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Morphological and molecular evidence places *Maronina* into synonymy with *Protoparmelia* (Ascomycota: *Lecanorales*)

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Abstract: The phylogenetic placement of the genus *Maronina* was studied, based chiefly on phenotypic characters such as thallus colour and anatomy, secondary chemistry, the anatomy of the excipulum and the ascus-type. DNA sequence data of mitochondrial and nuclear ribosomal loci from some of the species support the hypothesis that *Maronina* is nested within *Protoparmelia*. Hence, *Maronina* is reduced to synonymy with *Protoparmelia*. Comparison of genetic distances suggests that the two varieties within *M. orientalis* should be regarded as distinct species. Consequently, the new combinations *Protoparmelia australiensis* (Hafellner & R. W. Rogers) Kantvilas *et al.*, *P. corallifera* (Kantvilas & Papong) Kantvilas *et al.*, *P. hesperia* (Kantvilas & Elix) Kantvilas *et al.*, *P. multifera* (Nyl.) Kantvilas *et al.*, and *P. orientalis* (Kantvilas & Papong) Kantvilas *et al.* are proposed.

Key words: lichens, *Lecanoraceae*, *Parmeliaceae*, phylogeny

Introduction

The lichen genus *Maronina* (*Lecanoraceae*) was originally described for two tropical species known from Australia and South America [*M. australiensis* Hafellner & Rogers, *M. multifera* (Nyl.) Hafellner & Rogers] (Hafellner & Rogers 1990). Subsequently, two additional species were described from Australia and Thailand, respectively (Kantvilas & Elix 2007; Kantvilas *et al.* 2010). The genus is characterized by a crustose thallus with a trebouxioid photobiont, lecanorine apothecia, polyspored asci, hyaline, non-septate ascospores and bacilliform conidia (Hafellner & Rogers 1990; McCarthy 2004; Kantvilas & Elix 2007; Kantvilas *et al.* 2010). The asci of the different *Maronina* species are variable but are connected through intermediate types and can be regarded as belonging to the '*Maronina*-type': outer wall intensely amyloid; tholus well-developed, with intensely amyloid flanks and a generally weakly amyloid, fuzzy, masse axiale; ocular chamber poorly developed. A similar ascus type is found in *Protoparmelia*. The paraphyses of *Maronina* are likewise variable and range from robust, capitate and mainly simple, to slender, branched and anastomosing (Kantvilas *et al.* 2010).

When describing the genus, Hafellner & Rogers (1990) pointed out the close relationship of *Maronina* to *Protoparmelia* and noted that "*Maronina* may be regarded as a multispored derivative" (p. 102) of the latter genus. Interestingly, Nylander (1863) had already suggested a placement of *M. multifera* (as *Lecanora multifera* Nyl.) in the *Lecanora badia* group (now *Protoparmelia*).

The phylogenetic placement of *Maronina* and its relationships to *Protoparmelia* were unknown, and previous attempts to obtain DNA sequence data failed. Recently, the first author was able to collect fresh material of both taxa of *Maronina* present in Thailand and new attempts to obtain DNA sequences were successful. We have, however, not been able to obtain DNA sequence data from the type species of *Maronina*. We present the

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results of our phylogenetic analysis based on these sequence data, which, together with the morphological and chemical evidence obtained previously (Kantvilas & Elix 2007; Kantvilas *et al*. 2010), have enabled us to evaluate the phylogenetic relationship of this group of lichenized fungi.

Material and Methods

Morphological and chemical examination

The study is based on collections housed in MSUT, and on duplicates and reference specimens in F and HO. Anatomical and morphological observations and chemical studies were performed as described previously (Kantvilas *et al*. 2010).

Molecular methods

Sequence data of 15 species (with four samples for the two currently accepted varieties of *Maronina orientalis*) were assembled using sequences of the mitochondrial small subunit and nuclear ITS rDNA (Table 1), with newly obtained sequences from *Lecidea rubrocastanea, Maronina orientalis, M. orientalis* var. *corallifera, Protoparmelia capitata* and *P. isidiata*. Seven species of *Parmeliaceae* were included in the data set, since this family has been shown previously to be closely related to *Protoparmelia* and the genus is often classified within this family, albeit with reservations (Arup *et al*. 2007; Crespo *et al*. 2007, 2010; Lumbsch & Huhndorf 2010). We also included *Lecidea rubrocastanea*, another taxon that has been proposed to be closely related to *Parmeliaceae* (Spribille & Printzen 2007) in our sampling. Sample preparation, DNA isolation, PCR and direct sequencing were performed as described previously (Mangold *et al*. 2008; Wirtz *et al*. 2008). Primers for amplification were: mr SSU1 (Zoller *et al*. 1999) and MSU 7 (Zhou & Stanosz 2001) for mtSSU and ITS1F, and ITS4R (Gardes & Bruns 1993) for ITS rDNA. Sequence fragments obtained were assembled with SeqMan 4.03 (DNASTAR) and manually adjusted.

Sequence alignments and phylogenetic analysis

Alignments were done using Clustal W (Thompson *et al*. 1994). Ambiguously aligned regions were removed manually. The alignments were analyzed by maximum likelihood (ML) and a Bayesian approach (B/MCMC). Maximum likelihood analyses were performed using the program GARLI (Zwickl 2006), employing the general time reversible model of nucleotide substitution (Rodriguez *et al*. 1990), including estimation of invariant sites, and assuming a discrete gamma distribution with six rate categories. Bootstrapping (Felsenstein 1985) was performed based on 2000 replicates. The B/MCMC analysis was conducted using the MrBayes 3.1.2 program (Huelsenbeck & Ronquist 2001), using the same substitution model as in the ML analysis. A run with 20 000 000 generations, starting with a random tree and employing 4 simultaneous chains, was executed. Every 100th tree was saved into a file. The first 500 000 generations (i.e. the first 5000 trees) were deleted as the 'burn in' of the chain. We used AWTY (Nylander *et al*. 2007) to compare splits frequencies in the different runs and to plot cumulative split frequencies to ensure that equilibrium was reached. Of the remaining 390 000 trees (1925 000 from each of the parallel runs) a majority rule consensus tree with average branch lengths was calculated using the sumt option of MrBayes. Posterior probabilities were obtained for each clade. Only clades that received bootstrap support equal or above 70% under ML, and posterior probabilities $\geq 0{\cdot}95$ were considered as strongly supported. Phylogenetic trees were visualized using the program Treeview (Page 1996).

We employed alternative hypothesis testing to evaluate whether our data are sufficient to reject *Maronina* as an independent clade separate from *Protoparmelia*. Two different methods were used for the hypothesis testing: 1) Shimodaira-Hasegawa (SH) test (Shimodaira & Hasegawa 1999) and 2) expected likelihood weight (ELW) test following Strimmer & Rambaut (Strimmer & Rambaut 2002). The SH and ELW tests were performed using Tree-PUZZLE 5.2 (Schmidt *et al*. 2002) with the combined data set on a sample of 200 unique trees, the best trees agreeing with the null hypotheses, and the unconstrained ML tree. These trees were inferred in Tree-PUZZLE employing the GTR+I+G nucleotide substitution model. We also estimated genetic distances between the two varieties of *Maronina orientalis* on the ITS data set using Tree-PUZZLE under the GTR+I+G nucleotide substitution model.

Results and Discussion

Phenotypical evidence and phylogenetic analyses

The extent of overlap in many phenotypic characters of *Protoparmelia* and *Maronina* is startling. The similarity of ascus characters is discussed above and by Kantvilas *et al*. (2010). The anatomy of the cupulate excipulum as displayed by the species of *Maronina* and the type of *Protoparmelia, P. badia*, is identical and features a ± continuous layer of algal cells extending beneath the hypothecium (see also Henssen 1995). Also similar are the relatively narrow ascospores, the brownish thallus and the common occurrence of depsidones, such as alectoronic acid. Whereas no single character is unique to both genera, their occurrence in combination supports their close relationship. Indeed the only character that unequivocally separates the

TABLE 1. *Species and specimens used in the present study, with location, reference collection details and GenBank accession numbers. Newly obtained sequences are in bold*

Species name	Collector and Number	Location	GenBank Accession Numbers	
			mtSSU	ITS
Alectoria nigricans	–		GB	GB
Cetraria islandica	–		GB	GB
Cetrelia pseudolivetorum	–		GB	GB
Evernia prunastri	–		GB	GB
Lecidea rubrocastanea	*Spribille* 20749 (GZU)	USA, Montana, near Choteau	**JF821177**	GB
Lecidella meiococca	–		GB	GB
Maronina orientalis var. *corallifera*	*Papong* 7022 (MSUT)	Thailand, Muk Dahan Province, Phu Pha Kam (MSUT)	**JF821173**	**JF821178**
M. orientalis var. *corallifera*	*Papong* 6970 (MSUT)	Thailand, Muk Dahan Province, Phu Pha Kam	**JF821174**	**JF821179**
M. orientalis var. *corallifera*	*Papong* 6983 (MSUT)	Thailand, Muk Dahan Province, Phu Pha Kam	**JF821175**	**JF821180**
M. orientalis var. *corallifera*	*Papong* 6984 (MSUT)	Thailand, Muk Dahan Province, Phu Pha Kam	–	**JF821181**
M. orientalis var. *orientalis*	*Papong* 6922 (MSUT)	Thailand, Muk Dahan Province, Phu Pha Kam	**JF821176**	**JF821182**
Protoparmelia badia 1	–		GB	GB
P. badia 2	–		GB	GB
P. badia 2	–		GB	GB
P. capitata	*Lendemer* 9017 (NY)	USA, Alabama, Baldwin Co.	GB	**JF821183**
P. isidiata	*Lendemer* 9044 (NY)	USA, Alabama, Baldwin Co.	GB	**JF821184**
P. montagnei	–		–	GB
P. memnonia	–		–	GB
Usnea antarctica	–		GB	GB
Vulpicida pinastri	–		GB	GB
Xanthoparmelia semiviridis	–		GB	GB

genera is that of 8-spored versus polyspored asci, a feature seen within several other genera of lichenized fungi such as *Candelariella* and *Scoliciosporum*.

For the molecular analysis, the new sequences of *Maronina orientalis* and *Lecidea rubrocastanea*, *Protoparmelia capitata* and *P. isidiata* were aligned with sequences obtained from GenBank as listed in Table 1. A matrix of 938 unambiguously aligned nucleotide position characters was produced; 722 characters in the alignment were constant. ML analysis yielded a maximum likelihood tree that did not contradict the Bayesian tree topology. In the B/MCMC analysis of the combined data set, the likelihood parameters in the sample had the following mean (Variance): LnL = −3527·015 (0·31), while the likelihood of the ML tree was –3524·758.

Since the topologies of the ML and B/MCMC analyses did not show any strongly supported conflicts, only the tree from the ML analysis is shown (Fig. 1). The clustering of *Maronina orientalis* within *Protoparmelia* is strongly supported (ML-bootstrap support 99%, B/MCMC posterior probability 1·0); the two *Maronina orientalis* varieties form a strongly supported sister-group with a clade consisting of two corticolous tropical *Protoparmelia* species (*P. capitata, P. isidiata*). The clade with *Maronina* and the two corticolous *Protoparmelia* species itself forms a strongly

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supported sister-group relationship with a clade that includes three saxicolous, extratropical *Protoparmelia* species (*P. badia, P. montagnei, P. memnonia*); these three species also form a strongly supported monophyletic group. The *Parmeliaceae* excluding *Protoparmelia* forms a strongly supported monophyletic group. The poor taxon sampling from this group, however, does not allow any further conclusions for the phylogeny within this group and hence these relationships are not discussed further here. A potentially related genus is *Gypsoplaca* that was found by Arup *et al*. (2007) to be as closely related to *Parmeliaceae* s. str. as *Protoparmelia*. However, the genus was not included in this study since no DNA sequence of ITS and mtSSU rDNA is available in Genbank. We aligned the nuLSU rDNA sequences of *Gypsoplaca* and *Protoparmelia* (four sequences: DQ787365, DQ787366, DQ899296, DQ899298) and found them to be quite distinct (data not shown), supporting phenotypical evidence that the two genera are not closely related (Timdal 1990; Poelt & Gärtner 1992).

The alternative hypothesis testing significantly rejects a monophyly of *Protoparmelia* separate from *Maronina* ($P \leq 0{\cdot}001$ in SH and ELW tests).

Since the three samples of *Maronina orientalis* var. *corallifera* showed very low genetic variability, but were quite distinct from the sample of *M. orientalis* s. str. included in the combined analysis, we compared the genetic distances within and between *Maronina* varieties using the ITS data set with four samples for each taxon included (Table 1). The genetic variability within each variety was low (var. *corallifera:* 0·0092 [SD ±0·0041]; var. *orientalis:* 0·0059 [SD ±0·0023]) in comparison with the genetic distance between the two varieties (0·2872 [SD ±0·0087]). The latter is comparable to the distances to *P. capitata* (0·2891) and *P. isidiata* (0·2979). These results indicate that the morphologically different varieties are better distinguished at the species rank.

Within the genus *Protoparmelia*, the genetic distances found were high compared with those of taxa included in *Parmeliaceae* s. str. This may be caused by a younger age of the *Parmeliaceae* s. str. clade or an accelerated evolutionary rate in the *Protoparmelia* clade. With the data at hand we cannot address this issue any further.

Consequently, we propose here to enlarge the circumscription of *Protoparmelia* to include species with polyspored asci that were previously classified in the genus *Maronina*. Although we have not been able to obtain sequences of the type species of *Maronina*, we feel confident, based on phenotypical evidence, that *Maronina* is congeneric with *Protoparmelia*. Given the genetic distances and the morphological differences, we raise *Maronina orientalis* var. *corallifera* to the species level.

The revised classification

Protoparmelia M. Choisy

Bull. Soc. bot. Fr. **76:** 523 (1929); type species: *Protoparmelia badia* (Hoffm.) Hafellner, *Beih. Nova Hedwigia* **79:** 292 (1984).—*Verrucaria badia* Hoffm., *Deutschl. Fl.*, Zweiter Theil (Erlangen) (1796). =*Maronina* Hafellner & R.W. Rogers, *Bibl. Lichenol.* **38:** 100 (1990); type species: *M. australiensis* Hafellner & R.W. Rogers, *Bibl. Lichenol.* **38:** 102 (1990).

Protoparmelia australiensis (Hafellner & R. W. Rogers) Kantvilas, Papong & Lumbsch comb. nov.

MycoBank No.: MB561080

Bas.: *Maronina australiensis* Hafellner & R.W. Rogers, *Bibl. Lichenol.* **38:** 102 (1990).

Protoparmelia corallifera (Kantvilas & Papong) Kantvilas, Papong & Lumbsch comb. et stat. nov.

MycoBank No.: MB561083

Bas.: *Maronina orientalis* var. *corallifera* Kantvilas & Papong, *Lichenologist* **42:** 559 (2010).

Protoparmelia hesperia (Kantvilas & Elix) Kantvilas, Papong & Lumbsch comb. nov.

MycoBank No.: MB561084

Bas.: *Maronina hesperia* Kantvilas & Elix, *Bibl. Lichenol.* **96:** 138 (2007).

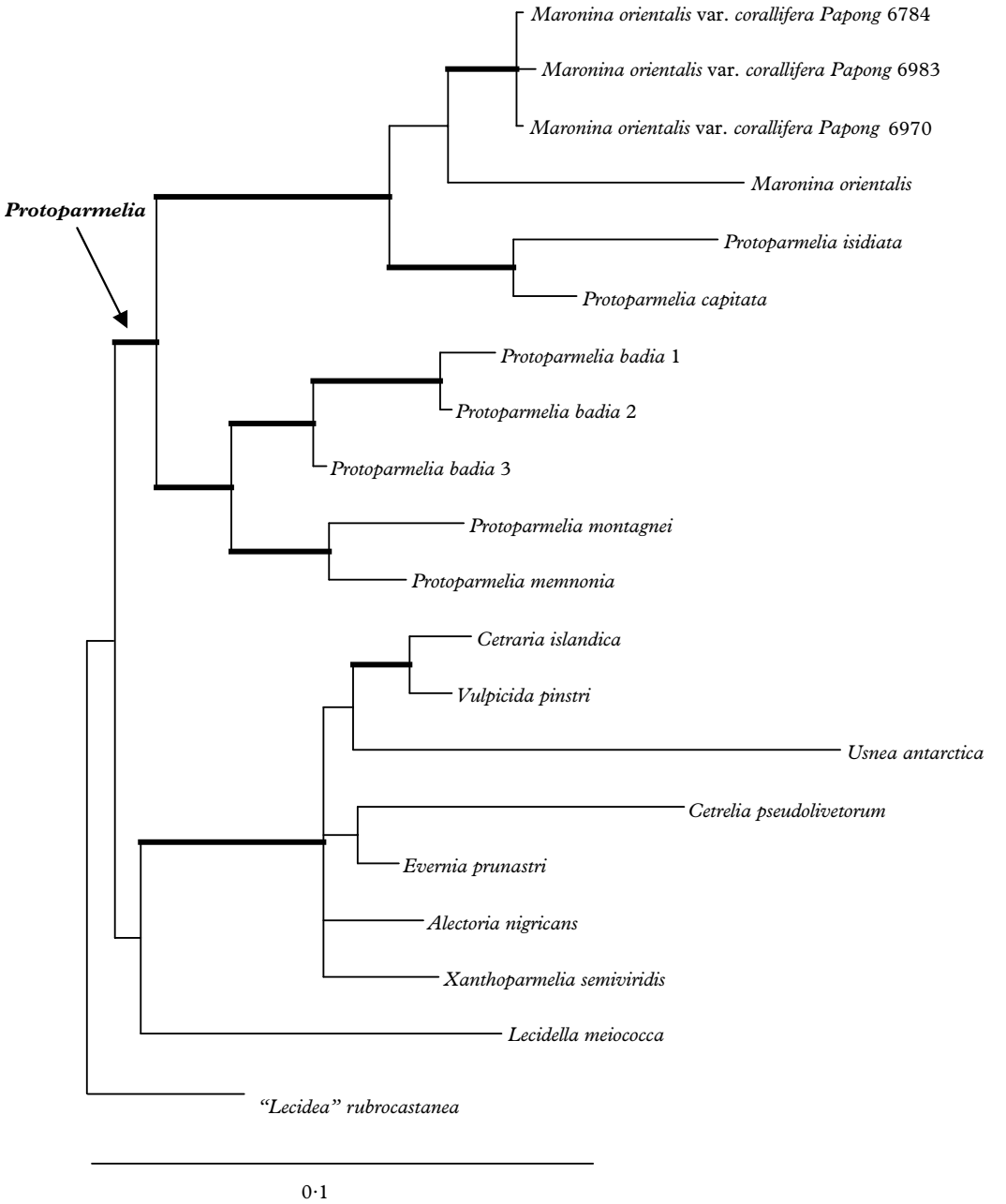

FIG. 1. Optimal tree under maximum likelihood analysis from a concatenated alignment of mtSSU, and ITS DNA sequences showing phylogenetic placement of *Maronina*. Branches in bold received likelihood bootstrap support values above 70%, and posterior probabilities equal or above 0·95. The enlarged genus *Protoparmelia* (including *Maronina*) is marked with an arrow.

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Protoparmelia multifera (Nyl.) Kantvilas, Papong & Lumbsch comb. nov.

MycoBank No.: MB561085

Bas.: *Lecanora multifera* Nyl., *Acta Soc. Sci. Fenn*. **7:** 445 (1863). —*Maronina multifera* (Nyl.) Hafellner & R.W. Rogers, *Bibl. Lichenol*. **38:** 106 (1990).

Protoparmelia orientalis (Kantvilas & Papong) Kantvilas, Papong & Lumbsch comb. nov.

MycoBank No.: MB561086

Bas.: *Maronina orientalis* Kantvilas & Papong, *Lichenologist* **42:** 557 (2010).

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