason why the masked analyses are not presented.

To further elucidate the phylogenetic relationships within the "*Lepidoderma peyerimhoffii* + *L. crustaceum* + *D. fallax* clade" in Figure S4, in which our cloned sequences intermingle with sequences of other species, subsequent ML and BI analyses were performed as already described (Fig. 2). In the case of the ML analysis, branch support values were assessed by using the standard non-parametric bootstrap method (- b). All alignments used in this study are available upon request.

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Appendix A. Supplementary Data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.protis.2019.125681.

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