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Research Article

In-Silico Molecular Phylogeny of Philippine Myxomycetes using 18S rRNA and small subunit rRNA (SSU) Gene Sequences

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ABSTRACT

Myxomycetes, commonly called plasmodial slime molds, are eukaryotic organisms usually thriving on terrestrial ecosystems, manifesting attributes of fungi and protists. The current taxonomy of myxomycetes poses serious problems and does not clearly reflect the evolutionary relationships of this group. The literature on myxomycetes in the Philippines has primarily focused on species identification, ecological and diversity studies. However, no attempts have been made yet to elucidate the evolutionary relationships of this class using molecular evidence. The use of 18S rRNA and small subunit rRNA (SSU) sequences in this study to construct phylogenies has revealed that certain taxa, traditionally considered monophyletic, exhibit inconsistencies that warrant further investigation. However, the molecular evidence that supports the division of myxomycetes into two distinct groupings (Lucisporidia and Columellidia) according to spore color remains consistent with the constructed phylogeny using 18S rRNA gene sequences. Therefore, the present study aims to assess Philippine myxomycetes' diversity and evolutionary relationships. In addition, the study aims to compare the conventional taxonomy and molecular phylogeny of myxomycetes species in the Philippines using two molecular barcode markers - the 18S rRNA and SSU sequences. The study demonstrated that it is not always possible for morphology to agree with molecular data. On the other hand, molecular data can be more beneficial in reconstructing phylogenies but only to a certain extent and should be further evaluated.

Keywords: In silico analysis, Myxomycetes, Phylogenetic analysis, Species problem

Introduction

Myxomycetes are eukaryotic organisms that are often found in terrestrial habitats. They are also referred to as myxogastria or plasmodial slime molds. The traditional classification of this class includes 5 orders, 15 families, 68 genera, and over 960 species [1]. Plasmodial slime molds are eukaryotic microorganisms commonly found on decaying leaf litter or logs in forests [2], distinguished by the capacity to generate complex spore-bearing structures known as sporocarps and the alternation of amoeboflagellate plasmodial vegetative phases [3]. In the current classification of living organisms, myxomycetes are regarded as a monophyletic taxon inside the Amoebozoa [4, 5]. Molecular approaches have not been used to study myxomycetes in nature. Only lately have the first molecular-based phylogenies been published [6-9].

It yielded two major clades: bright-spored clades (spores lightly tinted by diverse colors) and dark-spored clades (spores dark-colored by melanin, most of the cultivable species). Indeed, most taxa within this class are in critical need of revision [3]. In recent years, traditional classification systems have been both enhanced and challenged by molecular studies [10-13], most of which rely on partial or complete sequences of the nuclear ribosomal small subunit gene (SSU rRNA gene or 18S rRNA). The nuclear small subunit rRNA gene is a commonly used marker in phylogenetic and

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barcoding studies of Amoebozoa, including myxomycetes [14]. The SSU rRNA sequences have been found to be well-suited for phylogenetic analyses at various taxonomic levels, owing to their rapid evolution, sufficient variability among closely related species, and highly conserved regions that enable inference of both deeper and greater links [15]. On the other hand, the partial 18S rRNA sequences were used to effectively create phylogenies for most taxa within myxomycetes, to recover the systematic position of a species [10, 16], and to distinguish between species that are closely related [17].

Therefore, evaluating the diversity and evolutionary relationships of myxomycetes is crucial. The present study aims to achieve this by assessing the diversity and evolutionary relationships of Philippine myxomycetes, and comparing the conventional taxonomy and molecular phylogeny of myxomycetes species in the Philippines using two molecular barcode markers - the 18S rRNA and SSU sequences.

Material and Methods

Sequence data acquisition

A study has presented an updated list and records of myxomycetes in the Philippines [18]. The list of species was used as a reference and baseline data for the entirety of this study, and it contains records of 150 myxomycete species present in the Philippines. The 18S rRNA and SSU rRNA gene sequences of the sample species were obtained from the GenBank database, which provides public access to over a hundred thousand nucleotide sequences online and serves as a comprehensive repository of DNA sequence information [19]. Therefore, GenBank was selected as the primary source of genetic information for the species samples. The 150 species were manually screened to determine the availability of their 18S rRNA and SSU nucleotide sequences on the database. The nucleotide sequences with the highest number of base pairs were selected to maximize the results. The obtained sequences for both markers were recorded in FASTA format and subjected to multiple sequence alignment using MEGA 11. Forty-seven 18S rRNA nucleotide sequences were retrieved, accounting for 31.33% of the reference list; 75 SSU nucleotide sequences were retrieved, accounting for 50% of the reference list.

Selection of outgroup species

To assess the ingroup's monophyly and determine the root's position on the phylogenetic tree that has been generated, *Porphyra suborbiculata* sp. was incorporated as an outgroup. Furthermore, myxomycetes are commonly misunderstood as macrofungi due to their adaptations in their habitats. However, they are not fungi but rather a group of protists [20]. Hence, the *P. suborbiculata* sp. was chosen as the outgroup of the phylogenetic recon-struction tree for both sets due to its distinction as macrofungi compared to myxomycetes. The 18S rRNA and SSU sequence of this species were also retrieved from the GenBank database.

Sequence data analysis

Quality control of 18S rRNA and SSU nucleotide sequences was carried out by trimming and aligning the sequences to ensure good quality before they were subjected to MEGA 11. For the multiple sequence alignment, all sequences for both sets were recorded in FASTA format so that the MEGA 11 software could recognize the sequences. There is a high alignment level in the sequences that were processed. The aligned sequences were manually scanned for errors and trimmed down to avoid biased results in constructing the phylogenetic tree.

After that, the alignment was exported in MEGA format. The substitution models were selected through the "Find the best DNA/Protein Models (ML)" option in MEGA 11. The Kimura-2 parameter + discrete Gamma distribution (K2+G) and Tamura-3 parameter + discrete Gamma distribution models were selected as the best substitution model for 18S rRNA and SSU sequentially under the Bayesian Information Criterion (BIC). Both models were utilized for phylogenetic analyses through Maximum Likelihood, performed with 100 bootstrap replicates.

The Maximum Likelihood Tree, Maximum Parsimony Tree, Minimum-Evolution Tree, Neighbor-Joining Tree, and UPGMA Tree, were constructed. The Maximum Parsimony Tree was the best-fitting evolutionary tree on both sets due to its high bootstrap value compared to the other constructed trees. The trees on both datasets were analyzed for phylogenetic analysis.

Phylogenetic analysis

A total of 150 gene sequences together with the outgroup species were aligned using MUSCLE algorithm in MEGA 11 software. Genetic distance was calculated using Kimura-2 parameter (K2P) and Tamura-3 Parameter (T3P) as substitution model for 18S rRNA and SSU sequences, respectively.

Results and Discussion

Philippine Myxomycetes species

The 18S rRNA and SSU nucleotide sequences of all 150 myxomycetes were retrieved from the GenBank database. The list of myxomycetes selected for phylogenetic analysis, along with their 18S rRNA and SSU GenBank accession numbers, was presented in the study. The retrieved nucleotide sequences were aligned using MEGA 11, and errors in the alignment were manually corrected. Maximum Parsimony trees were then generated with 100 bootstrap replicates.

Morpho-species diversity of Myxomycetes using 18S rRNA sequences

Forty-seven species [13 species of bright-spored (Lucisporidia) and 34 species of dark-spored (Columellidia)] myxomycetes from 20 genera belonging to 9 families were subjected to phylogenetic analysis of the 18S rRNA dataset, accounting for 31.33% of the total myxomycetes species documented in the Philippines. The basic classification of these species, such as the group (superorder) and the genera, is indicated (Table 1).

The table also includes the number of species and their percentage for each genus, which allows for an evaluation of the morpho-species diversity of this dataset. As shown in Table 1, the genus *Physarum* dominates among other genera, with 13 species present and a percentage of 8.67% in the total myxomycetes list.

Morpho-species diversity of Myxomycetes using SSU sequences

Seventy-five species [15 species of bright-spored (Lucisporidia) and 60 species of dark-spored (Columellidia)] myxomycetes representing 20 genera belonging to 4 families were analyzed for phylogenetic analysis of the SSU dataset. This accounts for 50% of the 150 myxomycetes species recorded in the Philippines [18].

The group, genus, and record names, along with the number and percentage of current species in each genus, are also provided in Table 2 to assess the diversity of myxomycetes in this dataset.

Table 1. Morpho-species diversity of the assemblage of myxomycetes in the 18S rRNA dataset (group, genera, and records)

Group	Genus	Records	
		No.	%
Bright	*Alwisia*	1	0.67
Bright	*Arcyria*	2	1.33
Bright	*Calomyxa*	1	0.67
Bright	*Hemitrichia*	2	1.33
Bright	*Metatrichia*	1	0.67
Bright	*Oligonema*	1	0.67
Bright	*Perichaena*	4	2.67
Bright	*Trichia*	1	0.67
Dark	*Badhamia*	1	0.67
Dark	*Clastoderma*	1	0.67
Dark	*Diderma*	3	2.00
Dark	*Didymium*	7	4.67
Dark	*Echinostelium*	1	0.67
Dark	*Fuligo*	2	1.33
Dark	*Lamproderma*	2	1.33
Dark	*Lycogala*	1	0.67
Dark	*Meriderma*	1	0.67
Dark	*Physarella*	1	0.67
Dark	*Physarum*	13	8.67
Dark	*Stemonitopsis*	1	0.67
		47	**31.33%**

The dominance of the genus Physarum in this dataset is evident, with 29 species comprising 19.33% of the total myxomycetes species list (Table 2). Additionally, dark-spored (Columellidia) myxomycetes exhibit a considerable number compared to the bright-spored (Lucisporidia) group, as previously reported [18].

Molecular diversity of Myxomycetes using 18S rRNA sequences

This analysis included sequences from two molecular markers: the 18S rRNA and SSU sequences of 47 and 75 myxomycete species. The total length of the aligned sequences was 11,760 and 3,344 base pairs, respectively. *Porphyra suborbiculata* was the outgroup species due to its unrelatedness to myxomycetes [21]. A sequence identity matrix was generated for the 47 and 75 species of myxomycetes through MEGA 11 software. Sequence retrieval for 18S rRNA from GenBank yielded 18S rRNA partial sequences of varying lengths ranging from 322 to 3,962 base pairs.

Table 2. Morpho-species diversity of the assemblage of myxomycetes in the SSU dataset, including the group, genera, and records

Group	Genus	Records	
		No.	%
Bright	*Arcyria*	4	2.67
Bright	*Hemitrichia*	3	2.00
Bright	*Metatrichia*	1	0.67
Bright	*Oligonema*	1	0.67
Bright	*Perichaena*	1	0.67
Bright	*Trichia*	5	3.33
Dark	*Badhamia*	2	1.33
Dark	*Collaria*	1	0.67
Dark	*Craterium*	2	1.33
Dark	*Diachea*	2	1.33
Dark	*Diderma*	5	3.33
Dark	*Didymium*	8	5.33
Dark	*Enerthenema*	1	0.67
Dark	*Fuligo*	2	1.33
Dark	*Lamproderma*	1	0.67
Dark	*Meriderma*	1	0.67
Dark	*Physarella*	1	0.67
Dark	*Physarum*	29	19.33
Dark	*Stemonitis*	4	2.67
Dark	*Willkommlangea*	1	0.67
		75	**50%**

The phylogeny is the consensus of credible trees generated from the analysis of 18S rRNA sequences of myxomycetes. Due to the significant variance in sequence length seen in the initial alignment, only the aligned parts were chosen for phylogenetic analysis to improve the informative content of the dataset.

The Maximum Parsimony approach was utilized to determine evolutionary history. The evolutionary history of the taxa examined is shown by the bootstrap consensus tree based on 100 replications. Branches representing divisions replicated in fewer than 100% bootstrap replicates are flattened. The percentage of replicate trees in which the affiliated taxa grouped together in the bootstrap test (100 replicates) are shown next to the branches [22]. The MP tree was created using Subtree-Pruning-Regrafting (SPR) algorithm [23] with search level, in which the first trees were done by the random addition of sequences (10 replicates). This analysis comprises of forty-eight 18S rRNA nucleotide sequences and 76 SSU nucleotide sequences. The following codon locations were included: 1st+2nd+3rd+Noncoding. Gaps and incomplete data were cleared from all regions (complete deletion option). The resulting dataset had 243 (18S rRNA) and 44 (SSU) positions. Phylogenetic analysis was conducted on the 11,712-base pairs aligned dataset, including 47 taxa with *P. suborbiculata* defined as the taxonomic outgroup. It can be seen from the inferred maximum parsimony tree that most of the species were found to be highly grouped within their respective families (Figure 1).

However, some species belong to different families clustered into a monophyletic clade with other families. *P. suborbiculata* arises as the first taxon to have descended from the most recent common ancestor of the species; the rest of the myxomycete families are clustered into monophyletic clades.

A strong affinity is demonstrated between *Lycogala epidendrum* and *Alwisia bombarda*, which formed a notable monophyletic group and branched off as sister taxa despite belonging to two separate families – Enteridiaceae and Reticulariaceae, respectively. An earlydiverging taxon was identified in Trichiales, consisting of 12 species from eight genera (*Calomyxa, Perichaena, Metatrichia, Hemitrichia, Trichia, Oligonema, Perichaena,* and *Arcyria*). As shown in the tree, species of the Trichiaceae family (*P. depressa, P. chrysosperma, P. pedata, M. vesparium, O. schweinitzii, A. globosa, A. marginoundulata, H. serpula, P. corticalis, H. calyculata,* and *T. decepiens*) demonstrated a monophyletic assemblage having strong nodal support with *C. metallica*, a member of the Dianemataceae family. Furthermore, three sister taxa were formed with *P. chrysosperma* and *P. pedata*, *A. globosa* and *A. marginoundulata*, *H. calyculata*, and *T. decipiens*.

Physarum gyrosum is the basal taxa of the monophyletic clade composed of species from the families Physaraceae, Echinosteliaceae, Clastodermataceae, Stemonitidaceae, and Didymiaceae. This indicates that *P. gyrosum* is equally related to all representative species (*P. cinereum, P. didermoides, P. compressum, P. notabile, P. leucophaeum, D. minus, P. oblonga, M. cribrarioides, P. roseum, P. bivalve, P. flavicomim, P. rigidum, F. cinerea, P. polycephalum, B. utricularis, P. melleum, F. septica, E. minutum, C. debaryanum, S. typhina, L. cacographicum, D. chondrioderma, D. hemisphaericum, D. anellus, D. melanospermum, L. scintillans, D. ochroideum, D. nigripes, D. iridis, D. verrucosporum, D. fallax*) of the four

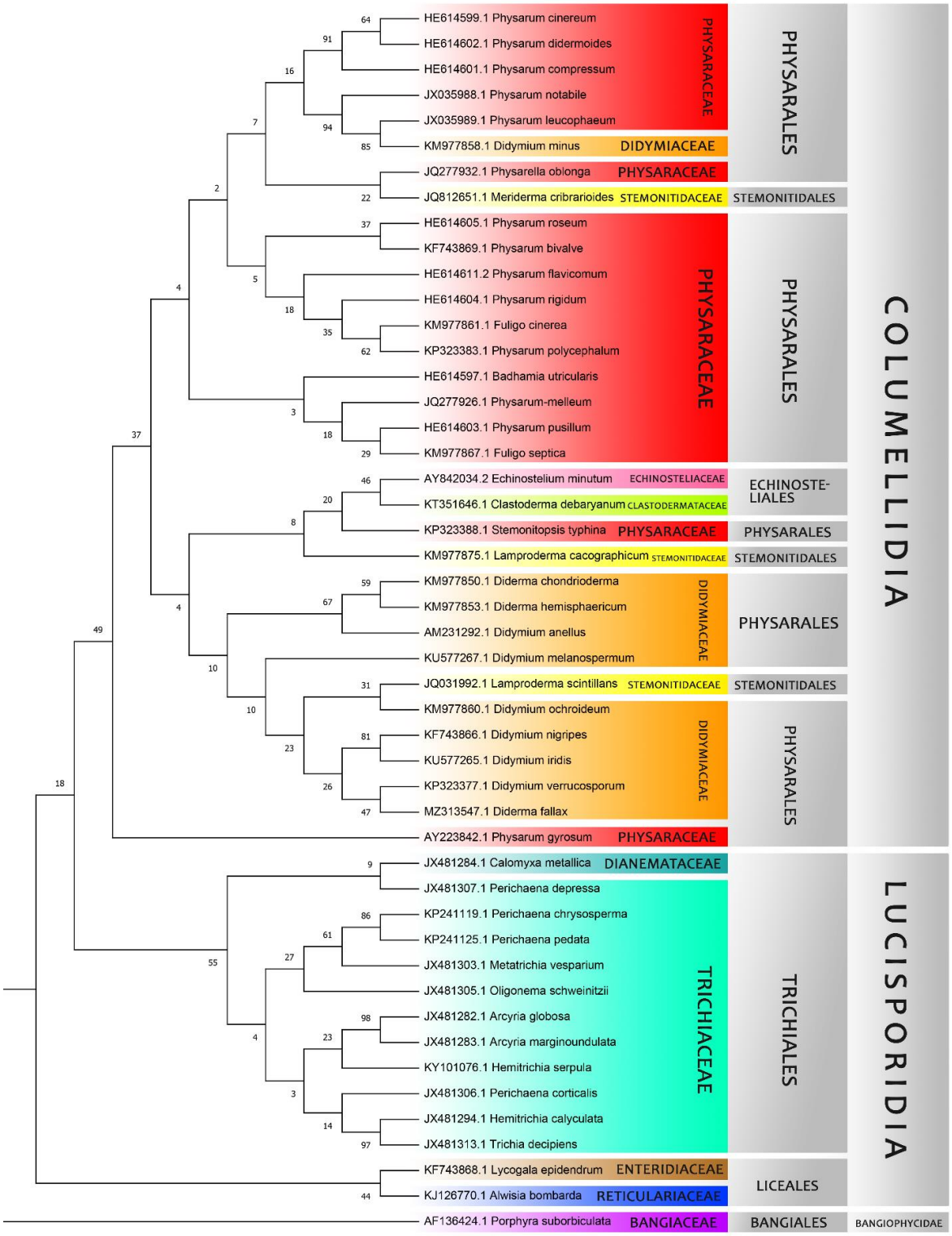

Figure 1. Maximum Parsimony Tree of the 18S rRNA sequences. The MP tree was inferred from 18S rRNA nucleotide sequences using MEGA11 with 100 bootstrap replicates

previously mentioned families; yet, it is significantly distantly related because it lacks a most recent common ancestor and its existing node indicates paraphyly. The six species under the Didymiaceae family (*D. melanospermum, D. ochroideum, D. nigripes, D. iridis, D. verrucosporum, D. fallax*) are grouped with the species belonging to the Stemonitidaceae family — *L.*

scintillans. It is also positioned as a sister to *D. ochroideum. D. verrucosporum* and *D. fallax, D. nigripes* and *D. iridis, D. chondrioderma* and *D. hemisphaericum* formed three sister taxas.

Lamproderma cacographicum spp., *Stemonitopsis typhina, Clastoderma debaryanum*, and *Echinostelium minutum* under four different families — Stemonitidaceae, Physaraceae, Clastodermataceae, and Echinosteliaceae all descended from a recent common ancestor, constituting a monophyletic clade with *L. cacographicum* in the basal position. *E. minutum* and *C. debaryanum* clustered into sister taxa. *E. minutum, C. debaryanum,* and *S. typhina* are more closely related to one another than *L. cacographicum* because the three species share a common ancestor that *L. cacographicum* does not share.

The ten species belonging to the Physaraceae family formed a strong monophyletic cluster (*F. septica, P. pusillum, P. melleum, B. utricularis, P. polycephalum, F. cinerea, P. rigidum, P. flavicomum, P. bivalve, P. roseum*). Furthermore, *M. cribrariodes* and *P. oblonga* from the Stemonitidaceae and Physareceae families likewise branched off as sister taxa. *Didymium minus*, a species under the family of Didymiaceae, is clustered into a monophyletic group with five species of the Physaraceae family (*P. leucophaeum, P. notabile, P. compressum, P. didermoides, P. cinereum*). *D. minus* is also positioned as a sister taxon to *P. leucophaeum. F. septica* and *P. pusillum, P. polycephalum* and *F. cinerea, P. bivalve* and *P. roseum, P. didermoides* and *P. cinereum* also diverged from a most recent common ancestor, resulting in the formation of four sister taxa in the phylogenetic tree.

Molecular diversity of Myxomycetes using SSU sequences

The retrieval of sequences from the GenBank database has resulted in SSU sequences of varying lengths, ranging from 325 to 3,994 base pairs. A high degree of variation was observed among the sequences during alignment, and efforts were made to minimize this variation and enhance the content of the sequences. Ultimately, 3,344 variable sites were identified as informative and were selected for phylogenetic analysis of this dataset.

The phylogeny of myxomycetes in this dataset does not conform to the existing traditional classification of this class. From the phylogenetic tree, it can be observed that some species are clustered together with species from different orders forming poly- and paraphyletic taxa (Figure 2).

This illustrates that the traditional classification, which recognizes five orders, namely: Echinosteliales, Stemonitidales, Trichiales, Liceales, and Physarales, are not all monophyletic. For example, the family Physaraceae belonging to the order Physarales and the family Stemonitidaceae of order Stemonitidales are clustered together, forming a paraphyletic group, as shown in the results. The divergence among these groups is seen from the order and becomes more intensified down to the species level of its current classification.

Parsimony analysis was conducted in this dataset with *P. suborbiculata*, a species of red algae and a member of the family Bangiaceae, as the taxonomic outgroup for both datasets. The tree was rooted against the outgroup, which formed a basal branch of the ingroup to *P. oblatum*, a member of the Physaraceae family. This suggests that Physaraceae is the basal family of this phylogeny. Followed by a branch that emerged to *S. splendens*, a member of the family Stemonitidaceae.

A clade from order Physarales was formed as an early diverging taxon with low support in the tree. It is primarily composed of 15 species belonging to three genera (*Diderma, Willkommlangea*, and *Physarum*). One species from Didymiaceae, *D. effusum*, branched, and the other 14 species from Physaraceae belong to the order Physarales. This indicates that this clade represents a monophyletic group.

The tree branched again to two species of Stemonitidiaceae, namely *M. cribrarioides* and *L. scintillans*. A monophyletic clade is formed with low support in the MP tree from five species belonging to three genera (*Fuligo, Physarella*, and *Physarum*). All of which are members of the Physaraceae family.

The successive branching of the tree from five species belonging to the family of Didymiaceae is formed. The first branch results in *D. nigripes*. The second branch formed a group between *D. megalosporum* and *P. cinerea*. And the species of *D. saundersii* and *D. hemisphaericum* formed the successive branches.

Five species from four genera (*Perichaena, Hemitrichia, Oligonema*, and *Trichia*) belonging to the order Trichiales were clustered with one species from the order Stemonitidales *C. arcyrionema* forming a paraphyletic clade with low sup-

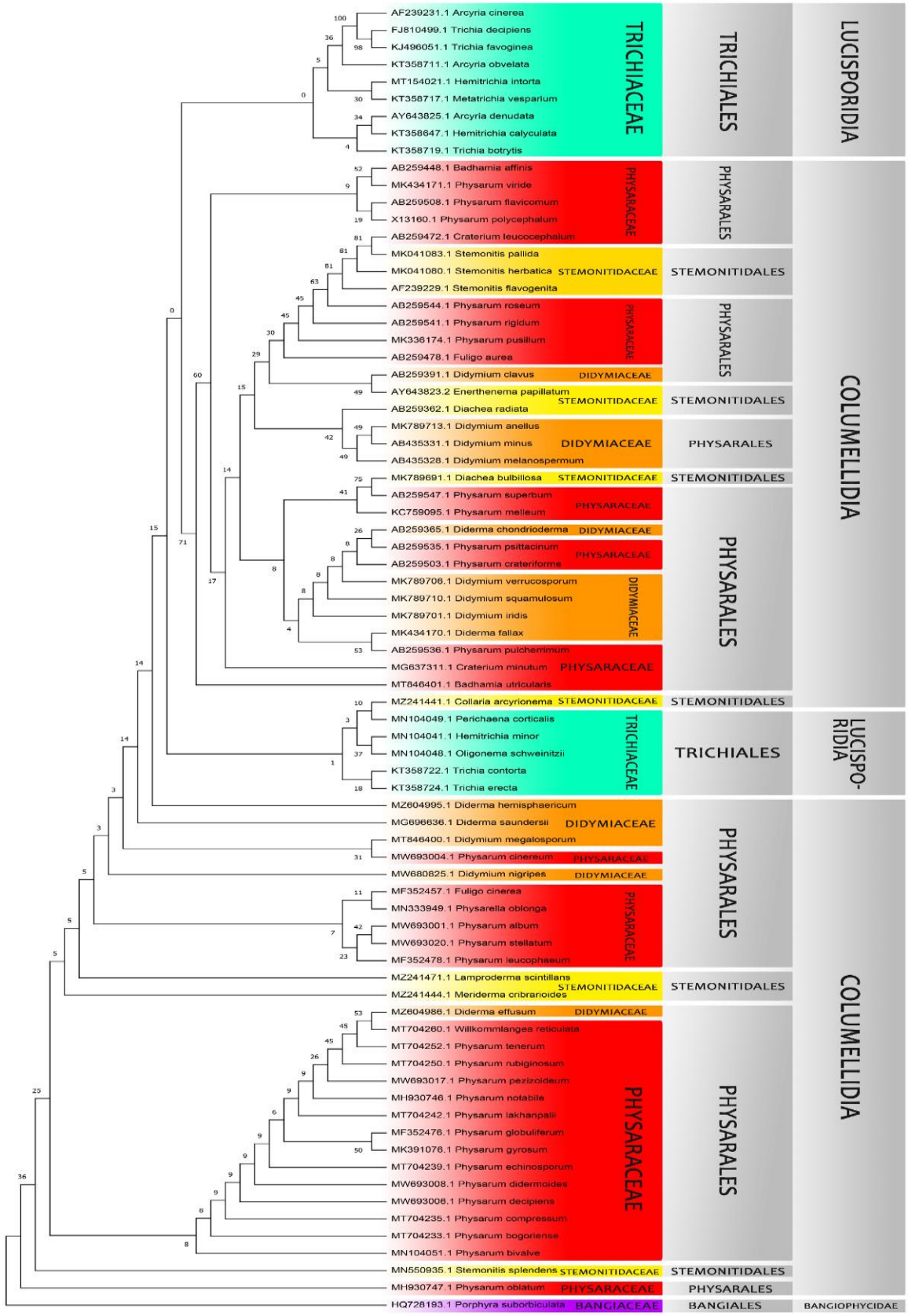

Figure 2. Maximum Parsimony Tree of the SSU sequences. The MP tree was inferred from SSU nucleotide sequences using MEGA11 with 100 bootstrap replicates.

port in the MP tree.

Further splitting of the tree resulted in the formation of two large groups. The first clade received no support in the MP tree. It is a monophyletic group consisting of nine species from four genera (*Arcyria, Trichia, Hemitrichia,* and *Metatrichia*), all of which are members of the family Trichidiaceae representing the order of Trichiales. The second clade is polyphyletic and received moderate support in the MP tree. *B. utricularis*, as species from Physaraceae, is found basal to this specific clade. The majority of the species in this clade originated from two orders, the Physarales and Stemonitidales. This polyphyletic clade also happens to have the most speciation events in the entirety of this phylogenetic tree.

The polyphyletic clade further splits into two groups. One group consists of two genera (Badhamia and Physarum) from the Physaraceae family. The other group comprises one branch and a further division of two clades, which compress 26 species from two orders of Physarales and Stemonitidales. The branch results in one species, namely *C. minutum.* The other branch results in two clades.

The first clade received low support in the MP tree and is found to be a polyphyletic group clustering 14 species belonging to two orders. This clade harbors five species from three genera (*Stemonitis, Enerthenema,* and *Diachea*) of the order Stemonatidales, grouped with nine species from four genera (*Craterium, Physarum, Fuligo*, and *Didymium*) representing the order Physarales. The second clade, however, showed a close relationship to the first clade, as they emerged as sister taxa. This clade comprises 11 species from three genera (Physarum, Didymium, and Diderma) belonging to the families Physaraceae and Didymiaceae, respectively. All of these species are members of the Physarales order, with the exception of one species, *D. bulbillosa*, which belongs to the Stemonitidales order. Despite its paraphyly and low support in the MP tree, this clade is a significant finding.

Conclusion

We used 18S rRNA and SSU gene sequences as molecular markers to explore the diversity and evolutionary relationships of myxomycete species in the Philippines. By applying molecular techniques to myxomycete taxonomy and systematics, we discovered both morphological and molecular evidence that enabled us to construct phylogenetic trees for the two molecular markers examined in this study. The resulting phylogeny from the two molecular markers provided a better understanding of species identification and comparison through phylogenetic analysis. We recommend using the 18S rRNA gene marker, which effectively differentiates and classifies the two groupings.

In addition, it is important to note that data obtained from various databases and software used for in-silico analysis can be useful and reliable up to a certain point. However, further subjective decisions are needed to obtain the required molecular information, which may introduce some bias into the analysis. Moreover, the molecular character states were examined and scored "as-is" and cannot be distinguished by comprehensive ultrastructural and developmental study, unlike superficially identical morphological states.

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