DNA-based identification of the lichen-forming genus Pertusaria (Pertusariales, Ascomycota) in the mangrove ecosystem of eastern Thailand

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ABSTRACT

The genus *Pertusaria* (Pertusariales, Ascomycota) is a diverse group of crustose lichens which is rich in secondary metabolites. However, studies concerning diversity of these lichens, especially in mangrove habitats are limited. Therefore, the present study aimed to explore and assess the diversity of the *Pertusaria* species occurring in the mangrove ecosystem of eastern Thailand. Their phylogenetic relationships were inferred using maximum likelihood (ML) and Bayesian analyses based on the mitochondrial SSU rDNA sequence data. Our results revealed that both ML and Bayesian tree topologies were congruent. Most of the *Pertusaria* species obtained form well-supported clades within the *Pertusaria* s.str. group, except for *Pertusaria* cf. *violacea* which is nested within the *Variolaria* group. Interestingly, Thai specimens of *P. pertusa* are not placed in the same clade with the type specimen. Based on our field observation, most species were found in the mid-intertidal *Rhizophora* zone, while *P. cicatricosa* was able to survive in the seaward *Avicennia-Sonneratia* zone. Additional studies with extended sampling will be necessary to address species boundaries of *Pertusaria* cf. *violacea* and *P. pertusa*.

Keywords: Lichenized fungi, mangrove ecosystem, mitochondrial SSU rDNA, *Pertusaria*, Pertusariales

INTRODUCTION

Lichens are symbiotic organisms which are composed of ascomycete and basidiomycete partners with microscopic green algae or cyanobacteria (Spribille *et al.* 2016). Traditionally, the growth form of lichens plays an important role in their classification. The shapes of lichens are determined by the organization of the fungal partners, which give rise to the names of the lichens. A challenge in lichen identification occurs as a result of the plasticity of thallus morphology and the diversity of secondary chemistry (Lumbsch *et al.* 2010; Parnmen *et al.* 2012*b*; Buaruang *et al.* 2015; Rangsiruji *et al.* 2016). In this study, we focused on warty crustose lichenized fungi in the genus *Pertusaria* which belongs to the family Pertusariaceae. Traditional classification of this genus is based on ascoma-morphology, amyloidity of ascus walls and hymenial gel, number of ascospores per ascus, and secondary metabolites (Schmitt & Lumbsch 2004). However, results of molecular phylogenetic studies suggested that lineages often do not correlate well with morphological and chemical features (Schmitt *et al.* 2010; Schmitt *et al.* 2012).

A recent checklist of lichenized fungi in Thailand has been documented, including 1,292 species (http://www.lichen.ru.ac.th/). Revisional studies of the genus *Pertusaria* revealed 103 taxa, of which 73 were new to science and 34 were new records in Thailand (Elix *et al*. 2008; Jariangprasert 2013). However, our knowledge of the diversity of this group of lichens in mangrove habitats is still inadequate. Hence, the objective of this study was to identify the species of *Pertusaria* in the mangrove ecosystem of eastern Thailand, and assess their phylogenetic placement using molecular data.

MATERIALS AND METHODS

Specimen collection, morphological and chemical evaluation

The study area was located in the mangrove forests in Trat Province. Specimens of *Pertusaria* were collected and deposited in RAMK. Morphological characteristics of the specimens were examined by using a low magnification stereomicroscope (Olympus SZ30), while anatomical characteristics were observed on free-hand sections. Anatomical investigations were conducted using a compound microscope (Olympus BX51) with 40-1,000 magnifications, and photographs were taken. Chemical constituents were identified using thin layer chromatography (White & James 1985; Elix & Ernst-Russell 1993).

DNA extraction, PCR amplification and DNA sequencing

For each specimen, small thallus fragments of 2-15 mg were ground in the 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 mitochondrial small subunit (mtSSU) rDNA. PCR primers and thermal cycling condition were employed as described previously by Rangsiruji *et al*. (2016). Amplification products were cleaned using the QIAquick Gel Extraction Kit (QIAGEN), and were sequenced with amplification primers.

Phylogenetic analyses

Sequence alignment of the *Pertusaria* specimens and other taxa of Pertusariales from GenBank was carried out using Geneious Pro 5.4.3 (http://www.geneious.com). To construct phylogenetic trees, maximum likelihood (ML) analysis was performed in the RAxML-HPC2 on XSEDE 8.1.11 of the CIPRES Science Gateway server (http://www.phylo.org/ portal2/login!input. action), using the GTRGAMMA model with 25 rate parameter categories (Stamatakis 2006). The bootstrap analysis was run with 1,000 pseudoreplicates. In addition, Bayesian analysis was conducted using MrBayes 3.1.2 with the GTR+G model (Huelsenbeck & Ronquist 2001). Posterior probabilities were evaluated by sampling trees using a variant of Markov Chain Monte Carlo (MCMC) method. Number of generations was 10 million. The first 25% of the generations were discarded as burn in. Of the remaining trees, a majority-rule consensus tree with average branch lengths was calculated using the sumt option of MrBayes. Only clades that received bootstrap support (BS) ≥75% under ML and posterior probabilities (PP) ≥0.95 were considered as strongly supported. The phylogenetic trees were depicted using the program FigTree 1.4.3 (http://tree.bio.ed.ac.uk/).

RESULTS AND DISCUSSION

Based on our field observation of the mangrove forests, six species of *Pertusaria* were recorded (Figure 1). Most species were found in the mid-intertidal *Rhizophora* zone. *Pertusaria cicatricosa* however, was able to survive in the seaward *Avicennia-Sonneratia* zone, indicating its resistance to salinity stress. Non-chlorinated xanthones such as lichexanthone and their derivatives were detected in all specimens. These substances are UV-protectant metabolites (Nguyen *et al.* 2013). Brief notes on the species collected, their morphological and anatomical characteristics as well as chemical constituents are listed in Table 1.
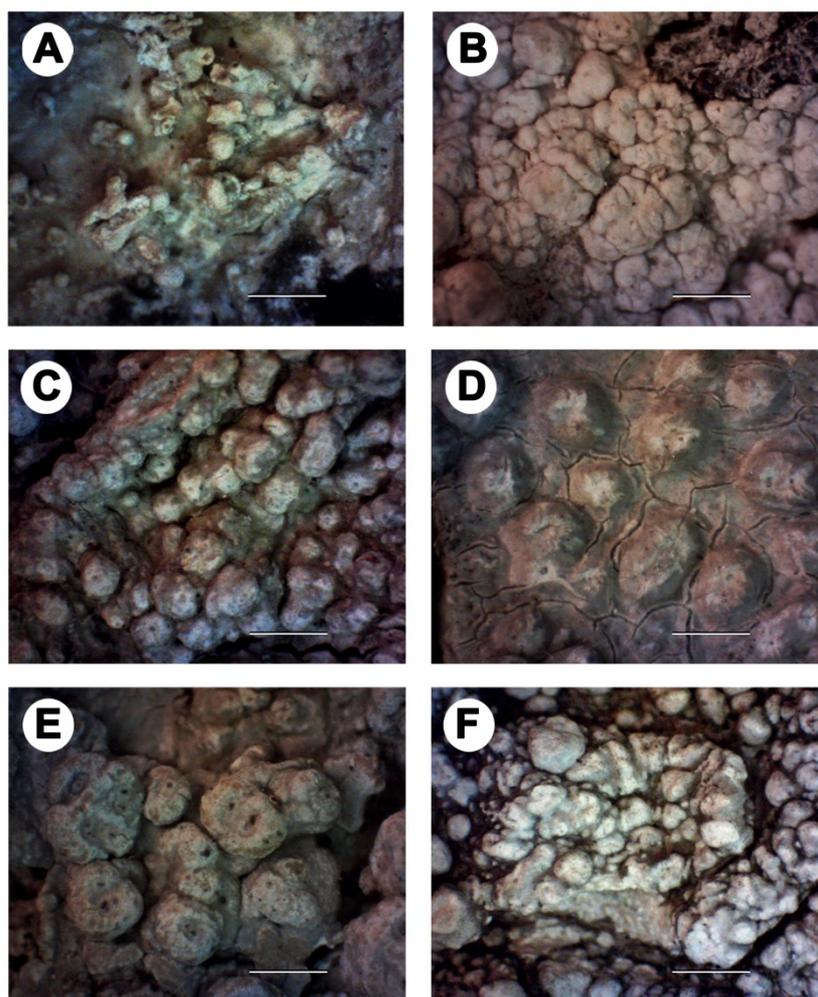

Figure 1 Habit photographs of *Pertusaria* species found in mangrove ecosystem of Thailand. (A) *Pertusaria balekensis* (B) *P. cicatricosa* (C) *P. denotanda* (D) *P. follmaniana* (E) *P. pertusa* (F) *Pertusaria* cf. *violacea*. Scale = 1 cm.

Table 1 Species collected, morphological/anatomical characteristics and chemical constituents.

| List of species	Morphological/anatomical characteristics	Chemical constituents
Pertusaria balekensis	Thallus crustose, corticolous, olivaceous green; surface smooth, cracked; isidia globose, burst on top; ascomata not seen.	Stictic acid, constictic acid, cryptostictic acid, hypostictic acid and 4,5-dichlorolichexanthone
Pertusaria cicatricosa	Thallus crustose, corticolous, olivaceous green; surface smooth, cracked; ascomata verruciform, conspicuous, constricted at the base, hemispherical, ostiole gray, ascospores 2-3 per ascus, hyaline, ellipsoid to cylindrical, rough wall, 39.5-72.4 μm long.	Stictic acid, constictic acid, cryptostictic acid, hypostictic acid, 2,4-dichlorolichexanthone, 2,5-dichlorolichexanthone and 2,4,5-dichlorolichexanthone
Pertusaria denotanda	Thallus crustose, corticolous, greenish gray; surface smooth, continuous; ascomata verruciform, conspicuous, constricted at the base, hemispherical, ostiole hyaline, ascospores 2 per ascus, hyaline, ellipsoid to cylindrical, smooth wall, 25-30 μm long.	Perlatolic acid and lichexanthone
Pertusaria follmaniana	Thallus crustose, corticolous, greenish gray; surface smooth, cracked; ascomata verruciform, conspicuous, constricted at the base, hemispherical, ostiole gray to brown, ascospores 4 per ascus, hyaline, ellipsoid to cylindrical, smooth wall, 20-30 μm long.	Stictic acid, constictic acid, cryptostictic acid, hypostictic acid and lichexanthone
Pertusaria pertusa	Thallus crustose, corticolous, greenish gray; surface smooth, continuous; ascomata verruciform, conspicuous, constricted at the base, hemispherical, ostiole dark brown to black, ascospores 2 (-3) per ascus, hyaline, ellipsoid, smooth wall, 20-27.5 μm long.	Stictic acid, constictic acid, cryptostictic acid, hypostictic acid and lichexanthone
Pertusaria cf. *violacea*	Thallus crustose, corticolous, grayish green; surface rough, cracked; soredia globose to hemispherical; ascomata not seen.	Haemathamnolic acid, deoxyhaemathamnolic acid and lichexanthone
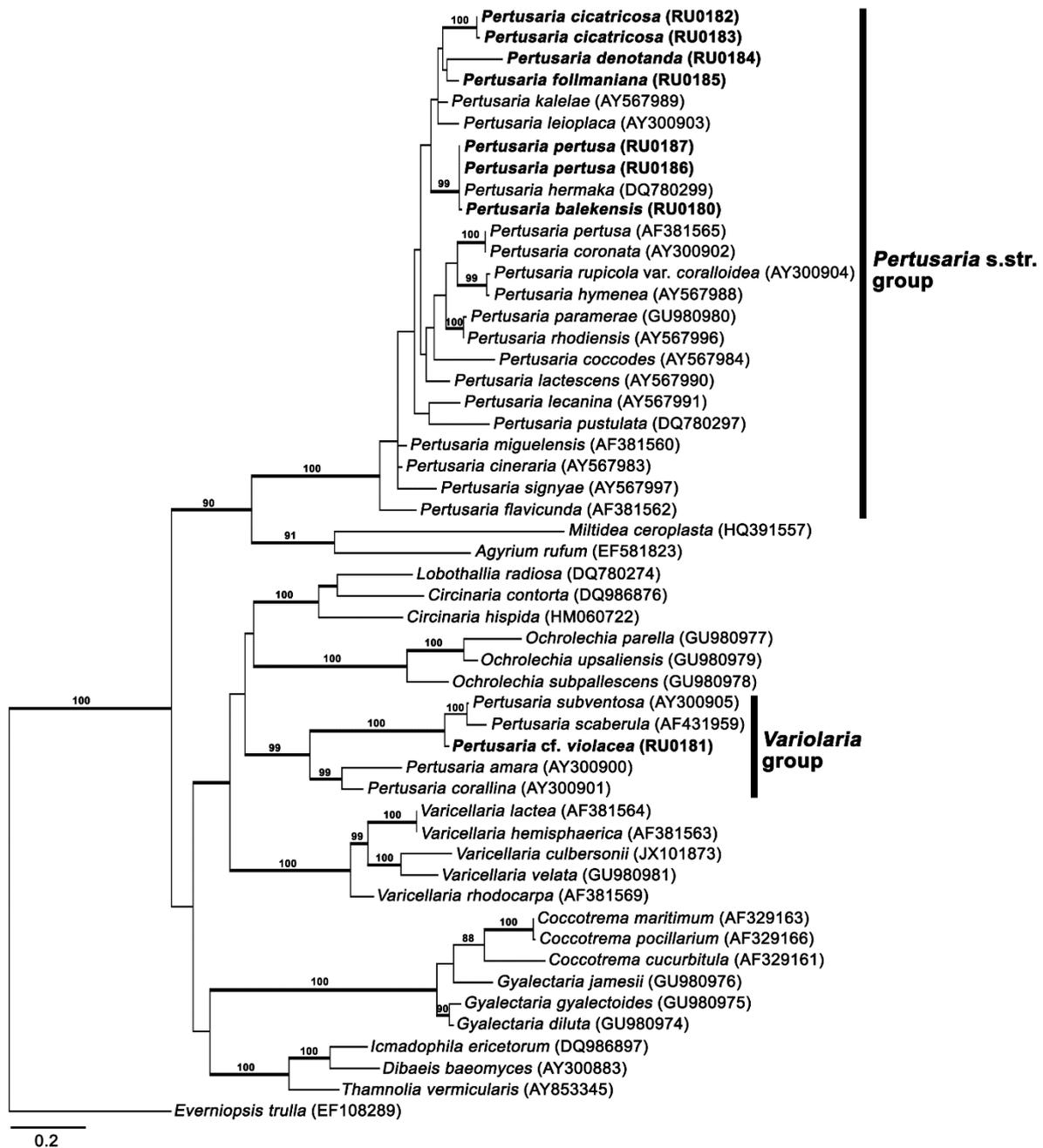
Figure 2 ML tree depicting relationships within Pertusariales based on mtSSU rDNA sequences. Only bootstrap values ≥75% are reported above the branches and posterior probabilities ≥0.95 are indicated as bold branches.

In terms of molecular study, the mtSSU rDNA sequence data were employed to identify the species of *Pertusaria*. This region of DNA has been widely used for the lichen species identification, helps in detection of cryptic species in various groups of lichenized fungi (Schmitt *et al.* 2010, 2012; Parnmen *et al.* 2012*a*; Rangsiruji *et al.* 2016), and hence, it accelerates biodiversity inventories. In this study, the length of the PCR products was *ca.* 800 bp. A matrix of 1,009 unambiguously aligned nucleotide position characters was constructed. The ML analysis yielded a tree with the final optimization likelihood of lnL = -8,286.575.

For the Bayesian analysis, the likelihood parameters in the samples possessed the following mean values (± standard deviation): base frequencies π(A) = 0.371 (±0.0001), π(C) = 0.133 (± 0.001), π(G) = 0.168 (±0.0001), π(T) = 0.328 (± 0.0001), lnL = -8,039.319 (±0.08), and the gamma shape parameter α = 0.215 (±0.001). The resulting tree topologies from both the ML and Bayesian analyses did not show any conflict. Therefore, only the ML tree is shown in Figure 2, with BS ≥75% and PP ≥0.95. Our molecular data demonstrated the polyphyly of the genus *Pertusaria*, and this was conformed with the results of the previous phylogenetic studies (Schmitt *et al.* 2010; Schmitt *et al.* 2012). The six *Pertusaria* species obtained form well-supported monophyletic clades within the *Pertusaria* s.str. and the *Variolaria* groups. Five species which belong to the *Pertusaria* s.str group (BS=100/PP=1.00) include *P. balekensis*, *P. cicatricosa*, *P. denotanda, P. follmaniana* and *P. pertusa*. This group also contains the mtSSU rDNA sequence of the type specimen of *Pertusaria*, *P. pertusa* (Schmitt & Lumbsch 2004). It is interesting to note, however, that our samples of *P. pertusa* are not clustered with the type specimen. *Pertusaria* cf. *violacea* on the other hand, is placed in the *Variolaria* group (BS=99/PP=1.00). This species is similar to *P. violacea*, except that it contains haemathamnolic acid instead of thamnolic acid. More samples of *Pertusaria* cf. *violacea* as well as *P. pertusa* are required to address their species boundaries.

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