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Original Article

Molecular systematics and species distribution of foliose lichens in the Gulf of Thailand mangroves with emphasis on *Dirinaria picta* species complex

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Abstract

Extensive surveys of mangrove foliose lichens in Caliciaceae and Physciaceae on the Gulf of Thailand revealed eight species of the genera *Dirinaria*, *Physcia*, and *Pyxine*. Species density was highest in the mid-intertidal zone (46%), followed by the landward and seaward zones (31% and 23%, respectively). Fifty-one new internal transcribed spacer sequences were generated and the resulting phylogenies based on maximum likelihood and Bayesian approaches yielded monophyletic lineages of all three genera. However, within the *Dirinaria* clade formation of polyphyletic assemblages among *D. aegialita*, *D. applanata*, and *D. picta* indicated the presence of homoplasies in certain morphological traits used to characterize them. To address species boundaries of these lichens in *Dirinaria picta* species complex, methods of DNA barcode-based delineation of putative species were employed. Additional sampling of the *Dirinaria* species from elsewhere is required to provide further insight into species delimitation of this heterogeneous genus.

Keywords: Caliciaceae, Gulf of Thailand, internal transcribed spacer, mangrove foliose lichens, Physciaceae

1. Introduction

Mangrove forests consist of different flora and fauna that flourish in estuarine ecosystems subjected to regular tidal flooding. Over 90% of these forests are located within developing countries (Reynolds, Er, Winder, & Blanchon, 2017). Mangroves have been categorized into three major zones that include the landward, the mid-intertidal *Rhizophora*, and the seaward *Avicennia-Sonneratia* zones based on dominant plant communities present (Waycott *et al*., 2011). They provide safe wildlife habitats as well as a variety of socio-economic services to people (Panda *et al*., 2017) and hence, are recognized as one of the world's most productive tropical ecosystems (Logesh, Upreti, Kalaiselvam, Nayaka, & Kathiresan, 2012). Despite the well accepted biodiversity value of mangroves across the globe, there has been a

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continued decline of the mangrove forests due to human influences (Reynolds *et al*., 2017).

Mangrove ecosystems are found in all 23 coastal provinces of Thailand covering areas of approximately 240,000 hectares (Aksornkoae, 2012). However, over the last three decades more than 50% of the total mangrove areas, especially along the Gulf of Thailand, have been depleted mainly due to shrimp farming, tourism, and industrial activities (Giri *et al*., 2008). Although under threatening conditions, these forests are hosts of manglicolous lichens commonly found as bark-dwelling species. Currently, an increasing number of lichen species has been recorded from Thailand (Buaruang *et al*., 2017), but there is still a lack of systematic studies of the manglicolous lichens. Therefore, this study focused on extensive surveys of foliose lichens, particularly those belonging to two closely related families, Caliciaceae and Physciaceae, present in the Gulf of Thailand mangroves. Firstly, it aimed to investigate phylogenetic placements of these lichens using internal transcribed spacer (ITS) regions of nuclear ribosomal DNA. Furthermore, a recent ITS-based phylogeny of the genus *Dirinaria* (Caliciaceae) revealed unresolved relationships among the species from South Korea as well as other countries (Jayalal *et al*., 2013). Thus, the present study also involved in elucidation of species boundaries within *Dirinaria* using ITS sequence data.

2. Materials and Methods

2.1 Specimens and phenotypic studies

The collecting localities were in the mangrove forests of several provinces along the Gulf of Thailand. These included Trat Province (Koh Chang Island: 11°59'N, 102°23'E and Koh Kood Island: 11°37'N, 102°32'E), Rayong Province (12°42'N, 101°41'E), Chachoengsao Province (13°32'N, 101° 00'E), and Chumphon Province (10°21'N, 99°13'E). The foliose lichens were randomly sampled from stem barks, branch barks, and aerial parts of ten host plants located in each type of the mangrove zonation. Forty-eight specimens were obtained and identified on the basis of their morphological, anatomical, and chemical characteristics, and vouchers were deposited in RAMK. Three additional specimens of *Dirinaria picta* (RAMK 030405-030407) were obtained from secondary forests in Khao Yai National Park (14°24'N, 101°22'E) in Nakhon Ratchasima Province. Morphologically, the specimens were examined for the presence or absence of vegetative propagules (dactyls and soredia) as well as colors and patterns of lobes using a low magnification stereomicroscope (Olympus SZ30). Free-hand sections were performed for anatomical studies of certain characteristics such as textures of the medulla and colors of the asci and ascospores using an Olympus BX51 compound microscope. Stained wet mounts and amyloidity tests were carried out using 10% potassium hydroxide and Lugol's iodine solution following White & James (1985). Photos were taken using a Canon PowerShot G12. In addition, chemical constituents were identified using thin layer chromatography with two solvent systems: A (Toluene [180 mL], dioxin [60 mL], and acetic acid [8 mL]) and G (Toluene [139 mL], ethyl acetate [83 mL], and formic acid [8 mL) (Elix, 2014).

2.2 DNA extraction, PCR amplification and DNA sequencing

Small thallus fragments (2–15 mg) excised from freshly collected specimens were ground in liquid nitrogen. Total genomic DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. The DNA obtained was used for PCR amplification of the ITS regions using the primers ITS1F (Gardes & Bruns, 1993) and ITS4 (White, Bruns, Lee, & Taylor, 1990). PCR reactions were performed in a thermal cycler (Eppendorf Mastercycler Gradient) and thermal cycling parameters followed Rangsiruji *et al*. (2016). Amplification products were cleaned using the QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's protocol. The purified PCR products were sequenced using the amplification primers.

2.3 Taxon sampling and phylogenetic analyses

Voucher information and GenBank accession numbers of the ITS sequences for the specimens obtained from the Gulf of Thailand coastline are shown in Table 1. This also included reference collection details and the GenBank accession numbers of other ITS-derived sequence data from elsewhere. An aligned data matrix of the ITS sequences was obtained using Geneious R8 (http://www.geneious.com). The removal of ambiguous regions was carried out using Gblocks with the less stringent setting (Castresana, 2000) and subsequently, the alignment was subjected to analyses under maximum likelihood and Bayesian approaches.

A maximum likelihood (ML) analysis was performed using RAxML-HPC2 on XSEDE (8.2.10) of the CIPRES Science Gateway server (Miller, Pfeiffer, & Schwartz, 2010) based on the GTRGAMMA model (Sta matakis, 2014). Bootstrap values for recovered nodes were estimated from the analysis of 1,000 pseudoreplicate datasets. Only clades that received bootstrap support (BS) ≥75% were considered strongly supported.

The Bayesian analysis was accomplished using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). The Markov Chain Monte Carlo (MCMC) algorithm for 10,000,000 generations was used for sampling trees from the distribution of posterior probabilities. These posterior probabilities of the phylogeny were determined by constructing a 50% majority-rule consensus of the remaining trees. Internodes with the posterior probabilities (PP) ≥0.95 were considered statistically significant. The resulting ML and Bayesian consensus trees were visualized with FigTree 1.3.1 software (http://tree.bio.ed.ac.uk/).

2.4 Phylogenies of selected *Dirinaria* species

The ITS sequence alignment of three *Dirinaria* species (36 specimens) was conducted using Geneious R8. Phylogenetic trees were reconstructed based on the ML and Bayesian analyses under the best-fit mode estimated above. *Pyxine asiatica* was defined as the outgroup. Two methods of sequence-based species delimitation, including the Automatic Barcode Gap Discovery (ABGD) and Bayesian Poisson Tree Processes (bPTP) were employed.

Table 1. Specimens obtained from this study were deposited in RAMK with GenBank accession numbers in bold. Other ITS sequences from elsewhere were retrieved from GenBank with their reference collection details.

Species	Family	Voucher information (country of origin)	GenBank accession number
Calicium adspersum	Caliciaceae	Prieto 3037	KX512907
Calicium lecideinum	Caliciaceae	Prieto	KX512911
Calicium nobile	Caliciaceae	Tibell 21968	KX512913
Calicium notarisii	Caliciaceae	Prieto 3007	KX512915
Calicium salicinum	Caliciaceae	Prieto	KX512919
Calicium trachylioides	Caliciaceae	Nordin 4002	KX512933
Dirinaria aegialita	Caliciaceae	Kawasaki id9 (Cambodia)	AB764068
Dirinaria aegialita	Caliciaceae	RAMK 031278 (Thailand)	**MK028196**
Dirinaria aegialita	Caliciaceae	RAMK 032083 (Thailand)	**MK028167**
Dirinaria aegialita	Caliciaceae	RAMK 032084 (Thailand)	**MK028168**
Dirinaria aegialita	Caliciaceae	RAMK 032085 (Thailand)	**MK028169**
Dirinaria aegialita	Caliciaceae	RAMK 032086 (Thailand)	**MK028170**
Dirinaria aegialita	Caliciaceae	RAMK 032087 (Thailand)	**MK028171**
Dirinaria aegialita	Caliciaceae	RAMK 032088 (Thailand)	**MK028172**
Dirinaria aegialita	Caliciaceae	RAMK 032089 (Thailand)	**MK028173**
Dirinaria applanata	Caliciaceae	MAF 9854 (Australia)	AY449727
Dirinaria applanata	Caliciaceae	Anonymous (India)	EU722342
Dirinaria applanata	Caliciaceae	Sipman 46067 (Singapore)	AF540512
Dirinaria applanata	Caliciaceae	Hur 041637 (South Korea)	EU670217
Dirinaria applanata	Caliciaceae	RAMK 032090 (Thailand)	**MK028175**
Dirinaria applanata	Caliciaceae	RAMK 032091 (Thailand)	**MK028176**
Dirinaria picta	Caliciaceae	Sipman 45628 (Singapore)	AF540514
Dirinaria picta	Caliciaceae	Hur 050536 (South Korea)	EU670223
Dirinaria picta	Caliciaceae	RAMK 032092 (Thailand)	**MK028177**
Dirinaria picta	Caliciaceae	RAMK 032093 (Thailand)	**MK028178**
Dirinaria picta	Caliciaceae	RAMK 032094 (Thailand)	**MK028179**
Dirinaria picta	Caliciaceae	RAMK 032095 (Thailand)	**MK028180**
Dirinaria picta	Caliciaceae	RAMK 032096 (Thailand)	**MK028181**
Dirinaria picta	Caliciaceae	RAMK 032097 (Thailand)	**MK028182**
Dirinaria picta	Caliciaceae	RAMK 032098 (Thailand)	**MK028183**
Dirinaria picta	Caliciaceae	RAMK 032099 (Thailand)	**MK028184**
Dirinaria picta	Caliciaceae	RAMK 032100 (Thailand)	**MK028185**
Dirinaria picta	Caliciaceae	RAMK 032101 (Thailand)	**MK028186**
Dirinaria picta	Caliciaceae	RAMK 032102 (Thailand)	**MK028187**
Dirinaria picta	Caliciaceae	RAMK 031273 (Thailand)	**MK028174**
Dirinaria picta	Caliciaceae	RAMK 031274 (Thailand)	**MK028192**
Dirinaria picta	Caliciaceae	RAMK 031275 (Thailand)	**MK028193**
Dirinaria picta	Caliciaceae	RAMK 031276 (Thailand)	**MK028194**
Dirinaria picta	Caliciaceae	RAMK 031277 (Thailand)	**MK028195**
Dirinaria picta	Caliciaceae	RAMK 031279 (Thailand)	**MK028197**
Dirinaria picta	Caliciaceae	RAMK 031280 (Thailand)	**MK028198**
Dirinaria picta	Caliciaceae	RAMK 031281 (Thailand)	**MK028199**
Dirinaria picta	Caliciaceae	RAMK 031282 (Thailand)	**MK028200**
Dirinaria picta	Caliciaceae	RAMK 031283 (Thailand)	**MK028201**
Dirinaria picta	Caliciaceae	RAMK 031284 (Thailand)	**MK028202**
Dirinaria picta	Caliciaceae	RAMK 030405 (Thailand)	**MK028188**
Dirinaria picta	Caliciaceae	RAMK 030406 (Thailand)	**MK028189**
Dirinaria picta	Caliciaceae	RAMK 030407 (Thailand)	**MK028190**
Dirinaria picta	Caliciaceae	RAMK 030408 (Thailand)	**MK028191**
Physcia adscendens	Physciaceae	Moberg 12260	EU682184
Physcia aipolia	Physciaceae	Hernandez & Sicilia XII.2002	EU682185
Physcia alnophila	Physciaceae	Ahti 64008	EU682210
Physcia atrostriata	Physciaceae	RAMK 031285 (Thailand)	**MK028203**
Physcia atrostriata	Physciaceae	RAMK 031286 (Thailand)	**MK028204**
Physcia atrostriata	Physciaceae	RAMK 031287 (Thailand)	**MK028205**
Physcia austrostellaris	Physciaceae	CBG:Elix 38829 (Australia)	GU074409
Physcia caesia	Physciaceae	Urbanavichus C-01566	EU682197
Physcia dubia	Physciaceae	BCN-Lich 17042	GU247179
Physcia leptalea	Physciaceae	BCN-Lich 16792	GU247176
Physcia stellaris	Physciaceae	Moberg 12012	EU682183
Physcia subalbinea	Physciaceae	Pykälä 9712	EU682186
Physcia tenella	Physciaceae	Odelvik & Hellström 0827	KX512932
Physcia tropica	Physciaceae	Elix 36320 (CANB) (Australia)	FJ822890

Table 1. Continued.

Species	Family	Voucher information (country of origin)	GenBank accession number
Physcia tropica	Physciaceae	Elix 37727 (CANB) (Australia)	FJ822889
Physcia undulata	Physciaceae	RAMK 031298 (Thailand)	**MK028206**
Physcia undulata	Physciaceae	RAMK 031299 (Thailand)	**MK028207**
Pyxine albovirens	Caliciaceae	Sipman 25/99-1 (Guayana)	AY143412
Pyxine asiatica	Caliciaceae	RAMK 031288 (Thailand)	**MK028208**
Pyxine asiatica	Caliciaceae	RAMK 031289 (Thailand)	**MK028209**
Pyxine coccifera	Caliciaceae	RAMK 031290 (Thailand)	**MK028210**
Pyxine coccoes	Caliciaceae	Prieto	KX512936
Pyxine retirugella	Caliciaceae	RAMK 031291 (Thailand)	**MK028211**
Pyxine retirugella	Caliciaceae	RAMK 031292 (Thailand)	**MK028212**
Pyxine retirugella	Caliciaceae	RAMK 031293 (Thailand)	**MK028213**
Pyxine retirugella	Caliciaceae	RAMK 031294 (Thailand)	**MK028214**
Pyxine retirugella	Caliciaceae	RAMK 031295 (Thailand)	**MK028215**
Pyxine retirugella	Caliciaceae	RAMK 031296 (Thailand)	**MK028216**
Pyxine retirugella	Caliciaceae	RAMK 031297 (Thailand)	**MK028217**
Pyxine sorediata	Caliciaceae	Wetmore 91254	KX512937
Pyxine subcinerea	Caliciaceae	AFTOL-ID 686	HQ650705
Xanthoria elegans	Teloschistaceae	Odelyik 04532	KX512947

The ABGD method aims to discover the existence of the DNA barcode gaps and estimate the number of species (Puillandre, Lambert, Brouillet, & Achaz, 2012). The analysis was performed on the ABGD website (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) with a prior *p* range of 0.005–0.01, and a relative gap width of 1.5, with the Kimura 2-parameter model.

The bPTP model is used for delimiting species boundaries on a rooted phylogenetic tree (Zhang, Kapli, Pavlidis, & Stamatakis, 2013). In this study, a rooted input tree obtained from the Bayesian approach was used and run on a web server (https://species.h-its.org/ptp/) with 500,000 MCMC generations, excluding the outgroup, following the remaining default settings.

3. Results and Discussion

3.1 Species distribution

The collected foliose lichens inhabited the mangroves in both the eastern and western parts of the Gulf of Thailand. The sampling localities encompassed four provinces, namely Chachoengsao, Rayong, Trat, and Chumphon. Eight foliose taxa of Caliciaceae and Physciaceae (Figure 1), belonging to three genera, viz. *Dirinaria* (*D. aegialita*, *D. applanata*, *D. picta*), *Physcia* (*P. atrostriata*, *P. undulata*) and *Pyxine* (*P. asiatica*, *P. coccifera*, *P. retirugella*) were discovered following in-depth investigations of their morphological, anatomical, and chemical characteristics (Table 2). From these eight species of lichens (48 specimens), the species density was determined among the different types of mangrove zonation. The highest species density was shown in the mid-intertidal zone (46%), followed by the landward and seaward zones (31% and 23%, respectively) (Figure 2A).

Within the family Caliciaceae (Figure 2B), *D. picta* was most abundant as it occurred in all zones, whereas *D. aegialita* and *P. asiatica* were restricted to the mid-intertidal *Rhizophora* zone. On the other hand, *D. applanata* appeared to inhabit two different life zones of the landward as well as the mid-intertidal *Rhizophora* zones. *P. retirugella* was distributed from the mid-intertidal *Rhizophora* to the seaward *Avicennia-Sonneratia* zones and *P. coccifera* was found in terrestrial forests. Studies by Nayaka, Upreti, and Ingle (2012) and Sethy, Pandit, and Sharma (2012) also revealed that both *Dirinaria* and *Pyxine* were among the most common foliose lichens in the mangrove forests of India.

In the family of Physciaceae (Figure 2B), *P. undulata* was widely distributed in the landward zone. In contrast, *P. atrostriata* occupied the mangrove zonation extending from the mid-intertidal *Rhizophora* to the seaward *Avicennia-Sonneratia* zones. Previously, the specimens of *P. undulata* obtained from Koh Chang Island were recorded as *Physcia crispa* var. *mollescens* by Vainio (1909). However, in the present study a voucher specimen was delivered from the University of Turku Herbarium (TUR, Finland) for a thorough re-examination. Based on the detailed morphological and anatomical studies as well as a chemical analysis of this specimen it was re-identified as *P. undulata* (Figure 3). This finding also supported Buaruang *et al*. (2017).

The presence of lichens in the mangrove ecosystems indicates their tolerance to harsh environmental conditions, including solar radiation, desiccation, salinity, and human interruptions (Delmail *et al*., 2013; Nayaka, Upreti, & Ingle, 2012). However, in Thailand the knowledge of lichen diversity in such ecosystems is still inadequate. Our previous study showed that the cyanolichen species (blue-green algae photobionts) were most abundant in the seaward zone (Rangsiruji *et al*., 2016). The present study revealed the existence of dense populations of another group of lichenized fungi with green algae phycobionts in the mid-intertidal zone. Moreover, three halotolerant lichen species, namely *D. picta*, *P. atrostriata*, and *P. retirugella*, were also discovered in the seaward zone. These species produced a major secondary metabolite, atranorin, as a photoprotective pigment to ensure efficient photosynthesis of the phycobionts. Furthermore, it has been shown that these phycobionts were able to produce polyols to enhance the desiccation tolerance of their mycobiont partners (Delmail *et al*., 2013).

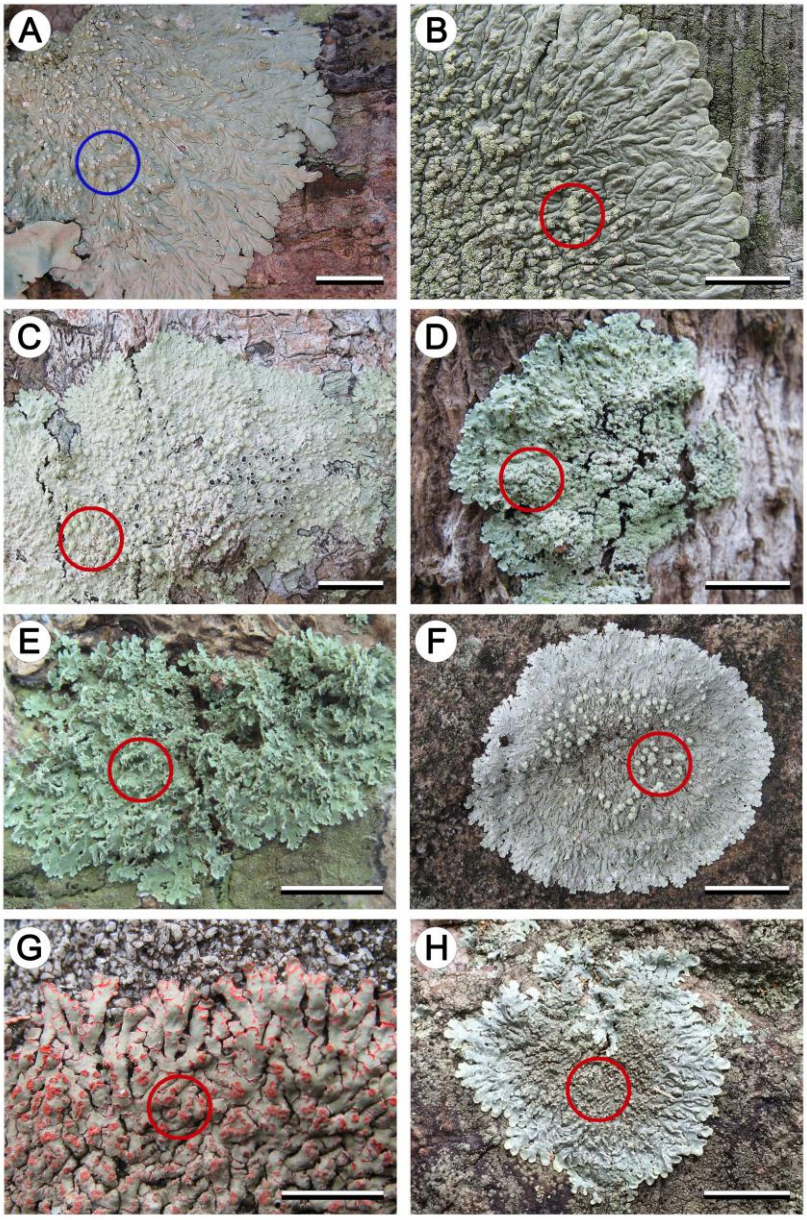

Figure 1. Habit photographs of foliose lichens in Caliciaceae and Physciaceae present in mangroves of Thailand. (A) *Dirinaria aegialita*, (B) *D. applanata*, (C) *D. picta*, (D) *Physcia atrostriata*, (E) *Physcia undulata*, (F) *Pyxine asiatica*, (G) *Pyxine coccifera*, and (H) *Pyxine retirugella*. Blue circle and red circles indicate dactyls and soredia, respectively. Scale = 1 cm.

Table 2. Species collected and their distinctive morphological, anatomical and chemical characteristics.

List of species	Morphological and anatomical characteristics	Chemical constituents
Dirinaria aegialita	Lobes with flabellate apices, thalline dactyls	Atranorin, divaricatic acid
Dirinaria applanata	Lobes with flabellate apices, farinose soredia	Atranorin, divaricatic acid
Dirinaria picta	Lobes with discrete apices, farinose soredia	Atranorin, divaricatic acid
Physcia atrostriata	Pruinose upper surface and brown-black lower surface, soredia	Atranorin, zeorin
Physcia undulata	Pruinose upper surface and white to pale brown lower surface, soredia	Atranorin, zeorin
Pyxine asiatica	Thallus adnate to tightly, soredia, white medulla	Atranorin, norstictic acid
Pyxine coccifera	Thallus adnate to loosely, red-pigmented soredia	Atranorin, norstictic acid
Pyxine retirugella	Thallus adnate, soredia, white or cream medulla	Atranorin, norstictic acid

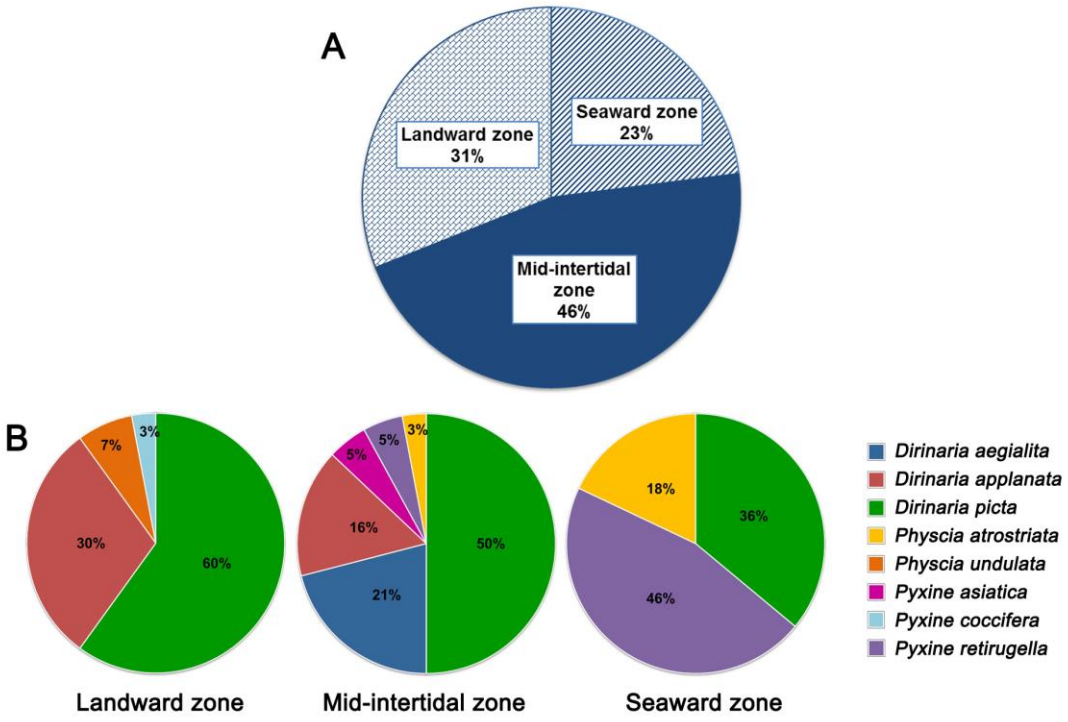

Figure 2. (A) Species density of foliose lichens compared among three types of mangrove zonation on the Gulf of Thailand. (B) Distribution of foliose lichen species in the landward, mid-intertidal, and seaward zones.

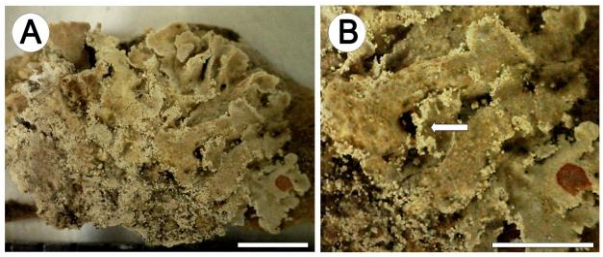

Figure 3. Specimen of *Physcia crispa* var. *mollescens* from Koh Chang Island listed by Vainio (1909) was re-identified in this study as *P. undulata*. (A) Thallus orbicular with frosted-pruinose and (B) soralia marginal (arrow). Scale = 0.2 cm.

3.2 Phylogenies of Caliciaceae and Physciaceae

Several groups of lichenized fungi are primarily distinguished on the basis of different growth forms, vegetative propagules, and secondary metabolites. However, classifications based on single vegetative characters have been shown to create non-monophyletic assemblages (Luangsupha bool *et al*., 2016; Parnmen, Lücking, & Lumbsch, 2012; Parnmen *et al*., 2012; Rangsiruji *et al*., 2016; Wedin, Döring, Nordin, & Tibell, 2000).

Previous phylogenetic studies showed that members of the mazaedia-producing family Caliciaceae were nested within the genera *Dirinaria*, *Pyxine*, and *Physcia* which were circumscribed in the family Physciaceae. Therefore, some authors treated all Caliciaceae and Physciaceae as one family, and the name Physciaceae was proposed for conservation (Wedin & Grube, 2002; Wedin, Baloch, & Grube, 2002; Wedin, Döring, Nordin, & Tibell, 2000). Recently, however, a two-family concept of Caliciaceae and Physciaceae was adopted and preferred (Gaya *et al*. 2012).

In this study, fifty-one new ITS sequences were generated and aligned with other sequences obtained from GenBank (Table 1). A matrix of 563 unambiguously aligned nucleotide position characters was analyzed. *Xanthoria elegans* was used as the outgroup. The ML tree had a likelihood of lnL = −6,930.534, while the Bayesian tree possessed a mean likelihood of lnL = −6,755.973 (±0.08). Both trees displayed similar topologies with two major clades. Thus, only the ML tree is shown here (Figure 4) with current phylogenetic placements of the specimens in agreement with those of Wedin *et al*. (2000, 2002).

Clade I (BS=70/PP=0.90) contains the monophyly of Caliciaceae, including *Dirinaria*, *Pyxine*, and *Calicium*. Two subclades consisting of the genera *Dirinaria* (100/1.00) and *Calicium* (89/0.99) were strongly supported, whereas the other subclade of the genus *Pyxine* was lacking support. Morphologically, this clade is characterized by the presence of *Bacidia*-type asci and ascospores without distinct wall thickenings and hypothecium pigmentation. A close-knit relationship between *Dirinaria* and *Pyxine* was observed and this was also revealed by Helms, Friedl, and Rambold (2003). Both genera possessed *Dirinaria*-type ascospores. They were differentiated based on excipulum types as well as secondary metabolite combinations. The presence of a thalline excipulum and a combination of atranorin and divaricatic acid were typical for *Dirinaria*, whereas the existence of a proper excipulum and a combination of atranorin and norstictic acid were common for *Pyxine*. Furthermore, this study demonstrated non-monophyletic lineages of *Dirinaria* species,

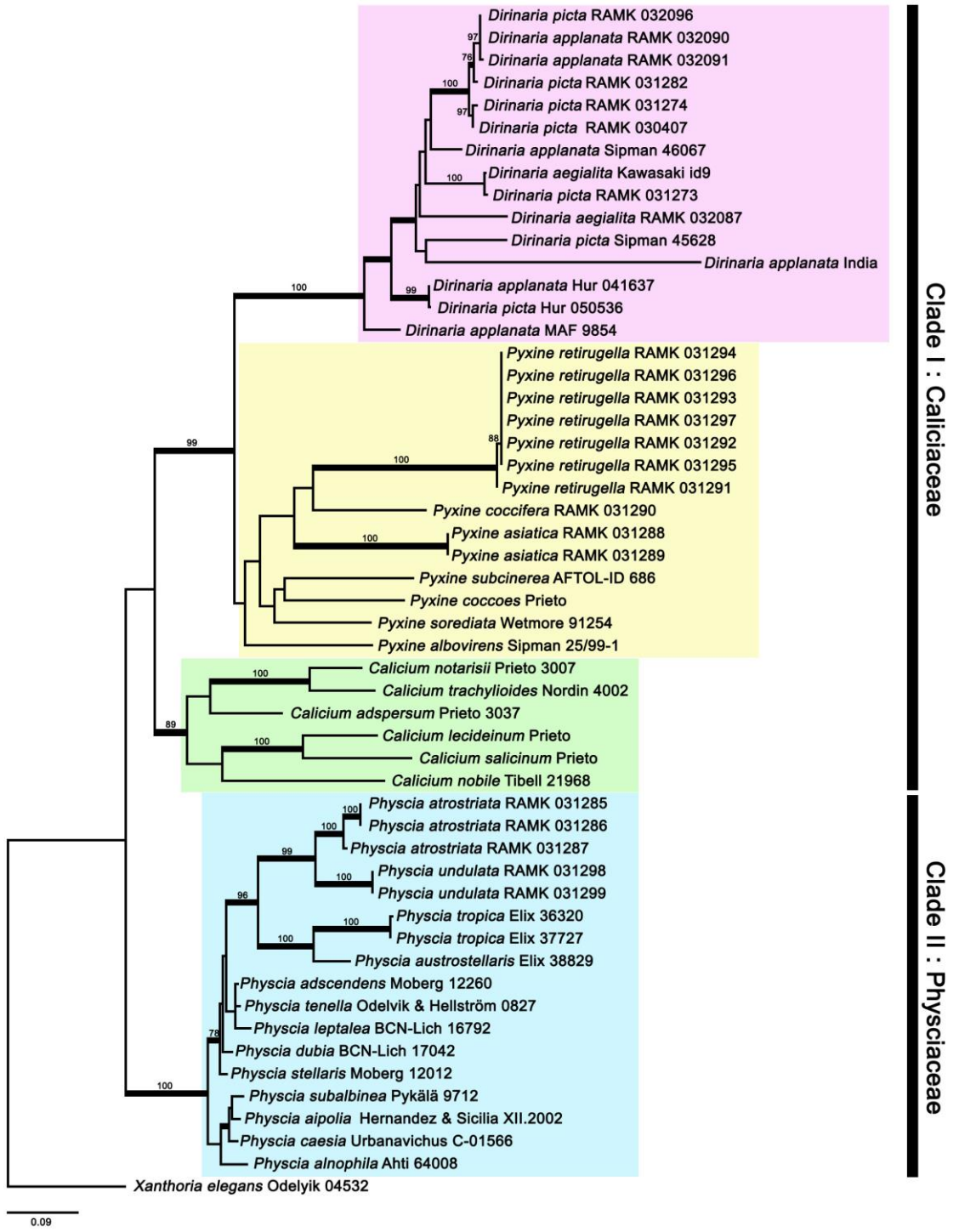

Figure 4. ML tree depicting relationships within Caliciaceae-Physciaceae based on ITS sequence data. Only ML bootstrap values ≥75% are reported above the branches and posterior probabilities ≥0.95 are indicated as bold branches.

despite their apparent spatial distribution pattern according to geographical origins.

Clade II (100/1.00) comprises members of the genus *Physcia*, representing the family Physciaceae. This clade is characterized by the presence of a pseudoparenchymatous upper cortex, *Physcia*-type ascospores, and cortical substances such as atranorin and zeorin. This study showed that *P. atrostriata* and *P. undulata* from Thailand form a distinct subclade with other species that originated in the southern hemisphere. Both of them belong to palaeotropical taxa in which most members contain soredia, and thus are rapidly dispersed (Galloway & Moberg, 2014).

3.3 *Dirinaria* phylogenies

The genus *Dirinaria* includes approximately 36 species with *D. picta* as the type species. They occur in pantropical and subtropical regions, as well as oceanic regions. Australia is the center of species diversity where 13 taxa of *Dirinaria* were recognized (Elix, 2009). In Thailand, eight species were reported to exist mostly in tropical and montane forests (Buaruang *et al*., 2017). Traditionally, the genus is characterized by the presence of vegetative propagules (soredia/dactyls) and patterns of lobe apices (flabellate/discrete) as well as a combination of secondary metabolites (atranorin, divaricatic acid, sekikaic acid, and xanthones) (Elix, 2009).

In this study, three species of *Dirinaria*, namely *D. aegialita*, *D. applanata*, and *D. picta*, were discovered. Taxonomically, these species are very similar. *Dirinaria aegialita* can be distinguished from the other two species mainly by the presence of dactyls and absence of orbicular soralia. On the other hand, *D. applanata* and *D. picta* are differentiated merely by the presence of flabellate and discrete apices, respectively (Elix, 2009). The morphological attributes of the three species are depicted in Figure 5.

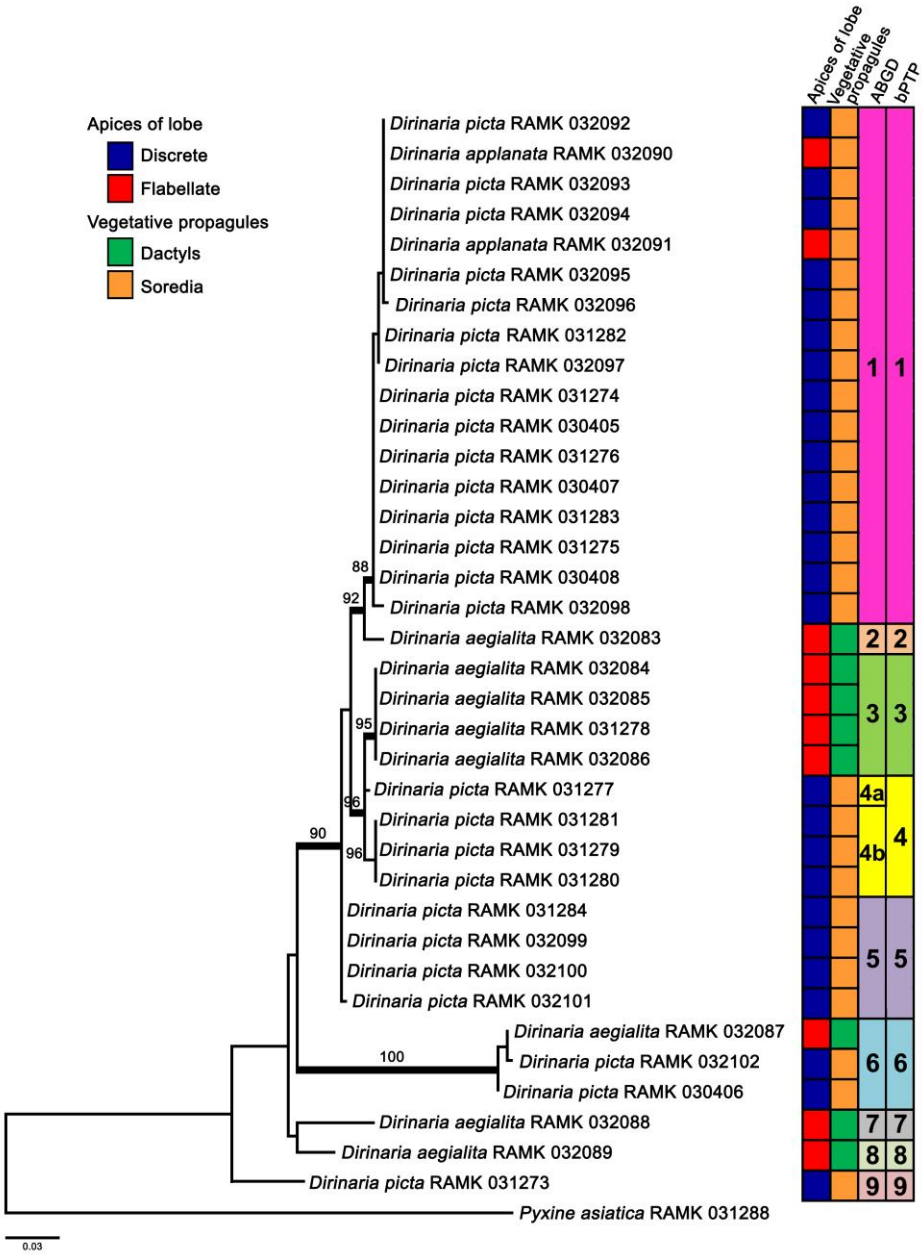

Figure 5. ML tree showing relationships within *Dirinaria picta* species complex based on ITS sequences. Only ML bootstrap values ≥75% are denoted above the branches and posterior probabilities ≥0.95 are demonstrated as bold branches. Phenotypic characters and species delimitation scenarios obtained from different methods are indicated in columns to the right. The proposed putative species are highlighted with different colors and corresponding numbers.

The present study revealed the ITS-based phylogenies at the infrageneric level of the genus *Dirinaria*. The phylogenetic estimates obtained from both the ML and Bayesian approaches were congruent. Thus, only the ML tree is illustrated and it revealed a large group of intermixed species of *Dirinaria*, giving rise to *Dirinaria picta* species complex. The presence of putative species (molecular operational taxonomic units) was considered based on the ABGD and bPTP analyses as well as the ML bootstrap values and PP support (Figure 5). The ABGD analysis identified a barcode gap with a prior intra-specific divergence at 0.04 (Figures 6A & 6B). In addition, it revealed 10 putative species in all recursive partitions with prior intra-specific genetic distance thresholds between 0.50 and 0.73% (Figure 6C). On the contrary, the bPTP analysis demonstrated 9 putative species. Eight putative species (1–3 & 5–9) were recognized by both methods. The ABGD method however, further divided the putative species 4 into two more putative species (4a & 4b). Although four putative species, namely 1, 2, 3, and 6, were strongly supported by the ML and PP values, the resulting posterior probabilities based on the bPTP analysis of putative species 1, 3, and 6 were rather low (0.20, 0.56, and 0.65, respectively). Clusters of seven putative species (2–5 & 7–9) were apparently associated with the apex structures of the thallus lobes. Other traits such as soredia and dactyls appeared to be homoplasious and thus, were not reliable for the species delimitation. Therefore, more phenotypically diagnostic characters possessing true synapomorphies are required to reinforce the existence of the proposed delineated species within the current *Dirinaria picta* species complex.

4. Conclusions

Our study showed that the foliose lichens in Caliciaceae and Physciaceae were present in different types of mangrove zonation along the Gulf of Thailand. Three genera

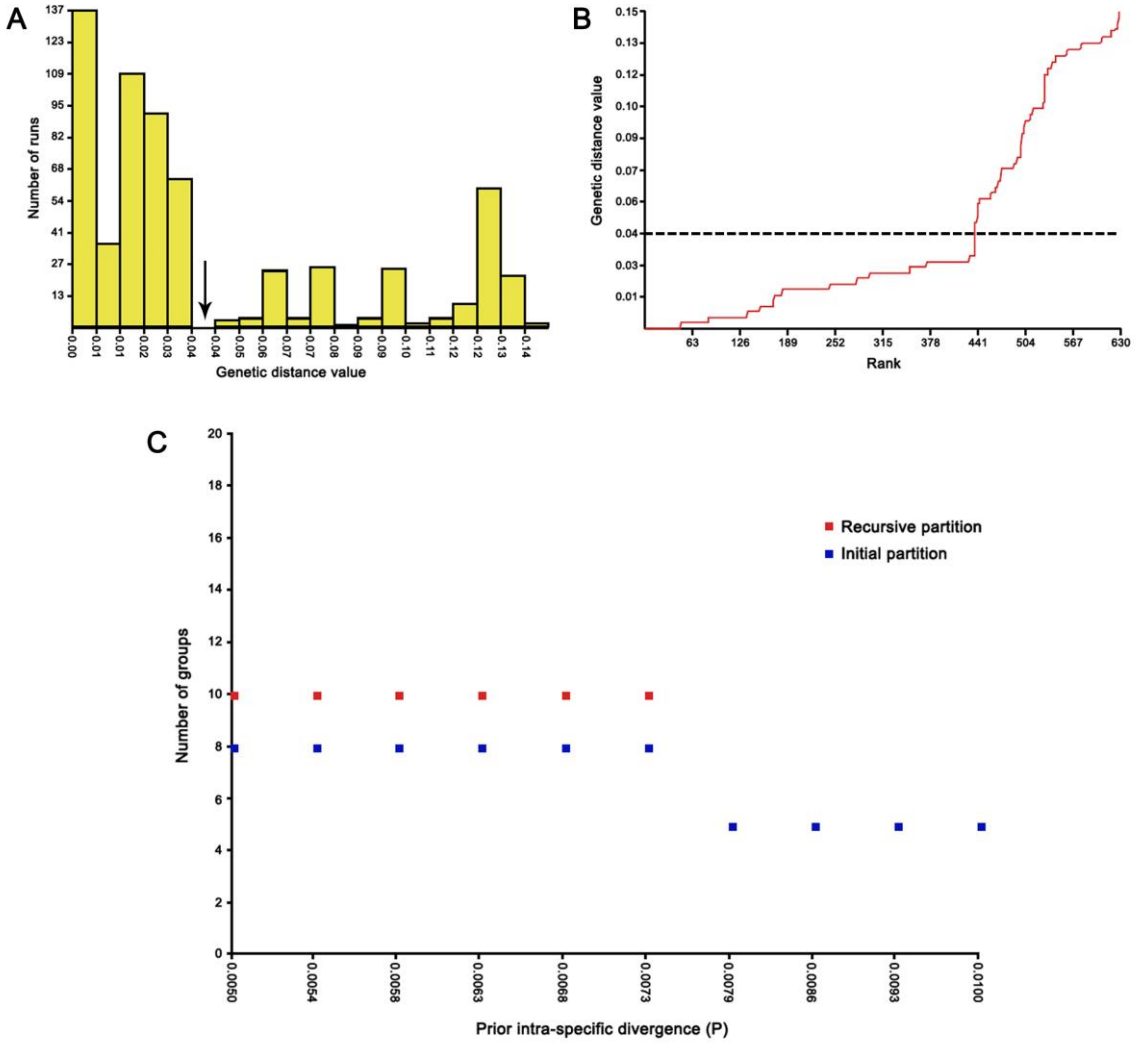

Figure 6. Automatic Barcode Gap Discovery (ABGD) outputs based on ITS sequences of *Dirinaria.* (A) Histogram showing distribution of genetic distances with an arrow indicating the threshold selected to separate between intra- and inter-specific divergences. (B) Ranked genetic distances with a dotted line showing approximate position of gap center. (C) Number of groups inside the partition as a function of the prior limit between intra- and inter-specific divergences.

were obtained that included *Dirinaria*, *Physcia*, and *Pyxine* to form monophyletic groups based on the molecular phylogenetic analyses. Phenotypically, the morphological, anatomical, and chemical characteristics of *Dirinaria* under study were in line with those described by Elix (2009). However, within the *Dirinaria* clade several species are clearly dispersed, yielding polyphyletic assemblages of taxa. Thus, the ABGD and bPTP methods were employed as the DNA barcode-based delineation of the putative species within the *Dirinaria picta* species complex. The results confirmed that some vegetative characters were homoplastic synapomorphies and should be avoided in taxonomy. Additional sampling of the *Dirinaria* species from elsewhere is required to provide more valuable diagnostic traits for a better understanding of the nature of this species complex.

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