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Differentiation of some myxomycetes species by ITS sequences

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Abstract: Seven different PCR primers targeting 18 different myxomycetes genera were designed and ribosomal DNA ITS1-5.8SrDNA-ITS2 regions of 52 morphologically characterized species from Turkey were amplified and sequenced. The studied species belong to the genera *Arcyria*, *Badhamia*, *Ceratiomyxa*, *Collaria*, *Comatricha*, *Cribraria*, *Diachea*, *Diderma*, *Didymium*, *Enerthenema*, *Lamproderma*, *Lycogala*, *Perichaena*, *Physarum*, *Reticularia*, *Stemonitis*, *Stemonitopsis*, and *Trichia*. Significant ITS region size and sequence variations were observed among the analyzed species. The ITS based phylogenetic tree did not reflect evolutionary relationships at genus or higher level. The only exception was the genus *Didymium*, which formed a clearly distinctive cluster in the phylogenetic tree containing 18 different genera. It was concluded that interspecies variation of the ITS1-5.8S-ITS2 region is high enough to differentiate species, but this region did not reflect the evolutionary relationships between the myxomycetes species. Hence, we suggested that sequencing of the ITS region could only be used as a supporting tool for the phenotypic identification, not for revealing the evolutionary relationships between the myxomycetes species.

Key words: Myxomycetes, ITS sequences, molecular characterization, Turkey

1. Introduction

The myxomycetes are a group of eukaryotic organisms and are usually present in terrestrial ecosystems. They are also called plasmodial slime molds or myxogastrids. The myxomycota contains 6 orders, 15 families, 63 genera and 958 species (Lado, 2014). The majority of these are probably cosmopolitan, but a few seem to be confined to the tropics or subtropics and some others have been collected only in temperate regions of the world.

The myxomycete life cycle involve two morphologically different trophic stages. One of the stages consists of uninucleate amoebae with or without flagella, and the other consists of a distinctive multinucleate structure, the plasmodium (Martin and Alexopoulos, 1969). Under favorable conditions, the plasmodium gives rise to fruiting bodies containing spores. The fruiting bodies of myxomycetes are somewhat suggestive of those produced by higher fungi, although they are considerably smaller. The spores of myxomycetes are, for most species, apparently wind-dispersed and complete the life cycle by germinating to produce uninucleate amoeboflagellate cells (Stephenson et al., 2008).

The morphological species concept in myxomycetes lends itself to the false description of variants from the norm as new species (Clark, 2004). Without knowing the mechanism by which the morphological characters are affected by environmental conditions, assumptions cannot be made as to the classification of these collections. It has long been recognized that DNA sequence diversity can be used to discriminate species. Single gene sequence analysis of ribosomal DNA was used to investigate evolutionary relationships at a high level (Woese and Fox, 1977), and mitochondrial DNA approaches dominated molecular systematics in the late 1970s and 1980s (Avise, 2001). Recently, Tautz et al. (2003) made the case for a DNA-based taxonomic system, but different sequences have been used for different taxonomic groups to assist species identifications.

Based on small-subunit ribosomal-DNA (SSU rDNA) sequence phylogeny, myxomycetes were included in the Protozoa (Cavalier-Smith, 1993). However, only one myxomycete species (*Physarum polycephalum*) 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.

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Martín et al. (2003) designed primers targeting *Physarum polycephalum* to amplify internal transcribed spacer (ITS) regions of rDNA, including the 5.8S gene segment from *Lamproderma* and *Fuligo* species. Their results showed that *Lamproderma* spp. contains ITS1-5.8S-ITS2 regions of approximately 900 bp, which is similar in size to most eukaryotes. However, the corresponding region in *Fuligo* spp. is more than 2000 bp due to the presence of large direct-repeat motifs in ITS1. Martín et al. (2003) concluded that myxomycetes rDNA ITS regions are interesting both as phylogenetic markers in taxonomic studies and as model sequences for molecular evolution.

Morphological, biochemical, and molecular data have not provided conclusive evidence to support one phylogenetic tree for myxomycetes (Clark, 2004). Furthermore, there are few studies examining if the morphological variation observed for myxomycetes species can be attributed to an accumulation of genetic variation (Winsett and Stephenson, 2008, 2011). In general, literature on the phylogeny of myxomycetes based on molecular methods is scarce. Therefore, in this study, new polymerase chain reaction (PCR) primers were designed and rDNA ITS regions of 52 morphologically characterized myxomycetes species in Turkey were amplified and sequenced. The studied species belong to the genera *Arcyria*, *Badhamia*, *Ceratiomyxa*, *Collaria*, *Comatricha*, *Cribraria*, *Diachea*, *Diderma*, *Didymium*, *Enerthenema*, *Lamproderma*, *Lycogala*, *Perichaena*, *Physarum*, *Reticularia*, *Stemonitis*, *Stemonitopsis*, and *Trichia*.

2. Materials and methods

2.1. Myxomycetes isolation and morphological characterization

The studied myxomycetes species were collected from terrestrial forest ecosystems located in different regions of Antakya (Hatay), Turkey. The specimens were on natural substrata, debris material, decaying barks, barks of living trees, wood, leaves, and litter. Natural mature fructifications were gently and directly collected from the substratum and placed in cardboard herbarium boxes. In addition, the fructifications of myxomycetes were obtained from moist chamber cultures in the laboratory. The cultures were moistened with distilled water. The moist chambers were examined every day under a dissecting microscope. When developing myxomycetes were found, the moist chamber was allowed to dry slowly and the myxomycetes were then dried for one week.

Microscopic and macroscopic features of the myxomycetes samples were determined in the laboratory. The examined morphological characters were fruiting bodies shape, size, and color; spore size and ornamentation; capillitium color and branching; lime crystal size and morphology; and stalk color and proportion. All data were comparatively evaluated for taxonomic classification of plasmodial slime mold specimens that was carried out based on the literature listed in the references (Farr, 1976; Neubert et al., 1993, 1995, 2000; Ing, 1999; Stephenson, 2000).

2.2. DNA extraction

A Biospeedy Fungal DNA Isolation Kit (Bioeksen Ltd. Co., Turkey) was used for the DNA extraction. Briefly, spores from four to five fruiting bodies were homogenized at 6000 rpm for 2 min in a buffer (2% CTAB -hexadecyltrimethylammonium bromide-, 100 mM Tris-HCl pH 8, 20 mM EDTA, 1.4 M NaCl) containing 0.1 mm diameter glass beads. The samples were then incubated at 98 °C for 10 min. The samples were centrifuged and the supernatant was combined with a binding buffer (final concentration of 3.4 M Guanidinium thiocyanate, 8 mM Tris-HCl pH 8.0, 25% isopropanol). The extracted DNA was captured using silica columns and then washed twice with a buffer containing 20 mM NaCl, 2 mM Tris-HCl, pH 7.5; 80% v/v ethanol. The DNA was eluted in 100 mM Tris-HCl pH 8.0 and stored at –20 °C.

2.3. Primer design

PCR primers were designed based on comparison of the available myxomycete rDNA ITS sequences retrieved from GenBank. Partial 18S rRNA gene; complete ITS 1, 5.8S rRNA gene, and ITS 2; and partial 28S rRNA gene sequences used for the primer design are given in Table 1. The sequences were aligned using Clustal Omega (www.ebi.ac.uk) and the primers were designed manually based on the principles described by Dorak (2007). The specificity of the primers was tested using Primer-Blast (www.ncbi.nlm.nih.gov). These primers were also used in amplification of the other genera's ITS sequences that were not found in GenBank.

2.4. Real time PCR (Q-PCR)

A Biospeedy EvaGreen Master Mix (Bioeksen Ltd. Co., Turkey) and Roche Light Cycler Nano (Roche Diagnostics GmbH, Germany) were utilized for all reactions. Reaction mixes contained 25 ng of template DNA, 6 mg/mL BSA, 5 mg/mL PEG 400, 0.25% Tween 20, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.1 U Proofreading Hot-Start DNA Polymerase and 200 nM of the each primer. The following thermocycling program was applied: 98 °C, 3 min; 35 cycles of 10 s at 95 °C, 5 s at 52 °C, and 20 s at 72 °C. A melt curve analysis was performed from 65 °C to 95 °C to determine if only one amplified product was generated during Q-PCR. Q-PCR runs were analyzed using Roche Light Cycler Nano Software.

2.5. DNA sequencing and phylogenetic analysis

The Q-PCR products larger than 100 bp were purified using a Biospeedy PCR Product Purification Kit (Bioeksen

Table 1. The PCR primer sequences and their template DNAs for the design.

Accession no.	Species	Forward Primer (5'-3')	Reverse Primer (5'-3')
HM101138.1	*Physarum melleum*	GAAGAGCGCAGCGAAATGCGATA	TTACTGATATGCTTAAGTTCAGC
HM101141.1	*Physarum loratum*		
HM101140.1	*Arcyria nigella*	AACTTTCAACAACGGATCTCT	CCTACCTGATCTGAGGTC
EF513148.1	*Arcyria cinerea*		
HQ604855.1	*Badhamia utricularis*	TCTCTTGGTTCTCGCATCGA	GACGGGCGGTACAAAATCAT
HQ450518.1	*Didymium squamulosum*	ACACCGTTAGGCGATGGATTGCTA	CCTCCGCTGACTAATATGCTTAAATT
JN123459.1	*Stemonitis splendens*	TTTTTKRTAYAGARGTSTKGC	GCTTWTCCWYWMWGATKKAA
JN123458.1	*Stemonitis flavogenita*		
JN123458.1	*Stemonitis flavogenita*	CCYTTTGTKAWCYTTKAGWCTTG	AACAGGCATRCCCTKWGGAA
JN123460.1	*Stemonitopsis typhina*		
AJ302669.1	*Lamproderma ovoideum*	TGCCCGTACTGGTGAACCTG	TGAAGGCAACGGAAAGACAG
AJ302668.1	*Lamproderma atrosporum*		

Ltd. Co., Turkey). The purified DNAs were sequenced using the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 377 DNA sequencer (Applied Biosystems, USA). The sequences were analyzed in Chromas (www.technelysium.com) and manually checked for reading errors. Homology searches of the sequences in DNA databases were performed with BLAST (www.ncbi.nlm.nih.gov). The sequences showing less than 100% similarity were submitted to GenBank. The accession numbers are given in Table 2. The ITS sequences and their closest matches in GenBank were aligned using Clustal Omega (www.ebi.ac.uk) followed by manual adjustments. Only unambiguously aligned base positions were used in the analysis. The maximum-likelihood method was performed with the MEGA software (www.megasoftware.net) to construct an unrooted phylogenetic tree. Branch robustness of the tree was estimated by bootstrap analysis of 1000 heuristic replicates.

3. Results

The morphologically characterized 52 species from 5 different orders (Ceratiomyxales, Liceales, Physarales, Stemonitales, and Trichiales), 7 different families (Ceratiomyxaceae, Cribrariaceae, Didymiaceae, Enteridiaceae, Physaraceae, Stemonitidaceae, and Trichiaceae), and 18 different genera are given in Table 2. Accession numbers of the sequenced DNAs and their closest matches in NCBI GenBank are also listed in Table 2.

The *Arcyria*, *Badhamia*, *Didymium*, *Lamproderma*, *Physarum*, *Stemonitis*, and *Stemonitopsis* targeted primers amplified the rDNA ITS regions of genomic DNAs from the relevant species. The *Arcyria* targeted primer pair was also able to amplify the ITS sequences from *Ceratiomyxa*, *Collaria*, *Comatricha*, *Cribraria*, *Enerthenema*, *Lycogala*, *Perichaena*, *Reticularia*, and *Trichia*. The sequencing of these DNAs revealed that all of the amplified DNA regions contained the complete ITS1-5.8S-ITS2 sequences of the myxomycetes species. These results showed that the designed primers specifically amplified the intended targets.

Significant ITS region size variation was observed among the analyzed species. The intergenera ITS region size variation was significantly higher than the intragenera variation. For example, ITS region sizes of the genera *Physarum* and *Badhamia* of the family *Physaraceae* were approximately 400 bp and 700 bp, respectively.

Using the ITS sequences, molecular operational taxonomic units (MOTUs) can be defined by a similarity threshold of ≤99% (Walther et al., 2013). None of the 52 different species analyzed formed a MOTU based on the ITS sequences. Furthermore, 50 of the isolates did not show ITS sequence similarity higher than 99% to the previously identified sequences in NCBI GenBank. The isolates HB31, HB38, and HB59 were morphologically characterized as *Stemonitis flavogenita*, *Didymium squamulosum*, and *Stemonitopsis subcaespitosa*. The ITS sequences of these isolates were 100% similar to the previously identified ITS sequences of *Stemonitis flavogenita*, *Didymium squamulosum*, and *Stemonitopsis subcaespitosa*. These results indicated that the ITS region can reliably be used for identifying myxomycetes species as long as their ITS sequences are present in the DNA data bank.

Table 2. Morphological characterization and the sequenced partial 18S rRNA gene; complete ITS 1, 5.8S rRNA gene, and ITS 2; and partial 28S rRNA gene sequences of the studied myxomycetes.

Isolate no.	Morphological characterization	Accession no. (this study)	The most similar sequence in NCBI GenBank		
			Accession no.	Organism	Similarity
HB23	*Arcyria affinis*	KF772265	EF513148.1	*Arcyria cinerea*	306/313(98%)
HB24	*A. cinerea*	-	EF513148.1	*A. cinerea*	313/313(100%)
HB10	*A. denudata*	KF772253	HM101140.1	*A. nigella*	322/334(96%)
HB2	*A. incarnata*	KF772247	HM101140.1	*A. nigella*	323/334(97%)
HB63	*A. minuta*	KF772289	EF513148.1	*A. cinerea*	305/313(97%)
HB39	*A. obvelata*	KF772276	HM101140.1	*A. nigella*	325/335(97%)
HB33	*A. pomiformis*	KF772271	HM101140.1	*A. nigella*	324/334(97%)
HB22	*A. versicolor*	KF772264	EF513148.1	*A. cinerea*	304/313(97%)
HB57	*Badhamia dubia*	KF772284	HQ604855.1	*Badhamia utricularis*	757/793(95%)
HB52	*B. macrocarpa*	KF772281	HQ604855.1	*B. utricularis*	759/792(96%)
HB16	*Ceratiomyxa fruticulosa*	KF772259	EF513148.1	*Arcyria cinerea*	298/313(95%)
HB44	*Collaria lurida*	KF772279	JN123461.1	*Stemonitopsis subcaespitosa*	329/346(95%)
HB6	*Comatricha elllae*	KF772249	EU146306.1	*S. subcaespitosa*	326/346(94%)
HB36	*C. laxa*	KF772274	EU146306.1	*S. subcaespitosa*	330/346(95%)
HB17	*C. nigra*	KF772260	EU146306.1	*S. subcaespitosa*	326/346(94%)
HB62	*Cribraria argillacea*	KF772288	EF513148.1	*Arcyria cinerea*	295/313(94%)
HB19	*C. cancellata*	KF772262	EF513148.1	*A. cinerea*	301/314(96%)
HB28	*C. intricata*	KF772269	EF513148.1	*A. cinerea*	296/313(95%)
HB14	*C. vulgaris*	KF772257	EF513148.1	*A. cinerea*	295/313(94%)
HB67	*Diachea leucopodia*	KF772293	AJ302669.1	*Lamproderma ovoideum*	421/462(91%)
HB11	*Diderma carneum*	KF772254	HQ450507.1	*Didymium squamulosum*	598/650(92%)
HB56	*D. hemisphaericum*	KF772283	HQ450515.1	*D. squamulosum*	646/680(95%)
HB48	*Didymium bahiense*	KF772280	HQ450518.1	*D. squamulosum*	593/619(96%)
HB34	*D. difforme*	KF772272	HQ450510.1	*D. squamulosum*	777/802(97%)
HB37	*D. megalosporum*	KF772275	HQ450507.1	*D. squamulosum*	614/659(93%
HB60	*D. melanospermum*	KF772286	HQ450510.1	*D. squamulosum*	766/802(96%)
HB38	*D. squamulosum*	-	HQ450518.1	*D.squamulosum*	619/619(100%)
HB5	*Enerthenema papillatum*	KF772248	EU146306.1	*Stemonitopsis subcaespitosa*	312/346(90%)
HB61	*Lamproderma scintillans*	KF772287	AJ302668.1	*Lamproderma atrosporum*	482/497(97%)
HB27	*Lycogala epidendrum*	KF772268	EF513148.1	*Arcyria cinerea*	285/314(91%)
HB40	*Perichaena corticalis*	KF772277	HM101140.1	*A. nigella*	311/334(93%)
HB42	*P. depressa*	KF772278	HM101140.1	*A. nigella*	307/334(92%)
HB65	*P. vermicularis*	KF772291	HM101140.1	*A. nigella*	307/334(92%)
HB25	*Physarum album*	KF772266	HM101141.1	*Physarum loratum*	301/308(98%)
HB1	*P. cinereum*	KF772246	HM101138.1	*P. melleum*	436/444(98%)
HB58	*P. compressum*	KF772285	HM101138.1	*P. melleum*	436/444(98%)
HB53	*P. contextum*	KF772282	HM101138.1	*P.melleum*	435/444(98%)
HB66	*P. viride*	KF772292	HM101138.1	*P. melleum*	436/444(98%)
HB12	*Reticularia lygoperdon*	KF772255	EF513148.1	*Arcyria cinerea*	286/314(91%)
HB13	*Stemonitis axifera*	KF772256	JN123458.1	*Stemonitis flavogenita*	394/411(96%)
HB31	*S. flavogenita*	-	JN123458.1	*S.flavogenita*	411/411(100%)
HB15	*S. fusca*	KF772258	JN123459.1	*S. splendens*	412/430(96%)
HB18	*S. herbatica*	KF772261	JN123458.1	*S. flavogenita*	395/414(95%
HB8	*Stemonitopsis amoena*	KF772251	JN123460.1	*Stemonitopsis typhina*	214/229(93%)
HB30	*S. hyperopta*	KF772270	JN123461.1	*S. subcaespitosa*	241/254(95%)
HB20	*S. reticulata*	KF772263	JN123459.1	*Stemonitis splendens*	421/433(97%)
HB59	*S. subcaespitosa*	-	JN123461.1	*Stemonitopsis subcaespitosa*	254/254(100%)
HB7	*Trichia botrytis*	KF772250	EF513148.1	*Arcyria cinerea*	293/313(94%)
HB26	*T. decipiens*	KF772267	EF513148.1	*A. cinerea*	296/313(95%)
HB35	*T. persimilis*	KF772273	HM101140.1	*A. nigella*	316/334(95%)
HB64	*T. varia*	KF772290	HM101140.1	*A. nigella*	313/334(94%)
HB9	*T. verrucosa*	KF772252	EF513148.1	*A. cinerea*	283/313(90%)

The tree showing phylogenetic relationships among the 52 isolates is shown in the Figure. Since only the unambiguously aligned base positions were used, the 250 bp region of ITS1-5.8S-ITS2 sequence was used to construct the tree. This sequence corresponds to the region between the 87th and 337th bases of the *Didymium squamulosum* rDNA ITS region sequence (GenBank Accession No. HQ450510).

Although the same species clustered together, the ITS1-5.8S-ITS2 based tree did not reflect evolutionary relationships at genus or higher level. *Arcyria*, *Stemonitopsis*, and *Physarum* genera formed more than one cluster with different genera. All of the *Stemonitis* species were involved in a cluster that also includes species from *Stemonitopsis* and *Lamproderma*. The two unclassified Myxogastria *Perichaena depressa* and *Perichaena vermicularis* clustered with *Arcyria nigella*. *Didymium* formed a distinct cluster that did not include any other genera.

4. Discussion

Literature on the phylogeny of myxomycetes, based on molecular methods, is very limited. The primary problem is the difficulty of isolating DNA from sporophores without cross-contamination. Although DNA isolation from a single sporophore (Martín et al., 2003) and use of only mature and distantly separated colonies of myxomycetes (Novozhilov et al., 2013) were reported to be successful in eliminating cross-contamination, the universal DNA databases are dominated by rDNA sequences from 7 different genera of myxomycetes. This is why it is very challenging to develop primers by using published sequence data from conserved rDNA genes of related myxomycetes species. In this study, the 7 different primer pairs newly designed specifically amplified the target ITS sequences from the 19 different genera. These primer sets possess a great potential to increase the number of ITS targeted molecular diversity studies on myxomycetes.

In this study, significant ITS region size and sequence variations were observed among the analyzed species. Martín et al. (2003) also reported a huge difference in ITS1-5.8S-ITS2 region sizes of *Lamproderma* and *Fuligo* species due to the presence of large direct-repeat motifs in ITS1. Winsett and Stephenson (2008) analyzed ITS sequences of *Didymium squamulosum* to show variation among geographically separated isolates. They concluded that heterogeneity in ITS was too great for any meaningful biogeographical conclusion. Winsett and Stephenson (2011) also examined fifty-six collections of *Didymium difforme* from three different geographically distant regions of the world for intraspecific variation using mitochondrial SSU rDNA sequences. The analysis of the sequences did not resolve the collections from each geographic region into separate groups.

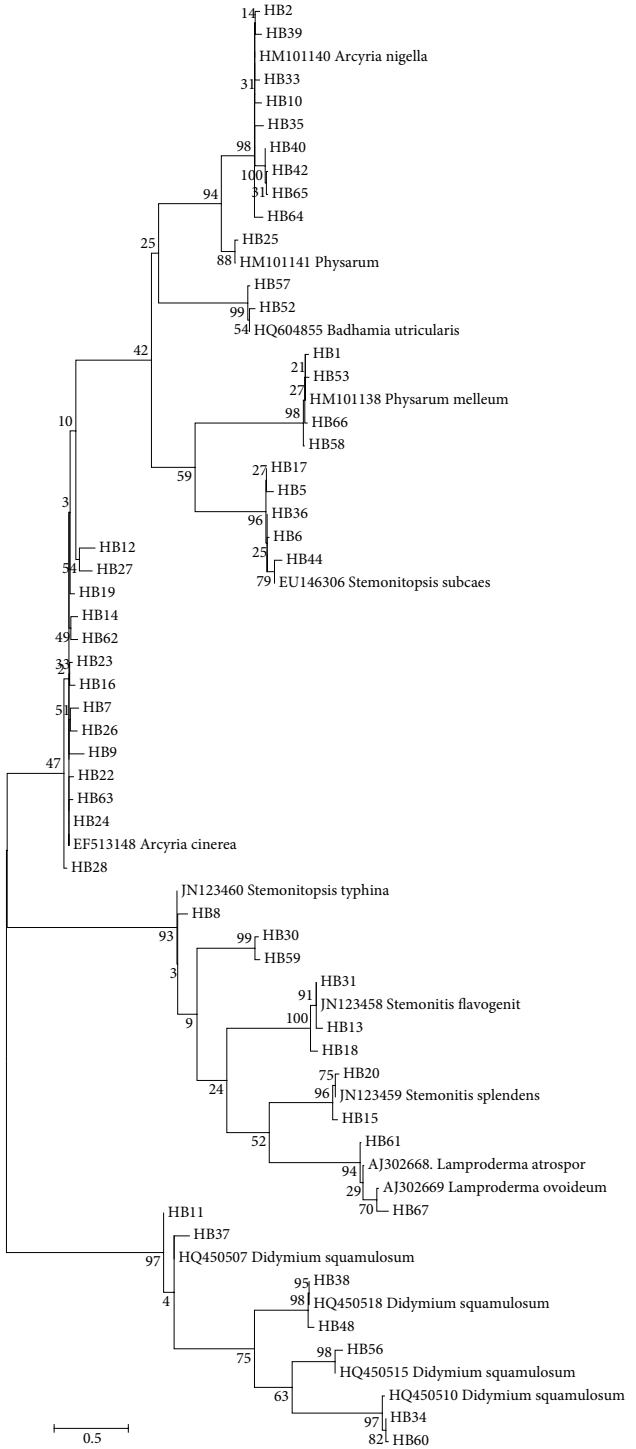

Figure. The unrooted phylogenetic tree using maximum-likelihood method. Bootstrap values are indicated above the branches.

It has been known for a long time that analyses based on any single molecule are inaccurate in constructing all higher-order taxonomic relationships for myxomycetes

(Baldauf, 1999). In this study, the ITS based phylogenetic tree did not reflect evolutionary relationships at genus or higher level. The only exception was the genus *Didymium*, which formed a clearly distinctive cluster in the phylogenetic tree containing 18 different genera.

Overall results implied that the ITS1-5.8S-ITS2 sequence could be used as a DNA barcode to identify myxomycetes species as long as their rDNA sequences are already present in the DNA databases. However, this marker did not meet the requirements to study molecular myxomycetes phylogeny in that the main goal is to identify an unknown sample in terms of a preexisting classification.

In conclusion, 7 different newly designed primer pairs targeting 18 different myxomycetes genera specifically amplified ITS1-5.8S-ITS2 regions of 52 different species. The sequencing and phylogenetic analyses revealed that interspecies variation of the ITS1-5.8S-ITS2 region is high enough to differentiate species, but this region did not reflect the evolutionary relationships between the myxomycetes species. Hence, we suggested that sequencing of the ITS region could only be used as a supporting tool for the phenotypic identification, not for revealing the evolutionary relationships between the myxomycetes species.

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