Mycosphere 9(2): 202–214 (2018) www.mycosphere.org ISSN 2077 7019
Article
Doi 10.5943/mycosphere/9/2/4

Phylogenetic diversity of 18S rDNA sequences of dictyostelids from Amnat Charoen Province, Thailand

Rukseree K[1], Wonganun K[2], Palittapongarnpim P[2,3], Ajawatanawong P[2*]

[1] *Department of Sciences and Liberal Arts, Mahidol University, Amnatcharoen Campus, Mueng, Amnat Charoen 37000, Thailand*
[2] *Department of Microbiology, Faculty of Science, Mahidol University, Rama VI Road, Phayathai, Bangkok 10400 Thailand*
[3] *National Science and Technology Development Agency (NSTDA), 111 Thailand Science Park, Phahonyothin Road, Khlong Nueng, Khlong Luang, Pathum Thani 12120, Thailand*
[*] *Corresponding Author*

Rukseree K, Wonganun K, Palittapongarnpim P, Ajawatanawong P 2018 – Phylogenetic Diversity of 18S rDNA Sequences of Dictyostelids from Amnat Charoen Province, Thailand. Mycosphere 9(2), 202–214, Doi 10.5943/mycosphere/9/2/4

Abstract

Dictyostelids, a group of social amoebae, are one of the major eukaryotic microbes in soil. They play an important role in the turnover of nutrients and minerals and also in bacterial population control in nature. Species diversity of these microbes has been surveyed globally, but surveys in Thailand are rare. We collected 73 isolates of dictyostelids from soils in two districts of Amnat Charoen Province, Thailand. The majority of dictyostelids recovered from Amnat Charoen Province belong to the genus *Dictyostelium*, with 48.0%. The 5'-end of the 18S rDNA gene was amplified and sequenced using the Sanger sequencing approach. Phylogenetic trees were reconstructed using three different methods. These were neighbour-joining (NJ), maximum likelihood (ML) and Bayesian inference (BI). The resulting phylogeny suggests that five genera of dictyostelids (*Cavenderia*, *Heterostelium*, *Raperostelium*, *Dictyostelium* and *Polysphondylium*) were found in Amnat Charoen Province, with *Dictyostelium* as the dominant group. These data are the first on the phylogenetic diversity of dictyostelids in north-eastern Thailand and will be useful in the management of natural and microbial resources.

Key words – dictyostelids – 18S rDNA – phylogenetics – diversity

Introduction

Dictyostelids are a group of cellular slime molds that live in soil and water. These microbes belog to the supergroup "Amoebozoa", which is the sister clade of the superkingdom Opisthokonta (Animals + Fungi) in the tree of life (Cavalier-Smith 2003, Fiore-Donno et al. 2010). Dictyostelids possess a complex life-cycle, with both unicellular and multicellular asexual stages, as well as sexual reproduction, which is rarely observed (Romeralo et al. 2012). The best-known species is *Dictyostelium discoideum*, which is a well-studied model organism for both its genotypic and phenotypic variation (Eichinger et al. 2005, Roberge-White & Katoh-Kurasawa 2011, Bozzaro 2013, Loomis 2016).

Submitted 4 February 2018, Accepted 10 March 2018, Published 15 March 2018
Corresponding Author: Pravech Ajawatanawong – e-mail – pravech.aja@mahidol.edu

The traditional classification of dictyostelid cellular slime molds is based solely on the morphology of their fruiting body (sorocarp). Based on this, they were divided into three genera, namely *Dictyostelium, Polysphondylium* and *Acytostelium.* In general, the genus *Dictyosteleum* produces unbranched or irregularly-branched sorophores, while the genus *Polysphondyleum* produces fruiting bodies with whorled or laterally branched sorophores, with or without sori at the tips of the whorl. Sorophores of both genera develop a cellular stalked structure. However, the genus *Acytostelium* is very different. They produce tiny sorophores with an acellular stalk (Raper 1984).

In 2018, a major taxonomic revision was carried out on the dictyostelids, based on 18S rDNA gene signature sequences and some morphological derived characteristics (synapomorphies). As a result, the taxon was divided into 12 genera (Sheikh et al. 2018). These 12 genera were classified into two major orders: Acetosteliales and Dictyosteliales. Apart from these two orders, a third taxon is also identified: genus *Synstelium* (formerly known as the "polycarpum complex" in Romeralo et al. (2010, 2011)). The order Acetosteliales comprises two families (Acytosteliaceae and Cavenderiaceae). Acytosteliaceae comprises three genera: *Acytostelium* (previously Group 2A in Romeralo et al. (2010, 2011), *Heterostelium* (previously Group 2B) and *Rostrostelium.* The family Cavenderiaceae has one genus (*Cavenderia*), which was previously known as Group 1. The order Dictyosteliales contains two families (Dictyosteliaceae and Raperosteliaceae) and a single genus (*Coremiostelium*). The family Dictyosteliaceae is separated into two genera, *Dictyostelium* (previously Group 4 in Romeralo et al. (2010, 2011)) and *Polysphondylium* (formerly the violaceum complex). The family Raperosteliaceae contains four genera: *Tieghemostelium* (formerly Group 3A), *Hagiwaraea* (formerly Group 3B), *Raperostelium* (formerly Group 3C) and one novel genus, *Speleostelium*.

There are a handful of reports on species diversity of dictyostelids in Southeast Asia, which is one of the world's biodiversity hotspots (Cavender 1976b, Romeralo et al. 2011, Yulo & dela Cruz 2011, Seephueak & Petcharat 2014). Studies of dictyostelid biodiversity in Thailand have been carried out only in the northern (Chiang Mai province) and southern (Nakhon Si Thammarat and Songkhla provinces) regions of the country (Cavender 1976b, Romeralo et al. 2011, Seephueak & Petcharat 2014). There are at least eight species isolated from soil sample from the north of Thailand—*Dictyostelium mucoroides* Bref. (1869), *D. purpureum* Olive. (1901), *Hagiwaraea lavandula* (Raper & Fennell) S. Baldauf, S. Sheikh & Thulin, comb. nov. (Sheikh et al. 2018) (formerly *D. lavandulum* (Raper & Fennell)), *Cavenderia bifurcata* (Cavender) S. Baldauf, S. Sheikh & Thulin, comb. nov. (Sheikh et al. 2018) (formerly *D. bifurcatum* Cavender (1976a)), *H. vinaceofusca* (Raper & Fennell) S. Baldauf, S. Sheikh & Thulin, comb. nov. (Sheikh et al. 2018) (formerly *D. vinaceo-fuscum* Raper & Fennell (1967)), *Coremiostelium polycephalum* (Raper) S. Baldauf, S. Sheikh, Thulin & Spiegel, comb. nov. (Sheikh et al. 2018) (formerly *D. polycephalum* Raper (1956)), *Heterostelium pallidum* (Olive) S. Baldauf, S. Sheikh & Thulin, comb. nov. (Sheikh 2018) (formerly *Polysphondylium pallidum* Olive (1901)) and *Polysphondylium violaceum* Bref. (1884) (Cavender 1976a). Another team of investigators isolated 10 species from the rubber-leaf litter from the south of Thailand, including *D. dichotomum* Vadell & Cavender (2007), *C. macrocarpa* (Vadell & Cavender) S. Baldauf, S. Sheikh & Thulin, comb. nov. (Sheikh 2018) (formerly *D. macrocarpum* Vadell & Cavender (2007)), *Tieghemostelium menorah* (Vadell & Cavender) S. Baldauf, S. Sheikh & Thulin, comb. nov. (Sheikh 2018) (formerly *D. menorah* Vadell & Cavender (2007)), *C. microspora* (H. Hagiw.) S. Baldauf, S. Sheikh & Thulin, comb. nov. (Sheikh 2018) (formerly *D. microsporum* H. Hagiw.(1978)), *Raperostelium minutum* (Raper) S. Baldauf, S. Sheikh & Thulin, comb. nov. (Sheikh 2018) (formerly *D. minutum* Raper (1941)), *D. mucoroides* Bref., *D. rosarium* Raper & Cavender (1968), *Heterostelium multicystogenum* (S. Kawak. & H. Hagiw.) S. Baldauf, S. Sheikh & Thulin, comb. nov. (Sheikh 2018) (formerly *P. multicystogenum* S. Kawakami & Hagiwara (2008)), *Heterostelium pallidum* (Olive) S. Baldauf, S. Sheikh & Thulin, comb. nov. and *P. violaceum* Bref. (Seephueak & Petcharat 2014). Moreover, there was a huge survey of the diversity of eumycetozoans, including dictyostelids, under the "Global Biodiversity Survey of Eumycetozoans" project during the last decade. They reported five new species from

Thailand, three of them are classified in the genus *Cavenderia* and the other two are classified in the family Raperosteliaceae (Romeralo et al. 2011).

Herein, we surveyed the phylogenetic diversity of dictyostelid cellular slime molds in two districts in Amnat Charoen Province in north-eastern Thailand. A total of 73 isolates of dictyostelids were identified, based on the partial sequences of the 18S rDNA gene. Those sequences were then used to reconstruct phylogenetic trees using three different methods in order to molecularly identify the lineages of dictyostelids that were recovered from Amnat Charoen Province.

Materials & Methods

Study Sites and Soil Samples

We collected eight soil samples from two districts (Phana and Mueang) in Amnat Charoen Province, north-eastern Thailand (Fig. 1). The province is located in the Mekong Valley. The average altitude of Amnat Charoen Province is about 68 meters above sea level. Most of the areas are covered with loamy sand and lateritic soil. The climate in Amnat Charoen is classified as tropical savanna. The dry season runs from February to April, the rainy season runs from May until October, and the winter is between November and January. The average temperature over the year is 28.7°C and the accumulated rainfall averages 158.1 millimetres per year.

Two of the soil samples were collected from Phana District, and other six samples were collected from Mueang District (Table 1). All soil samples were collected in December 2014 and July 2015. Soil was collected from a depth of about 0.5–1 cm from the surface of the ground under shrubs or trees. Each sample of 10–20 g of soil was collected and stored in a Ziplock plastic bag at room temperature without exposure to light. All samples were delivered to the laboratory for subsequent analyses within 24 hours.

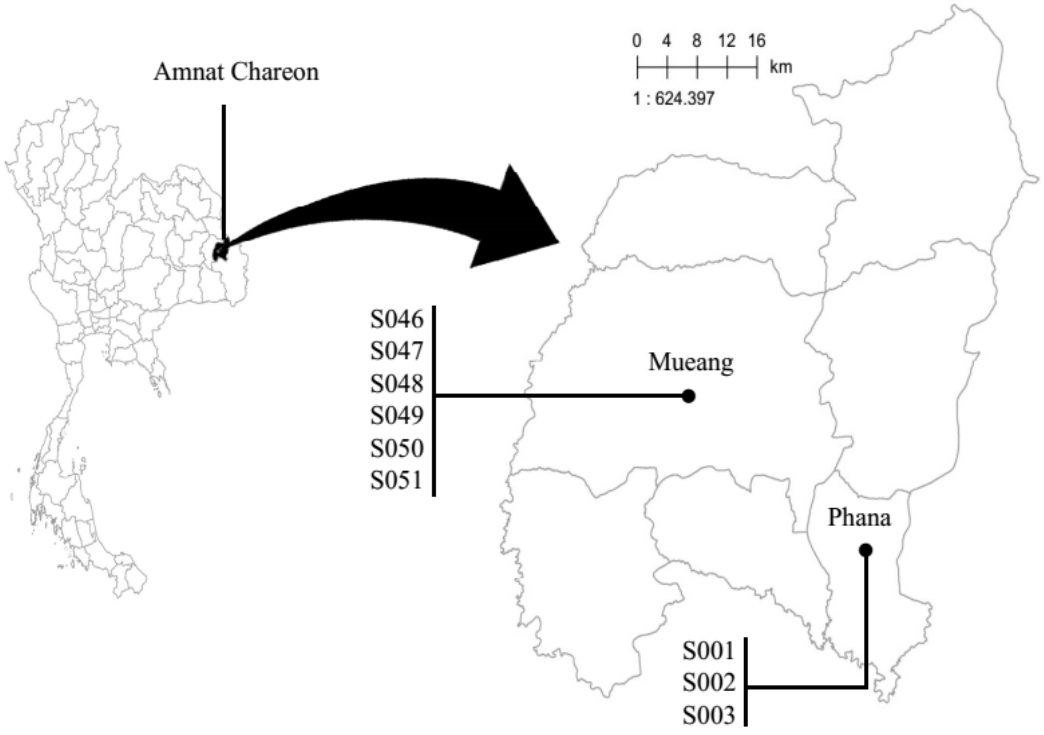

Figure 1 – Map of Thailand and the sampling sites. Map on the left-handed side shows the location of Amnat Charoen Province (labeled in black) in the northeast of Thailand. The map on the right-handed side shows the districts in Amnat Charoen Province. Six soil samples (S046, S047, S048, S049, S050 and S051) were collected from Mueang District and two samples (S002 and S003) were collected from Phana District.

Table 1 Geographical locations and collection dates of soil samples from eight sites in Amnat Charoen Province.

District	Sample Number	Geographical Location			Collection Date
		Latitude	Longitude	Elevation[a] (m)	
Phana	S002	15°40'13.45"N	104°51'35.54"E	N/A	Dec 2014
Phana	S003	15°40'10.98"N	104°51'35.47"E	N/A	Dec 2014
Mueang	S046	15°53'29.0"N	104°37'17.9"E	179	Jul 2015
Mueang	S047	15°53'31.0"N	104°37'16.6"E	179	Jul 2015
Mueang	S048	15°53'32.7"N	104°37'19.0"E	182	Jul 2015
Mueang	S049	15°53'25.7"N	104°37'24.8"E	179	Jul 2015
Mueang	S050	15°53'24.6"N	104°37'26.1"E	174	Jul 2015
Mueang	S051	15°53'29.4"N	104°37'16.7"E	168	Jul 2015

[a] above sea level
N/A not available

Isolation of Dictyostelids

To isolate dictyostelids from soil samples, five grams of each soil samples were suspended in 50 ml sterilized water to get a final soil suspension concentration of 10% weight per volume. Two-hundred microliters of the soil suspension were then mixed with 300 µl of *E. coli* ATCC 8739 before it was spread on hay infusion (HI) agar plates. The HI plates were incubated at 22ºC for four days before they were checked for dictyostelid fruiting bodies. The HI plates were observed for 10 days before discarding. Individual dictyostelid fruiting bodies were selected and transferred to non-nutrient agar (NNA) plates along with 300 µl of *E. coli* ATCC 8739. The *E. coli* ATCC 8739 was cultured in Luria-Bertani (LB) broth at 37ºC overnight before use. All isolates were serially transferred to new NNA plates until the culture was deemed pure. The concentration of dictyostelids in the soil was calculated by counting the number of clones and multiplying this number by the dilution factor (×50) to get the number of dictyostelids per gram of soil. This method was modified from the one previous described (Cavender & Raper 1965, Yulo & de la Cruz 2012).

DNA Extraction, Amplification and Sequencing of the 18S rDNA Gene

All isolates of dictyostelids were co-cultured with *E. coli* ATCC 8739 on NNA plates at 22ºC for four days before harvesting dictyostelid cells for genomic DNA extraction. To avoid over-contamination of the *E. coli* genome, the dictyostelid cells were removed from the zone of clear *E. coli* growth. The cells were then suspended in 200 µl of TE buffer. The genomic dictyostelid DNA was then extracted with the AxyPrep Blood Genomic DNA Miniprep Kit (Axygen Biosciences, Union City, CA).

An oligonucleotide primer pair, 18S_FA (AAC CTG GTT GAT CCT GCC AG) and 18S_RB (TGA TCC TTC TGC AGG TTC AC), was used for amplification of the dictyostelid 18S rDNA gene (Medlin et al. 1988). Each PCR reaction contained 5 µl of 10x PCR buffer (Biotechrabbit GmbH, Germany), 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM of each primer, 1.25 U of *Taq* polymerase (Biotechrabbit GmbH, Germany), 5 ng of genomic DNA template, and the final volume of the reaction was adjusted to 50 µl by adding sterile deionized water. The reaction was incubated at 95°C for 5 min for pre-PCR, followed by 95°C for 30 sec, 56°C for 1 min, and 72°C for 2 min for 30 cycles, and finally 72°C for 10 min. PCR amplicons were purified using the FavorPrep^{TM} GEL/PCR Purification kit (Favorgen Biotech Corporation, Taiwan) before they were directly sequenced at Macrogen (Korea) using the 18S_FA primer.

Sequence Analyses

We identified the putative genus of all 73 dictyostelid isolate sequences by comparing the acquired sequence to previously deposited sequences using GenBank's BLASTn feature (Altschul et al. 1990). To prepare the dataset for phylogenetic analysis, the 73 newly acquired 18S rDNA partial

sequences (accession number MG754973–MG755045) were combined with 43 published sequences that were broadly sampled from the ten genera (Romeralo et al. 2011) (Table 2).

Table 2 List of the 43 previously published nucleotide sequences used in the dataset. Accession numbers refer to the NCBI database (Altschul et al. 1990).

Genus	Former Clade	Species	Strain Name	Accession Number	References
Cavenderia	Group 1	*C. aureostipes*	YA6	AM168083	Schaap et al. 2006
		C. deminutiva	MexM19A	AM168092	Schaap et al. 2006
		C. exigua	TNS-C-199	AM168085	Schaap et al. 2006
		C. fasiculata	SH3	AM168087	Schaap et al. 2006
		C. macrocarpa	MGE2	HQ141519	Romeralo et al. 2011
		C. microspora	TNS-C-38	AM168090	Schaap et al. 2006
		C. multistipes	UK26b	AM168070	Schaap et al. 2006
		Dictyostelium sp.[a]	TAS30A	HQ141516	Romeralo et al. 2011
		Dictyostelium sp.[a]	TH1A	HQ141515	Romeralo et al. 2011
		Dictyostelium sp.[a]	TH18B	HQ141517	Romeralo et al. 2011
		Dictyostelium sp.[a]	TH39A	HQ141518	Romeralo et al. 2011
		Dictyostelium sp.[a]	THC11X	HQ141523	Romeralo et al. 2011
Acytostelium	Group 2A	*A. amazonicum*	HN1B1	HQ141511	Romeralo et al. 2011
		A. singular	FDIB	HQ141514	Romeralo et al. 2011
Heterostelium	Group 2B	*H. asymmetricum*	Landolt HN20C	HQ141503	Romeralo et al. 2011
		H. pallidum	TNS-C-98	AM168103	Schaap et al. 2006
		H. album	PN500	AM168104	Schaap et al. 2006
		Polysphondylium sp.[a]	TH12A	HQ141504	Romeralo et al. 2011
Tieghemostelium	Group 3A	*T. menorah*	M1	AM168073	Schaap et al. 2006
Hagiwaraea	Group 3B	*H. rhizopodium*	AusKY-4	AM168063	Schaap et al. 2006
Raperostelium	Group 3C	*R. gracile*	TNS-C-183	AM168078	Schaap et al. 2006
		R. minutum		AY040332	direct submission
		Dictyostelium sp.[a]	TH14B	HQ141491	Romeralo et al. 2011
Dictyostelium	Group 4	*D. citrinum*	OH494	AM168033	Schaap et al. 2006
		D. discoideum	91HO9	AM168040	Schaap et al. 2006
		D. mucoroides	Sweden20	HQ141482	Romeralo et al. 2011
		D. mucoroides	S28b	AM168054	Schaap et al. 2006
		D. mucoroides var. *stoloniferum*	FOII1	AM168055	Schaap et al. 2006
		D. purpureum	cavender	HQ141481	Romeralo et al. 2011
		D. rosarium	M45	AM168065	Schaap et al. 2006
		Dictyostelium sp.	Laos1	HQ141483	Romeralo et al. 2011
		Dictyostelium sp.	Laos5	HQ141484	Romeralo et al. 2011
Synstelium	Polycarpum Complex	*S. polycarpum*	VE1b	AM168057	Schaap et al. 2006
		S. polycarpum	Ohio	AM168058	Schaap et al. 2006
Coremiostelium	Polycephalum Complex	*C. polycephalum*	Landolt #2132 B-9c	HQ141489	Romeralo et al. 2011
		C. polycephalum	Landolt #1675 GUAM	HQ141490	Romeralo et al. 2011
		C. polycephalum	Landolt #1130 SS3B	HQ141488	Romeralo et al. 2011
		C. polycephalum	MY1-1	AM168056	Schaap et al. 2006
Polysphondylium	Violaceum Complex	*P. laterosorum*	AE4	AM168046	Schaap et al. 2006
		P. patagonicum	Cavender H-H 1	GQ496156	Vadell et al. 2011
		P. violaceum	209	HQ141486	Romeralo et al. 2011
		P. violaceum	P6	AM168108	Schaap et al. 2006
		Dictyostelium sp.[a]	Laos4	HQ141485	Romeralo et al. 2011

[a] Taxonomy of these sequences was not updated in the GenBank taxonomy database.

The sequences in the dataset were aligned by MUSCLE (Edgar 2004) via SeaView version 4.6.1 (Gouy et al. 2010). After the alignment was manually edited, the conserved regions were manually identified and extracted from the DNA multiple sequence alignments (MSAs) based on the guideline suggestion from the conserved module in SeqFIRE version 1.0.1 (Ajawatanawong et al. 2012). The edited alignment was used to perform the phylogenetic analysis with three different methods: (i) neighbour-joining (NJ), (ii) maximum likelihood (ML) and (iii) Bayesian Inference (BI). The sequence alignment matrix and all phylogenetic trees were submitted to TreeBase under the accession http://purl.org/phylo/treebase/phylows/study/TB2:S22363.

The NJ tree was reconstructed using the BioNJ tree building algorithm in SeaView. The '*nucleotide substitution matrix*' was calculated from the original sequence alignment (Observed). For ML, we used PhyML version 3.0 (Guindon et al. 2010) via the SeaView package with the GTR model whereby the '*nucleotide equilibrium frequencies*' and '*proportion of the invariable sites*' were optimized by the program. The tree searching was done using nearest-neighbour interchange (NNI). The bootstrap analyses were done using 1,000 replications of pseudo-alignment data in both the NJ and ML methods. The BI analyses were performed with MrBayes version 3.2.6 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). The Bayesian tree was run with the GTR+I+Γ model of nucleotide substitution and tree searching was performed with four simultaneous chains for 2 million generation. The first 25% of generations were discarded as the burn-in to prevent sampling before reaching stationarity.

Results

We isolated 73 dictyostelid cellular slime molds from six of the eight soil samples taken in Amnat Charoen Province, Thailand during December 2014 and July 2015. Four samples from Mueang District and two samples from Phana District contained dictyostelids. Identification of the dictyostelids was done for all 73 isolates. Results showed that the dictyostelids recovered from Amnat Charoen Province belonged to five genera (Table 3). Thirty-nine isolates (53.4%) were recovered from Phana District and 34 isolates 46.6%) were recovered from Mueang District.

Table 3 Overview of the dictyostelids recovered in this study, according to the new taxonomy (Sheikh et al. 2018). The genera of all dictyostelid isolates from Amnat Charoen Province were identified using molecular phylogenetic analyses of partial sequences of the 18S rDNA gene.

Genus	Number of Isolates (%)		Total Number (%)
	Phana District	Mueang District	
Cavenderia	10 (13.7)	7 (9.6)	17 (23.3)
Acytostelium	0 (0.0)	0 (0.0)	0 (0.0)
Rostrostelium	0 (0.0)	0 (0.0)	0 (0.0)
Heterostelium	0 (0.0)	1 (1.4)	1 (1.4)
Speleostelium	0 (0.0)	0 (0.0)	0 (0.0)
Tieghemostelium	0 (0.0)	0 (0.0)	0 (0.0)
Hagiwaraea	0 (0.0)	0 (0.0)	0 (0.0)
Raperostelium	0 (0.0)	5 (6.8)	5 (6.8)
Dictyostelium	14 (19.2)	21 (28.8)	35 (48.0)
Polysphondylium	15 (20.5)	0 (0.0)	15 (20.5)
Coremiostelium	0 (0.0)	0 (0.0)	0 (0.0)
Synstelium	0 (0.0)	0 (0.0)	0 (0.0)
Total	39 (53.4)	34 (46.6)	73 (100.0)

Phylogenetic analyses of Dictyostelids in Amnat Charoen Province

In order to reconstruct the tree using the 5'-end regions of the 18S rDNA gene, we tested the specificity of clade identification using the 5'-end partial sequence of the 18S rDNA gene from the 43 published isolates (data not show). Then, all 73 novel dictyostelid isolates and 43 published

sequences were subjected to phylogenetic analyses with three different methods: neighbor-joining (NJ), maximum likelihood (ML) and Bayesian inference (BI).

Although the trees reconstructed using the different methods of analysis were different in the deep branching patterns, they all clustered the novel isolates into 10 monophyletic groups. The taxa within each clade were consistently clustered together in all three phylogenetic analyses. This suggests that the partial sequences of the 18S rDNA gene has enough phylogenetic signal to group dictyostelids into the appropriate clade. The novel dictyostelid isolates were found in five genera: *Cavenderia*, *Heterostelium*, *Raperostelium*, *Dictyostelium*, and *Polysphondylium.* These findings were supported with both significant bootstrap values and posterior probabilities (Fig. 2). However, we did not find any isolates from Amnat Charoen Province that belong to the genera *Coremiostelium* (previously known as the polycephalum complex), *Hagiwaraea*, *Tieghemostelium* (previously Group 3), *Speleostelium*, *Synstelium* (previously called the polycarpum complex), *Acetostelium* (previously Group 2A) or *Rostrostelium* (Fig. 2, Table 3).

Cavenderia (Formerly Group 1)

From the tree, we see that this clade contains 29 isolates of *Cavenderia* spp., with 17 isolates from Amnat Charoen Province (Figure 2). All taxa in this genus from Amnat Charoen Province form a monophyletic group together with other four new species of Thai isolates of *Cavenderia* (presented as *Dictyostelium* in the tree) from another study (Romeralo et al. 2011). Within the Thai subclade, the sequences from Amnat Charoen Province split into two unique clusters. One cluster contains 10 isolates (ACR003, ACR004, ACR005, ACR020, ACR021, ACR022, ACR023, ACR024, ACR031 and ACR032) from Phana District (Table 3), sharing 100% sequence similarity. The Phana clade is a sister group of *Dictyostelium* sp. TH1A. Another seven isolates of *Cavenderia* spp. (ACR051, ACR052, ACR056, ACR059, ACR066, ACR068 and ACR070) are from Mueang District (Table 3). These sequences were also 100% similar but they appeared to branch early relative to other taxa in the subgroup.

Heterostelium (Formerly Group 2)

Only one isolate (ACR072) of a dictyostelid was recovered from Mueang District (Table 3) in Amnat Charoen Province which was identified to the genus *Heterostelium* sp. (previously classified as Group 2). This isolate is a sister taxon of the clade including *Polysphondylium* sp. TH12A from Thailand, *Heterostelium album* PN500 (formerly *Polysphondylium pallidum* PN500) and *Heterostelium asymetricum* HN20C. The posterior probability support for the genus *Heterostelium* in this study was 1.00.

Raperostelium (Formerly Group 3)

Five isolates (ACR054, ACR063, ACR064, ACR067 and ACR071) of dictyostelids from Mueang District (Table 3) in Amnat Charoen Province were classified as belonging to genus *Raperostelium*. All *Raperostelium* taxa from Amnat Charoen Province form a monophyletic group with statistically significate support (1.00 Bayesian inference posterior probability, 100% ML bootstrap support and 100% NJ bootstrap support) with 100% sequence similarity. Another new species from Thailand identified in an earlier study (*Dictyostelium* sp. TH14B; Romeralo et al. 2011) was the sister clade of the Amnat Charoen cluster, which also grouped with *Dictyostelium* sp. TH14B and *Raperostelium gracile* TNS-C-183. The sequences of the isolates AusKY4 and M1 were reclassified to be two different genera, *Hagiwaraea rhizopodium* AusKY4 and *Tieghemostelium* M1, respectively.

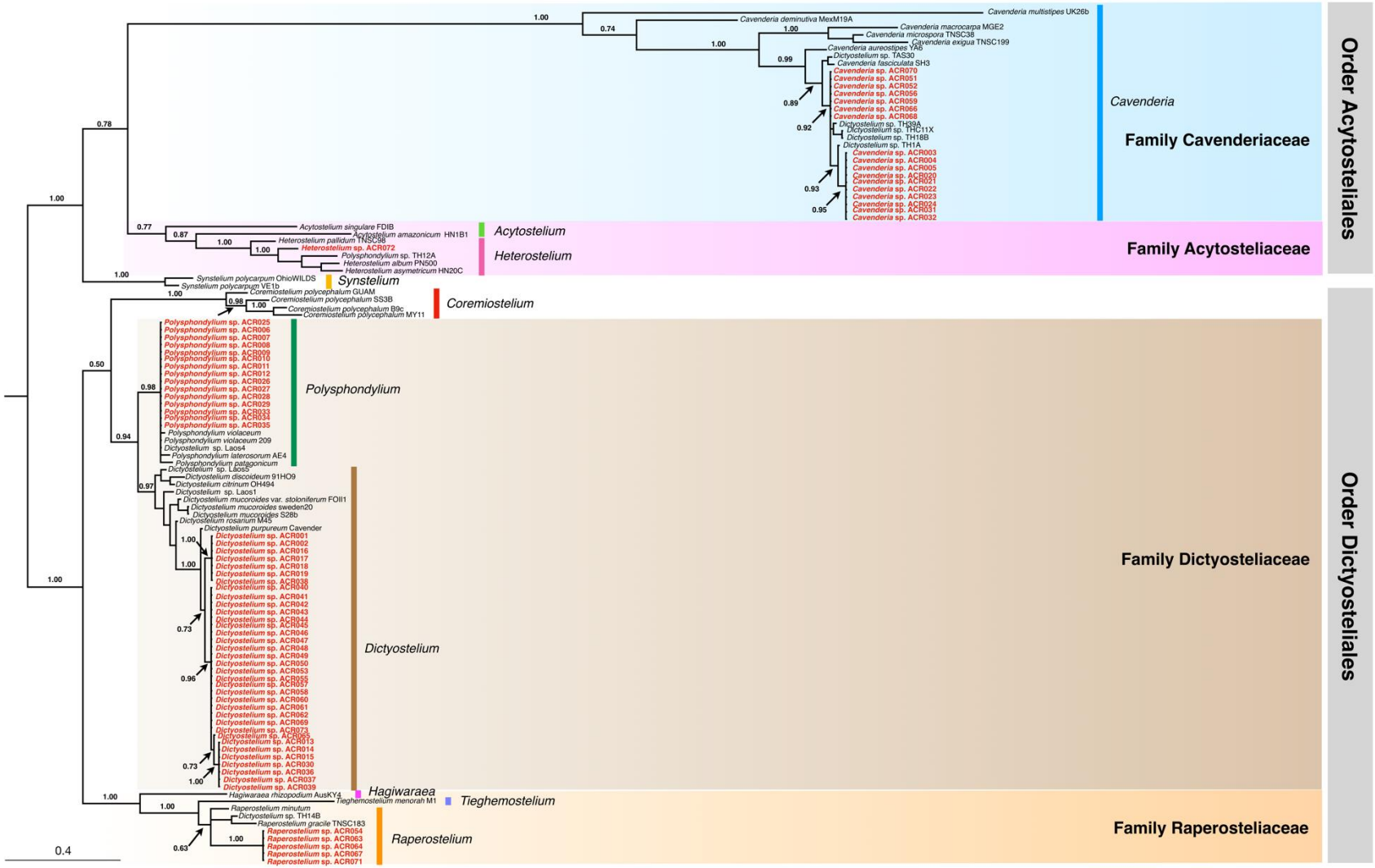

Figure 2 – The phylogeny of 73 novel dictyostelid isolates from Amnat Charoen Province based on a partial sequence of the 18S rDNA gene. The tree was reconstructed using Bayesian inference. All taxa from Amant Charoen are shown in red. The tree was rooted using order Dictyosteliales (Baldauf et al. 2018, Sheikh et al. 2018). Each genus is labeled with a different bar color. Families are indicated by colored boxes. The grey boxes show the orders. The posterior probabilities were shown on the deep branches. The scale bar indicates the rate of nucleotide substitution per site.

Dictyostelium (Formerly Group 4)

The genus *Dictyostelium* is the largest group of dictyostelids (*N*=35 isolates) in Amnat Charoen Province. All Amnat Charoen isolates form a unique subclade, which has *D. purpureum* Cavender as a sister taxon. Within the Amnat Charoen subclade, the isolates are divided into two subgroups. All taxa in the first group, including the isolates ACR001, ACR002, ACR016, ACR017, ACR018, ACR019 and ACR038, were isolated from soil in Phana District. They form a monophylytic group and are the first split from the other Amnat Charoen isolates with significant statistical support (1.00 Bayesian inference posterior probability, 97% ML bootstrap support and 78% NJ bootstrap support). The second and third groups are sister clades to each other. The second group is a mixed-taxa group comprised of the 28 remaining taxa isolated and also has significant statistical support (0.96 Bayesian inference posterior probability, 71% ML bootstrap support and 58% NJ bootstrap support). Some isolates from Phana (ACR013, ACR014, ACR015, ACR030, ACR036, ACR037 and ACR039) also form another clade. The average sequence similarity of the partial 18S rDNA sequences of all 35 novel isolates is 99.5%.

Polysphondylium (Formerly Violaceum Complex)

We found 15 isolates from Phana District in Amnat Charoen Province that were clustered into the genus *Polysphondylium* (previously called the violaceum complex). Both morphological and molecular approaches identified the taxa to the genus *Polysphondylium*. The average sequence similarity of all Amnat Charoen isolates in this taxon is 100%. The tree shows that the nucleotide sequences of Amnat Charoen isolates have high similarity with other published sequences from this genus.

Phylogenetic Diversity of Dictyostelids in Amnat Charoen Province

We found three dictyostelid genera (genus *Cavenderia*, *Dictyostelium* and *Polysphondylium*) in Phana District, and four genera (genus *Cavenderia*, *Heterostelium*, *Raperostelium* and *Dictyostelium*) in Mueang District (Table 3). Genus *Dictyostelium* is the most abundant in the province (48%). Three of these dictyostelid genera from Amnat Charoen are found either in only Phana or Mueang District. Isolates of *Heterostelium* and *Raperostelium* were found only in Mueang District with 1.4% and 6.8% relative frequency, respectively. Inversely, isolates of *Polysphondylium* were found only in Phana District (20.5%).

The number of dictyostelids recovered from each genus varied from site to site (Table 4). Three soil samples (S002 from Phana District; S046 and S051 from Mueang District) contained dictyostelids from several genera. The average number of dictyostelid from soils in Phana and Mueang Districts are 105.85 and 237.5 clones per gram of soil, respectively. Dictyostelids belonging to the genus *Dictyostelium* were found in five of the eight soil samples (62.5%).

Discussion

We isolated 73 dictyostelids from six soil samples collected from two districts (Phana and Mueang) in Amnat Charoen Province, Thailand (Fig. 1, Table 1). Comparison of homologous sequences in NCBI's GenBank database suggests that isolates belonging five dictyostelid genera were found in Amnat Charoen Province (Table 3). Further phylogenetic analysis of the partial sequences of the 18S rDNA gene identified the dictyostelids in Amnat Charoen into five genera, with high statistical support. These are *Cavenderia* (previously Group 1), *Heterostelium* (previously Group 2A), *Raperostelium* (previously Group 3C), *Dictyostelium* (previously Group 4) and *Polysphondylium* (previously the violaceum complex) (Fig. 2). Two of the five genera (*Cavenderia* and *Dictyostelium*) were found in both districts in Amnat Charoen Province, whereas *Heterostelium* and *Raperostelium* were only found in Mueang District and *Polysphondylium* was found only in Phana District (Table 3). *Dictyostelium* appeared to be the most abundance genus in Amnat Charoen (Table 4). In addition, some soil samples contain dictyostelids belonging to more than one genera, whereas others contain dictyostelids from a single genus (Table 4).

Table 4 Approximate number of dictyostelid cellular slime molds from soils in Phana and Mueang Districts in Amnat Charoen Province, according to the recently revised taxonomy (Sheikh et al. 2018). The dictyostelid abundances were calculated as number of clones per gram of soil. The codes S002 and S003, and S046–S051 represent the soil samples from Phana and Mueang Districts, respectively.

Genus	**Relative abundance of dictyostelids (CPG)**								**Total (net) abundance**
	Phana Dist.		**Mueang District**						
	S002	**S003**	**S046**	**S047**	**S048**	**S049**	**S050**	**S051**	
Cavenderia	100.0	0.0	175.0	0.0	0.0	0.0	0.0	0.0	275.0
Acytostelium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Rostrostelium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Heterostelium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	50.0	50.0
Speleostelium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tieghemostelium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Hagiwaraea	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Raperostelium	0.0	0.0	75.0	0.0	100.0	0.0	0.0	0.0	175.0
Dictyostelium	16.7	45.0	325.0	0.0	0.0	0.0	50.0	175.0	611.7
Polysphondylium	50.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	50.0
Coremiostelium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Synstelium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Number of genera	3.0	1.0	3.0	0.0	1.0	0.0	1.0	2.0	
Total Abundance	166.7	45.0	575.0	0.0	100.0	0.0	50.0	225.0	1,166.7

In this study, we identified the phylogenetic position of the novel isolates of dictyostelids from the partial sequences of the 18S rDNA gene. The phylogenetic relationships among the dictyostelids were inferred from nearly complete sequences of the 18S rDNA (SSU) gene (Schaap et al. 2006, Romeralo et al. 2010, Romeralo et al. 2011, Baldauf et al. 2018, Sheikh et al. 2018). This study showed that the phylogenetic signal in this gene region is strong enough to classify the dictyostelids into the appropriate genera even with only the 5'-end partial sequences of the gene. This is supported by initial analyses on the generic identification of known taxa using the 5'-end partial sequence of previously published sequences, which were correctly identified through three different molecular phylogenetic reconstruction methods (data not shown). Although the phylogenetic signal in this region is powerful enough to differentiate and identify the appropriate genus of sequences, the signal is not sufficient for resolving the deep branching patterns of dictyostelids (Romeralo et al. 2011, Sheikh et al. 2018).

The major contribution of this study is its description of the phylogenetic diversity of dictyostelids in Amnat Charoen Province, which was apparently higher than any previous surveys in Thailand (Schaap et al. 2006, Romeralo et al. 2010, Romeralo et al. 2011). This is the first attempt to reveal the basic population structure of dictyostelids in this province using phylogenetic analyses. A previous report by Romeralo et al. (2011) identified three genera of dictyostelids in Thailand (*Cavenderia*, *Heterostelium* and *Raperostelium*), while we found five genera of dictyostelids (*Cavenderia*, *Heterostelium*, *Raperostelium*, *Dictyostelium* and *Polysphondylium*). This suggests that the phylogenetic diversity of dictyostelids in Thailand is still poorly known.

The most abundant genus in Amnat Charoen is *Dictyostelium*, which is consistent with previous reports of the distribution of dictyostelids in the north of Thailand, where *D. purpureum* and *D. mucoroides* Bref. were found to be the most abundant taxa in Chiang Mai and Chiang Dao (Cavender 1976b). Surprisingly, *Dictyostelium* is less frequently found in the south of Thailand (Seephueak & Petcharat 2014). It possible that *Dictyostelium* may be the most common genus of dictyostelids in Thailand. The genus *Cavenderia* is the second most commonly found in both Amnat Charoen Province and southern Thailand. As shown in the previous report, *Cavenderia macrocarpa* (Vadell & Cavender) S. Baldauf, S. Sheikh & Thulin, comb. nov. (previously *D. macrocarpum* Vadell &

Cavender)) and *C. microspora* (H. Hagiw.) S. Baldauf, S. Sheikh & Thulin, comb. nov. (previously *D. microsporum* H. Hagiw.) are the second most commonly found species in southern Thailand as well (Seephueak & Petcharat 2014). However, *C. bifurcatum* (Cavender) S. Baldauf, S. Sheikh & Thulin, comb. nov. (formerly *D. bifurcatum* Cavender), is less frequently found in the north of Thailand (Cavender 1976b).

The genus *Tieghemostelium* and *Raperostelium* were less frequently recovered in our study compared to another study in the south of Thailand, which reported that *Tieghemostelium menorah* (Vadell & Cavender) S. Baldauf, S. Sheikh & Thulin, comb. nov. (previously *D. menorah* Vadell & Cavender) and *R. minutum* (Raper) S. Baldauf, S. Sheikh & Thulin, comb. nov. (previously *D. minutum* Raper) are more common (Seephueak & Petcharat 2014). Surprisingly, these genera have never been reported from the north of Thailand, at least in Chiang Mai (Cavender 1976b, Romeralo et al. 2011). The genus *Polysphondylium* is rare throughout Thailand (Cavender 1976b, Seephueak & Petcharat 2014).

Conclusion

This is the first report of dictyostelids in Amnat Charoen Province, Thailand. We found five genera of dictyostelids in northeastern Thailand. Among those genera, *Dictyostelium* and *Cavenderia* are the most common, which is consistent with patterns seen throughout the country. Our study revealed a clearer picture of the phylogenetic diversity of dictyostelids in Thailand. However, more surveys are needs to represent the entire distribution of dictyostelids. A full understanding of the dictysotelid distribution will be useful for natural resource management, and particularly for microbial diversity management.

Acknowledgements

We thank Pensri Whiting for facilitating soil collection in Phana District. We also thank Nalumon Thadtapong and Phajongjit Karraphan for the preparation and maintenance of dictyostelid stock cultures. This work was funded by Mahidol University and also supported by the Talent Management program of Mahidol University, Thailand.

References

Ajawatanawong P, Atkinson GC, Watson-Haigh NS, MacKenzie B, Baldauf SL. 2012 – SeqFIRE: a web application for automated extraction of indel regions and conserved blocks from protein multiple sequence alignments. Nucleic Acids Research 40, W340–W347.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990 – Basic local alignment search tool. Journal of Molecular Biology 215, 403–410.

Baldauf SL, Romeralo M, Fiz-Palacios O, Heidari N. 2018 – A Deep Hidden Diversity of Dictyostelia. Protist 169, 64-78.

Bozzaro S. 2013 – The model organism *Dictyostelium discoideum*. Methods in Molecular Biology 983, 17–37.

Brefeld O. 1869 – *Dictyostelium mucoroides* Ein neuer Organismus aus der Venvandschaft der Myxomyceten. Abhandlungen der Senckenbergischen Naturforschenden Gesellschaft 7, 85.

Brefeld O. 1884 – *Polysphondylium violaceum* und *Dictyostelium mucoroides* nebst Bemerkungen zur Systematik der Schleimpilze. Untersuchungen aus dem Gesammtgebiet der Mykol 6, 1.

Cavalier-Smith T. 2003 – Protist phylogeny and the high-level classification of Protozoa. European Journal of Protistology 39, 338–348.

Cavender JC. 1976a – Cellular slime molds of Southeast Asia. I. Description of new species. American Journal of Botany 63, 60–70.

Cavender JC. 1976b – Cellular slime molds of Southeast Asia. II. Occurrence and distribution. American Journal of Botany 63, 71–73.

Cavender JC, Raper KB. 1965 – The Acrasieae in nature. I. Isolation. American Journal of Botany 52, 294–296.

Edgar RC. 2004 – MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32, 1792–1797.
Eichinger L, Pachebat JA, Glöckner G, Rajandream MA et al. 2005 – The genome of the social amoeba *Dictyostelium discoideum*. Nature 435(7038), 43–57.
Fiore-Donno AM, Nikolaev SI, Nelson M, Pawlowski J et al. 2010 – Deep phylogeny and evolution of slime moulds (mycetozoa). Protist 161, 55–70.
Gouy M, Guindon S, Gascuel O. 2010 – SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Molecular Biology and Evolution 27, 221–224.
Guindon S, Dufayard JF, Lefort V, Anisimova M et al. 2010 – New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Systematic Biology 59, 307–321.
Huelsenbeck JP, Ronquist F. 2001 – MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17, 754–755.
Kawakami SI, Hagiwara H. 2008 – *Polysphondylium multicystogenum* sp. nov., a new dictyostelid species from Sierra Leone, West Africa. Mycologia 100, 347–351.
Loomis WF. 2016 – A better way to discover gene function in the social amoeba *Dictyostelium discoideum*. Genome Research 26, 1161–1164.
Medlin L, Elwood HJ, Stickel S, Sogin ML. 1988 – The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene 71, 191–199.
Olive EW. 1901 – A preliminary enumeration of the Sorophorae. Proceedings of the American Academy of Arts and Science 37, 340.
Raper KB. 1941 – *Dictyostelium minutum*, a second new species of slime mold from decaying forest leaves. Mycologia 33, 633–649.
Raper KB. 1984 – Dictyostelids. (Princeton University Press, Princeton, N).
Raper KB. 1956 – *Dictyostelium polycephalum* n. sp.: a new cellular slime mould with coremiform fructifications. Journal of General Microbiology 14, 716–732.
Raper KB, Fennell DI. 1967 – The crampon-based Dictyostelia. American Journal of Botany 54, 515–528.
Roberge-White E, Katoh-Kurasawa M. 2011 – Plasticity in the development and dedifferentiation of *Dictyostelium discoideum*. Development, Growth & Differentiation 53, 587–596.
Romeralo M, Cavender JC, Landolt JC, Stephenson SL, Baldauf SL. 2011 – An expanded phylogeny of social amoebas (Dictyostelia) shows increasing diversity and new morphological patterns. BMC Evolutionary Biology 11, 84.
Romeralo M, Escalante R, Baldauf SL. 2012 – Evolution and diversity of dictyostelid social amoebae. Protist 163, 327–343.
Romeralo M, Spiegelb FW, Baldauf SL. 2010 – A Fully Resolved Phylogeny of the Social Amoebas (Dictyostelia) Based on Combined SSU and ITS rDNA Sequences. Protist 161, 539–548.
Ronquist F, Huelsenbeck JP. 2003 – MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.
Schaap P, Winckler T, Nelson M, Alvarz-Curto E et al. 2006 – Molecular phylogeny and evolution of morphology in the social amoebas. Science 134, 661–663.
Seephueak P, Petcharat V. 2014 – The biodiversity of Dictyostelid cellular slime molds in rubber tree leaf litter in Southern Thailand. Mycosphere 5, 805–813.
Sheikh S, Thulin M, Cavender JC, Escalante R et al. 2018 – A new classification of the Dictyostelids. Protist 169, 1-28.
Vadell EM, Cavender JC, Romeralo M, Edwards SM et al. 2011 – New species of dictyostelids from Patagonia and Tierra del Fuego, Argentina. Mycologia 103, 101–117.
Vadell EM, Cavender JC. 2007 – Dictyostelids living in the soils of the Atlantics forest, Iurazú region, Misiones, Argentina: description of new species. Mycologia 99, 112–124.
Yulo PRJ, de la Cruz TEE. 2011 – Cellular slime molds from Lubang Island, Occidental Mindoro, Philippines. Mycosphere 2, 565–573.

Yulo PRJ, de la Cruz TEE. 2012 – Diversity and distribution of cellular slime molds (dictyostelids) in Lubang Island, Occidental Mindoro, Philippines. Mycology: An International Journal on Fungal Biology 3(3), 188–194.