

# Evolution of complex symbiotic relationships in a morphologically derived family of lichen-forming fungi

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## Summary

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• We studied the evolutionary history of the Parmeliaceae (Lecanoromycetes, Ascomycota), one of the largest families of lichen-forming fungi with complex and variable morphologies, also including several lichenicolous fungi.

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- We assembled a six-locus data set including nuclear, mitochondrial and low-copy protein-coding genes from 293 operational taxonomic units (OTUs).
- The lichenicolous lifestyle originated independently three times in lichenized ancestors within Parmeliaceae, and a new generic name is introduced for one of these fungi. In all cases, the independent origins occurred c. 24 million yr ago. Further, we show that the Paleocene, Eocene and Oligocene were key periods when diversification of major lineages within Parmeliaceae occurred, with subsequent radiations occurring primarily during the Oligocene and Miocene.
- Our phylogenetic hypothesis supports the independent origin of lichenicolous fungi associated with climatic shifts at the Oligocene–Miocene boundary. Moreover, diversification bursts at different times may be crucial factors driving the diversification of Parmeliaceae. Additionally, our study provides novel insight into evolutionary relationships in this large and diverse family of lichen-forming ascomycetes.

## Introduction

Mutualistic systems include two or more partners that provide services to each other in order to maximize the net fitness of all partners (Bronstein, 1994). Lichens represent an iconic example of mutualistic interactions. However, relatively little is known of the factors driving partner selection in these systems. In many cases, lichenized fungi can form symbiotic associations with a range of photobiont species. For example, under extreme conditions, lichen-forming fungi have been shown to establish symbioses with a broad range of locally available photobionts (Wirtz *et al.*, 2003; Jones *et al.*, 2013). In some cases, multiple distinct algal species may even be found within a single thallus (Del Campo *et al.*, 2013; Muggia *et al.*, 2013; Dal Grande *et al.*, 2014; Sadowska-Des *et al.*, 2014). Furthermore, some fungal genera include both lichen-forming species and species with different biologies (Hawksworth, 2005), and there are single species that can live either in a symbiotic association with algae or alternatively as saprobes on bark (Wedin *et al.*, 2004; Muggia *et al.*, 2011).

A number of studies suggest that the evolution of lichen symbioses occurred independently several times in Ascomycota (Gargas *et al.*, 1995; Gueidan *et al.*, 2008; Schoch *et al.*, 2009). Within some lichen-forming fungal lineages, especially ascomycetes, a few authors have suggested that nonlichenized fungi have evolved from lichenized ancestors (Eriksson, 1981, 2005; Hawksworth, 1982a; Lutzoni *et al.*, 2001), implicitly suggesting that the lichen symbiosis is labile at an evolutionary scale. Kranner & Lutzoni (1999) argued that transitions from a lichenized to a nonlichenized lifestyle would be more likely, with more losses of lichenization than gains, than vice versa, because lichenization would involve complicated physiological adaptations of both partners. However, experimental evidence indicates that shifts to mutualism can happen within a short time frame given suitable ecological conditions (Hom & Murray, 2014). Such shifts have been considered to occur in other fungal nutritional systems, such as endophytes and plant pathogens (Arnold *et al.*, 2009). The presence of both lichenized and nonlichenized forms in several ascomycete clades clearly indicates that transitions from mutualistic to nonmutualistic

lifestyles and/or vice versa must have happened during fungal evolution. However, the pathways of such transitions remain largely unsettled.

Lutzoni and coworkers (Lutzoni *et al.*, 2001; Arnold *et al.*, 2009) have proposed that lichenicolous fungi play an important role in the transition from lichenized to other nonlichenized nutritional modes. It has also long been recognized that a single fungal genus can include species with different nutritional strategies (Santesson, 1967; Wedin *et al.*, 2004; Hawksworth, 2005). Lichenicolous fungi represent an ecological group of over 1800 species that form obligate associations with lichens, as parasites, saprotrophs, or commensals (Hawksworth, 1982b, 2003; Richardson, 1999; Lawrey & Diederich, 2003). Some species are clearly pathogenic, such as *Clypeococcum hypocenomyces*, which causes necrosis and degeneration of the host thallus (Hawksworth, 1980), whereas others can form galls or hardly perturb the thallus with no obvious harmful effects, such as some *Nesolechia* or *Phacopsis* species (Triebel *et al.*, 1995; Peršoh & Rambold, 2002). In the latter case, it has been hypothesized that these lichenicolous fungi have a mutualistic relationship with the photobiont of the lichen, whereas there is a competitive relationship with the primary fungal partner (Poelt & Vězda, 1984; Friedl, 1987; Rambold & Triebel, 1992; Peršoh & Rambold, 2002).

While the lichenicolous lifestyle has been suggested to facilitate the transition to different nutritional modes in fungi (see above), there have been multiple origins of the lichenicolous lifestyle, with lineages including mainly or exclusively lichenicolous fungi, and being unrelated to lichen-forming lineages, in both Basidiomycota (Lawrey *et al.*, 2007; Millanes *et al.*, 2011) and Ascomycota (Diederich *et al.*, 2012; Suija *et al.*, 2015). Some transitions from lichenized to lichenicolous lifestyles have, however, been suggested (Diederich *et al.*, 2012; Frisch *et al.*, 2014).

The aim of this paper was to elucidate phylogenetic relationships within Parmeliaceae and to test whether a transition from lichenized to lichenicolous lifestyles happened within a morphologically and chemically diverse clade of lichenized fungi. Parmeliaceae is one of the largest families of lichen-forming ascomycetes with c. 2800 species, representing c. 15% of the

total species diversity in lichenized fungi. The family has a world-wide distribution, with the highest diversity in the tropics, but members occur across a broad range of habitats, from hyperarid deserts to polar or alpine regions. The family is characterized morphologically by a specific type of ascoma ontogeny and the presence of an ascomatal feature termed the cupulate exciple (Henssen *et al.*, 1981). Most genera in this family form lichens with large and often complex thalli, having either foliose or fruticose growth forms. Thus it was surprising when internal transcribed spacer (ITS) and nuclear ribosomal small subunit (SSU) rDNA data revealed a phylogenetic affiliation of the lichenicolous genera *Phacopsis* and *Nesolechia* with this morphologically complex family (Peřoh & Rambold, 2002). While this placement was initially questioned (Grube & Hawksworth, 2007), it was subsequently confirmed using additional molecular loci (Crespo *et al.*, 2007, 2010). Here, we assess the phylogenetic placement and the age of origin of the lichenicolous habit within Parmeliaceae, in addition to elucidating the broader evolutionary history of other genera in the family. To this end, we have assembled an extended multilocus data set from 293 operational taxonomic units (OTUs) representing 72 of the 80 genera in Parmeliaceae and included five samples from two lichenicolous species. One additional lichenicolous species was included in a more comprehensive single locus (ITS) data set. Based on the results of this study, we discuss the hypothesis that the lichen symbiosis is labile and that lichenicolous fungi can evolve from lichenized ancestors. We also provide an updated hypothesis of phylogenetic relationships and divergence time estimates for Parmeliaceae.

## Materials and Methods

### Data assembly

Molecular analyses were based on a six-locus data set (two nuclear ribosomal markers: ITS and the nuclear ribosomal large subunit (nuLSU); the mitochondrial SSU (mtSSU) marker; and three protein-coding loci: the largest subunit of RNA polymerase II (*RPB1*), the DNA replication licensing factor mini-chromosome maintenance complex component 7 (*Mcm7*) and the pre-rRNA processing *Trypanosoma* serine-arginine 1 protein (*Tsr1*)) generated from 293 OTUs with representatives from the families Parmeliaceae, Gypsoplacaceae, Lecanoraceae and Cladoniaceae (Supporting Information Table S1). Species from the family Cladoniaceae were used to root the tree following Crespo *et al.* (2007). The sampling focused on the family Parmeliaceae and included 274 species representing 72 of the 80 accepted genera in this family (Thell *et al.*, 2012). DNA sequences of six loci (Table S1) represented a compilation of sequences from previous studies and others generated specifically for this study. The ITS data set included 297 OTUs. Primer sequences and annealing conditions are reported in Table S2. Detailed materials and methods sections, including gene amplification and DNA sequencing, sequence alignments, phylogenetic analyses, hypothesis testing, ancestral state reconstruction, divergence time estimates, and phylogenetic informativeness (PI) are provided in Methods S1.

## Results

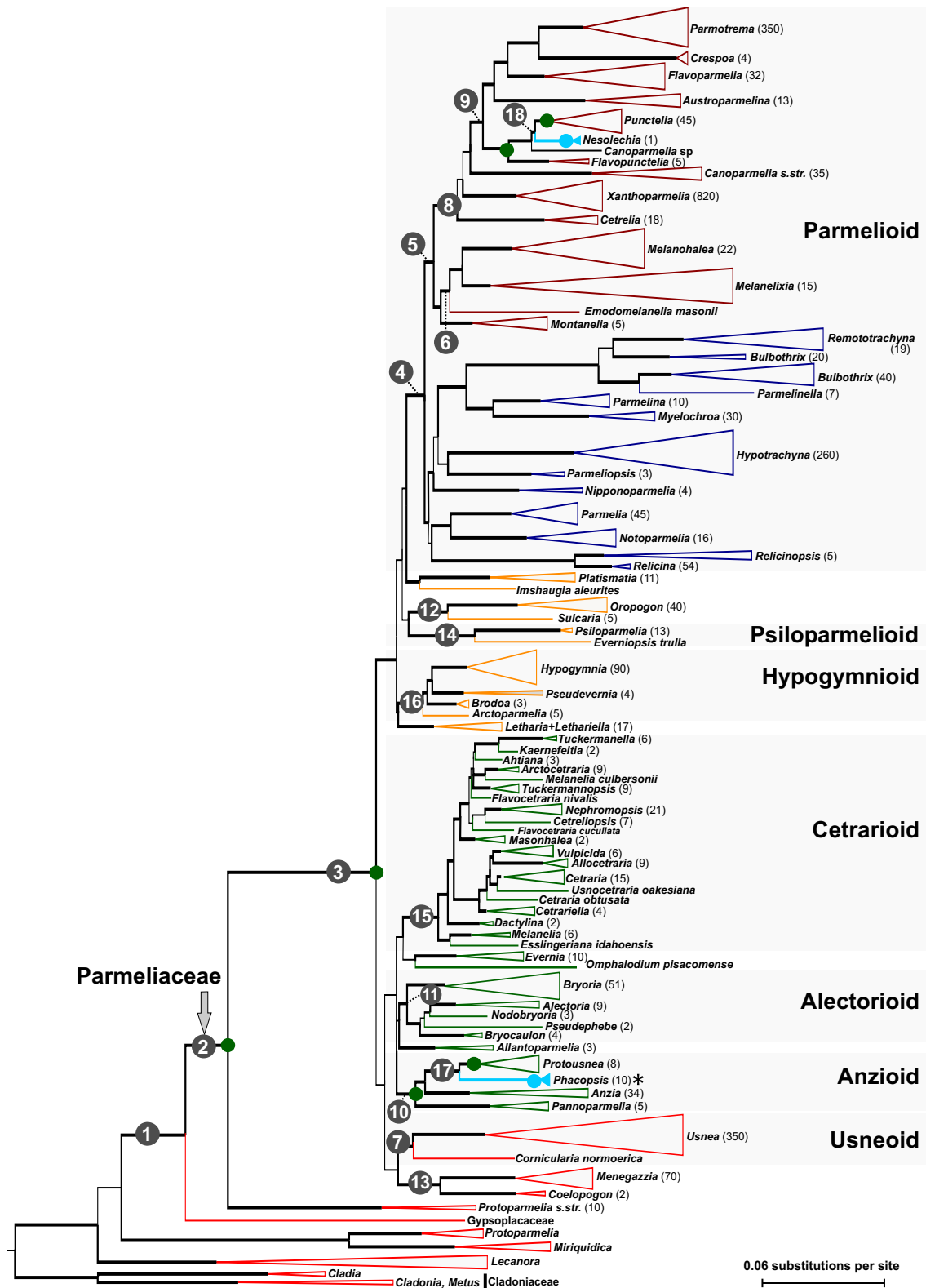
### Phylogenetic analysis

The number of unambiguous nucleotide positions in each data set, variable and parsimony informative sites, and the best-fitting models of evolution selected in jMODELTEST (Darriba *et al.*, 2012) are summarized in Table S3. Newly generated sequences (582) of ribosomal DNA (ITS, nuLSU, and mtSSU) and low-copy protein-coding genes (*RPB1*, *Mcm7*, and *Tsr1*) for this study are deposited in GenBank under accession numbers KP888160–KP888313 and KR995270–KR995697 (Table S1). Testing for topological incongruence showed no strongly supported conflicts (results not shown) and hence the concatenated six-locus data matrix was used for all subsequent analyses. Effective sample sizes (ESSs) of all estimated parameters were well above 200 in the Bayesian analyses, and the ‘compare plot’ produced by ‘Are We There Yet?’ (AWTY) indicated that parallel Markov chain Monte Carlo (MCMC) runs achieved topological convergence (results not shown). A simplified tree depicting phylogenetic relationships at the generic level is shown in Fig. 1, and the full tree containing all terminal taxa is provided in Fig. S1. While our best topology is largely in agreement with the existing phylogenetic reconstructions for the family Parmeliaceae that were based on fewer loci (Crespo *et al.*, 2007, 2010), our results provide improved resolution and increased nodal support for a number of key groups.

Parmeliaceae s. lat. (node 2) was strongly supported as monophyletic, and the sister-group relationship of *Protoparmelia* s. str. with all other Parmeliaceae (node 3) was also strongly supported. The largest clade within Parmeliaceae, the Parmelioid clade (node 4), was strongly supported. Within the Parmelioid group, a previously unsupported relationship of the *Cetrelia*, *Parmotrema*, and *Xanthoparmelia* clades (node 8) received strong support. Also, a clade consisting of the *Cetrelia*, *Melanohalea*, *Parmotrema*, and *Xanthoparmelia* clades (node 5) received strong support. The genus *Usnea* formed a strongly supported sister group to the monotypic genus *Cornicularia* (node 7). *Menegazzia* spp. formed a monophyletic group with *Coelopogon*, which was strongly supported (node 13). Also, the genera *Oropogon* and *Sulcaria* formed a strongly supported monophyletic group (node 12). The Alectorioid (node 11), Cetrarioid (node 15), Hypogymnioid (node 16), and Psiloparmelioid (node 14) groups were all recovered with strong support. A new clade, the Anzioid group, encompassing species from the genera *Anzia*, *Pannoparmelia*, *Phacopsis* and *Protousnea*, was also recovered with strong support (node 10).

### Phylogenetic placement of lichenicolous species

In the six-locus data set, specimens from the lichenicolous genera *Nesolechia* and *Phacopsis* were represented by a single species of each genus. Additionally, another species of *Phacopsis* was included in the ITS data set. Our results confirmed that both genera belong to Parmeliaceae. However, the two lichenicolous genera were recovered in distantly related lineages: *Nesolechia*



**Fig. 1** Cartoon tree showing phylogenetic relationships among major lineages of Parmeliaceae. The tree is derived from a six-locus phylogeny (see Supporting Information Fig. S1). Supported nodes are collapsed to generic level where applicable. The number of species currently accepted in each genus is shown in parentheses. Branches that received strong support in RAxML (bootstrap values  $\geq 70\%$ ) and/or Bayesian inference (posterior probabilities  $\geq 0.95$ ) are in bold. Strongly supported principal nodes are indicated as 1–18. All triangle colors correspond to single figures in Fig. S1. Lichenized (green circles) and lichenicolous (blue circles) ancestral character states are plotted on the node of interest over the tree. \**Phacopsis huuskonenii* (placed in the new genus *Raesaenenia* in this paper).



*oxyspora* (two samples) formed a well-supported sister group with the foliose genus *Punctelia* in the Parmelioid group (node 18), whereas '*Phacopsis*' *huuskonenii* (three samples) was a sister to the genus *Protousnea* (node 17) in the Anzioid group (Fig. 1) and clearly represents a genus distinct from *Phacopsis vulpina*. That was not surprising as the ascospores in the two species are quite different (Hawksworth, 1978). The new generic name *Raesaenenia* is therefore introduced for *P. huuskonenii* here (Box 1). *Phacopsis vulpina*, represented by a single ITS sequence, formed a sister relationship with the *Relicina* + *Pseudoparmelia* clade in the Parmelioid group in the single-locus ITS analysis (data not shown). Alternative hypothesis testing strongly rejected monophyly of these lichenicolous species ( $P < 0.001$  in Shimodaira–Hasegawa (SH) and expected likelihood weight (ELW) tests). Ancestral character reconstruction analyses under maximum parsimony and maximum likelihood optimization criteria estimated the common ancestors of nodes 17 and 18 as being lichenized, therefore suggesting that a transition from lichenized to lichenicolous lifestyle occurred independently in each of the three clades.

### Divergence time estimates

Overall, the estimated ages for major clades in Parmeliaceae are similar to the estimations from a previous study based on a more limited sampling (Amo de Paz *et al.*, 2011), and thus the results are not repeated here. Rather, we focus on clades that were not supported in the previous phylogenetic analysis, as well as on dating the origin of lichenicolous lifestyle within the family.

The estimated ages for selected nodes are listed in Table S4 and shown in Fig. 2. Within the Cretaceous, the split of Parmeliaceae from its sister group Gypsoplacaceae was estimated at 126 million yr ago (Ma; 95% highest posterior density (HPD) = 101.21–151.77 Ma; node 1), the split of core Parmeliaceae from *Protoparmelia* at 112 Ma (95% HPD = 92.97–135.47 Ma; node 2), and the split of the *Parmotrema* + *Xanthoparmelia* + *Cetrelia* clades from the *Melanohalea* clade at 68 Ma (95% HPD = 56.87–81.74; node 5). During the Paleocene, the split of *Emodomanelia* from *Melanelixia* + *Melanohalea* was estimated at 62 Ma (95% HPD = 50.77–74.63 Ma; node 6) and the split of the *Austroparmelina* + *Flavoparmelia* + *Parmotrema* clade from the *Nesolechia* + *Flavopunctelia* + *Punctelia* clade at 55 Ma (95% HPD = 44.85–65.69 Ma; node 9). The crown ages of both the Anzioid and Alectorioid clades were estimated at *c.* 54 Ma (95% HPD = 46.98–61.98 and 46.07–64.26 Ma; nodes 10 and 11). The crown ages of three major groups in Parmeliaceae were estimated at 38 Ma for the Cetrarioid (95% HPD = 30.02–46.16 Ma), 38 Ma for the Hypogymnioid (95% HPD = 27.44–48.90 Ma), and 46 Ma for the Psiloparmelioid (95% HPD = 39.68–62.45 Ma), which dates them to the Eocene. The splits of the lichenicolous species of *Nesolechia* and *Phacopsis* studied from their sister taxa (*Punctelia* and *Protousnea*, respectively) were estimated to have occurred *c.* 25 Ma (95% HPD = 18.34–31.75 and 13.51–41.25 Ma; nodes 18 and 17).

**Box 1** *Raesaenenia*: a new generic name for *Phacopsis huuskonenii*.

***Raesaenenia*** D. Hawksw., Boluda & H. Lindgr., **gen. nov.**  
Mycobank MB 812847

**Etymology:** In honor of the astute Finnish lichenologist Veli Johannes Paavo Bartholomeus Räsänen (1888–1955) who first described the type species.

**Diagnosis:** Ascomata resembling those of *Phacopsis* in structure, but differing in the subcylindrical ascospores with thickened caps of wall tissue at each end.

**Type species:** *Raesaenenia huuskonenii* (Räsänen) D. Hawksw. *et al.* (syn. *Phacopsis huuskonenii* Räsänen).

***Raesaenenia huuskonenii*** (Räsänen) D. Hawksw., Boluda & H. Lindgr., **comb. nov.**  
Mycobank MB 812848

**Basionym:** *Phacopsis huuskonenii* Räsänen, *Lichenoth. Exs.*, fasc. 21 no. 525 (1949).

**Type:** Finland: Savonia borealis: Pielavesi, Sävia, Lähdemäki, on *Bryoria capillaris* on *Picea excelsa*, 6 March 1949, K. Huuskonen (Räsänen, *Lichenoth. Exs.*, fasc. 21 no. 525) (K-IMI 209424 – isotype).

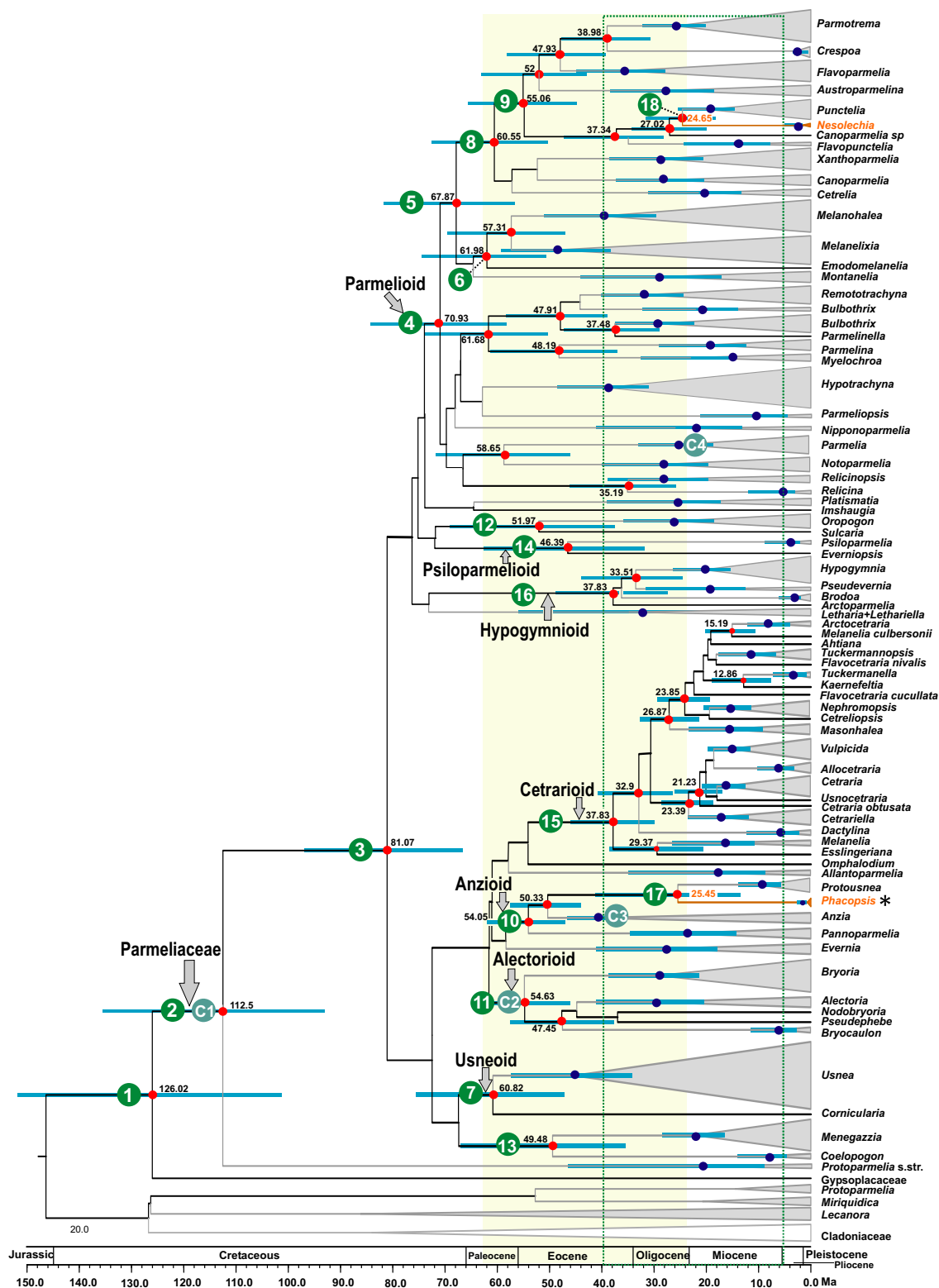
**Descriptions and illustrations:** Hawksworth (1978), Hafellner (1987), Triebel & Rambold (1988), and Triebel *et al.* (1995).

### Phylogenetic informativeness

Based on a per-site comparison, the *Tsr1* gene fragment produced higher PI across relative time units compared with *Mcm7*, *RPB1*, ITS, mtSSU and nuLSU (Fig. S2). *Mcm7* had higher PI values than *RPB1*, ITS, mtSSU, and nuLSU. *RPB1* had a higher PI for older time units (beyond 40 Ma) and ITS had a higher PI for younger time units (before 30 Ma). The two ribosomal markers (nuLSU and mtSSU) showed lower PI values.

### Discussion

This study is the first to give conclusive support to the hypothesis that lichenicolous fungi evolved several times within the predominantly lichen-forming fungal family Parmeliaceae, as first suggested by Peršoh & Rambold (2002). The evolution of lichenicolous fungi in a large family that otherwise includes large and morphologically derived lichen-forming fungi is consistent with the hypothesis that there are cases where the lichen symbiosis is labile and fungi with different lifestyles can evolve from lichenized ancestors. These fungi appear to have a mutualistic relationship with the photobionts of the lichens but an antagonistic relationship to the primary fungal partner through competition for resources provided by the photobiont (Poelt & Vězda, 1984; Friedl, 1987; Peršoh & Rambold, 2002). However, the ultrastructural relationship between the partners has not been investigated in these cases, as it has in some other lichenicolous species (de los Ríos & Grube, 2000). The possibility that these fungi are actually lichenized and share the algal partner with the



**Fig. 2** Timing of Parmeliaceae diversification. Chronogram derived from the maximum clade credibility tree estimated with the uncorrelated Bayesian relaxed molecular clock method in *BEAST* (Drummond *et al.*, 2012). Mean ages and their 95% highest posterior density bars are shown above nodes. The nodes indicated by (C) represent calibration nodes: C1, crown node of Parmeliaceae; C2, crown node of Alecatorioid clade; C3, crown node of Anzia; C4, crown node of *Parmelia*. Supported nodes are collapsed to generic level where applicable. The node splits are indicated in red circles and radiations in blue circles. Major splits period are highlighted with pale yellow rectangle and radiation periods with a green dotted line. Strongly supported principal and other interesting nodes are indicated as 1–18. \**Phacopsis huuskonenii* (placed in the new genus *Raesaenenia* in this paper).

lichen's fungal partner cannot be ignored, as several lichenized lichenicolous fungi are known (Hawksworth, 1988, 2003; Rambold & Triebel, 1992).

According to our estimates, the two distantly related lichenicolous genera in Parmeliaceae originated around the same time (*c.* 25 Ma in the late Oligocene). In the late part of the Oligocene, the Earth experienced a warming period, after a long cooling period in the early Oligocene that resulted in growth of the Antarctic ice sheets (Zachos *et al.*, 2008). This warming period, however, was interrupted by cooling periods, such as the Mi-1 glaciation (Zachos *et al.*, 2001; Wilson *et al.*, 2008) at the Oligocene–Miocene boundary *c.* 24 Ma. It has been shown previously that major splits within Parmeliaceae are associated with these climatic shifts (Amo de Paz *et al.*, 2011), and the origin of lichenicolous taxa in Parmeliaceae appears to be related to a major shift in the Earth's climate as well. A phylogenetic analysis of ITS data, including sequences from the lichenicolous *Phacopsis vulpina* (the type species of the genus), suggests the possibility of a third transition within the family. This species formed an independent lineage, sister to the *Relicina* + *Pseudoparmelia* clade (data not shown). However, despite several attempts, we were unable to obtain additional loci from the type species *P. vulpina* and other species of *Phacopsis* because of difficulties in obtaining fresh material, culturing the material, and obtaining PCR products and uncontaminated sequences. Therefore we could not verify the possibility of additional transitions from lichen-forming to lichenicolous lifestyles within the family with additional loci.

The monophyly of Parmeliaceae and its sister relationship with the monotypic family Gypsoplacaceae were strongly supported. Similar relationships have been found in previous studies (Arup *et al.*, 2007; Crespo *et al.*, 2007, 2010; Singh *et al.*, 2013). By contrast, in a recent class-wide study of Lecanoromycetes, the sister relationship between Parmeliaceae and Gypsoplacaceae was not recovered, and the alternative affiliation of the latter family with Malmideaceae and other families within Lecanorineae received high bootstrap (BS) support in selected analyses (Miadlikowska *et al.*, 2014). Here, we provide evidence of a strongly supported sister-group relationship of Gypsoplacaceae and Parmeliaceae within Lecanorineae using a larger sampling of loci and taxa (BS = 100% and posterior probability (pp) = 1.00; Fig. 1). However, the sister relationship of Parmeliaceae is dependent on the selection of the outgroup and the rooting of the tree. While phylogenetic relationships within the family were largely similar to those reported previously using a four-locus data set (Crespo *et al.*, 2010), our new analyses showed an increased number of resolved nodes and previously unrecognized relationships, which are discussed here. The Parmelioid, Hypogymnioid, and Psiloparmelioid clades, and the *Oropogon* + *Sulcaria* and *Platismatia* + *Imshaugia* clades formed a well-supported monophyletic group (pp = 0.97) while the remaining taxa in Parmeliaceae clustered within an unsupported group (Figs 1, S1). While our study supports the placement of the genera *Alectoria*, *Bryoria*, *Bryocaulon*, *Nodobryoria*, and *Pseudephebe* in the Alectorioid clade, the genus *Sulcaria*, considered a member of the Alectorioid clade in previous studies, is shown to be outside the Alectorioid clade and closely related to

*Oropogon*. The close relationship between *Oropogon* and *Sulcaria* is not surprising as both genera are characterized morphologically by having septate to muriform brown ascospores and cyphellae-like perforations. The beard lichens, classified in the genus *Usnea*, formed a sister-group relationship with *Cornicularia*, whereas *Menegazzia* spp., which previously formed a sister-group relationship with *Usnea* (Crespo *et al.*, 2010), formed a well-supported sister-group relationship with *Coelopogon* (Fig. 1). The Cetrarioid core group was reconstructed here as monophyletic with strong support, with *Melanelia* and *Esslingeriana* as sister to the other cetrarioid genera. This core group including *Melanelia* was either unsupported or weakly supported in previous studies (Thell *et al.*, 2009; Miadlikowska *et al.*, 2014). Within the Cetrarioid core group, two well-supported clades were recovered in our analysis: the *Cetraria* clade and the *Nephromopsis* clade, the latter unsupported in earlier studies (Thell *et al.*, 2009; Crespo *et al.*, 2010; Nelsen *et al.*, 2011). Within the Parmelioid clade, two major groups were recovered here for the first time: a strongly supported group (BS = 86%; pp = 1.00) including the *Parmotrema*, *Xanthoparmelia*, *Cetrelia*, and *Melanohalea* clades, including almost 80% of the total species diversity of Parmelioid lichens; and a clade that received support in the MRBAYES (Huelsenbeck & Ronquist, 2001) analysis only (pp = 0.95), comprising the rest of the Parmelioid species, including the *Nipponoparmelia*, *Hypotrachyna*, *Parmelia*, and *Parmelina* clades (Figs 1, S1). Furthermore, a novel strongly supported clade (BS = 100; pp = 1.00) which included species of the genera *Anzia*, *Pannoparmelia*, and *Protousnea*, and the lichenicolous species of *Phacopsis*, was recovered in Parmeliaceae for the first time and recognized as the Anzioid clade. The genus *Anzia* was part of the *Parmelina* clade in Miadlikowska *et al.* (2014).

Our results provide evidence for the divergence between the species-rich Parmeliaceae and the monotypic Gypsoplacaceae to have occurred in the early Cretaceous (mean age = 126 Ma; 95% HPD = 101–151 Ma; Fig. 2; node 1; Table S4). Divergence estimates between these families have not been inferred in previous molecular dating studies because of a lack of Gypsoplacaceae in the data set (Amo de Paz *et al.*, 2011). The origin of the family Parmeliaceae, represented by the divergence of the crustose genus *Protoparmelia* s.str. (node 2; Fig. 2) from the remaining part of the family (node 3), was here estimated to have occurred in the early Cretaceous (mean age = 112 Ma, 95% HPD = 92–135 Ma) which is almost the same as previously estimated (108 Ma; Amo de Paz *et al.*, 2011). Divergence time estimates for the Parmelioid clade were largely similar to those estimated before (Amo de Paz *et al.*, 2011) and thus are not discussed further here. The origin of the Cetrarioid clade was represented by an initial split of *Melanelia* + *Esslingeriana* at *c.* 37 Ma. However, the earliest divergent lineage was not recovered in the Parmelioid crown, and this may be attributable to the occurrence of lineage extinction events in this clade. The origin of the Usneoid clade (node 7) was estimated as mid-Paleocene, with *Cornicularia* representing the earliest divergent lineage; the Anzioid clade (node 10) in the early Eocene, with *Pannoparmelia* representing the earliest divergent lineage; the Psiloparmelioid clade (node 14) in the mid-Eocene; and the Hypogymnioid clade (node 16) originated at the

Eocene–Oligocene boundary. The age of the Alectorioid clade was estimated to be slightly older (*c.* 54 Ma; Table S4) than the previous estimates (*c.* 47 Ma, Amo de Paz *et al.*, 2011; 49 Ma, Abbas & Guo, 2015), although both estimates fall within the same stratigraphic intervals. A general trend emerging from these data is that the Paleocene, Eocene and Oligocene were key periods when diversification of major lineages within Parmeliaceae occurred, with subsequent radiation happening primarily during the Oligocene and Miocene (see Fig. 2). This may also be linked to the separation of the Southern Hemisphere landmasses (Abbas & Guo, 2015). Diversification bursts at different times may also be crucial factors driving the diversification of Parmeliaceae (Edwards & Donoghue, 2013; Christin *et al.*, 2014). Parmeliaceae shows relatively recent diversification patterns in comparison with other studied lichenized fungal groups (Prieto & Wedin, 2013; Beimforde *et al.*, 2014). High levels of species diversity are also found in many recently evolved groups of angiosperms (Magallon & Sanderson, 2001), with clades such as Apocynaceae, Arecaceae, Burseraceae, Casuarinaceae and Oleaceae, which were reported to have pronounced diversification in the Oligocene and Miocene (Magallon, 2010; De-Nova *et al.*, 2012; Bacon *et al.*, 2012).

The increased taxon and locus sampling, especially the addition of low-copy protein-coding markers such as *RPB1*, *Mcm7* and *Tsr1*, substantially improved the level of phylogenetic resolution and support within Parmeliaceae. Previous comparative studies have shown that low-copy protein-coding markers provide better nodal support than ribosomal markers (Schoch *et al.*, 2009). The *Mcm7* and *Tsr1* loci have been shown to outperform other genetic markers in resolving phylogenetic relationships in Ascomycota (Aguileta *et al.*, 2008; Schmitt *et al.*, 2009). Use of these protein-coding genes has become increasingly common in systematic studies within Ascomycota, including lichen-forming fungi (James *et al.*, 2006; Hofstetter *et al.*, 2007; Crespo *et al.*, 2010; Schmitt *et al.*, 2010; Leavitt *et al.*, 2013; Otálora *et al.*, 2013; Miadlikowska *et al.*, 2014). The PI of ribosomal markers (nuLSU, nuSSU, and mtSSU) and protein-coding genes (*RPB1*, *RPB2*, and *Mcm7*) was assessed for the higher level relationships in the Ascomycota tree of life (Schoch *et al.*, 2009; Raja *et al.*, 2011); however, it was never profiled for any of the major groups of lichenized fungi. The results of our PI analyses showed that *Tsr1* (625 bp) had the highest PI among the tested markers at this phylogenetic scale (Fig. S2). Moreover, *Tsr1* was the main contributor in resolving clades at both the higher and lower taxonomic levels. In our PI analysis, *Mcm7* (512 bp) performed worse than *Tsr1*, but better than *RPB1* and ITS (Fig. S2). ITS (345 bp) performed better than *RPB1* (663 bp) at the species level, whereas *RPB1* outperformed ITS at the generic and higher taxonomic levels. The commonly used ribosomal markers, nuLSU (791 bp) and mtSSU (724 bp), were outperformed by all other markers assessed here (Fig. S2). Thus, our results suggest that the phylogenetic power of *Tsr1* has a great potential to contribute significantly toward more stable relationships among lichenized fungi in Lecanoromycetes.

While our study provides an improved level of phylogenetic resolution within Parmeliaceae, some deep-level relationships,

at the backbone and among some of the major clades, still remained unresolved. Whether this is a result of adaptive radiations in the early evolution of Parmeliaceae is unclear. Phylogenomic approaches have been shown to help to resolve deep-level node relationships in different organisms, including fungi (Soltis *et al.*, 2011; Ebersberger *et al.*, 2012; Timme *et al.*, 2012; Zhou *et al.*, 2012; Shen *et al.*, 2013; Ampio *et al.*, 2014), and thus a phylogenomic approach is a logical next step to elucidate deep-level relationships within the Parmeliaceae in the future.

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Phylogenetic relationships among almost all accepted genera of Parmeliaceae.

**Fig. S2** Phylogenetic informativeness profiles for six loci.

**Table S1** Voucher information and GenBank accession numbers of the samples studied

**Table S2** Primers and annealing conditions used for amplification and sequencing

**Table S3** Genetic variability of the genes used in this study

**Table S4** Mean and range of divergence time estimations for Parmeliaceae

**Methods S1** Materials and methods.

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