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Vahliellaceae, a new family of cyanobacterial lichens (*Peltigerales*, Ascomycetes)

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Abstract: The recently described genus *Vahliella* (*Peltigerales*, Ascomycetes) has repeatedly appeared outside the *Pannariaceae* in molecular phylogenies. Here we include data from additional species of the genus and utilize mtSSU rDNA and RPB1 sequences to confirm its placement as the sister to a group consisting of *Lobariaceae*, *Massalongiaceae*, *Nephromataceae* and *Peltigeraceae*, in the *Peltigerales*. The new family *Vahliellaceae* Wedin, P. M. Jørg. & S. Ekman is described for the genus, and its morphological characteristics are briefly discussed.

Key words: classification, fungi, Lecanoromycetes, phylogeny

Introduction

Most fungi forming symbioses with cyanobacteria ('cyanolichens') are currently classified in the order *Peltigerales* (Lecanoromycetes), a group comprising a number of large and conspicuous macrolichen families, including the *Pannariaceae* (Tehler & Wedin 2008; Lumbsch & Huhndorf 2010). The *Pannariaceae* at present contains *c*. 20 genera (Jørgensen 2003, 2008; Passo *et al*. 2008), two of which are *Fuscopannaria* and *Vahliella*.

Fuscopannaria leucophaea (Vahl) P. M. Jørg. and related species were recognized as a distinct subgenus, *Fuscopannaria* subgenus *Micropannaria*, by Jørgensen (1994). The members differ from the subgenus *Fuscopannaria* [type *F. leucosticta* (Tuck.) P. M. Jørg.] mainly in ascus characteristics, having an apical amyloid internal sheet instead of an amyloid tube structure, but also by lacking secondary compounds by TLC, by lacking a distinct epispore, and by having irregularly developed thalline ascoma margins. In several recent phylogenetic investigations of cyanobacterial lichens, samples of *Fuscopannaria leucophaea* appear not only outside *Fuscopannaria* in the strict sense but also outside a monophyletic *Pannariaceae* (Ekman & Jørgensen 2002; Wedin & Wiklund 2004; Wedin *et al*. 2007). This led Jørgensen (2008) to describe the new genus *Vahliella* for *Fuscopannaria* subgenus *Micropannaria*, including eight very closely related species. Jørgensen (2008) hesitated to exclude *Vahliella* from *Pannariaceae* until further studies had confirmed this placement. A much enlarged study of the *Peltigerales* based on three molecular markers and covering many more taxa (Wedin *et al*. 2009) resulted in a similar topology as previous studies regarding the placement of *Vahliella*, but did not add further *Vahliella* samples. Wedin *et al*. (2009) concluded that additional samples and species of *Vahliella* should be included in future analyses to test this relationship further. They also concluded that if such investigations confirmed that *Vahliella* was the sister group to the rest of *Peltigerales* suborder *Peltigerineae sensu* Miądlikowska & Lutzoni (2004), then a new family needed to be described. A study including another *F. leucophaea* sample (Högnabba *et al*. 2009) supported the conclusion that *Vahliella* did not belong to the *Pannariaceae* implicitly, but the authors did not discuss it. Here, we provide an extended study of this problem.

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We have increased the sampling of taxa in *Vahliella*, and test the placement of the genus in the *Peltigerales* to see if a new name at family level is needed as indicated by the placement in earlier investigations.

Material and Methods

DNA extractions, amplification, and sequencing

DNA extractions, amplification and sequencing of new sequences follow the methods and settings described in Wedin *et al.* (2009). The markers selected were the mitochondrial SSU rDNA (mtSSU rDNA), and the gene coding for the largest subunit of RNA polymerase II (RPB1). These markers have proved useful in resolving phylogenetic relationships in the group, and to produce congruent results, in our earlier studies of cyanolichens in the *Peltigerales*.

Sequence alignment and phylogenetic analyses

New protein coding RPB1 sequences were unambiguously aligned to the alignment used by Wedin *et al.* (2009). This alignment contained very few gaps (four internal amino acid alignment sites) and most of the variation was found to be synonymous. mtSSU rDNA sequences were aligned using the Q-INS-i algorithm (Katoh & Toh 2008*a*) of the multiple alignment software MAFFT version 6.611 (Katoh *et al.* 2002; Katoh & Toh 2008*b*). The gap opening cost was set to 1·53 and the offset to 0 (i.e., default parameters). Ambiguous alignment in the mtSSU rDNA was identified and removed using Gblocks version 0.91b (Castresana 2000) with the relaxed condition parameters suggested by Talavera & Castresana (2007).

Phylogenetic analyses

To estimate dataset incongruence between the single-gene matrices the markers were analyzed separately by maximum parsimony bootstrapping and compared. Incongruence was defined as high bootstrap support ($\geq$ 70 %; Hillis & Bull 1993) for incompatible associations (i.e., groups in conflict with each other between the single-gene analyses). No incongruence was identified and we consequently proceeded with a phylogenetic analysis of the concatenated data.

We used Bayesian inference of phylogeny as implemented in the software BayesPhylogenies 1.1 (Pagel & Meade 2004). We used a combination of a pattern heterogeneity mixture model (Pagel & Meade 2004, 2005) and a branch-length set mixture model (Meade & Pagel 2008; Pagel & Meade 2008) in order to test if the phylogenetic position of *Vahliella* reported by Wedin *et al.* (2009) could be an artefact from improper data partitioning or unaccounted for by heterotachy (rate variation across a tree). For both types of model, we used reversible-jump Markov chain Monte Carlo (MCMC) to reduce parameter redundancy and to allow *a priori* ignorance about the number of substitution rate matrices, the specific partitioning of the data, as well as the number of branch length sets. We allowed an unspecified number of independently parameterized general time-reversible (GTR) rate matrices coupled with gamma (Γ) distributed rate heterogeneity across sites modelled as four discrete categories. The maximum number of branch length sets allowed was two. BayesPhylogenies operates with fixed priors that cannot be modified by the user. Priors were accounted for by Pagel & Meade (2008), except that the prior on branch lengths in the publicly available version of BayesPhylogenies 1.1 is exponentially distributed with mean 1 (A. Meade, personal communication). We performed six identical MCMC runs without Metropolis-coupling. Each run was 20 million generations in length, and trees and parameters were sampled every 1000 generations. The first half of each converged run was removed as burn-in. The marginal likelihood of the data was calculated using importance sampling (Newton & Raftery 1994; Suchard *et al.* 2003) with 10 000 bootstrap replicates, as implemented in Tracer 1.4 (Rambaut & Drummond 2007).

We further used maximum parsimony and parsimony bootstrap, performed using PAUP* 4.0b10 (Swofford 2002). Heuristic search settings: gaps treated as 'missing', 1000 random addition sequence replicates, TBR branch swap, steepest descent off, collapse branches if minimum length is 0, MulTrees on. Parsimony bootstrap: bootstrap settings: 1000 bootstrap replicates, full heuristic search, retain groups with frequency >50%; heuristic search settings: as above, but 10 random addition replicates. Uninformative characters were excluded from the analyses.

Results

Taxon sampling and DNA sequencing

We sampled taxa representing most families currently belonging in *Peltigerales* (*Collemataceae*, *Lobariaceae*, *Massalongiaceae*, *Nephromataceae*, *Pannariaceae*, *Peltigeraceae*, and *Placynthiaceae*). We obtained 13 new mtSSU rDNA and 13 RPB1 sequences (Table 1). The genus *Solorina* (*Peltigeraceae*) was represented by an mtSSU rDNA sequence of *Solorina saccata* (L.) Ach. and an RPB1 sequence of *S. crocea* (L.) Ach. The sequences were aligned together with sequences from our earlier studies, deposited in GenBank. *Lecidea fuscoatra* (L.) Ach. functioned as outgroup to root the tree.

Phylogenetic analyses

We compiled matrices including 25 taxa. The MAFFT alignment of mtSSU rDNA

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TABLE 1. *Specimens and sequences included in this study (newly produced sequences in bold)*

Species	Specimen data for newly produced sequences	mtSSU rDNA	RBP1
Lecidea fuscoatra		AY756401	AY756408
L. silacea		AY756402	AY756409
Collema nigrescens		GQ259020	GQ259049
Fuscopannaria leucosticta		DQ900631	GQ259055
F. praetermissa		GQ259026	GQ259056
Leptogium lichenoides		GQ259032	DQ917414
Lobaria pulmonaria		AY340503	GQ259068
Massalongia carnosa		AY340509	GQ259071
Nephroma parile		AY340512	GQ259072
Pannaria rubiginosa		AY340513	GQ259073
Parmeliella triptophylla		AY652623	GQ259075
P. miradorensis	Spain (La Gomera), *Perez & Hernandez* July 2007 (BG)	**HQ268592**	**HQ258591**
Peltigera aphthosa		AY340515	DQ915598
Placynthium nigrum		AY340518	GQ259079
Protopannaria pezizoides		AY340519	GQ259081
Pseudocyphellaria aurata		AY340520	GQ259082
Psoroma hypnorum		AY340523	GQ259085
Solorina crocea		–	DQ973066
S. saccata		AY340524	–
Sticta fuliginosa		AY340529	GQ259089
Vahliella californica	Canada, *Tønsberg* 26316 & *Goward* (BG)	**HQ268594**	**HQ268593**
V. leucophaea		AY652621	GQ259090
V. leucophaea SE460	Norway, *Tønsberg* 31574 (BG)	**HQ268596**	**HQ268595**
V. leucophaea MWE26	Sweden, *Wedin* 8131 (S)	**HQ268598**	**HQ268597**
V. leucophaea MWE84	USA, *Nash* 39445 (ASU)	**HQ268600**	**HQ268599**
V. saubinetii	Croatia, *Nordin* 2794 (UPS)	**HQ268602**	**HQ268601**

was 1100 nucleotides of which 390 were suggested for removal by Gblocks. RPB1 had no ambiguous sites. The concatenated matrix included 1432 characters after removal of ambiguous sites (RPB1 722 and mtSSU rDNA 710 nucleotides) of which 670 were variable and 502 parsimony-informative. Among the 383 variable characters in the RPB1 partition, 328 were parsimony-informative, and 222 out of 287 variable characters in the mtSSU rDNA were parsimony-informative.

In the Bayesian analyses, three out of six MCMC runs were found to suffer from poor mixing (as revealed by low effective sample sizes) or convergence problems (as revealed by biologically unrealistic parameter values), and were consequently excluded from further analysis. The three other MCMC runs were found to display excellent mixing properties and seemed to have converged on the same likelihood and parameter values. The samples drawn from these three runs were pooled and used for further analyses. Two independent GTR+dΓ4 models were fitted to the data with 100 % probability. The 95 % highest posterior density (a Bayesian equivalent of the most frequent confidence interval) of the number of branches requiring a second branch length ranged from zero to ten, the median being 3·9. No individual branch in the MCMC tree sample had a probability exceeding 50% of having a second length. The majority-rule consensus tree based on 30 000 trees is presented in Fig. 1. The MP analyses resulted in 6 most parsimonious trees of 1927 steps, CI = 43 and RI = 59. Groupings significantly supported in both analyses ($\geq$95 Bayesian PP and $\geq$70% MP-BS) are indicated with thick internodes.

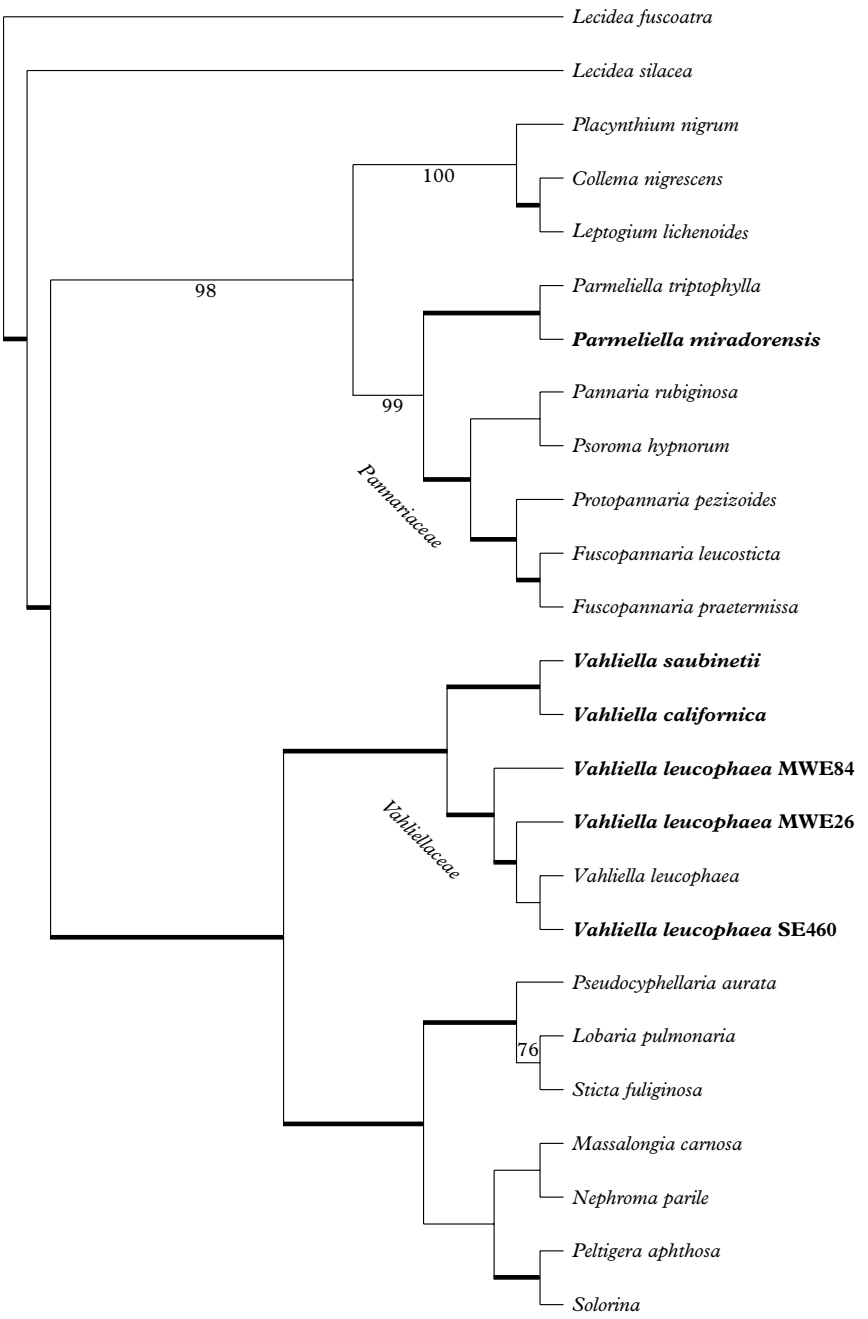

FIG. 1. Phylogenetic relationships among the *Peltigerales* resulting from Bayesian MCMC analysis (majority-rule consensus tree based on 30 000 trees, ln likelihood = −10648.661±0.169(SE)) based on combined mtSSU rDNA and RPB1 data sets. Samples newly sequenced here are indicated in bold type. Thick internodes received significant support in both types of analysis conducted (Bayesian inference PP ≥95%, MP-BP ≥70%). Nodes supported in the Bayesian inference only are indicated with the PP value below the internode, and one node supported in MP-BS only is indicated with the BS value above the internode. Branch lengths are not indicated, as two distinct branch length sets were allowed.

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Discussion

The phylogenies and the well-supported groups recovered correspond very well with topologies retrieved in earlier studies. All samples of *Vahliella* form one distinct and well-supported monophyletic group (Fig. 1), which is the sister group to one consisting of *Lobariaceae* (*Lobaria*, *Sticta* and *Pseudocyphellaria*), *Massalongiaceae*, *Nephromataceae*, and *Peltigeraceae* (*Peltigera* and *Solorina*). This topology is unlikely to be an artefact caused by poor model assumptions, since our model included mixtures on substitution rate matrices as well as branch lengths. Our study thus confirms that *Vahliella* is not closely related to the morphologically similar *Pannariaceae*, where it is currently classified.

Vahliellaceae Wedin, P. M. Jørg. & S. Ekman, fam. nov.

Differt a familia *Pannariacearum* in absentia acidorum lichenicorum, excipulis thallinis male evolutis et ascis cum stratis amyloideis apicalibus.

Typus: *Vahliella* P. M. Jørg.

Vahliellaceae differs from the related *Peltigeraceae*, *Nephromataceae*, and *Lobariaceae* in general morphology and chemistry, and in ascus structure. *Peltigeraceae* has a very distinctive apical tube, whereas *Nephromataceae* has no amyloid apical structure. *Lobariaceae* has a rather indistinct amyloid layer, which is not very similar to the structure in *Vahliellaceae*. These three families are all large, foliose lichens, normally with a complex thalline chemistry. *Vahliellaceae* can be somewhat similar to squamulose members of *Massalongiaceae*, which also have a somewhat similar ascus structure. Species of *Vahliellaceae*, however, usually have a distinct bluish black hypothallus that the *Massalongiaceae* lack. For a detailed discussion of the species of *Vahliella*, generic characteristics and morphological affinities, see Jørgensen (1994, 2008).

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