Expanded taxon sampling disentangles evolutionary relationships and reveals a new family in Peltigerales (Lecanoromycetidae, Ascomycota)

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Abstract Lichens that incorporate cyanobacterial symbionts (cyanolichens) are an ecologically key group of species used as biomonitors at all latitudes. Cyanolichen evolution is however based on intense studies of few keystone species and the bulk of species diversity, especially of small species in cold climates, has yet to be accounted for in phylogenetic studies. We assembled an expanded data set including members of all nine currently accepted Peltigeralean families as well as hitherto undersampled representatives of small, radially symmetrical, placodioid cyanolichen genera from the Northern and Southern Hemispheres. Bayesian and maximum likelihood consensus trees from our multilocus analyses (nuSSU, nuLSU and mtSSU) recovered the genera *Koerberia*, *Vestergrenopsis* and *Steinera* as a new, fully supported, family-level clade within the Peltigerales. This clade is further supported by *a posteriori* morphological analysis and we describe it here as the new family Koerberiaceae. The recently described and physiognomically similar genus *Steineropsis*, by contrast, is recovered as sister to *Protopannaria* in the Pannariaceae (Collematineae). Previous analyses have recovered strong monophyletic groups around Pannariaceae, Lobariaceae and Peltigeraceae. We discuss in detail the phylogenetic relationships of all these taxa, provide a pan-Peltigeralean overview of phenotypic characteristics and illustrate all major ascus apical structures. Our topology provides strong backbone support for the sister relationship of Peltigerineae to Collematineae as well as for most currently recognized families of the Peltigerales. The following new combinations are made: *Steinera symptychia* (Tuck.) T. Sprib. & Muggia, and *Vestergrenopsis sonomensis* (Tuck.) T. Sprib. & Muggia.

Introduction

Cyanolichens, lichens in which the primary photosynthetic partner is a cyanobacterium, have long fascinated ecologists and taxonomists alike. Cyanolichens are more sensitive to air pollution than many lichens with green algal photobionts and thus include some of the most important species used as early-warning biomonitors at all latitudes, as well as some of the highest priority species for conservation (Wolseley 1991; Gauslaa 1995; Goward and Arsenault 2000; Richardson and Cameron 2004). However, compared to other macrolichen groups such as the intensely studied family Parmeliaceae (e.g. Crespo et al. 2010, 2011), comparatively few studies have been dedicated to evolutionary relationships of cyanolichens. Even before the recognition of the symbiotic nature of lichens, taxonomists in the mid-19th century held the non-stratified foliose cyanolichens to be separate from other lichen groups (as Lichenes Gelatinosae, or Phycolichenes; see Lange and Wagenitz 2003). Stratified species with cyanobacteria, however, including well known genera such as *Sticta* and *Pannaria*, were often classified alongside *Parmelia* species as leafy macrolichens, the "Lichenes Phylloblasti" of e.g. Körber (1865). The recognition of embedded blue-green cells as cyanobacteria (Schwendener 1869) opened the door for

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photobiont-based classifications that eventually saw all lichens with cyanobacteria united under a single grouping, called the "Cyanophili" by Reinke (1896). Reinke's classification and detailed diagnoses of individual genera one year prior (Reinke 1895) laid the groundwork for the description of the Peltigerales by Watson (1929) and thereby the scaffolding for the modern taxonomy of cyanolichens.

The term "cyanolichen" was probably introduced by Paul Bubrick in his work in the 1970s on the cyanobacteria-rich Lichinaceae of the Negev Desert (Bubrick and Galun 1984) and quickly came to be applied to all of the species considered by Reinke (1896) to belong to the "Cyanophili". It is now generally accepted however that cyanolichens independently arose at least three times in the course of evolution. The mostly dryland-dwelling Lichinaceae on which Bubrick worked are now assigned to their own order (Lichinales; Eriksson and Hawksworth 1986) and class (Lichinomycetes) outside the Lecanoromycetes (Reeb et al. 2004; Miadlikowska et al. 2006), while a small group earlier believed to belong in Peltigerales (Miadlikowska and Lutzoni 2004), the Arctomiaceae, has since been recovered in the Ostropomycetidae (Lumbsch et al. 2005; Schmitt et al. 2009, 2010). The remaining core of cyanolichens, including the best known groups of terricolous and epiphytic species, was retained by Miadlikowska and Lutzoni (2004) in the order Peltigerales of the Lecanoromycetes. Concepts of evolutionary relationships within Peltigerales, first postulated in crude cladograms by Reinke (1896), took shape in a scheme of families treated in detail e.g. by Henssen and Jahns (1974). Miadlikowska and Lutzoni (2004) chronicled the 20th century evolution in family concepts within Peltigerales and recognized eight families. One of these (Solorinaceae) is now accepted to be part of the Peltigeraceae (Lumbsch and Huhndorf 2010). With the recent description of two new families by Wedin et al. (2007, 2011) this now makes for nine accepted families in the order.

Molecular studies of evolutionary relationships and testing of monophyly of genera and families in the Peltigerales have until now focused on species-to family-level questions in major, well known groups (Miadlikowska and Lutzoni 2000; Lohtander et al. 2002; Ekman and Jørgensen 2002; Goffinet et al. 2003; Miadlikowska et al. 2003; Wedin et al. 2007, 2011; Otálora et al. 2010). Wiklund and Wedin (2003) and Miadlikowska and Lutzoni (2004) were the first to produce broad-based phylogenies of Peltigerales. These have been followed by broad overviews of the relationships between major families by Wedin et al. (2009) and Muggia et al. (2011). Only a small proportion of the described genera of cyanolichens have been included in multilocus phylogenetic studies, but when taxon sampling has been broad, results have often been surprising and contradict past classifications. For instance, recent studies have shown that strikingly similar species arose independently in each of the two suborders of Peltigerales (Peltigerineae and Collematineae) in the case of the genus pairs *Fuscopannaria*/*Vahliella* (Wedin et al. 2011) and *Polychidium*/*Leptogidium* (Muggia et al. 2011). To date, only about two thirds of the 45 putative Peltigeralean genera have been sampled for molecular studies (based on a sample of genera listed by Lumbsch and Huhndorf 2010 and checked against GenBank). Given the wide array of photobiont-mycobiont combinations and diverse ecologies, it is easy to see how small Peltigerales represent a large and unexplored resource for molecular studies of the evolution of cyanolichens.

The present study is the product of a Rube-Goldberg-like series of events and questions that was set off by the discovery of a new genus of placodioid cyanolichens on cold, wet rock surfaces in maritime Alaska, and subsequently named *Steineropsis* (Spribille et al. 2010). Our original aim of contrasting *Steineropsis* to the Subantarctic genus *Steinera* soon grew to include comparison of other genera of temperate to subpolar, small, brown cyanolichens, few of which had ever had been sampled for a molecular phylogeny. In the process, we obtained one of the broadest high level DNA taxon samples to date in cyanolichens, strengthening the backbone of the Peltigerineae, and documenting at least two further separate cases of convergent body plan evolution. One of the groups central to this study clearly constitutes a morphologically well supported but hitherto overlooked family-level group. The purpose of the present paper is to summarize our most recent findings in the broader context of evolutionary relationships within the Peltigerales.

Materials and methods

Taxon sampling In order to study the phylogenetic position of the genera *Koerberia*, *Steinera*, *Steineropsis* and *Vestergrenopsis* we sampled 75 taxa (Supplementary Table S1) representing nine families currently recognized as constituting the Peltigerales (Wedin et al. 2007, 2009, 2011; Muggia et al. 2011). We generated new sequence data for a total of 12 taxa, including the genera named above and the species *Coccocarpia palmicola*, *Pannaria hookeri*, *Santessoniella saximontana*, a probably undescribed *Fuscopannaria* sp. not considered in previous studies, and a new specimen of *Vahliella leucophaea* (Table 1). Cited material is in the herbaria cited following abbreviations used in Index Herbariorum, with the exception of KLGO, which represents the herbarium of Klondike Gold Rush National Historical Park in Skagway, Alaska.

DNA extraction, amplification and sequencing DNA was extracted according to Cubero et al. (1999). The phylogenetic affiliation of the lichen mycobionts was studied with

Table 1 Newly analysed specimens included in the phylogenetic inference of Fig. 1. Dashes stand for absence of sequence data

Species	Geographic origin/voucher	Isolate	GenBank accession number		
			nuLSU	nuSSU	mtSSU
Coccocarpia palmicola	**Chile.** Region X, Senda Darwin Biological Research Station, *Wheeler & Nelson* 103 (CONC)	L873	JX464116	JX464148	-
Fuscopannaria sp.	**Canada.** British Columbia, Cassiar Highway, Dease River area, *Spribille* 25117 (CANL)	L854	JX464120	JX464152	JX464136
Koerberia biformis	**U.S.A.** Arizona, Gila County, along Ellison Creek, *Nash* 43229 (GZU)	L851	-	-	JX464132
Koerberia biformis	**Croatia.** Raba, along the road between Dubrovnik and Opuzen, *Tønsberg* 36505 (BG)	L860	JX464117	JX464149	JX464133
Pannaria hookeri	**U.S.A.** Alaska, White Pass, *Spribille* 29292 (KLGO)	L896	JX464118	JX464150	JX464134
Santessoniella saximontana	**Canada.** British Columbia, Albert River, *Spribille* 21173 (GZU, isotype)	L761	JX464119	JX464151	JX464135
Steinera radiata	**Chile.** Region X, Parqua Nacional Hornopiren, slopes of Volcan Yates, *Wheeler & Nelson* 2473a (CONC)	L874	JX464121	JX464153	JX464137
Steinera symptychia	**Chile.** Region X, Parque Pumalin, 3 km S of Cascada escondida trail, *Wheeler & Nelson* 3169 (CONC)	L872	JX464122	JX464154	JX464138
Steineropsis alaskana	**U.S.A.** Alaska, west side of White Pass, *Spribille* 26955 (KLGO)	L769	JX464123	JX464155	JX464139
Steineropsis alaskana	**U.S.A.** Alaska, White Pass, *Spribille* 26809 (US, holotype)	L809	JX464124	JX464156	JX464140
Vahliella leucophaea	**Canada**. British Columbia, Selkirk Mountains, Incompleux Canyon, *Spribille* 15422 (CANL)	L766	JX464125	JX464157	JX464141
Vestergrenopsis elaeina	**U.S.A.** Alaska, White Pass, *Spribille* 24525 (KLGO)	L757	JX464126	JX464158	JX464142
Vestergrenopsis isidiata	**U.S.A.** Alaska, White Pass, *Spribille* 29249 (KLGO)	L756	JX464127	JX464159	JX464143
Vestergrenopsis isidiata	**U.S.A.** Alaska, Chilkoot Pass, *Spribille* 29177 (KLGO)	L759	JX464128	JX464160	JX464144
Vestergrenopsis isidiata	**U.S.A.** Alaska, Chilkoot Pass, *Spribille* 29090 (KLGO)	L760	JX464129	JX464161	JX464145
Vestergrenopsis sonomensis	**U.S.A.** Montana, Sanders Co., Cabinet Mtns., Goat Rocks, *Spribille* 21008 (GZU)	L808	JX464130	JX464162	JX464146
Vestergrenopsis sonomensis	**U.S.A.** Washington, Okanogan County, Aeneas Creek, *Björk* 19079 (UBC)	L946	JX464131	JX464163	JX464147

sequences of the nuclear large, partial nuclear small and mitochondrial small ribosomal subunits (nuLSU, nuSSU and mtSSU). The nuLSU fragment was obtained in two pieces using primers ITS1F (Gardes and Bruns 1993) and LR5 for the first half, and LR7 (Vilgalys and Hester 1990) and LR3R for the second (http://www.biology.duke.edu/fungi/mycolab/primers.htm). The nuSSU was amplified using nuSSU0072 and nuSSU0852 (Gargas and Taylor 1992), and mtSSU was obtained with mtSSU1 and mtSSU3R (Zoller et al. 1999). PCR conditions were as in previous studies (Muggia et al. 2010, 2011). Complementary strands were sequenced, and sequences were run by Macrogen, Inc. (Korea) and assembled and edited in BioEdit (Hall 1999).

Alignment and phylogenetic analysis In order to assess heterogeneity in phylogenetic signal between the different genetic markers (Buckley et al. 2002), we first analysed each locus separately and second we combined them in a multi-locus alignment, as performed in previous studies (Kauff and Lutzoni 2002; Miadlikowska et al. 2006). The concatenation of the three genetic markers was possible as no incongruence was found among the phylogenetic hypothesis obtained for each individual locus. The combined dataset was used to infer the phylogenetic relationships of the selected taxa using both Bayesian and maximum likelihood

(ML) approaches. The optimal nucleotide substitution model was estimated with the program MrModeltest v3.7. The Bayesian Markov Chain Monte Carlo (B/MCMC) algorithm of MrBayes 3.1.2 (Huelsenbeck and Ronquist 2003; Ronquist et al. 2005) was performed with the General Time Reversible substitution model (Rodriguez et al. 1990) with estimation of invariant sites and assuming a gamma distribution with four categories (GTR+I+Γ). The algorithm ran with six chains simultaneously, each initiated with a random tree, for 10 million generations, and trees were sampled every 100th generation for a total sample of 100,000 trees. We plotted log-likelihood scores against generation time using Tracer 1.4 (Rambaut and Drummond 2007) to determine when stationarity of likelihood values had been reached as a guide for where to set the burn-in stage (Ronquist et al. 2005). We set the burn-in to 5,000,000 generations (50,000 trees) and a majority rule consensus tree was calculated from the posterior sample of 50,001 trees. The programs RAxML 7.0.4 (Stamatakis et al. 2005) and the online program PhyML 3.0 (Guindon and Gascuel 2003; http://atgc.lirmm.fr/phyml/) were used for the maximum likelihood analyses and estimation of bootstrap support. The ML analysis in RAxML was performed with a GTRMIX model of molecular evolution for three different gene partitions and 500 bootstrap replicates. The analysis with PhyML

was performed with the GTR substitution model, starting from a random tree, with estimated proportion of invariable site and estimated gamma shape parameter. The phylogenetic trees were visualized in TreeView (Page 1996).

Morphological analysis In order to provide a baseline against which to evaluate morphology across the families of the Peltigerales, we tabulated anatomical and chemical characters for all described families based on our own observations as well as by drawing from cited literature sources. For describing ascus apical apparati an inconsistent terminology is in use in the literature and it was necessary to reassess basic types as the Peltigeralean fungi have not been systematically surveyed for ascus type morphology (notwithstanding photos and schematized sketches by Keuck 1977 and e.g. Jørgensen 2008). We sampled asci in representative genera across the Peltigerales. Asci were studied on hand-cut apothecia sections pretreated with and squashed in KOH and subsequently stained with Lugol's solution, with emphasis placed on immature asci in which initial spore shapes could be made out (Hafellner 1984).

Thallus morphology was studied using a Leica Wild M3Z dissecting microscope and ascus type and other microanatomical features were studied using a Zeiss Axioscop light microscope at 100×/1.03× magnification. Photographs were taken with a ZeissAxioCam MRc5 digital camera (Zeiss, Jena); images of growth habit were digitally optimized using CombineZM open source image processing software (www.hadleyweb.pwp.blueyonder.co.uk/CZM/).

Results of the phylogenetic analysis

We obtained forty-seven new sequences for the phylogenetic analysis (15 nuLSU, 16 nuSSU, 16 mtSSU) for a final matrix including 82 taxa and 4,638 nucleotide positions (2,303 for nuLSU, 1,635 for nuSSU, and 700 for mtSSU following exclusion of introns and ambiguous regions). All new sequences have been deposited in GenBank under the accession numbers listed in Table 1. The General Time Reversible substitution model with estimation of invariant sites and assuming a gamma distribution with four categories (GTR+I+Γ) was the optimal nucleotide substitution model selected for the genetic analyses. The B/MCMC and ML approaches returned congruent phylogenies (Fig. 1). Analysis of B/MCMC log likelihood tracer plots indicated that convergence had been reached between ca. 1.5 and 2 million generations. The average standard deviation across runs for splits with a frequency of at least 0.1 was 0.003732.

We obtained two strongly supported core groups within Peltigerales, corresponding to the suborders Collematineae and Peltigerineae. Within the Collematineae the families Coccocarpiaceae, Collemataceae, Pannariaceae and Placynthiaceae are fully supported; Coccocarpiaceae is unresolved in the Collematineae. Our newly sequenced sample of *Coccocarpia palmicola* groups in the Coccocarpiaceae, whereas *Pannaria hookeri*, *Santessoniella saximontana*, *Fuscopannaria* sp. and *Steineropsis alaskana* group within the Pannariaceae.

In the Peltigerineae the families Lobariaceae, Massalongiaceae, Nephromataceae, Peltigeraceae and Vahliellaceae are monophyletic, fully supported and completely resolved. The newly investigated samples of *Koerberia*, *Steinera*, and *Vestergrenopsis* are resolved in their own monophyletic clades which in turn form a major clade that we recognize here as the new family Koerberiaceae (see below).

Discussion

Phylogenetic reconstructions of the evolution of lineages within the Peltigerales have advanced significantly in the 15 years since Goffinet and Bayer (1997) published the first rDNA sequence reads from *Peltigera*. The most fundamental split of Peltigerales into two main groups, recognized by Miadlikowska and Lutzoni (2004) and reproduced in subsequent taxon samplings (Wedin et al. 2009, 2011; Muggia et al. 2011) is again confirmed by the present results. However, not all relationships strongly supported in Miadlikowska and Lutzoni's (2004) phylogeny are strongly supported. This is especially true of the Collematineae. Here, the sister group relationship between the Collemataceae/Placynthiaceae clade and Pannariaceae recovered by Miadlikowska and Lutzoni falls apart when Coccocarpiaceae are included in the sampling. The relationships between the higher level clades in Collematineae continue to be unresolved in our present phylogeny as in the previous work of Muggia et al. (2011) and contrary to the results of Wedin et al. (2009), who recovered a sister-group relationship between Collemataceae/Placynthiaceae/Coccocarpiaceae, on the one hand, and Pannariaceae, on the other.

The situation in the Peltigerineae is clearer and appears to have benefited from the expanded taxon sampling in the present study. Here the most noteworthy rearrangement is the recognition of *Koerberia* and *Steinera*, which had previously been unresolved in the phylogenies of Wedin et al. (2009) and Muggia et al. (2011), as a strongly supported and resolved clade sister to the rest of the Peltigerineae (Fig. 1). Other family relationships in the Peltigerineae have not stabilized across the four major studies to date, especially the relationship of the Lobariaceae/Nephromataceae clade to Peltigeraceae. In two previous studies (Miadlikowska and Lutzoni 2004; Muggia et al. 2011) Massalongiaceae were recovered as the closest relative of Lobariaceae/
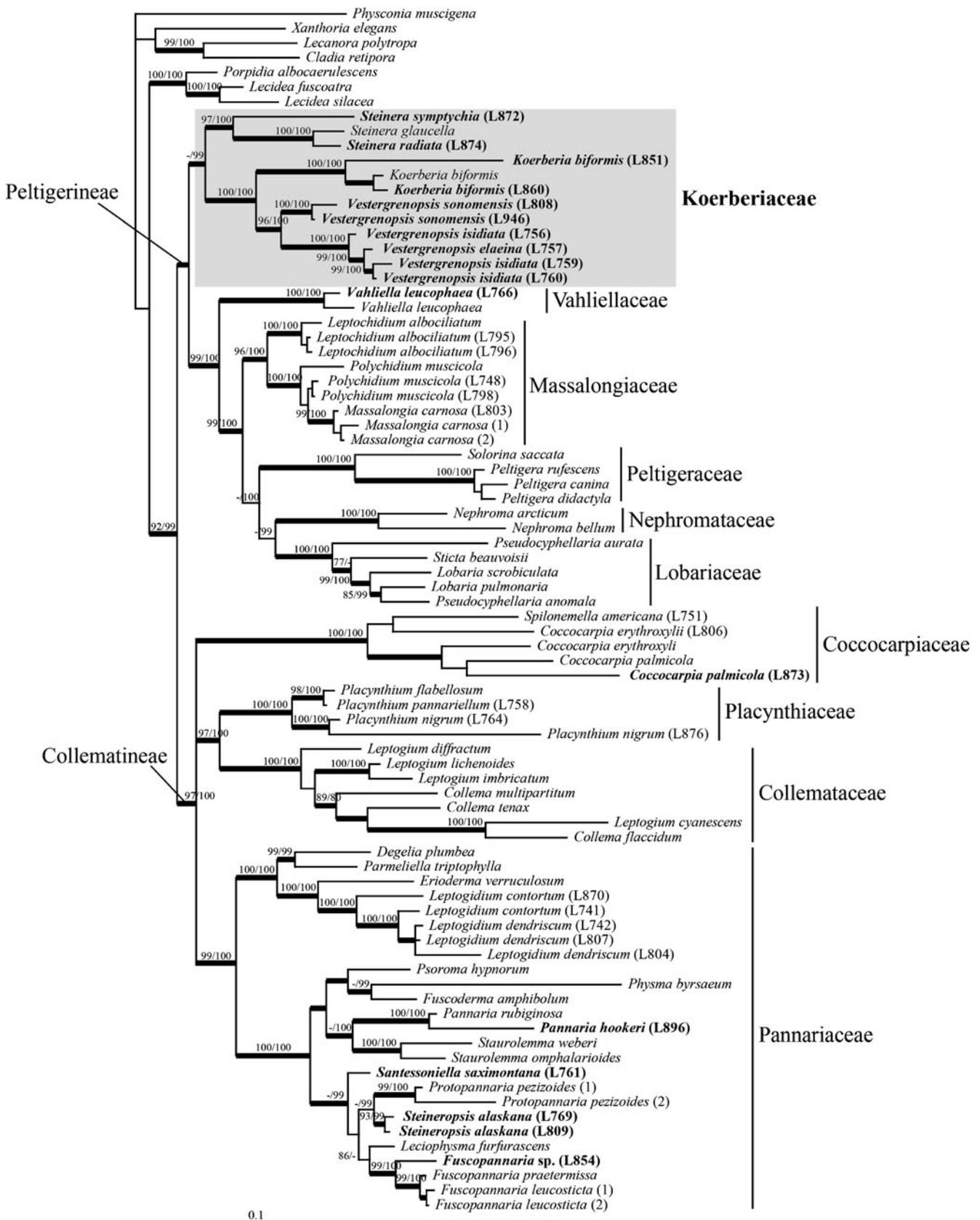
Fig. 1 Multigene phylogenetic hypothesis of Peltigerales. Majority rule consensus tree of a B/MCMC analysis with branch length based on the combined nuLSU, nuSSU and mtSSU dataset (Table 1, Supplementary Table S1). Bold branches denote Bayesian PP>95 %. ML values of the RAxML and PhyML analyses greater than 70 % are reported above branches

Nephromataceae, while Wiklund and Wedin (2003) recovered *Massalongia* as sister only to *Nephroma* and, with a larger taxon sampling, Wedin et al. (2009) recovered Peltigeraceae as the strongly supported closest relative of Lobariaceae/ Nephromataceae and Massalongiaceae as strongly supported sister to this three-family clade. In the present study we obtained the same result as Wedin et al. (2009), though the sister-group relationship between Lobariaceae/Nephromataceae and Peltigeraceae is supported only by maximum likelihood analyses. This however constitutes a discrepancy between our present results and those of Muggia et al. (2011), where the relationships of Massalongiaceae and Peltigeraceae are reversed. This topological "wobble" appears to be attributable to taxon and locus sampling. Resolving relationships and obtaining improved support will require more loci and could benefit from a cumulative supermatrix approach similar to what has been applied by Gaya et al. (2012) to the Teloschistales.

Convergence and parallelism in Peltigerineae and Collematineae

When Miadlikowska and Lutzoni (2004) segregated Collematineae from the rest of the order, the two suborders appeared to contain very different lineages of lichens. All gelatinous species and those with small, squamulose thalli ("pannarioids") came out in the Collematineae, whereas the large, broadly lobed macrolichens ("lobarioids" and "peltigeroids") formed the Peltigerineae, with the exception of a single sequence of *Massalongia*, corroborating the earlier result of Wiklund and Wedin (2003), and indicating that this coarse morphological dichotomy might not hold. The recovery of *Massalongia* in this group however proved to hold up to further scrutiny and ultimately Wedin et al. (2007) showed that *Massalongia* not only is well supported within the Peltigerineae but that this clade also includes the genera *Leptochidium* and *Polychidium*.

Recent studies have uncovered more examples of species groups or genera thought to belong to Collematineae in fact harbouring a hidden Peltigerineae lineage or vice versa. The first of these was found in the so-called pannarioid lichens (aggregates of the classical form genus *Pannaria*), with Jørgensen (2008) segregating *Vahliella* from *Fuscopannaria* based primarily on its different ascus apical apparatus despite no obvious outward differences in thallus morphology. Wedin et al. (2011) recognized this group as constituting its own family, Vahliellaceae, as sister to the lineage containing Lobariaceae, Peltigeraceae, Massalongiaceae and Nephromataceae, and our results corroborate this. The next example was the recognition that the genus *Polychidium*, morphologically characterized by radially symmetric dendroid thalli, in fact harbours species that arose independently from relatives of *Erioderma* in the Collematineae (Muggia et al. 2011) in addition to *Polychidium* s.str., which is in the Peltigerineae. The present paper

adds at least two more examples to this: *Degelia* (Collematineae) as circumscribed by Jørgensen (2004) harbours at least one species that evidently also acquired similar morphology within the genus *Steinera* and the recently described placodioid genus *Steineropsis* (Spribille et al. 2010), notwithstanding its superficial similarity to species such as *Steinera radiata* (Peltigerineae), is in fact more closely related to *Protopannaria* (in the Collematineae; see below).

Our study reveals numerous contradictions to previous morphology-based classifications, especially with regard to assignment to family. This in part reflects the fluidity of family delimitations in the past as well as the selective emphasis on certain morphological characteristics. The main consequences can be summarized under seven points:

1) *Koerberia* and *Vestergrenopsis* are not in the Placynthiaceae

The only systematic treatments of the genera *Koerberia* and *Vestergrenopsis* were published by Aino Henssen nearly 50 years ago (Henssen 1963a,b). Like *Massalongia* (Henssen 1963c), Henssen placed these two genera in a broadly circumscribed Peltigeraceae on account of their hemiangiocarpic apothecia and thus was early to recognize the affinities to what is now Peltigerineae. It was only later that Henssen and Jahns (1974) and Keuck (1977) resorted to placing these two genera formally in Placynthiaceae. This classification persists to the present in, e.g., Lumbsch and Huhndorf (2010). Our results show a clear split between *Placynthium*, which is recovered as sister to the Collemataceae and *Koerberia* and *Vestergrenopsis*, which we recognize as part of the new family Koerberiaceae (see below).

All Koerberiaceae share as synapomorphy the presence of radiating lines of photobiont below the upper cortex separating by minute furrows (Fig. 2). The character is difficult to see in some species and specimens (e.g., *Koerberia*) but can almost always be seen in well preserved or young material, and is spectacularly developed in *Steinera* and *Vestergrenopsis* (Fig. 2b, f). It is caused by photobiont bundling just beneath the upper cortex that results in thin lines with the cyanobiont separated by bundles of cyanobiont-free medullary hyphae. This character has often been cited as diagnostic for *Vestergrenopsis* and has been overlooked in the past in *Steinera*.

Already Henssen (1963a,b) recognized the close relationship between *Koerberia* and *Vestergrenopsis* and suggested that polyspory in *Vestergrenopsis* is derived (Henssen 1963c). Our results clearly support the eight-spored *Koerberia sonomensis* with its 1-septate ascospores resolved in a strongly supported clade with *Vestergrenopsis elaeina*. The 1-septate spores may be an evolutionary precursor to polyspory. The generic circumscriptions of *Koerberia* and *Vestergrenopsis* are easily modified to accommodate this finding (Table 3).

2) *Steinera* is not in the Coccocarpiaceae

The genus *Steinera* as circumscribed by Henssen and James (1982) includes a group of rare, Subantarctic and New Zealand

Fig. 2 Representative members of the Koerberiaceae: thalli and detail of the lobes. **a**, **b**) *Vestergrenopsis isidiata* (*Spribille* 29090, KLGO); **c**, **d**) *Koerberia biformis* (*Nash* 43229, GZU); **e**, **f**) *Steinera symptychia* (*Wheeler & Nelson* 3169, CONC); **g**, **h**) *Steinera radiata* (*Mayrhofer* 9736, GZU). Bar=1 mm (**b**, **d**, **f**, **h**), 2 mm (**c**, **g**), 4 mm (**a**, **e**)
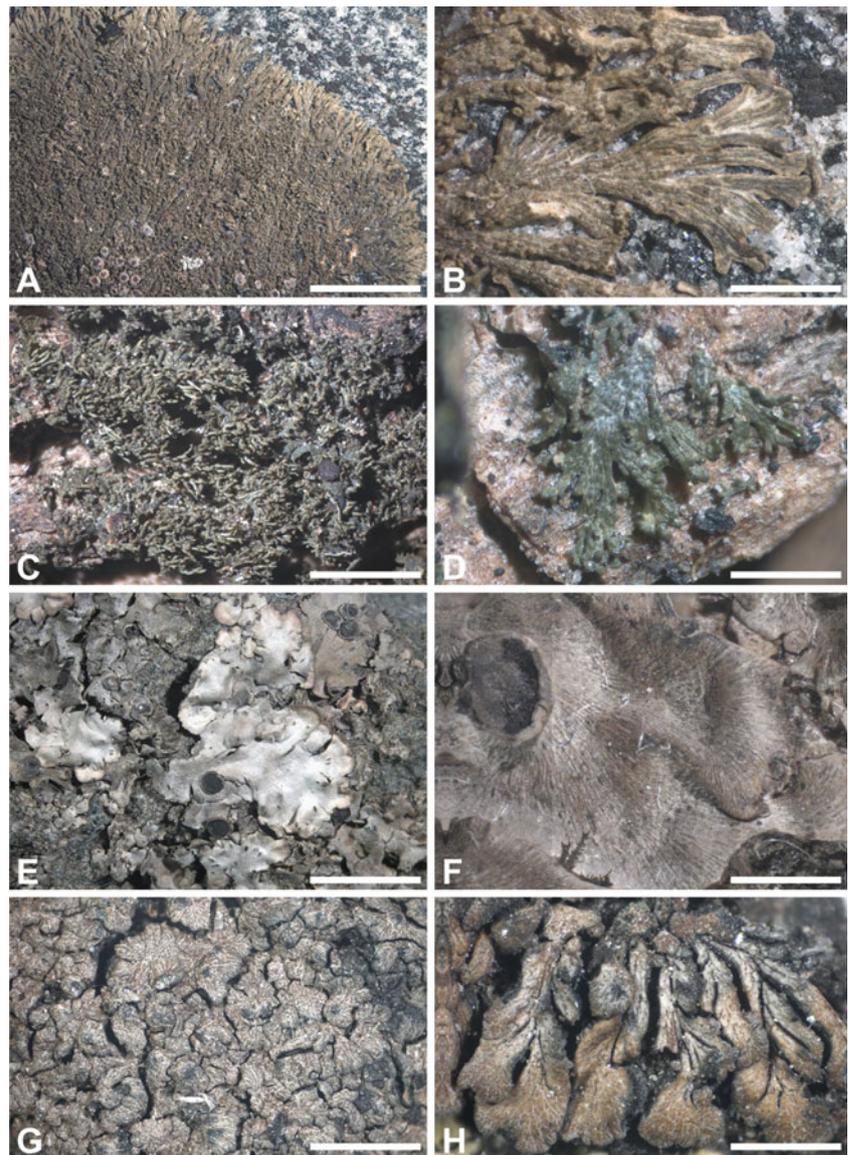
species with placodioid thalli on rock. Although members of the genus actually exhibit a wide range of ascomatal development types, the authors argued that the apothecia in the type species *Steinera glaucella* develop through the aggregation of short cells supplemented by adjacent thallus hyphae, as in the Coccocarpiaceae, and accordingly placed it in that family. *S. glaucella* was first included in a phylogeny by Wiklund and Wedin (2003) and Lumbsch et al. (2004) based on a specimen from Kerguelen Island, and is clearly unrelated to Coccocarpiaceae in our phylogeny. *Steinera sorediata* and *S. polymorpha*, the two species of the genus with pointed fusiform to acicular ascospores, have been found to belong to the Arctomiaceae in the Ostropomycetidae and will be treated elsewhere (P. Nelson and T. Spribille, in prep.).

3) Recovery of *Degelia symptychia* in *Steinera*

One of the more surprising results of our sequencing effort is the recovery of the southern South American endemic *Degelia symptychia* in *Steinera*. *Degelia symptychia* has been variously treated as *Pannaria* and *Parmeliella* and first placed in *Degelia* by Jørgensen and James (1990, as *D. fluviatilis*). Jørgensen (2004) discussed the nomenclatural issues and priority of Tuckerman's name. *A posteriori* examination of *D. symptychia* reveals the typical striations of Koerberiaceae and the typical ascus of *Steinera*, and we thus transfer it to the latter genus, where it becomes the largest known member (see taxonomic innovations). *Degelia subcincinnata* (Jørgensen 2004) likewise possesses Koerberiaceae-like thallus striations and is likely closely related to *Steinera symptychia*. Our results with regard to the circumscription of *Steinera* and convergent evolution in Peltigerineae and Collematineae strongly recommend a review of the taxonomy of those small endemic lineages of pannarioids in Australia and South America treated by Jørgensen (2001, 2004) and Jørgensen and Sipman (2006).

4) Split in the Pannariaceae

Ekman and Jørgensen (2002) were the first to observe a split in the Pannariaceae as classically defined with *Degelia* sect. *Amphiloma* recovered outside the core part of the family. They used this observation as the basis for rejecting the monophyly of Pannariaceae, but Wiklund and Wedin (2003) were the first to include Pannariaceae in a wider taxon sampling of Peltigerales, and later a more broadly defined Pannariaceae was again accepted (Wedin et al. 2007, 2009). Our results echo those of Wedin et al. (2009) and Muggia et al. (2011) in finding a strongly supported clade with *Degelia, Parmeliella, Erioderma* and *Leptogidium* (and possibly *Joergensenia*: Passo et al. 2008) as sister to core Pannariaceae. This group of four genera may warrant recognition as a distinct family but we refrain from taking this step while other research groups continue to study Pannariaceae.

5) Polyphyly of *Santessoniella*

Santessoniella was erected by Henssen (1997) to accommodate a series of small species formerly placed in *Pannaria*, *Parmeliella* and *Placynthium*. Ekman and Jørgensen (2002) recovered the type species of the genus, *S. polychidioides*, in *Psoroma* and Spribille et al. (2007) suggested that the genus consists of disparate elements. So far only ITS sequence data are available for *S. polychidioides* and thus were non-homologous with our sampled loci; DNA extractions were not available to us to amplify other loci. However, that *Santessoniella* species come out in at least two different places of the Pannariaceae can be triangulated from the fact that *S. saximontana* in our phylogeny occupies a relatively isolated position from *Psoroma*.

6) *Pannaria hookeri* is indeed a *Pannaria*; *Leciophysma* is not Collemataceae

We recovered the Arctic saxicole *Pannaria hookeri* close to the type species *P. rubiginosa*, contrary to Jørgensen's (2000) claim that it is likely not closely related to *Pannaria*. Our finding of *Leciophysma furfurascens* in the Pannariaceae agrees with the results for that species and *L. finmarkicum* in Wedin et al. (2009) and contradicts the current classification of that genus in the Collemataceae by Lumbsch and Huhndorf (2010).

7) Position of *Steineropsis*

We recently described the new genus *Steineropsis* to accommodate a newly discovered placodioid species in southeastern Alaska (Spribille et al. 2010), and promised forthcoming evidence of its relatedness to *Protopannaria*, which we provide here. *Steineropsis* is strikingly similar to *Steinera*, which occupies similar fog-drenched, maritime rock surfaces in the Southern Hemisphere. However, *Steineropsis* differs from *Steinera* amongst other things in its stratified thallus. The recent discovery of fertile material further underlines the affinities of the genus with Pannariaceae (T. Spribille and L. Muggia, in prep.). Its sister group relationship to *Protopannaria* is strongly supported in maximum likelihood analysis but marginally supported by Bayesian inference.

Congruence with morphology and internal anatomy

Detailed comparative anatomical analyses have been carried out numerous times for the families of the Peltigerales (Reinke 1895; Henssen 1963d, in part; Henssen and Jahns 1974; Keuck 1977). All of these analyses are however in narrative style and in German, and to our knowledge the salient morphological features distinguishing the major groups of Peltigerales have never been systematically tabulated or scored. A synthesis of the main characteristics of the families of Peltigerales (Table 2) highlights some of the challenges that systematists in the pre-molecular era had in circumscribing families. It also highlights areas where more anatomical work is needed. For instance, ascocarp ontogeny was invoked by Aino Henssen as a strong indication of common ancestry. Henssen did not however have the benefit of molecular insight and was influenced by a priori classifications of genera, and thus played down differences in ascomatal development e.g. in *Pannaria* s.lat. (Henssen and Jahns 1974) and *Steinera* (Henssen and James 1982), genera now known to consist of disparate elements. Similarly, the study of ascomatal development in Placynthiaceae by Keuck (1977) was based on analyses of the hemiangiocarpic genera *Koerberia* and *Vestergrenopsis* on the assumption that those genera belonged to that family. A reassessment of original observations on ascomatal ontogeny may reveal key differences between Peltigerineae and Collematineae. Currently, there seems to be a strong trend towards hemiangiocarpy in Peltigerineae and various expressions of gymnocarpy in Collematineae, deserving of further study. Similarly, the understanding of biont evolution in the Peltigerales would profit from a rigourous molecular assessment of photobionts in use across known lineages; initial indications suggest that nearly all Peltigerineae harbour *Nostoc* (with the exception of *Koerberia* and *Vestergrenopsis*) while Scytonemataceae are common in Collematineae (though Collemataceae s.str. also contains *Nostoc*). Confirming these patterns would require examination of large amounts of material and additional analyses that are beyond the scope of the present paper.

Another character that has been fraught with inconsistency is ascus structure. We were compelled to propose a new terminology for ascus morphology to overcome the inconsistency of terms currently in use. Although staining patterns differ slightly from genus to genus and are difficult to shoehorn into rigid types, we find a broad typology useful for assessing patterns and generating future evolutionary hypotheses for Peltigerales. To this end, we recognize five

Table 2 Families of the Peltigerales and their key morphological characteristics

	Koerberiaceae	Vahliellaceae	Massalongiaceae	Peltigeraceae	Nephromataceae	Lobariaceae	Coccocarpiaceae	Placynthiaceae	Collemataceae	Pannariaceae
Ascomatal development	hemiangiocarpic	hemiangiocarpic	hemiangiocarpic	hemiangiocarpic	hemiangiocarpic	hemiangiocarpic	gymnocarpic; cell differentiation into ascoma takes place on thallus surface	hemiangiocarpic[1]	gymnocarpic; cell differentiation begins deep within thallus and protrudes upward on a vertical stalk	gymnocarpic; cell differentiation begins below the thallus cortex above the algal layer, breaking cortex long before maturation
Position of ascomata	laminal	laminal	laminal	on lobe tips, rarely lobe undersurfaces[2], laminal in *Solorina*	on undersurface of lobe tips	on lobe tips	laminal	laminal	laminal	laminal
Ascus apical apparatus	*Pannaria* type, *Micarea*-type (*Steinera*)	*Vahliella*-type	*Vahliella*-type	*Micarea*-type with thickened basal amyloid section	*Pannaria*-type	*Vahliella*-type	*Coccocarpia*-type	*Micarea*-type, some species *Vahliella*-type	*Micarea*-type	*Pannaria*-type, *Micarea*-type, *Vahliella*-type (in *Erioderma*)
Ascospores	simple to 3-septate	simple	1-multiseptate	3- or more septate	3-septate	1-7-septate	simple	1-multiseptate to muriform	1-multiseptate to muriform	simple
Proper exciple	rudimentary	well developed	well developed	weakly developed to absent (*Solorina*)	weakly developed	weak to well developed	poorly distinguished from hymenial hyphae, sometimes byssoid	well developed	well developed	well developed
Thallus secondary metabolites	none known	none known	none known	depsides, hopane triterpenoids, quinones (*Solorina*)	diphenylethers, hopane triterpenoids	diphenylethers, depsides, depsidones, hopane triterpenoids	depsides[3]	none known	none known	depsides, depsidones, fatty acids, unidentified terpenoids, biphenyl ethers[4]
Thallus form	placodioid to ribbon-like	squamulose	squamulose, dendroid-fruticose to microfoliose	large foliose	large foliose	large foliose or dendroid-fruticose ("*Dendriscocaulon*")	foliose to microfruticose	microfoliose to microfruticose	foliose to squamulose	small foliose, squamulose or nearly crustose, dendroid-fruticose
Thallus stratification	weakly stratified, true upper and lower cortex lacking	stratified with upper cortex, lower surface ecorticate	stratified with upper cortex, lower surface ecorticate	stratified, upper cortex well developed (also lower cortex in *Solorina*)	stratified with upper and lower cortex	stratified with upper and ±lower cortex	stratified with upper cortex, lower cortex in some species	stratified with upper cortex	strongly to weakly stratified to homoiomerous	stratified with upper cortex, lower cortex present or absent
Hypothallus and lower surface	poorly developed to lacking	well developed, bluish black	lacking	rhizines on lower surface	lacking	lacking	well developed, bluish black	well developed, bluish black	lacking	well developed, bluish black to lacking
Cyanobiont	*Scytonemataceae*[5], *Nostoc*[6]	*Nostoc*	*Nostoc*	*Nostoc*	*Nostoc*	*Nostoc*	*Scytonema*, *Hyphomorpha*[7]	Rivulariaceae, Scytonemataceae[8]	*Nostoc*	*Nostoc*, *Scytonema*
Chlorobiont	lacking	lacking	lacking	*Coccomyxa* in some species	*Coccomyxa* in some species	*Dictyochloropsis* (or *Coccomyxa*[9]) in some species	lacking	lacking	lacking	*Myrmecia* (in *Psoroma*)

Table 2 (continued)

	Koerberiaceae	Vahliellaceae	Massalongiaceae	Peltigeraceae	Nephromataceae	Lobariaceae	Coccocarpiaceae	Placynthiaceae	Collemataceae	Pannariaceae
Primary biont arrangement	strongly bundled, appearing as striations	not bundled	not bundled	not bundled	not bundled	not bundled	not bundled	not bundled	not bundled	not bundled
Conidia	bacilliform	bacilliform	bacilliform, sometimes with end thickenings	ellipsoid to pyriform or truncate	bacilliform	bacilliform to long-bacilliform with end thickenings	oblong to bacilliform	bacilliform, sometimes with end thickenings	oblong to bacilliform, with or without end thickenings	bacilliform

[1] stated by Henssen 1963e, but detailed anatomical analysis by Keuck (1977) focused on *Vestergrenopsis*, now in Koerberiaceae; [2] Keuck 1977:57; [3] Atranorin reported from *C. erythroxyli* by Keuck (1977); [4] Elix et al. 1978; [5] based on Henssen (1963a,b); [6] Henssen and James (1982); [7] in two species of *Spilonema* (Henssen 1981); [8] Henssen 1963e; [9] in *Lobaria silvae-veteris* (Goffinet and Goward 1998).

broadly defined types that account for all ascus structures we have seen or seen reported in Peltigerales:

a) **Pannaria-type**, with no visible, densely amyloid zone in the apical apparatus and lacking a thick, gelatinous sheath (Fig. 3a, b, g, n, p); typically referred to as an ascus lacking internal amyloid structures (Keuck 1977; Jørgensen 2007);

b) **Micarea-type**, with an amyloid inner tube penetrating the tholus (Fig. 3c, f, m, o) and also called "plug" (e.g., Arvidsson and Galloway 1981; Passo and Calvelo 2006), "ring structure" (Jørgensen 2000, 2007), "Ringstruktur" (Keuck 1977) or "annulus" (Vitikainen 2007; Smith et al. 2009). We see no fundamental difference in structure to various expressions of this architecture in Pilocarpaceae including genera such as *Micarea* (Hafellner 1984); the variant in *Peltigera* with a dense, widened amyloid zone at the base of the tube (*Peltigera*-type of Vitikainen 2007; Smith et al. 2009, but note discrepancy between the illustration in the latter and our Fig. 3f) appears to be only a variation on the theme. In *Collema* it is typical that the amyloid zone around the axial tube flares towards the upper end of the masse axiale, recalling *Porpidia* (Hafellner 1984).

c) **Vahliella-type**, with a thin densely amyloid layer capping the endoascus and an amyloid exoascus, but no other densely amyloid internal structures (Fig. 3d, e, h, i). Also variously called "amyloid sheet" (Jørgensen 1994, 2007) and "apical cap" (Arvidsson and Galloway 1981; Wedin et al. 2007). We could not convince ourselves of the difference between the "indistinct" amyloid layer in Lobariaceae and the "apical cap" reported in *Massalongia* by Wedin et al. (2007), nor the difference between Lobariaceae and Vahliellaceae emphasized by Wedin et al. (2011), as both endo- and exoascal amyloid layers appear to be present in the entire array. We note that *Sticta* can possess in addition a thick gelatinized sheath (Fig. 3i) but maintains otherwise similar ascus staining as *Lobaria*. See also the illustrations of Letrouit-Galinou (1971).

d) **Coccocarpia-type**, with an axial body that does not penetrate through the D-layer, thus recalling a *Biatora* with a particularly wide masse axiale (Hafellner 1984), but with a strongly thickened gelatinous sheath (Fig. 3j, k). We could see no way of reconciling internal ascus stains in *Coccocarpia* with the illustration by Keuck (1977: 98).

e) **Joergensenia-type**, with a completely amyloid axial chamber, recalling *Catillaria*. We have not seen this structure, reported only from *Joergensenia* (Pannariaceae), but it is well illustrated by Passo et al. (2008), who also provide molecular data.
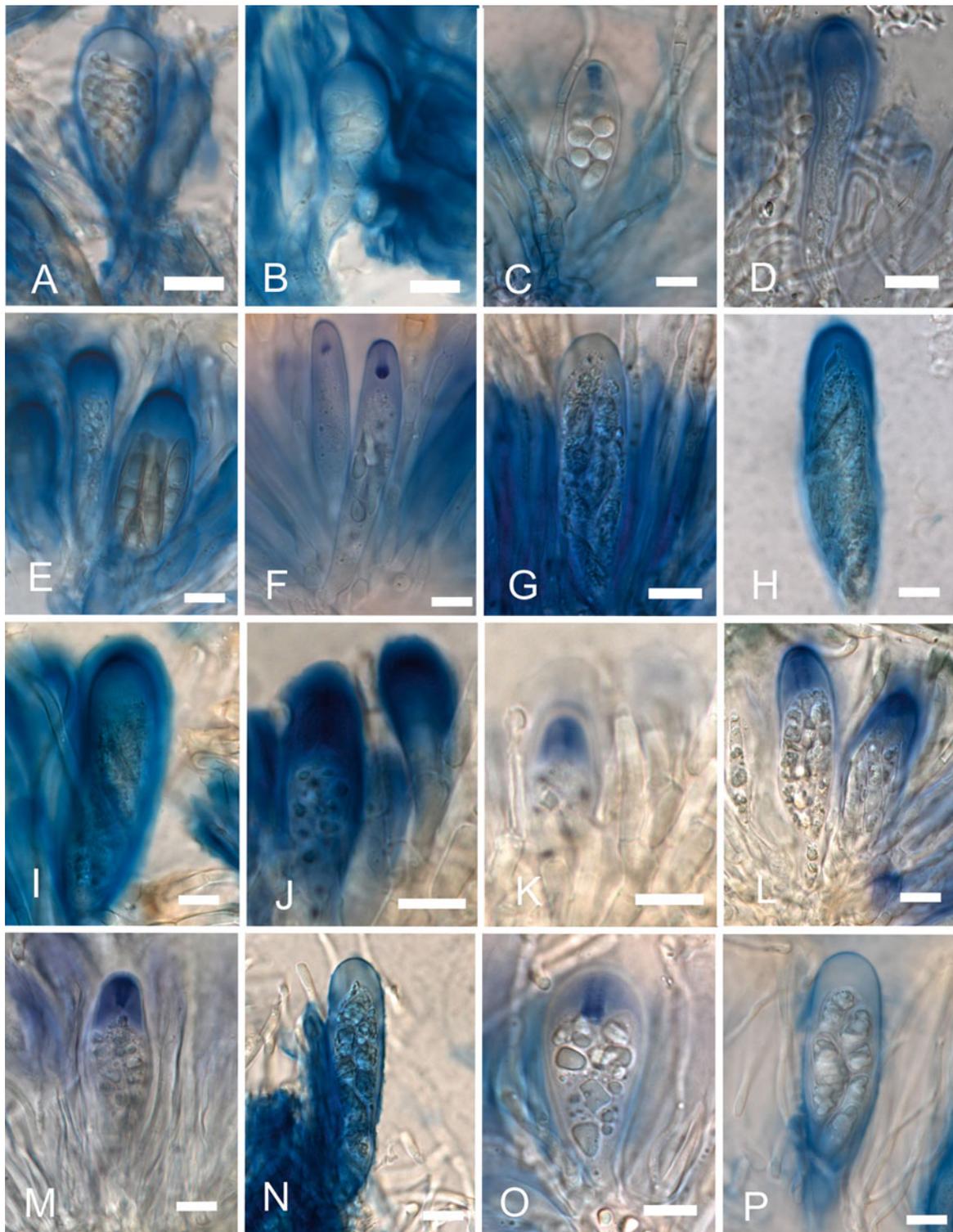
Fig. 3 Diversity of ascus apical apparati from selected taxa across the phylogeny of the Peltigerales shown in Fig. 1. **a**. *Koerberia biformis* (*Nash* 43229, GZU); **b**. *Vestergrenopsis elaeina*, eight-spored variant (*Spribille* 24525, KLGO); **c**. *Steinera radiata* (*Wheeler* 2473a, CONC); **d**. *Vahliella leucophaea* (*Spribille* 15422, GZU); **e**. *Massalongia carnosa* (*Spribille* 28815, KLGO); **f**. *Peltigera neopolydactyla* (*Spribille* 14843, GZU); **g**. *Nephroma helveticum* subsp. *sipeanum* (*Spribille* 12160, GZU); **h**. *Lobaria pulmonaria* (*Spribille* 11203, GZU); **i**. *Sticta damaecornis* (*Vivant* s.n. in Vězda, *Lich. Rar. Exs.* 149, GZU); **j**–**k**. *Coccocarpia erythroxyli*, (**j**) in full concentration staining Lugol's solution and (**k**) snapshot during dissipation of solution with KOH (*Hafellner* 29499, GZU); **l**. *Placynthium nigrum* (*Hafellner & Hafellner* 26383, GZU); **m**. *Collema polycarpon* (*Muggia & Hafellner* s.n., TSB-37278); **n**. *Pannaria rubiginosa* (*Hafellner* 48249, GZU); **o**. *Santessoniella saximontana* (*Spribille* 21173, isotype, GZU); **p**. *Protopannaria pezizoides* (*Spribille* 26651, GZU)

Table 3 Characteristics of genera of Koerberiaceae following recircumscription of *Vestergrenopsis* and *Steinera*, highlighting the intermediate morphology of *V. sonomensis*

	Koerberia	*Vestergrenopsis sonomensis*	*Vestergrenopsis*	*Steinera*
Ascus apical apparatus	*Pannaria*-type	*Pannaria*-type	*Pannaria*-type	*Micarea*-type
Number of ascospores per ascus	8, spirally twisted	8, biseriate	(8-)12-16	8
septation	0	1	0	0-3-septate
shape	acicular	fusiform	narrowly ellipsoid	broadly ellipsoid
Size	43–55×1.5–2 μ	10–17×3.5–4 μ	7–10×4–6 μ	8–21×6–7.5
Thallus	Small, ribbon-like, loosely attached	Ribbon-like thalli, closely appressed (except for lobules)	Ribbon-like thalli on rock, closely appressed	Placodioid thalli, closely appressed
Substrate ecology	bark	rock	rock	rock
Macroecology	Mediterranean-Californian	Temperate to Atlantic	Oceanic subpolar	Oceanic Subantarctic
Photobiont	Scytonemataceae	Scytonemataceae	*Scytonema*	*Nostoc*
Conidia	bacilliform	bacilliform	unknown	bacilliform

Comparison of these types across the Peltigerales (Table 2) hints at broad patterns. Seemingly identical ascus structures such as the amyloid tube appear to be homoplasious across the Peltigerales, echoing ascus type homoplasy in other lichenized fungi (Lumbsch et al. 2007; Ekman et al. 2008). Interestingly, the *Vahliella*-type is found only in Peltigerineae with the exception of its report in *Erioderma* and some species of *Placynthium* (*P. aspratile*, *P. rosulans*; Keuck 1977). The similarity between *Vahliella*-type and the ascus staining patterns of the closely related Lecideales (*Lecidea*-type, Hafellner 1984; Smith et al. 2009) is possibly not coincidental and the two should perhaps be treated as evolutionarily related.

Taxonomic innovations

Koerberiaceae T. Sprib. & Muggia, **fam. nov.**

Mycobank Number MB 800590

Familia nova ad Peltigerales subordinem Peltigerineas pertinet. Fungi in symbiosem cum cyanobacteriis aut Nostocacearum aut Scytonematacearum vivent. Thalli minute foliosi aut placodioidei arcte appressi, ad superficiem fissuris aut lineis radialiter dispositis muniti. Ontogenia apotheciorum hemiangiocarpa. Asci apicaliter cum aut sine structura tubiformi amyloidea, octo- vel multispori. Ascosporae simplices ad multiseptatae, ellipsoideae ad aciculares.

Thalli small squamulose to placodioid, strongly dorsiventral, bearing longitudinal furrows or lines on the upper surface that radiate from the thallus centre. **Photobiont** cyanobacterial, *Nostoc* or Scytonemataceae, bundled in rows beneath the upper cortex. **Apothecia** round, hemiangiocarpic in their development, with persistent "lecanorine" margin. **Asci** cylindrical, with or without amyloid tube.

Ascospores 8 to many per ascus, simple to multiseptate, ellipsoid to acicular. **Pycnidia** immersed, flask-shaped. **Conidia** bacilliform.

Type genus: *Koerberia* Massalongo, Geneacaena Lichenum, p. 5 (1854).

The Koerberiaceae encompass with the present circumscription the genera *Koerberia*, *Vestergrenopsis* and *Steinera* (Table 3). The new family is compared to the other nine recognized families of the Peltigerales in Table 2. Two species of Koerberiaceae originally described on adjacent pages by Tuckerman (1877) require new combinations:

Steinera symptychia (Tuck.) T. Sprib. & Muggia, **comb. nov.**

Mycobank Number 800591

Basionym: *Pannaria symptychia* Tuck., Proc. Amer. Acad. Arts Sci. 12, new ser., vol. 4: 168 (1877) Type: **CHILE**. Juan Fernández, *T. Hill* (FH, holotype seen by Jørgensen 2004).

Vestergrenopsis sonomensis (Tuck.) T. Sprib. & Muggia, **comb. nov.**

Mycobank Number 800592

Basionym: *Pannaria sonomensis* Tuck., Proc. Amer. Acad. Arts Sci. 12, new ser., vol. 4: 169 (1877). Type: **U.S.A.** California, *Bolander* (FH, seen by Henssen 1963a).

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