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Massalongiaceae fam. nov., an overlooked monophyletic group among the cyanobacterial lichens (*Peltigerales*, Lecanoromycetes, Ascomycota)

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Abstract: In this investigation we utilized parsimony and Bayesian analyses of mtSSU and nuLSU rDNA sequence datasets to show that the lichenized ascomycete genera *Leptochidium* and *Polychidium* (formerly classified in *Placynthiaceae*) form a well-supported monophyletic group with *Massalongia* (*Peltigerales*, *Lecanoromycetes*, *Ascomycota*). This group is also supported by morphological characteristics (ascus type, ascoma ontogeny and anatomy), but does not have a formal name on any level. We describe it here as the family *Massalongiaceae*. *Massalongiaceae* is related to a group consisting of *Peltigeraceae-Nephromataceae*, and *Lobariaceae*, but the detailed relationships within this group are not resolved with convincing support.

Key words: ascomycetes, classification, *Lecanorales*, lichens, new taxa, *Peltigerales*, phylogeny, systematics, taxonomy

Introduction

Ascomycetes forming lichen symbioses with cyanobacteria as primary symbionts ('cyanobacterial lichens') are currently classified (Eriksson 2006) in three distinctly unrelated taxonomic groups, *Lichinales* (Schultz *et al*. 2001), the small family *Arctomiaceae* (Lumbsch *et al.* 2005; Wedin *et al*. 2005) and *Peltigerales* (Wiklund & Wedin 2003; Miądlikowska & Lutzoni 2004; for a period usually treated as a suborder within *Lecanorales*, *Peltigerineae*, but recently again often treated on ordinal level).

There are some smaller genera within *Peltigerales*, which have not been satisfactorily classified and which have continuously shifted their position in different families. This is particularly true for *Massalongia*, which for a very long time was included in the *Pannariaceae*, for example in Zahlbruckner (1926), and later referred to *Peltigeraceae* (see Henssen 1963*a*).

Though noting differences in the apothecial ontogeny, Henssen (1963*a*) placed *Massalongia* close to *Peltigera* owing its hemiangiocarpic ascoma development, and later in Henssen & Jahns (1973) it was included in *Peltigerineae*. Henssen (1963*b*) also discussed another of the uncertain genera, *Polychidium*. She pointed out that this genus, in its strict sense, is related to *Massalongia*. She also excluded *Leptochidium* from the genus *Polychidium*, based on thalline characters.

Massalongia, however, deviates from *Peltigeraceae* in ascus structure, in having a distinct apical cap-structure instead of the typical *Peltigeraceae*-tube. This led Hafellner *et al*. (1993) to place *Massalongia* in an artificial group G where the members all had asci with cap-structures. Other genera placed in this artificial group were *Degelia* and *Erioderma* [commonly classified in *Pannariaceae*, which is also supported by the studies of Wiklund & Wedin (2003) and Wedin & Wiklund (2004)], *Spilonema*

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(*Coccocarpiaceae*), and *Leptochidium* and *Polychidium* (*Placynthiaceae*). The classification by Hafellner *et al.* (1993) was unfortunately never properly published, but was still extremely influential among lichenologists. It was to a very large extent based on ascus characteristics, a feature given very high weight in lichen classification since the work of Hafellner (1984).

Recent phylogenetic studies utilizing DNA sequence data have not yet clarified the relationships of *Massalongia*. In the larger studies of Wiklund & Wedin (2003) and Miądlikowska & Lutzoni (2004), *Massalongia* grouped with *Nephroma*, but without significant support. All molecular phylogenies where *Massalongia* has been included have placed it in a group together with *Peltigeraceae*, *Nephromataceae* and *Lobariaceae* (the '*Peltigerineae*' of Miądlikowska & Lutzoni 2004) but the relationship of *Massalongia* within this group was not resolved with high confidence in any of these studies. It is currently treated as a genus 'incertae sedis' in the classification by Eriksson (2006).

The similarities between *Massalongia*, *Leptochidium*, and *Polychidium* in the detailed construction of the asci (Keuk 1977; Hafellner *et al*. 1993), have for some time led us to speculate about the possible close relationships between these genera in spite of their different thalline morphology and anatomy. *Leptochidium* and *Polychidium* are currently classified in *Placynthiaceae*, but this family deviates in ascus type, having asci with a distinct tube, similar to the tube structure present in *Collemataceae* (Rambold & Triebel 1992: 58). *Placynthiaceae* is the sister-group to the gelatinous lichens in *Collemataceae* and is not closely related to *Massalongia* (Wiklund & Wedin 2003).

Here, we test our current working hypothesis on the close relationship of *Massalongia*, *Leptochidium*, and *Polychidium*, which is based on considerable morphological and anatomical similarities. We do this by producing DNA sequence data representing the nuLSU rRNA and mtSSU rRNA genes from these genera, integrating this into the data matrices of Wiklund & Wedin (2003) and performing phylogenetic analyses utilizing parsimony and Bayesian MCMC analyses. We thus hope to contribute to the knowledge of the phylogeny, evolution and classification of these fascinating lichens.

Materials and Methods

DNA extractions, amplification, and sequencing

Total DNA was extracted using the Qiagen DNeasy Plant Mini Kit according to the manufacturer's instructions, with the exception that the DNA was eluted in sterile water. Polymerase chain reaction (PCR) amplifications were performed using Amersham Pharmacia Biotech Ready-To-Go PCR Beads according to the manufacturer's instructions. Fungal nuclear LSU rDNA and the mitochondrial SSU rDNA were amplified with the following settings: initial denaturation 94°C for 5 min, followed by five cycles (94°C for 30 s, 55°C for 30 s and 72°C for 60 s) and finally 30 cycles (94°C for 30 s, 52°C for 30 s, and 72°C for 60 s) with a final extension of 72°C for 300 s. Fungal nuLSU was amplified using combinations of the primers ITS1F (Gardes & Bruns 1993), nu-LSU-155-5′ (Döring *et al.* 2000) and LR3, LR5, and LR6 (http://www.biology.duke.edu/fungi/mycolab/primers.htm), and fungal mtSSU rDNA was amplified using the primers mrSSU1 and mrSSU3R (Zoller *et al.* 1999). The PCR products were sequenced using the DYEnamicET terminator cycle sequencing kit (Amersham Pharmacia Biotech) with the following settings: 28 cycles of 95°C for 20 sec, 50°C for 15 sec and 60°C for 60 sec. The samples were run on an automated sequencer (ABI Prism 377, PE Biosystems).

Sequence alignments

The data matrix is based on revised versions of the matrices utilized by Wiklund & Wedin (2003) and Wedin & Wiklund (2004). Newly produced nuLSU rDNA sequences were aligned into these matrices by hand, utilizing a crude alignment resulting from analysis by the ClustalV algorithm (as implemented in MegAlign v5.03 in the LaserGene 1.66 package; DNASTAR Inc.) as a starting point followed by manual optimization. Major insertions in the nuLSU rDNA were then identified and 381 bp-sites excluded from this alignment. Ambiguously aligned regions in both the nuLSU and mtSSU rDNA alignment were subsequently identified by employing an alignment procedure that uses a linear Hidden Markov Model as implemented in the software SAM (Karplus *et al.* 1998). Regions not aligned with statistical confidence were excluded from analyses.

Phylogenetic analyses

The data-matrices were analysed with parsimony analysis using PAUP* 4.0b10 (Swofford 2002), with the following settings; Heuristic search settings: gaps

are treated as missing data, 1000 random addition sequence replicates, TBR branch swap, steepest descent off, collapse branches if minimum length is 0, multiple trees saved. Uninformative characters and major insertions were identified and excluded from the analyses. Four representatives of the lecanoralean crown-group were used as outgroup.

Parsimony jack-knifing for rapid identification of well-supported monophyletic groups (Farris *et al.* 1997) was performed in PAUP*, with the following settings; Heuristic search settings: 10 random addition replicates; Jack-knife settings: 1000 jack-knife replicates with 'JAC'-emulation, nominal deletion of characters 37%, full heuristic search, retain groups with frequency >70%.

A Bayesian analysis (Huelsenbeck *et al.* 2001) was performed where posterior probabilities were approximated by sampling trees using a MCMC method. MrBayes 3.1.1 (Ronquist & Huelsenbeck 2003) was employed using default settings and assuming a discrete gamma distribution with six rate categories (GTR+I+G) for both genes, allowing both partitions to have their own model parameters; no molecular clock assumed. The model was selected using a likelihood ratio test (Huelsenbeck & Crandall 1997) with Modeltest 3.7 (Posada & Crandall 1998). MCMC sampling was performed with two parallel runs, four chains, one million generations, and every 100th tree saved into a file. The first 6000 saved trees were discarded as burn-in, before a stable equilibrium was reached.

The combinability of data sets was investigated by comparing the amount of conflict in terms of significantly supported nodes in parsimony jack-knife trees based on the single-gene partitions (De Queiroz 1993). Jack-knife support (j) $\geq$70% and posterior probabilities (pp) $\geq$95% were considered significant and are indicated in Fig. 1.

Results

We obtained 8 new nuLSU rDNA and 12 new mtSSU rDNA sequences (Table 1), and the final data matrix was composed of 1771 aligned nucleotide sites, 950 in the nuLSU rDNA and 821 in the mtSSU rDNA partition. Of these 540 were parsimony informative; 240 in the nuLSU rDNA and 300 in the mtSSU rDNA partition. The parsimony analysis resulted in 8 most parsimonious trees of 2215 steps; CI 0.41, RI 0.65, RC 0.26. The parsimony jack-knifing and Bayesian analyses (Fig. 1; j/pp given at the nodes) were not in conflict with each other or with the strict consensus tree from the parsimony analysis, nor were single-gene jack-knife majority rule consensus trees (not shown) in conflict.

Discussion

Massalongia, *Leptochidium* and *Polychidium* form a well-supported monophyletic group, which is also characterized by morphology. They have a similar hemiangiocarpic ascoma ontogeny where only a few 'cover cells' (Henssen 1963*a*) are produced, they have similarly built apothecia, and similar asci with an amyloid apical cap. This group currently lacks a formal name, and we propose to recognize it as the family *Massalongiaceae*.

Massalongiaceae Wedin, P. M. Jørgensen & E. Wiklund fam. nov.

A familia *Peltigeraceae* differt in thallo squamuloso vel fruticuloso, sine acidae lichenosae. Apothecia circularia; asci cum tunicis apicalis amyloides.

Typus: *Massalongia* Körb.

This family differs from the closely related *Peltigeraceae*, *Nephromataceae* and *Lobariaceae* in general morphology and chemistry, and particularly in the ascus structure. *Peltigeraceae* has a most distinctive apical tube, whereas *Nephromataceae* has no amyloid apical structure. *Lobariaceae* has a rather indistinctive amyloid layer which is not comparable to the apical cap in *Massalongiaceae*. These three families are also all large, foliose lichens, normally with complex thalline chemistry. The detailed relationship between *Massalongiaceae* and the other three families in this group is unresolved and needs data from other parts of the genome to be clarified. The mtSSU rDNA data-set alone, however, supports a relationship between *Massalongiaceae* and the *Nephromataceae-Peltigeraceae* clade (j=90, tree not shown). From a morphological perspective, the hemiangiocarpic ascoma ontogeny, in particular, is more similar to the development in *Peltigeraceae* and *Nephromataceae* than the development in *Lobariaceae*, where the ascogonia and paraphysoids are different (Keuck 1977).

Placynthiaceae, where *Leptochidium* and *Polychidium* have been formerly classified, is apparently not closely related to *Massalongiaceae*, and deviates also in ascus type. *Pannariaceae*, where *Massalongia* was classified

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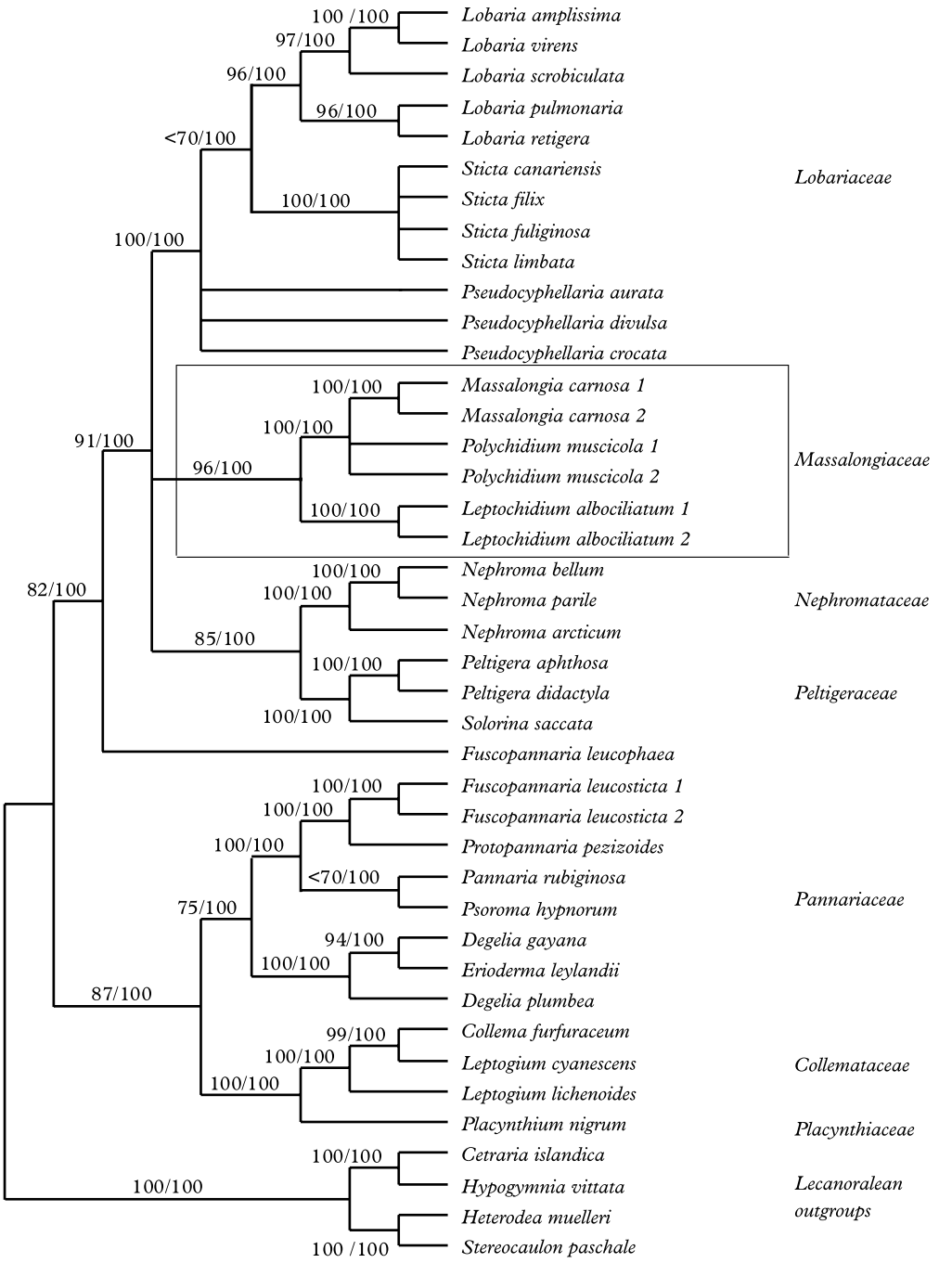

FIG. 1. The phylogenetic hypothesis resulting from our analyses, summarizing groupings found in the 70% majority rule consensus parsimony jack-knifing tree, and groupings obtaining 95% posterior probability or more in the Bayesian MCMC sampling procedure (j/pp). The new family *Massalongiaceae* is indicated with a box.

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TABLE 1. *Sequences used in this investigation, with GenBank accession numbers, and voucher data for newly produced sequences (highlighted in bold typeface)*

Taxon	Family classification	Voucher	mtSSU rDNA	nuLSU rDNA
Cetraria islandica (L.) Ach.	*Parmeliaceae*		AY340486	AY340539
Heterodea muelleri (Hampe) Nyl.	*Cladoniaceae*		AY340492	AY340545
***Hypogymnia vittata* (Ach.) Parrique**	***Parmeliaceae***	**Sweden, *Wedin* 15/7/00 (UPS)**	**DQ900629**	**DQ900637**
Stereocaulon paschale (L.) Hoffm.	*Stereocaulaceae*		AY340525	AY340568
Collema furfuraceum (Arnold) Du Rietz	*Collemataceae*		AY340488	AY340541
***Degelia gayana* (Mont.) Arv. & D. J. Galloway**	***Pannariaceae***	**Chile, *Wedin* 6112 (UPS)**	AY652619	**DQ900638**
Degelia plumbea (Lightf.) P. M. Jørg. & P. James	*Pannariaceae*		AY340491	AY340543
***Erioderma leylandii* (Taylor) Müll. Arg.**	***Pannariaceae***	**Chile, *Wedin* 6003 (UPS)**	AY340492	**DQ900639**
Fuscopannaria leucosticta* (Tuck.) P. J. Jørg. *1	***Pannariaceae***	**USA, *Harris* 33159 (S)**	**DQ900630**	**DQ900640**
Fuscopannaria leucosticta 2	***Pannariaceae***	**USA, *Nordin* 4090 (UPS)**	**DQ900631**	**DQ900641**
***Fuscopannaria leucophaea* (Vahl) P. M. Jørg.**	*?*	**Sweden, *Wedin* 6849 (UPS)**	AY652621	**DQ900642**
Leptochidium albociliatum* (Desm.) M. Choisy *1	***Massalongiaceae***	**Spain, La Gomera, *Hafellner* 34116 (UPS)**	**DQ900633**	**DQ900643**
Leptochidium albociliatum 2	***Massalongiaceae***	**USA, *Tønsberg* 29087 (BG)**	**DQ900632**	**DQ900644**
Leptogium cyanescens (Rabh.) Körb.	*Collemataceae*		AY340469	AF356672
***Leptogium lichenoides* (L.) Zahlbr.**	***Collemataceae***	**Norway, *Wedin* 6206 (UPS)**	AY340498	**DQ900645**
Lobaria amplissima (Scop.) Forssell	*Lobariaceae*		AY340500	AY340546
Lobaria pulmonaria (L.) Hoffm.	*Lobariaceae*		AY340503	AY340548
Lobaria retigera (Bory) Trevis.	*Lobariaceae*		AY340505	AY340550
Lobaria scrobiculata (Scop.) DC.	*Lobariaceae*		AY340506	AY340551
Lobaria virens (With.) J. R. Laundon	*Lobariaceae*		AY340508	AY340553
Massalongia carnosa (Dicks.) Körb. *1*	*Massalongiaceae*		AY340509	AY340554
Massalongia carnosa 2	***Massalongiaceae***	**Norway, *Wedin* 7229 (UPS)**	**DQ900635**	**DQ900646**
Nephroma arcticum (L.) Torss.	*Nephromataceae*		AY124172	AY286828
Nephroma bellum (Spreng.) Tuck.	*Nephromataceae*		AY300895	AY300844
Nephroma parile (Ach.) Ach.	*Nephromataceae*		AY340512	AY340557
Pannaria rubiginosa (Ach.) Bory	*Pannariaceae*		AY340513	AY340558
Peltigera aphthosa (L.) Willd.	*Peltigeraceae*		AY340515	AF286759
Peltigera didactyla (With.) J. R. Laundon	*Peltigeraceae*		AY124164	AF286807
Placynthium nigrum (Huds.) Gray	*Placynthiaceae*		AY340518	AF356674
Polychidium muscicola* (Sw.) Gray *1	***Massalongiaceae***	**Austria, *Obermayer* 8547 (UPS)**	**DQ900634**	**DQ900647**
Polychidium muscicola 2	***Massalongiaceae***	**Norway, *Tønsberg* 32049 (BG)**	**DQ900636**	**DQ900648**
Pseudocyphellaria aurata (Ach.) Vain.	*Lobariaceae*		AY340520	AY340562
Pseudocyphellaria crocata (L.) Vain.	*Lobariaceae*		AY340521	AY340563
Pseudocyphellaria divulsa (Taylor) Imshaug	*Lobariaceae*		AY340522	AY340564
Psoroma hypnorum (Vahl) Gray	*Pannariaceae*		AY340523	AY340565
Solorina saccata (L.) Ach.	*Peltigeraceae*		AY340524	AY424199
Sticta canariensis (Bory) Delise	*Lobariaceae*		AY340527	AY340570
Sticta filix (Sw.) Nyl.	*Lobariaceae*		AY340528	AY340571
Sticta fuliginosa (Hoffm.) Ach.	*Lobariaceae*		AY340529	AY340572
Sticta limbata (Sm.) Ach.	*Lobariaceae*		AY340531	AY340574

for a long time, is likewise not closely related. It is rather similar in also containing squamulose lichens. The ascus structure varies considerably in this group, but *Protopannaria* has amyloid caps that are rather similar to the structure in *Massalongiaceae*. The ascoma development is not hemiangiocarpic in *Pannariaceae*, however, and they sometimes have a secondary thalline ascoma margin. Most of the species also have distinct thalline chemistry.

The general topology resulting from our phylogenetic analyses (Fig. 1) is very similar to the pattern revealed in Wiklund & Wedin (2003), Miądlikowska & Lutzoni (2004) and Wedin & Wiklund (2004). The order *Peltigerales* is composed of two distinct groups. *Lobariaceae*, *Massalongiaceae*, *Nephromataceae*, *Peltigeraceae* and *Fuscopannaria leucophaea* form one of these, corresponding to the *Peltigerineae* in the sense of Miądlikowska & Lutzoni (2004). The *Pannariaceae*, *Collemataceae* and *Placynthiaceae* likewise form a distinct group, corresponding to the *Collematineae sensu* Miądlikowska & Lutzoni (2004). *Pannariaceae* is polyphyletic, as suggested by Ekman & Jørgensen (2002) and Wedin & Wiklund (2004). *Fuscopannaria leucophaea* is not closely related to the type of *Fuscopannaria*, which is included here (*F. leucosticta*), and should be excluded from *Pannariaceae*.

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