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Parmotrema clavuliferum and *P. reticulatum* are independent species

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Parmotrema clavuliferum (Räsänen) Streimann and *P. reticulatum* (Taylor) M. Choisy (Parmeliaceae, lichenized Ascomycotina) have similar morphological characters such as wide lobes with soralia, reticulate-maculate on upper surface of lobes, and marginal cilia. In addition, they both produce salazinic acid and atranorin as major chemical substances. These similar properties found in both species have led to confusion in recognizing them as distinct species; however, *P. clavuliferum* is distinguished from the latter by capitate soralia on short laciniae which are always mottled white and by the production of fatty acid and gyrophoric acid together with salazinic acid. In addition, the most likely phylogenetic tree of these species shows that they are nested in well-supported monophyletic groups. Therefore, it is reasonable to recognize them as two separate species, *P. clavuliferum* and *P. reticulatum*.

Keywords: *Parmotrema clavuliferum*, *Parmotrema reticulatum*, separated species, lichens

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Introduction

Parmotrema clavuliferum (Räsänen) Streimann and *P. reticulatum* (Taylor) M. Choisy were once treated under the genus *Rimelia* (Hale and Fletcher, 1990; Kurokawa, 1991); however, based on molecular phylogenetic studies, Crespo *et al*. (2001) and Blanco *et al*. (2005) declared that *Rimelia* was synonymous with *Parmotrema*. In addition, Divakar *et al*. (2005) treated *P. clavuliferum* as re-synonymous with *Parmotrema reticulatum*.

Parmotrema reticulatum was first described as *Parmelia reticulata* Taylor based on a specimen from Kerry, Ireland (Taylor, 1836). This species is characterized by having sorediate lobes, white reticulate-maculate on the upper surface of the lobes, black lower surfaces; simple to branched rhizines; and the presence of atranorin and salazinic acid. This species is widely distributed throughout tropical and subtropical areas of the world. On the other hand, *Parmotrema clavuliferum* was described by Räsänen (1944) as *Parmelia clavulefera* Räsänen based on a specimen from Tahiti. *Parmotrema clavuliferum* is distinct in having capitate soralia on short laciniae, which are always mottled white on the lower surface, especially below the sorediate lobules. It contains atranorin and salazinic acid as major chemical substances. It is also widely distributed in tropical and subtropical areas of the world.

Parmotrema clavuliferum and *P. reticulatum* have similar morphological and chemical characteristics as shown above. It was because of these common characteristics that Hale and Fletcher (1990) synomized *Parmelia clavulifera* as *R. reticulata* (= *P. reticulatum*). On the other hand, *P. clavuliferum* is clearly distinguished from *P. reticulatum* by capitate soralia on short laciniae, a mottled white lower surface, and the production of fatty acid and gyrophoric acid as accessory substances; and *P. clavuliferum* has been treated as a separated species by many researchers (e.g. Park, 1990; Kurokawa, 1991; Moon, 1999; Moon *et al*., 2000; 2001; Kashiwadani *et al*., 2000; 2002; Kurokawa and Lai, 2001; Elix, 2001; Komine *et al*., 2014 under the genus *Rimelia*; and Streimann, 1986; Barbosa *et al*., 2010; Ohmura *et al*., 2012 under the genus *Parmotrema*).

In a phylogenetic study, Divakar *et al*. (2005) asserted that *P. clavuliferum* and *P. reticulatum* are synonyms because of their morphology, distribution, and formation of a monophyletic group. In contrast, Del-Prado *et al*. (2011) reported that the *Parmotrema reticulatum* - *P. pseudoreticulatum* complex includes several species that remain taxonomically controversial. Del-Prado *et al*. (2011) also reported that *P. reticulatum* (s. lat.) was a polyphyletic group including well-supported clades within it. Although they used molecular analysis on this group, only a few specimens from Eastern Asia were treated.

In this study, the *P. clavuliferum* - *P. reticulatum* complex is revised morphologically, chemically, and phylo-

genetically, based in particular on Korean and Japanese materials, one Portuguese specimen. Sequences of internal transcribed spacer regions of nuclear ribosomal DNA (nu ITS rDNA) and mitochondrial small subunit (mt SSU) were used to analyze its phylogeny.

Materials and Methods

The specimens used for this study were based on the specimens preserved in the herbarium of the NIBR unless otherwise stated. The secondary products of the specimens examined were determined by thin layer chromatography (TLC) using the amended procedures of Culberson and Johnson (1982).

Specimens examined (Voucher specimens): ***P. clavuliferum*. KOREA. Incheon**, Ongjin-gun, Jangbong Island, Bukdo-myoun, Jangbong-1-ri, East-south seaside of Janbong Island, around Ongam Harbor, on rocks, elevation about 17 m, September 21, 2012, K.H. Moon 13488. **Prov. Gyeongsangbuk-do**, Cheongsong-gun, Budong-myeon, Sangui-ri, Mt. Juwang, en route from Daegeon temple to Mt. Janggun-bong via Backryong-am (hermitage), on bark, elevation 270-680 m, October 13, 2013, K.H. Moon 13818; the same locality, on bark of *Taxus cuspidate*, K.H. Moon 13824; Gyongju city, Jinhyon-dong, around Bulguk-sa temple, on bark of *Zelkova* sp., elevation ca. 250 m, October 14, 2013, K.H. Moon 13833; Pohang-shi, Nam-gu, Homigot-myun, Gangsa-ri, beside of R. Ilchul, on rocks, elevation about 20 m. September 15, 2012, K.H. Moon 13443, 13447, 13448 and 13450. **Prov. Gyungsangnam-do**, Tongyoung-shi, Sanyang-eup, Shinjeon-ri, seaside, on rocks, elevation about 5 m. September 12, 2012, K.H. Moon 13383 and 13384; Tongyoung-shi, Bonpyung-dong, around summit area, on rocks, elevation about 450 m, September 12, 2012, K.H. Moon 13397; Gerje-shi, Nambu-myun, Galgot-ri, around the Windy Hill, on rocks, elevation about 100 m, September 13, 2012, K.H. Moon 13428. **Prov. Jeollanam-do**, Goheung-gun, Jeomam-myeoun, St. Palbong-gil, Mt. Palyeoung, around Yuyoung-bong, on bark, elevation 430-490 m, October 17, 2013, K.H. Moon 13854 and 13855; Goheung-gun, Podu-myeoun, Namseoung-ri, en route from Mabok-sa temple to summit area, on bark, elevation 200-500 m, October 20, 2013, K.H. Moon 13920; Goheung-gun, Gwayeok-myeon, Backil-ri, Ils. Backil-do, seaside, on rocks, elevation 2 m, October 20, 2013, K.H. Moon 13971 and 13972; Goheung-gun, Bongrae-myeoun, Yeanae-ri, Ial. Oinaro-do, seaside, on rocks, elevation 5 m, October 20, 2013, K.H. Moon 13989. **Prov. Jeju**, Jeju-shi, Jochon-up, Seonhul-ri, around Mt. Dongbackdong-san, on rocks (lava), elevationa about 180 m, May 24, 2012, K.H. Moon 13026. **JAPAN. Honshu. Prov. Izu (Pref. Shizuoka)**, Izu-city, Mochikoshi, on bark of *Prunus yezoensis*, elevation about 345 m, February 10, 2015, K.H. Moon 14821; Kamo-gun, Ohnabe, Komori shrine, on rocks, elevation about 151 m, February 10, 2015, K.H. Moon 14826; the same locality, on bark of *Podocarpus macrophyllus*, K.H. Moon 14832; Kamo-gun, Minami-izu-cho, Cape Irohzaki, on rocks, elevation about 55 m, February 11, 2015, K.H. Moon 14835; Kamo-gun, Minami-izu-cho, Teishi, Tsukima shrine, on rocks (granite), elevation about 5 m, February 11, 2015, K.H. Moon 14846. **Prov. Ohmi (Pref. Shiga)**, Takashima-city, Otowa, Ohi shrine, on bark of *Cinnamomum camphora*, elevation about 100 m, June 27, 2013, K.H. Moon 13620; Takashima-city, Hata, Hachiman shrine, on rocks, elevation about 185 m, June 27, 2013, K.H. Moon 13626; Takashima-city, Machino-cho, Nishihama, on bark of *Pinus thunbergii*, elevation about 90 m, June 27, 2013, K.H. Moon 13631; Maibara-city, Ueno, Mt. Ibuki, Ibukisan 3-gome, on bark of *Acer* sp., elevation about 770 m, June 28, 2013, K.H. Moon 13640. **Prov. Mikawa (Pref. Gifu)**, Ibi-gun, Ibi-cho, Kasugakawai, Mt. Ibuki, along Ibukisan Drive Way, on bark of *Cornus controversa*, elevation 890 m, June 26, 2013, K.H. Moon 13600. **PORTUGAL.** Sintra area, on mosses, November 30, 2014. H.W. Kwon (K.H. Moon, no. 14854)

***P. reticulatum*. KOREA. Prov. Gangwon.** Hongcheon-gun, Nae-myeoun, Myunggae-ri, Odaesan National Park, Mt. Odae, en route from Naemyeoun Information Center to Pass Duro-ryeong, on rocks along the Geybang stream, elevation 700-900 m, October 17, 2014, K. H. Moon 14669B. **Prov. Gyeongsangbuk-do**, Cheongsong-gun, Budong-myeon, Sangui-ri, Mt. Juwang, en route from Daegeon temple to Mt. Janggun-bong via Backryong-am (hermitage), on bark, elevation 270-680 m, October 13, 2013, K.H. Moon 13794; same locality, on rock with mosses, K.H. Moon 14000; Cheongsong-gun, Budong-myeon, Sangui-ri, Mt. Juwang, en route from Yongyeoun Fall (3rd Fall) to Gumungwangui, on rocks, elevation ca. 380 m, October 12, 2013, K.H. Moon 13741; Pohang-shi, Nam-gu, Homigot-myun, Gangsa-ri, beside of R. Ilchul, on rocks, elevation about 20 m, September 15, 2012, K.H. Moon 13437 and 13449. **Prov. Gyungsangnam-do**, Gerje-shi, Jangsungpo-dong, on bark of *Pinus thunbergii*, elevation about 50 m, September 12, 2012, K.H. Moon 13366. **JAPAN. Honshu. Prov. Izu (Pref. Shizuoka)**, Kamo-gun, Minami-izu-cho, Cape Irohzaki, on rocks, elevation about 55 m, February 11, 2015, K.H. Moon 14834; Kamo-gun, Ohnabe, Komori shrine, on rocks, elevation about 151 m, February 10, 2015, K.H. Moon 14825. **Prov. Ohmi (Pref. Shiga)**, Takashima-city, Otowa, Ohi shrine, on bark of *Cinnamomum camphora*, elevation about 100 m, June 27, 2013, 13618 and 13619; Maibara City, Ibuki, Taka-cho, Ibuki

Shrine, on bark of *Zelkova serrata*, elevation about 180 m, June 28, 2013, K.H. Moon 13638.

***P. tinctorum*. KOREA. Prov. Gyungsangbuk-do**, Pohang-shi, Nam-gu, Homigot-myun, Gangsa-ri, beside of R. Ilchul, on rocks, elevation about 20 m, September 15, 2012, K.H. Moon 13446. **Prov. Jeollanam-do**, Goheung-gun, Gwayeok-myeon, Backil-ri, Ils. Backil-do, seaside, on rock with mosses, elevation 2 m, October 20, 2013, K.H. Moon 13978. **JAPAN. Honshu. Prov. Ohmi (Pref. Shiga)**, Takashima-city, Katsuno, Hiyoshi shrine, on bark of *Cryptomeria japonica*, elevation about 100 m, June 27, 2013, K.H. Moon 13611.

Molecular phylogenetic analyses in this study were based on 45 samples collected in Japan, Korea, and Portugal from 2012 to 2014. The samples included several species of *P. clavuliferum*, *P. reticulatum*, and *P. tinctorum*. The GenBank accession numbers for the species are shown in Table 1.

DNA extraction, PCR, and sequencing: Small samples from freeze-dried specimens were ground with TissueLyser II (Qiagen, Germany). Total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Dilutions of 1 : 10 of the total DNA were used for PCR amplifications of the nu ITS rDNA and mt SSU rDNA regions. Fungal nu ITS rDNA was amplified using the primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White *et al*., 1990), while mt SSU rDNA was amplified using the primers mrSSU1 and mrSSU3R (Zoller *et al*., 1999).

Amplifications were performed in AccuPower PCR tube (Bioneer) with a total mixture volume of 20 μL consisting of 1 μL of extracted DNA solution, 1 μL each of 10 pmol/μL of primer, and 17 μL of deionized sterile water (Bioneer). For high quality PCR amplification, the stepdown PCR method (Hecher and Roux, 1996) was used. The program for the PCR reaction, using a Mastercycler® thermal cycler (Mastercycler ep gradient S; Eppendorf), was three cycles of 95°C for 1 min, 68°C for 1min, and 72°C for 1 min 30 sec; three cycles of 95°C for 1 min, 65°C for 1 min, and 72°C for 1 min 30 sec; four cycles of 95°C for 1 min, 60°C for 1 min, and 72°C 1 min 30 sec; and 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 sec. Amplification of the PCR products was checked electrophoretically with a molecular weight standard (Optima) in 1.0% agarose gel stained with YBR® (Invitrogen). The PCR products were then cleaned using AccuPrep® Purification Kit (Bioneer) according to the manufacturer's instructions.

The cleaned PCR products were determined with the same amplification primers using ABI 3730XL automated sequencer (Applied Biosystems, USA). Partial nu SSU rDNA, including an intron at the end of the 3' (SSU), was removed before the alignment.

Sequence alignment and phylogenetic analysis: The forward and reverse sequences were assembled and checked using BioEdit version 7.2.5 (Hall, 1999). Sequence alignment was performed using Clustal X ver 2.1 (Thompson *et al*., 1997).

Maximum likelihood (ML) analysis, maximum parsimony (MP), and Bayesian Markov Chain Monte Carlo approach (B/MCMC) were conducted. ML analysis was performed using the program RAxML 8 (Stamatakis, 2014). We selected the GTRGAMMA model. For the bootstrap analysis of the ML tree, the "rapid bootstrapping" option with 1000 replicates was conducted.

A maximum parsimony (MP) analysis was also performed using Paup* 4.0b10 (Swofford, 2003). Heuristic searches with 1000 random taxon addition replicates were conducted with the tree-bisection-reconnection (TBR) branch-swapping and MulTrees options in effect, equally weighted characters, and gaps treated as missing data. The bootstrap method (Felsenstein, 1985) was used to estimate the robustness of the clades. Bootstrap values were estimated from 1000 replicates. Random addition sequences and the MulTrees option were in effect when the analyses were performed.

Bayesian inference with the Markov chain Monte Carlo (B/MCMC) method (Larget and Simon, 1999) was also performed using MrBayes ver 3.0b4 (Huelsenbeck and Ronquist, 2001) to assess the level of confidence for relationships revealed by the ML and MP search. The nucleotide substitution model selected with the jModeltest ver. 0.1 (Posada, 2008) was the Tamura-Nei model of nucleotide substitution (Tamura and Nei, 1993) including estimation of invariant sites (TrN + I) with six rate categories for single-genes and combined analysis with its base frequencies A = 0.2946, C = 0.1894, G = 0.2175, T = 0.2986; substitution rate matrix A-C = 1.0000, A-G = 2.7967, A-T = 1.0000, C-G = 1.0000, C-T = 5.4550, and G-T = 1.0000; and proportion of invariable sites = 0.8120. One out of every 100 trees was sampled for a total of 2,000,000 generations with DNA substitution parameters estimated during the search. The first 5,000 trees from 25,000 sampled trees were discarded to avoid trees that might have been sampled prior to convergence of the Markov chains. Separate analyses of the two data sets were performed. Because no conflict was evident, we assembled a two-locus data set consisting of ITS rDNA and mt SSU rDNA.

Results

We generated 45 new nu ITS rDNA sequences and 45 mt SSU sequences for this study (Table 1). After removing alignment sites with gaps, there were 497 sequence sites in nu ITS, 830 sequences in mt SSU. The processed

Table 1. *Parmotrema* species and GenBank accession numbers of sequences used in the DNA analyses. Bold specimens represent specimens for a phylogenetic tree among same alphabet character specimens (i.e. they share identical sequences).

Species	Collection accession no.	Genbank accession no.		Represented specimen
		nu ITS	mt SSU	
P. clavuliferum	**13026**	**KU354435**	**KU354448**	**A**
	13383	KU354436	KU354449	a
	13384	KU354437	KU354450	a
	13397	KU354438	KU354451	a
	13428	**KU354439**	**KU354452**	**C**
	13443	**KM983341**	**KM983365**	**D**
	13447	KM983342	KM983366	c
	13448	KM983343	KM983367	d
	13450	KU354440	KU354453	c
	13488	KM983345	KM983369	a
	13600	KU354441	KU354454	
	13620	KM983348	KM983372	a
	13626	KM983349	KM983373	a
	13631	KM983350	KM983374	
	13640	KM983352	KM983376	c
	13818	KM983355	KM983379	a
	13824	KM983356	KM983380	c
	13833	KM983357	KM983381	a
	13854	KU354442	KU354455	c
	13855	KU354443	KU354456	
	13920	KU354444	KU354457	
	13971	KM983359	KM983383	c
	13972	KM983360	KM983384	c
	13989	KM983361	KM983385	a
	14821	KP942517	KP942524	a
	14826	KP942518	KP942525	a
	14832	KP942519	KP942526	
	14835	KP942520	KP942527	a
	14846	KP942521	KP942528	a
	14854	KU354445	KU354458	
P. reticulatum	**13366**	**KM983339**	**KM983363**	**E**
	13437	KM983340	KM983364	e
	13449	KM983344	KM983368	e
	13618	KM983346	KM983370	
	13619	KM983347	KM983371	
	13638	**KM983351**	**KM983375**	**H**
	13741	**KM983353**	**KM983377**	**G**
	13794	KM983354	KM983378	e
	14000	KM983362	KM983386	
	14669B	KU354446	KU354459	h
	14825	KP942515	KP942522	
	14834	KP942516	KP942523	g
P. tinctorum	13446	KM817766	KM983387	
	13611	**KM817767**	**KM983388**	**F**
	13978	KU354447	KU354460	f

alignments were 1327 nucleotides including 102 variable sites and 90 parsimony-informative sites. The MP analysis of the data matrix resulted in 2402 most parsimonious trees (tree length = 121 steps, CI = 0.909, RI = 0.955). For the Bayesian analysis the LnL value was −2593.67 with a standard deviation of ±0.04.

A 50% majority-rule consensus tree of the Bayesian tree sampling is shown (Fig. 1) with MP and ML bootstrap values. Because ML, MP, and Bayesian analysis trees had the same topology, a single tree is presented here. The tree was rooted by *P. tinctorum* and has four clades, all are strongly supported in both the ML, MP and Bayesian analyses. Because some specimens share identical sequences (Table 1), only a representative sequence was used for phylogenetic analysis.

Clade I forms a well-supported monophyletic group

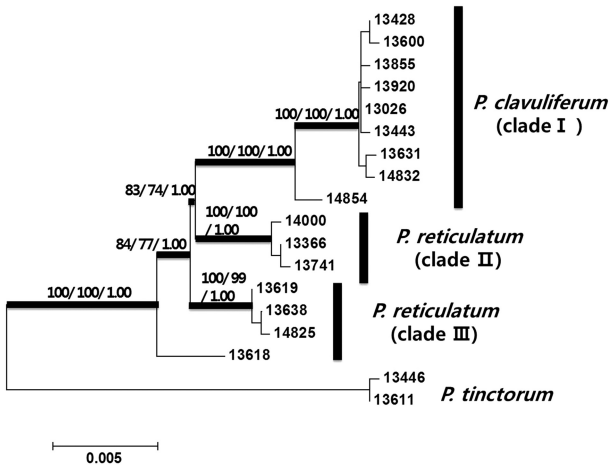

Fig. 1. 50% majority-rule consensus tree of the molecular phylogenetic relationships in *Parmotrema clavuliferum* and *P. reticulatum*, based on 15,000 trees from the B/MCMC tree-sampling procedure and from the dataset of nu ITS and mt SSU sequences. Branches that were strongly supported in all three analyses (i.e., PP $\geq$ 0.95 in the B/MCMC analysis and $\geq$ 70% in both bootstrap values of the ML and MP) are indicated in solid bold line. Numbers on branches indicate bootstrap values of ML (left), MP (middle), and B/MCMC posterior probabilities (right).

(100/100/1.00), including only *P. clavuliferum* specimens. Those specimens of *P. clavuliferum* are from Korea, Japan, and Portugal. Seven *P. reticulatum* specimens formed a different clade (clade II), three specimens of *P. reticulatum* from Japan were assigned to clade III, and one *P. reticulatum* specimen was separated from the others. Both clade II and clade III were strongly supported with ML, MP, and Bayesian values.

The results show that all *P. clavuliferum* specimens form a single clade, while *P. reticulatum* specimens split into clade II, clade III, and clade IV.

Discussion

As discussed above, *P. clavuliferum* is easily distinguished from *P. reticulatum* by the presence of capitate soralia on short laciniae, which always have white lower surfaces; *P. reticulatum* has marginal pulvinate soralia, which are always black on the lower side as discussed by Moon *et al.* (2001), and Spielmann and Marcelli (2009).

Phylogenetically, Divarkar *et al.* (2005) supported the opinion of the synonymy of *P. reticulatum* and *P. clavuliferum*. After that, Del-Prado *et al.* (2011) concluded that the *P. reticulatum* complex (including *P. clavuliferum* and *P. reticulatum*) has eight clusters.

Usually, cryptic species are species that are well defined phylogenetically, but separation of the species is not supported by morphological characters and they show no obvious features to separate them (Crespo and Pérez-Ortega, 2009). Our molecular analysis is based on two independent parts of different genomes (nu ITS and mt SSU rDNA). ITS rDNA and mt SSU are widely used for species confirmation in lichens (Divarkar *et al.*, 2005; Ohmura *et al.*, 2008; Del-prado *et al.*, 2011; Lendemer and Hodkinson, 2012; Aptroot *et al.*, 2014). Especially ITS rDNA is a suitable universal DNA barcode marker for fungi (Schoch *et al.*, 2012).

Parmotrema clavuliferum formed a strongly supported (100/100/1.0 pp) monophyletic lineage, while *P. reticulatum* was segregated into three groups. These latter three groups could be considered as "cryptic species"; however, we cannot find any morphological and chemical differences within these latter three groups. Consequently additional studies are necessary to discriminate these polyphyletic lineages. As well as being distinguished by molecular characters, *P. clavuliferum* is distinguished also by morphological and chemical characters: capitate soralia on short laciniae, which have a mottled white lower surface, and the production of fatty acid and gyrophoric acid. Therefore, it is reasonable to recognize them as two separate species, *P. clavuliferum* and *P. reticulatum*.

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