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Species in section *Peltidea* (*aphthosa* group) of the genus *Peltigera* remain cryptic after molecular phylogenetic revision

Jolanta Miadlikowska[1*], Nicolas Magain[1,2], Carlos J. Pardo-De la Hoz[1], Dongling Niu[3], Trevor Goward[4], Emmanuël Sérusiaux[2] & François Lutzoni[1]

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Abstract. Closely related lichen-forming fungal species circumscribed using phenotypic traits (morphospecies) do not always align well with phylogenetic inferences based on molecular data. Using multilocus data obtained from a worldwide sampling, we inferred phylogenetic relationships among five currently accepted morphospecies of *Peltigera* section *Peltidea* (*P. aphthosa* group). Monophyletic circumscription of all currently recognized morphospecies (*P. britannica, P. chionophila, P. frippii* and *P. malacea*) except *P. aphthosa*, which contained *P. britannica*, was confirmed with high bootstrap support. Following their re-delimitation using bGMYC and Structurama, BPP validated 14 putative species including nine previously unrecognized potential species (five within *P. malacea*, five within *P. aphthosa*, and two within *P. britannica*). Because none of the undescribed potential species are corroborated morphologically, chemically, geographically or ecologically, we concluded that these monophyletic entities represent intraspecific phylogenetic structure, and, therefore, should not be recognized as new species. Cyanobionts associated with *Peltidea* mycobionts (51 individuals) represented 22 unique *rbcLX* haplotypes from five phylogroups in Clade II subclades 2 and 3. With rare exceptions, *Nostoc* taxa involved in trimembered and bimembered associations are phylogenetically closely related (subclade 2) or identical, suggesting a mostly shared cyanobiont pool with infrequent switches. Based on a broad geographical sampling, we confirm a high specificity of *Nostoc* subclade 2 with their mycobionts, including a mutualistically exclusive association between phylogroup III and specific lineages of *P. malacea*.

Key words: cyanolichen, molecular systematics, morphospecies, *Nostoc*, phylogeny, species delimitation, specificity, symbiosis

Introduction

The lichen-forming genus *Peltigera* (*Peltigerales*, *Ascomycota*) has been divided into eight sections based on morpho-chemical data and the large subunit ribosomal RNA gene (Miadlikowska & Lutzoni 2000). Section *Peltidea* (*P. aphthosa* group) is unique in containing both bimembered and trimembered species in which, respectively, the mycobiont associates either with a cyanobiont (*Nostoc* spp.) or with a chlorobiont (*Coccomyxa* spp.) and a cyanobiont (*Nostoc* spp., encapsulated in external cephalodia that sometimes can grow and form bimembered thalli, also referred to as photomorph). Currently, this section includes five accepted morphospecies (Holtan-Hartwig 1993; O'Brien et al. 2009; Miadlikowska et al. 2014; Magain et al. 2017a) of which three are trimembered: *P. aphthosa*, the type species of the section and one of the earliest described lichens (as *Lichen aphthosus*; Linneus 1753), *P. britannica* (Tønsberg & Holtan-Hartwig 1983), and *P. chionophila* (Goward & Goffinet 2000); and two are bimembered: *P. frippii* (Holtan-Hartwig 1988) and *P. malacea* (Funck 1827). Phylogenetically, section *Peltidea* clusters (with low bootstrap support; Miadlikowska et al. 2014; Magain et al. 2017a) with the remaining trimembered sections in the genus, i.e., section *Chloropeltigera* (*P. latiloba, P. leucophlebia* and *P. nigripunctata*) and section *Phlebia* (*P. venosa*) (Miadlikowska & Lutzoni 2000). Members of section *Peltidea* are widespread and relatively common in the arctic and boreal

[1] Department of Biology, Duke University, Durham, NC 27708-0338, USA
[2] Evolution and Conservation Biology, University of Liège, B-4000 Liège, Belgium
[3] Life Science School, Ningxia University, Yinchuan, Ningxia Hui Autonomous Region 750021, China
[4] UBC Herbarium, Beaty Museum, University of British Columbia, Vancouver, BC V6T 1Z4, Canada
[*] Corresponding author e-mail: jolantam@duke.edu

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biomes, as well as in mountainous regions of Eurasia and North America. They are found across a broad ecological spectrum, ranging from hygrophytic to xerophytic habitats, but they are most abundant in habitats with mesic conditions. They are almost absent from southern temperate lowland areas (except rare records of *P. malacea*: Vitikainen 1994; Martínez et al. 2003). Section *Peltidea* can be easily distinguished from other *Peltigera* sections based on ITS sequences from the mycobiont (e.g., Goffinet & Bayer 1997). Although rarely tested, no evidence of gene flow or hybridization has been observed between the recognized species (*P. frippi* was not examined), and fixed differences in nucleotide substitutions were found for at least two of the three loci of the collections studied from British Columbia (O'Brien et al. 2009).

In response to environmental factors, *P. aphthosa* and *P. britannica* may form photomorphs (Brodo & Richardson 1978; Armaleo & Clerc 1991; Stocker-Wörgötter & Türk 1994; Stocker-Wörgötter 1995; Goffinet & Bayer 1997), i.e., morphologically distinct thalli with different photobionts (where cephalodia of trimembered thalli with *Coccomyxa* and *Nostoc* can develop into bimembered thalli with *Nostoc* only) – a phenomenon reported also in section *Phlebia* (*P. venosa*; Tønsberg & Holtan-Hartwig 1983; Ott 1988; Miadlikowska 1998) and in other genera within the order *Peltigerales* (e.g., Stenroos et al. 2003; Magain et al. 2012; Moncada et al. 2013), as well as from lichenized *Arthoniomycetes* (Ertz et al. 2018). Except *P. venosa* (section *Phlebia*), which has a very distinctive thallus appearance, trimembered *Peltigera* with broad and leafy thalli (the *P. aphthosa* group sensu lato including members of section *Chloropeltigera*; Holtan-Hartwig 1993; Miadlikowska & Lutzoni 2000) can have similar morphology and chemistry, and therefore are sometimes difficult to identify without sequence data. Presence of a continuous cortex on the lower side of apothecia allows the identification of fertile thalli of section *Peltidea* (*P. aphthosa*, *P. britannica* and *P. chionophila*) in comparison with often co-occurring *P. leucophlebia* and *P. latiloba*, which have ecorticate or patchily corticate apothecia (Holtan-Hartwig 1993; Goward et al. 1995) and belong to section *Chloropeltigera* (*P. nigripunctata* is restricted to Southeast Asia; Goward et al. 1995; Martínez et al. 2003). However, some species such as *P. britannica* and *P. chionophila* are rarely found with apothecia and overall many collections of trimembered species are sterile. Although typical specimens can be identified based on other diagnostic features, including the thallus habit and lobe margin, degree of venation (evenly rounded boat shape lobes with fused veins in *P. aphthosa*), or the shape of the cephalodia (peltate in *P. britannica*), a tremendous morphological plasticity that has been documented in all trimembered species obscures species boundaries. Their overlapping geographical ranges and field ecology further contribute to difficulties in species recognition (e.g., *P. aphthosa* and *P. chionophila* inhabit similar snow prolonged sites in British Columbia, Canada; Goward et al. 1995). For example, in Norway, Holtan-Hartwig (1993) distinguished three morphotypes and the corresponding sets of chemotypes (with the same chemotype present in more than one morphotype) in *P. aphthosa* and *P. malacea*. Although overall secondary chemistry was shown to be useful and supportive of species circumscriptions across *Peltigera* (e.g., Holtan-Hartwig 1993; Vitikainen 1994; Kotarska 1999; Miadlikowska & Lutzoni 2000; Magain et al. unpubl.), their recognition in sections *Peltidea* and *Chloropeltigera* cannot rely solely on chemotypic variation because the same set of triterpenoids was reported from different species (e.g., chemotype III of *P. aphthosa* is identical to chemotype I of *P. malacea*; Holtan-Hartwig 1993), which can be morphologically similar (e.g., *P. leucophlebia* chemotype is similar to *P. latiloba* chemotype II; Holtan-Hartwig 1993), but phylogenetically unrelated (e.g., *P. leucophlebia* chemotype is similar to *P. aphthosa* chemotype III; Vitikainen 1994).

Molecular phylogenies, sometimes in combination with phenotypic data, allowed the evaluation of the alleged monophyly of described morphospecies. In some cases, this led to the discovery of new species in many lichen-forming groups of fungi, including the genus *Peltigera* (e.g., Goffinet & Miadlikowska 1999; Miadlikowska et al. 2003; Goffinet et al. 2003; Sérusiaux et al. 2009; Han et al. 2013; Han et al. 2015; Manoharan-Basil et al. 2016; Magain et al. 2016; Jüriado et al. 2017). O'Brien et al. (2009) used ITS data (in combination with population genetic methods) to show that *P. leucophlebia* (section *Chloropeltigera*) in British Columbia contains at least three well-supported monophyletic groups, which might represent putative species. More recently, Magain et al. (2017a, b) reported a high level of seemingly cryptic diversity in section *Polydactylon*, which was discovered by implementing multiple species delimitation and validation methods on multilocus data including three newly developed Collinear Orthologous Regions (containing intergenic spacers): COR1b, COR3, and COR16 that provided higher levels of phylogenetic signal than the fungal barcode ITS (Schoch et al. 2012). Furthermore, the authors showed that each of the three iconic and broadly recognized species-specific morphotypes: *P. scabrosa*, *P. dolichorhiza* and *P. neopolydactyla* represented multiple and sometimes unrelated phylogenetic lineages, and therefore questioning the current delimitation of common morphospecies in section *Polydactylon*.

Regardless of the focus group and sequenced loci, most studies implementing species delimitation and validation methods revealed multiple undescribed species with much narrower geographic ranges than traditionally circumscribed morphospecies, whereas an overestimation of species richness based on phenotypic traits was rarely reported. Most importantly, molecular phylogenetics enables the reevaluation of characters regarded as taxonomically important (e.g., Leavitt et al. 2011a, b, c, 2013; Lücking et al. 2014; Saag et al. 2014; Singh et al. 2015; Kirika et al. 2016 and references therein; but see Wei et al. 2016). For example, in the scabrosoid clade of section *Polydactylon*, seven of ten delimited species were putatively new, whereas in the dolichorhizoid clade 22 potentially new species were delimited for a total of 27 (Magain et al. 2017a, b). For *Peltigera hydrothyria* s.l.,

the only aquatic member of the genus (section *Hydrothyriae*), the ITS region alone provided sufficient genetic information to distinguish three cryptic species (Lendemer & O'Brien 2011), which were later confirmed by analyzing additional loci (Miadlikowska et al. 2014). Phylogenetic inferences based on multilocus data including intergenic spacers (COR) and the implementation of different species delimitation methods (bGMYC, bPTP, BPP) suggested the presence of 88 species in section *Peltigera*, including 50 species potentially new to science, hence uncovering a surprisingly high proportion of previously unnoticed, but mostly cryptic biodiversity (Magain et al. 2018). However, poor sampling, i.e., restricted to few localities that are separated by long distances, can also mislead species recognition and validation methods in detecting an artificially high number of species. Collections for areas between these isolated sampling sites can reveal intermediary patterns of variation and signatures of gene flow, which led to the circumscription of two subspecies (ssp. *udeghe* and ssp. *polydactylon*) in the broadly distributed *P. polydactylon*, which better reflects the evolution of these populations and speciation mechanisms than describing new species (Magain et al. 2016).

A wide range of specificity has been reported for *Peltigera-Nostoc* associations depending on the phylogenetic breadth of the mycobiont and the spatial scale. Viewed at an intercontinental (global) scale, for example, most species in section *Peltigera* show a relatively low level of specificity, such that widely distributed species usually recruit a broader selection of *Nostoc* phylogroups (up to eight phylogroups) than species with more limited distributions (usually with a single phylogroup: Magain et al. 2018). At an intrabiome scale, more specifically within the boreal zone, *Peltigera* species mostly associate with one or sometimes two cyanobiont phylogroups, which themselves have broader distribution than their mycobiont partners and associate with many *Peltigera* species (Lu et al. 2018). In general, reciprocal mutually exclusive specificity at the species level seems to be a rare phenomenon in cyanolichens, demonstrated to date in the isidiated (producing vegetative propagules containing both symbionts) species of *Leptogium* and *Collema* (*Collemataceae*, *Peltigerales*; Otálora et al. 2010), and for a few species of *Peltigera* including *P. malacea*, which was found to associate predominantly with *Nostoc* from phylogroup III of subclade 2 (mostly records from British Columbia and a few collections from Europe and Asia; O'Brien et al. 2013), *P. neopolydactyla* 5, *P.* sp. 11 and *P. vainioi* associated with phylogroups XIb, IX, XXVIa respectively (Magain et al. 2017a, 2018).

For practical purposes, this study constitutes Part 3 (after section *Polydactylon* and *Peltigera*) in our ongoing worldwide phylogenetic revision of *Peltigera*. Its purpose is to: 1) provide a comprehensive multilocus phylogeny for section *Peltidea*; 2) evaluate the currently recognized species in a phylogenetic context using multiple discovery and validation methods; 3) reassess the validity of species-specific phenotypic characters including secondary chemistry; 4) reevaluate the high mutually exclusive specificity reported for *P. malacea* and their *Nostoc* partners (O'Brien et al. 2013); and 5) compare patterns of symbiont specificity in section *Peltidea* to other sections of the genus *Peltigera*.

We sampled members of section *Peltidea* across their ranges, but with special emphasis on North America (from arctic regions southward through the Rocky Mountains and in the southern Appalachians) where trimembered species of *Peltidea* co-occur with somewhat phenotypically similar and morphologically variable species from section *Chloropeltigera*, *P. leucophlebia* s.l. and *P. latiloba*. We identified the mycobiont using four markers (β-tubulin, COR1b, COR3, and ITS) and the cyanobiont with *rbcLX*, i.e., the last 82 amino acids of the RUBISCO large subunit (*rbcL*), a putative chaperone gene (*rbcX*), and two intergenic spacers (Li & Tabita 1997). We inferred phylogenetic relationships for each partner, i.e., *Peltigera* and *Nostoc*. We delimited *Peltigera* species using multiple species discovery and validation methods and discussed the results within a phylogenetic framework.

Materials and methods

Taxon sampling and data acquisition

Based on examined collections from various fungaria (AMNH, B, BG, CONN, DUKE, GZU, H, LG, MIN, NY, OSU, QFA, UBC, UPS) and freshly collected specimens (e.g., fieldtrips to Norway, Canada: Québec, USA: Alaska in 2011; Canada: Alberta; USA: Michigan in 2013), we selected 196 specimens representing a wide morphological spectrum and broad geographical ranges within each species currently classified in section *Peltidea*: *P. aphthosa* (83), *P. britannica* (21), *P. chionophila* (11), *P. frippii* (5), and *P. malacea* (76). For each specimen, the internal transcribed spacer (ITS) region, which is the universal fungal barcode marker (Schoch et al. 2012) was sequenced. We also sequenced three other loci: β-tubulin for 173 specimens, and one intergenic spacer from each of the two CORs (Magain et al. 2017b): COR1b for 125 and COR3 for 70 specimens (Appendix 1). We supplemented the newly acquired sequences with ITS and β-tubulin data from GenBank (mostly from O'Brien et al. 2009) for an additional 78 individuals: 34 for *P. aphthosa*, 21 for *P. britannica*, two for *P. chionophila*, and 21 for *P. malacea*. Four specimens from the closely related section *Chloropeltigera* (two from *P. leucophlebia* s.l. and two from *P. latiloba*) were chosen as the outgroup (Miadlikowska et al. 2014; Magain et al. 2017a) in subsequent phylogenetic analyses. These four markers were sequenced for these outgroup specimens. Overall, we obtained 568 new sequences, specifically 196 of ITS, 177 of β-tubulin, 125 of COR1b and 70 of COR3. The data matrix contains 274 OTUs (including the outgroup) of which 53 were represented by all markers, 79 by three markers (mostly ITS, β-tubulin, and COR1b), 106 by two markers (mostly ITS and β-tubulin) and 36 by the ITS locus only. COR3 was the least represented marker among the four loci. In addition, we sequenced *rbcLX* for the cyanobacterial partners associated with 51 of the 196 selected *Peltigera* specimens and obtained sequences from GenBank for 21 of the 78 individuals from section *Peltidea* included in the

study of O'Brien et al. (2009) for a total of 72 specimens for which we had sequences from the mycobiont and associated *Nostoc* (Appendix 1). Information about the extraction protocol, amplification conditions, primers and reagents used, as well as Sanger sequencing are provided in Magain et al. (2017a, b).

All newly acquired sequences were subjected to BLAST searches to confirm their fungal or cyanobacterial origin. They were assembled and edited using the software package Sequencher™ 4.1 (Gene Codes Corporation, Ann Arbor, MI, USA) and aligned manually with MacClade 4.08 (Maddison & Maddison 2005). The "Nucleotide with AA color" option was used for guiding (delimiting exons and introns) all alignments of protein-coding genes. Ambiguously-aligned regions (sensu Lutzoni et al. 2000) were delimited manually and excluded from subsequent analyses.

Data sets and phylogenetic analyses

For the mycobionts, we assembled a separate data set for each locus: β-tubulin (228 OTUs), ITS (274 OTUs), COR1b (125 OTUs), and COR3 (70 OTUs). Single locus alignments were concatenated and reduced to 125 unique multilocus sequence types (H; Appendix 1) where each OTU was represented by at least one locus (1- to 4-locus data set; ML1) using in-house PERL scripts (combine.pl and collapse_multi.pl, respectively; Magain 2018). In addition to this most inclusive data set for 125 OTUs, we compiled a reduced data set for 92 OTUs where each OTU was represented by at least three loci (3- to 4-locus data set; ML2) using in-house PERL scripts (compare_and_choose.pl; Magain 2018). For cyanobionts from species of section *Peltidea*, the 51 new *rbcLX* sequences were filtered to 23 unique haplotypes (using collapse_multi.pl) including one haplotype from *P. latiloba*, a member of section *Chloropeltigera* (part of the outgroup). We assembled *rbcLX* data set (ML3) containing the 23 newly added and 11 previously published *Nostoc* haplotypes (HT; O'Brien et al. 2013) from section *Peltidea*, in addition to free-living, non-lichen-associated, and lichen-associated *Nostoc* (from the remaining sections of *Peltigera* and from other cyanolichens, mainly *Peltigerales*) for a total of 318 OTUs (see Appendix 1 for *rbcLX* sequences from section *Peltidea*).

Maximum likelihood analyses using RAxMLH-PC-MPI-SSE3 (Stamatakis 2006; Stamatakis et al. 2008) were performed (at the nucleotide level) on each locus separately and each concatenated data set (ML1 and ML2) for the mycobiont, and on the cyanobiont *rbcLX* (ML3) (Appendix 1). Optimal tree and bootstrap searches were conducted with the rapid hill-climbing algorithm for 1000 replicates with GTR substitution model (Rodríguez et al. 1990) and gamma distribution parameter as implemented in the Mobyle SNAP Workbench version 1.0.5, a portal for evolutionary and population genetics analyses (North Carolina State University online facilities) developed as part of the Dimensions of Biodiversity project (DoB; Monacell & Carbone 2014). For the analyses of *rbcLX* and β-tubulin, the 1st, 2nd, and 3rd codon positions, and introns in the latter locus were treated as different subsets; two subsets were defined for the ITS (ITS1+ITS2 vs. 5.8S) and no partition for each, COR1b and COR3 locus analyses. For the concatenated analyses, a partition of four and three subsets was estimated in ML1 and ML2 data sets, respectively by implementing PartitionFinder v. 1.1.0 (Lanfear et al. 2012) greedy search and using the Bayesian information criterion (BIC) for model selection (ML1: ITS1 + ITS2, 5.8S + β-tubulin 1st, β-tubulin 2nd, and β-tubulin 3rd + β-tubulin introns + COR1b + COR3; ML2: ITS1 + ITS2, 5.8S + β-tubulin 1st and 2nd, and β-tubulin 3rd + β-tubulin introns + COR1b + COR3). Relationships receiving bootstrap support above 70% were considered strongly supported.

Species delimitation and validation analyses

Sequences from 88 individuals representing section *Peltidea* (ML1 data set excluding the outgroup) were subjected to two species discovery methods, Structurama (Huelsenbeck et al. 2011), and bGMYC (Reid & Carstens 2012). The delimited species were validated using BPP (Yang & Rannala 2010). Structurama analyses were performed separately on two data sets (the *P. malacea* clade: 36 specimens, and the *P. aphthosa* clade: 52 specimens). In preparation for the Structurama analyses, we used an in-house PERL script (prepstructurama.pl; Magain 2018) to code the alleles represented in each sequenced locus (ITS, β-tubulin, COR1b, COR3) for each individual using 100% similarity as the criterion for collapsing haplotypes into a single allele. We ran Structurama on each data set for two million generations, sampling every 1000th generation and tested several gamma hyperpriors on the expected number of populations (a gamma scale varying from 1 to 3 and a gamma shape varying from 1 to 6). We selected a gamma scale value of 1 and a gamma shape value of 3 for the final analyses on the *P. malacea* and the *P. aphthosa* clades, respectively. We performed bGMYC analyses on chronograms derived from BEAST v. 1.8 (Drummond & Rambaut 2007) analyses (as implemented on the CIPRES portal; Miller et al. 2010) on each of the four following loci individually (ITS, β-tubulin, COR1b, COR3). We ran BEAST for each locus for 10,000,000 generations, sampling every 1000th generation. We used a strict molecular clock and the models GTR+G for ITS, GTR for β-tubulin, HKY+G for COR1b, and HKY for COR3, respectively (as determined by a MrModelTest v2.3 analysis; Nylander 2004). From each single-locus BEAST analysis, we selected 200 chronograms from the posterior tree distribution. Each file initially contained 10,000 trees, of which we kept one out of every 50th sampled trees using an in-house PERL script (lignes.pl; Magain 2018) to generate a final file of 200 trees. We ran bGMYC on each set of 200 trees for 50,000 generations per chronogram. We discarded 40,000 generations as burn-in with a thinning value of 100 and threshold values (corresponding to the interval of possible number of species) from 2 to 30. We considered a species to be well-delimited by bGMYC when the probability of grouping haplotypes together was higher than the probability of any alternative groupings that included at least one haplotype from this putative species. We ran BPP v. 2.2

on four single-locus alignments implementing a consensus species delimitation (numbered clades in Fig. 2) and a guide-tree corresponding to the ML2 topology (Fig. 2). We used the species delimitation algorithm and kept all sites containing missing data. We estimated the relative rates of evolution for single loci based on substitution rates from the ML analyses. For the τ prior we set the gamma shape to 1 and the gamma scale to 1000, estimating the height of the root based on the branch lengths in the RAxML single locus trees. For the θ prior, we set the gamma shape to 1000, and tested shape values of 0.5, 2, 5 and 10, so that the mean of the θ prior was 0.0005, 0.002, 0.005 and 0.01, respectively. We run the analyses for 50,000 generations, with a burn-in of 2000 generations, sampling every 2nd generation. We selected a θ mean of 0.005 for the final analysis and run it for 1 million generations, sampling every 100th generation, and discarding 200,000 generations as burn-in.

Chromatography

A total of 101 specimens (Appendix 1) were subjected to thin-layer chromatography (TLC) following the protocol by Orange et al. (2001). The lichen extracts were eluted in the solvent systems C (referred to as TA in Holtan-Hartwig 1993) and G (Culberson et al. 1981). Identification of substances was made in comparison with the results of Holtan-Hartwig (1993), Vitikainen (1994), and Kotarska (1999). Identified terpenoids (only well visible spots were considered) and chemotypes were numbered following Holtan-Hartwig (1993) and Kotarska (1999).

Results

Phylogenetic relationships and identity of the mycobiont and cyanobiont

Among the 196 sequenced representatives of *Peltigera* section *Peltidea*, we identified 125 unique multilocus sequence types across the four molecular markers (ML1 data set), with 92 of them being represented by at least three loci (ML2 data set). ML1 and ML2 phylogenies provided concordant relationships. Bootstrap support went up especially for the internal groupings (relationships among individuals) within *P. malacea* and *P. aphthosa* (Figs. 1 and 2) when we reduced the number of missing sequences by keeping only specimens with three and four loci. All presently recognized species in the section, except *P. aphthosa*, represent monophyletic highly supported lineages (Figs. 1 and 2; all specimens of *P. frippii* were represented by a single sequence type). In both phylogenies, *P. britannica*, a highly-supported clade, was found nested within *P. aphthosa* (one of the branches was so short that their relationship is shown as a polytomy in Fig. 1). Therefore, their sister relationship cannot be ruled out because the main divisions in the *P. aphthosa* s.l. clade received low bootstrap support (Figs. 1 and 2). The first phylogenetic split within section *Peltidea* separates the bimembered species (*P. frippii* and *P. malacea*) from trimembered species (*P. chionophila* sister to *P. aphthosa* including *P. britannica*). Robust intraspecific phylogenetic structure was detected within *P. malacea*, which comprises five strongly supported monophyletic groups.

Altogether, the sequenced cyanobionts (51 individuals) associated with species from section *Peltidea* represented 22 unique haplotypes (with one additional haplotype detected in *P. latiloba* section *Chloropeltigera*; H1-23; Appendix 1) representing four phylogroups (III-VI) and one undetermined lineage (Fig. 3; Appendix 1). The most frequent haplotype is H5, which was found in 13 specimens of *P. malacea* collected in several localities in North America and Norway. Four other haplotypes were encountered multiple times (H3, H6, H8, and H9; N = 3–5) in different species, including *P. aphthosa, P. britannica, P. frippii* and *P. malacea* in North America, northeastern Asia and Norway. The remaining 17 haplotypes were rare, i.e., recorded from one or two lichen thalli. All *Nostoc* strains associated with section *Peltidea* represent Clade II, subclades 2 (BS = 90%) and 3 (BS = 57%). All cyanobionts from *P. malacea* are grouped in subclade 2, mostly phylogroup III (BS = 71%), which exclusively contains *Nostoc* from this species. A few *Nostoc* strains from *P. malacea* including a haplotype (H8) shared with *P. aphthosa* and *P. frippii* were placed in phylogroup IV (BS < 70%), together with *Nostoc* associated with *P. aphthosa* and *P. britannica*, as well as with *P. neopolydactyla* 4 and other taxa from *Peltigerineae* including *Sticta* and *Nephroma arcticum* (sequences from GenBank). Subclade 2 contains a third poorly supported clade with *Nostoc* from other members of the order *Peltigerales* (*Scytinium lichenoides* [formerly *Leptogium lichenoides*] from *Collemataceae* and *Protopannaria pezizoides* from *Pannariaceae*). The remaining sequences of *Nostoc* (mostly from *P. aphthosa* and *P. britannica*) were found in subclade 3, phylogroups V (BS = 68%) and VI (BS < 60%) together with many cyanobionts from other tri- and bimembered sections of *Peltigera*. A single representative of *Nostoc* from *P. aphthosa* (P1330) was placed outside of the delimited phylogroups in the part of the tree where the phylogenetic relationships are very unstable (possibly part of phylogroup I; Fig. 3).

Species delimited and validated

The number of species delimited in *Peltigera* section *Peltidea* by bGMYC analyses performed on each locus separately varied from 12 (β-tubulin and COR3) to 13 (COR1b) and 15 (ITS), whereas Structurama grouped all individuals into 12 species based on the concatenated 3- to 4-locus data set (ML2). Of the 14 species tested, all were validated by BPP with high posterior probabilities (Fig. 2; Appendix 2). The overlap among species boundaries established on each locus and by different methods varied across the section. We observed complete agreement in delimitation of *P. frippii* and *P. chionophila* (except the ITS data, which supported the recognition of P1140 as an additional species), some degree of similarity in species circumscriptions within *P. malacea* s.l. (four to seven putative species, many of which were delimited by multiple inferences, but only M2 was consistently recognized); and a noticeable discrepancy in species assignment within *P. aphthosa* and *P. britannica* (although the

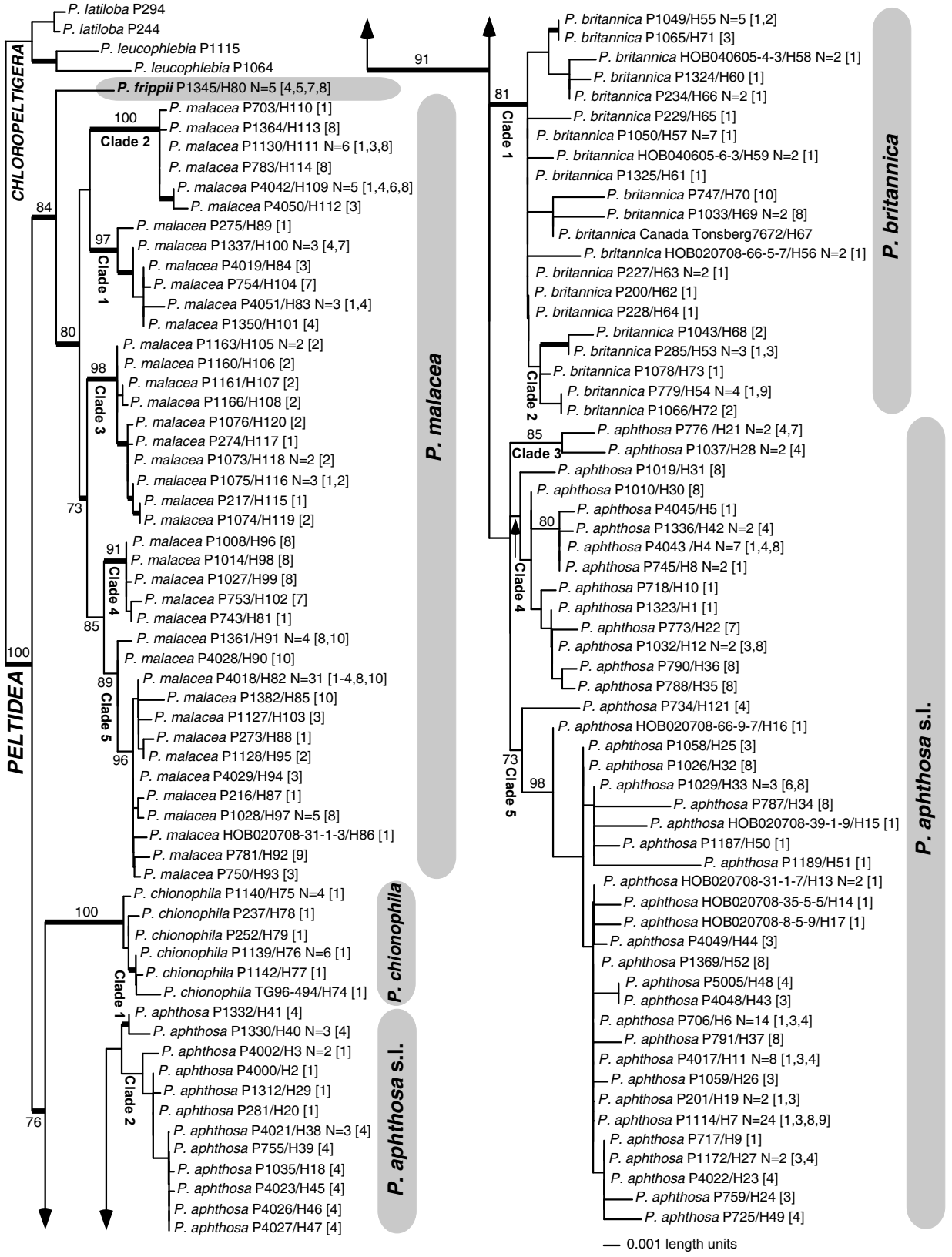


Figure 1. Phylogenetic relationships among members of section *Peltidea* as revealed by a maximum likelihood analysis of the concatenated 4-to1-locus data set (ML1: ITS + β-tubulin + COR1b + COR3; 2706 nucleotides; taxa represented by one, two, three and four loci) for 125 OTUs including four outgroup taxa from section *Chloropeltigera* (used to root the tree; Miadlikowska et al. 2014). Support values that resulted from the bootstrap analysis of the ML1 data set are provided on thick internodes when values are ≥ 70 %. The remaining bootstrap values provided for thin internodes, which were poorly supported in ML1 (ML-BP below 70%), are derived from bootstrap analyses of the ML2 data set (92 taxa with 3- to 4-locus data set; Fig. 2). Grey bars show current circumscription of species in section *Peltidea*. Clades within each recognized species are numbered in accordance to species delimitations validated by BPP (Fig. 2; Appendix 2). Each terminal represents a distinct mycobiont sequence type (based on four combined loci). For each terminal we provide current species name, DNA extraction number (P) or voucher id (for specimens represented by GenBank sequences), mycobiont sequence type designation (H), the number of individuals represented by each sequence type (N), and their geographical origin (in square brackets) (see Appendix 1 and Fig. 2).

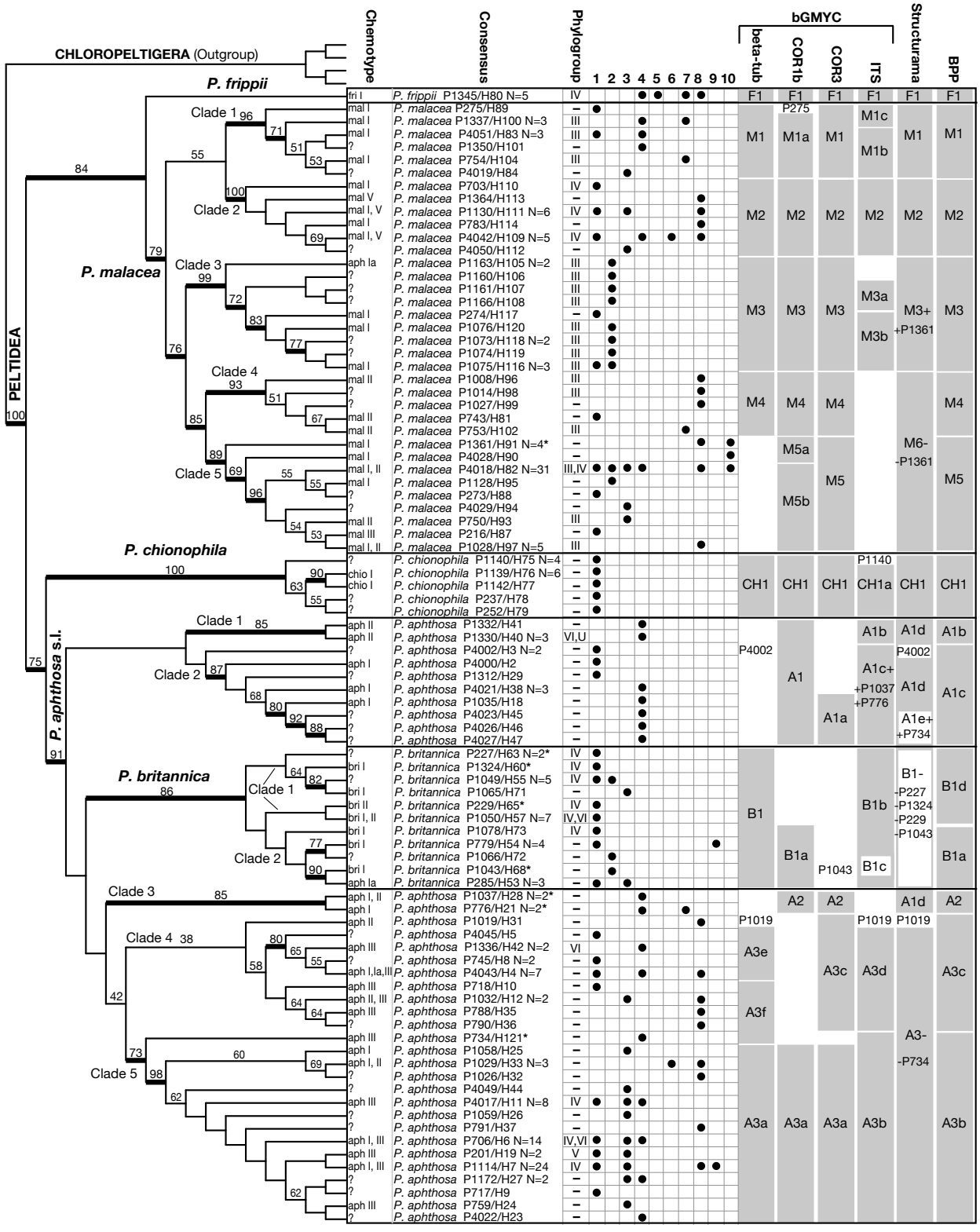


Figure 2. Summary of species delimitation (bGMYC and Structurama) and validation (BPP), chemotypic variation, cyanobiont phylogroup, and geographic origin (1-10) of the examined specimens within a phylogenetic framework (ML2 cladogram) for *Peltigera* section *Peltidea*. Phylogenetic relationships were inferred based on maximum likelihood analyses of a concatenated 3- to 4-locus data set (ML2: ITS + β-tubulin + COR1b + COR3; 2706 nucleotides; taxa represented by three and four loci) for 92 OTUs including four outgroup taxa from section *Chloropeltigera* (used to root the tree; Miadlikowska et al. 2014). Bootstrap values > 50% are provided. Internodes with ML bootstrap support (ML-BP ≥ 70%) are shown with thick branches. Within each recognized species, clades were numbered in accordance with species delimitations validated by BPP (see also Fig. 1; Appendix 2). Panels next to the cladogram (from left to right) represent: chemotype designation; taxonomic name according to the consensus species recognition and associated information; *Nostoc* phylogroup designation; geographic origin of sampled specimens; species delimitation by bGMYC based on β-tubulin, COR1b, COR3, and ITS loci; species delimitation by Structurama (with gamma shape of 1 and 3 for the *P. aphthosa* and the *P. malacea* clades, respectively); and species validation by BPP. Each terminal represents a distinct mycobiont multilocus sequence type. Chemotype designation (Fig. 4; Appendix 1) based on Thin Layer Chromatography (TLC) is provided for all individuals examined within each multilocus sequence type. Question marks indicate terminals for which TLC data are not available. Each terminal name

→

Figure 2. Continued.

contains the DNA extraction number (P) or voucher id (GenBank sequences) for the representative individual, mycobiont multilocus sequence type designation (H; after a slash), and the number of individuals represented by each sequence type (N) (see Fig. 1; Appendix 1). Asterisks indicate terminals assigned to polyphyletic or paraphyletic species delimited by bGMYC based on ITS and Structurama. *Nostoc* phylogroups were defined following O'Brien et al. (2013), Magain et al. (2017a), and this study (U indicated the placement outside of defined phylogroups; Fig. 3; Appendix 1). For the chemotype designations, aph refers to *P. aphthosa*, bri to *P. britannica*, chio to *P. chionophila*, fri to *P. frippii*, and mal to *P. malacea,* followed by the chemotype number (Fig. 4; see also Holtan-Hartwig 1993 and Vitikainen 1994). Black dots represent (single or multiple) specimens sampled in this study from the following geographic regions: 1) West Coast: Oregon, Washington, British Columbia, Alaska, Yukon; 2) Rocky Mountain Region: Montana, Wyoming, Colorado; 3) Midwest Region: Minnesota, South Dakota, Michigan, Alberta, Manitoba, Ontario; 4) Northeastern Region: Newfoundland, New Brunswick, Nova Scotia, Québec, Labrador; 5) Nunavut; 6) Central Northern Asia (Siberia): Krasnoyarsk Territory; 7) Eastern Northern Asia (Far Eastern District): Khabarovsk Territory, Kamchatka, Yakutia; 8) Fennoscandia; 9) Iceland; 10) remaining Europe including North Caucasus. Gray boxes show species delimited by bGMYC and Structurama and validated by BPP (for species abbreviations and posterior probability values see Appendix 2). White boxes within grey ones indicate nested species (e.g., species B1c is nested within species B1b as delimited by bGMYC based on ITS locus alone). Empty space indicates specimens that could not be assigned to any species because they were included in an alternative species delimitation with a higher posterior probability value or were not represented in a single gene matrix (missing data; Appendix 1). Singletons (species containing a single representative) are indicated by a unique DNA number (e.g., P275). Consensus species delimitations reflecting current species circumscriptions are indicated by black frames (notice that *P. britannica* as currently recognized, is nested within *P. aphthosa* s.l., but without strong bootstrap support).

number of recognized species was in overall agreement, their delimitations varied drastically; Fig. 2; Appendix 2). A high level of uncertainty (low likelihood and probability values) in species delimitation occurred in selected clades of *P. malacea* s.l. based on ITS and β-tubulin and in general across *P. aphthosa/britannica* complex (based on β-tubulin, COR1b and COR3). bGMYC on the ITS and Structurama analyses resulted in a few polyphyletic species (e.g., A1c+P1037+P776, M3+P1361 in *P. malacea* s.l., A1e+P734 in *P. aphthosa* s.l.) and species nested within broadly delimited ones (e.g., B1c within B1b, A1e+P734 within A1d) (Fig. 2). Depending on the data sets used, several singletons were recognized as independent species (e.g., P275, P1140, P4002, P1043, and P1019). According to BPP, the following species were validated with high probability: *P. frippii*, *P. chionophila*, five putative species within each, *P. malacea* and *P. aphthosa* (all of them are monophyletic and well supported except two of *P. aphthosa*, which received bootstrap support below 70%), and two potential species in *P. britannica* (one paraphyletic and both poorly supported) (Figs. 1 and 2).

Chemotypic diversity

All specimens subjected to TLC analyses contained secondary metabolites. We focused on conspicuous and tractable spots corresponding to terpenoids identified by Holtan-Hartwig (1993). In many cases, a direct comparison of our results with published chemical profiles was difficult or impossible because we did not use EHF solvent (contains diethyl ether, highly volatile and flammable), which was developed especially for *Peltigera* (Tønsberg & Holtan-Hartwig 1983). Although the EHF solvent resembles system G of Culberson et al. (1981), which we used in this study, it gives a better separation of terpenoids and enhances the visibility of terpenoids present in relatively low concentrations. Among the 101 specimens examined, we detected seven chemotypes previously reported from members of *Peltidea* by Holtan-Hartwig (1993) (Fig. 4): *P. aphthosa* I (aph I) from *P. aphthosa*, *P. chionophila* (chio I), and *P. frippii* (fri I); *P. aphthosa* II (aph II) from *P. aphthosa* and *P. britannica* (bri II); *P. aphthosa* III (aph III) from *P. aphthosa*, and *P. malacea* (mal I); *P. britannica* I (bri I) from *P. britannica*; *P. malacea* II (mal II), *P. malacea* III (mal III), and *P. malacea* V (mal V) from *P. malacea*. Additionally, we detected a potentially new chemotype (aph Ia) from *P. aphthosa, P. britannica* and *P. malacea*, which resembles aph I with the addition of terpenoid 12. All examined specimens of *P. chionophila* and *P. frippii* were represented by a single chemotype (chio I and fri I, respectively), while three chemotypes (aph Ia, bri I, bri II) were detected in *P. britannica*, four chemotypes (aph I, aph Ia, aph II, aph III) in *P. aphthosa*, and four chemotypes (mal I, mal II, mal III, mal V) were found in *P. malacea* (Figs. 2 and 4; Appendix 1). For three species, specimens representing the same multilocus sequence type contained more than one (up to three) chemotypes. For example, chemotypes mal I and mal V were detected among individuals of *P. malacea* multilocus sequence types 111 (H111) and 109 (H109), and chemotypes aph I, aph Ia, and aph III were detected in *P. aphthosa* multilocus sequence type 4 (H4) (Fig. 2; Appendix 2). Depsides aggregate and methyl gyrophorate were clearly visible on plates across most samples, especially in the G solvent.

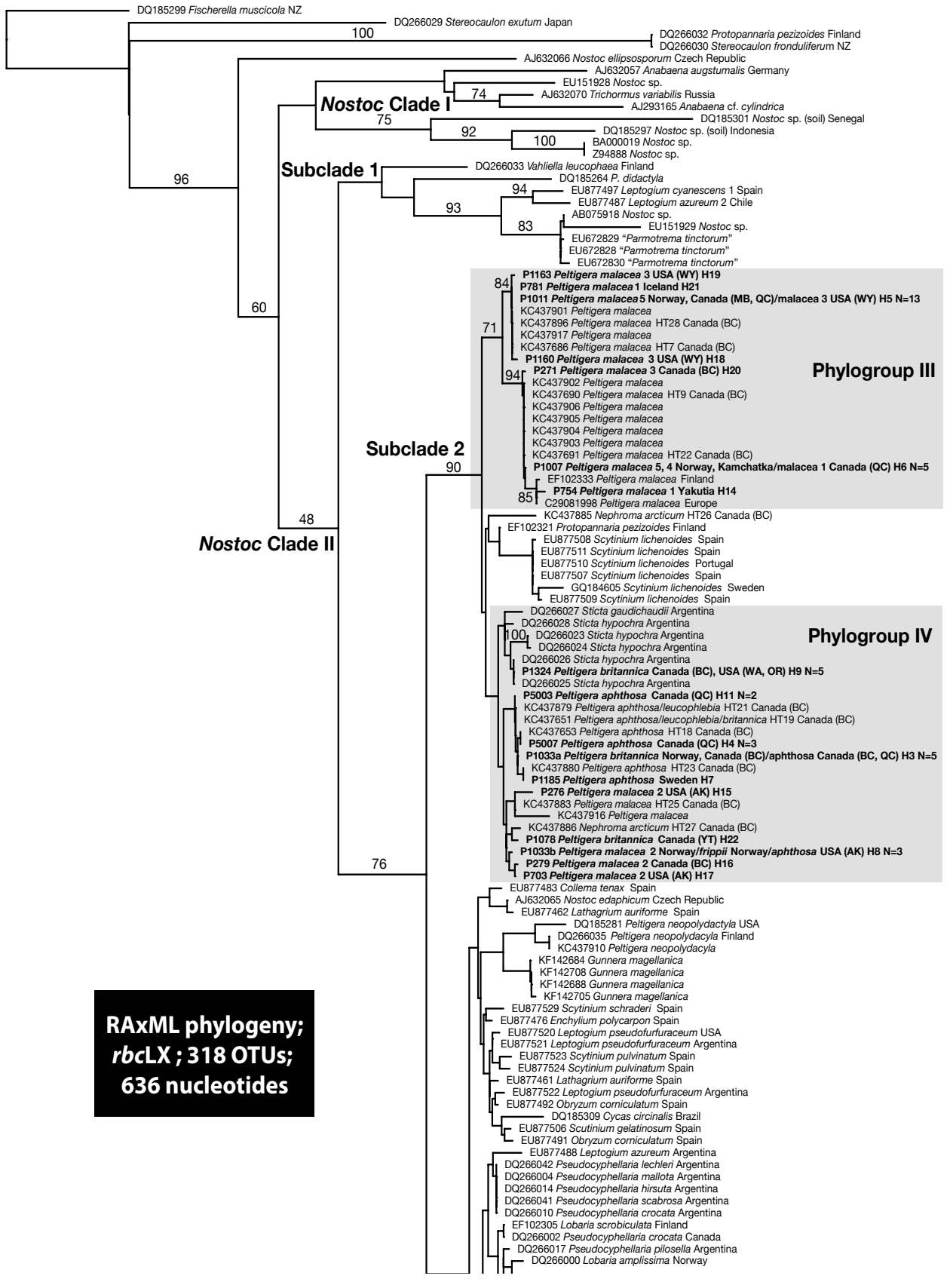
Discussion

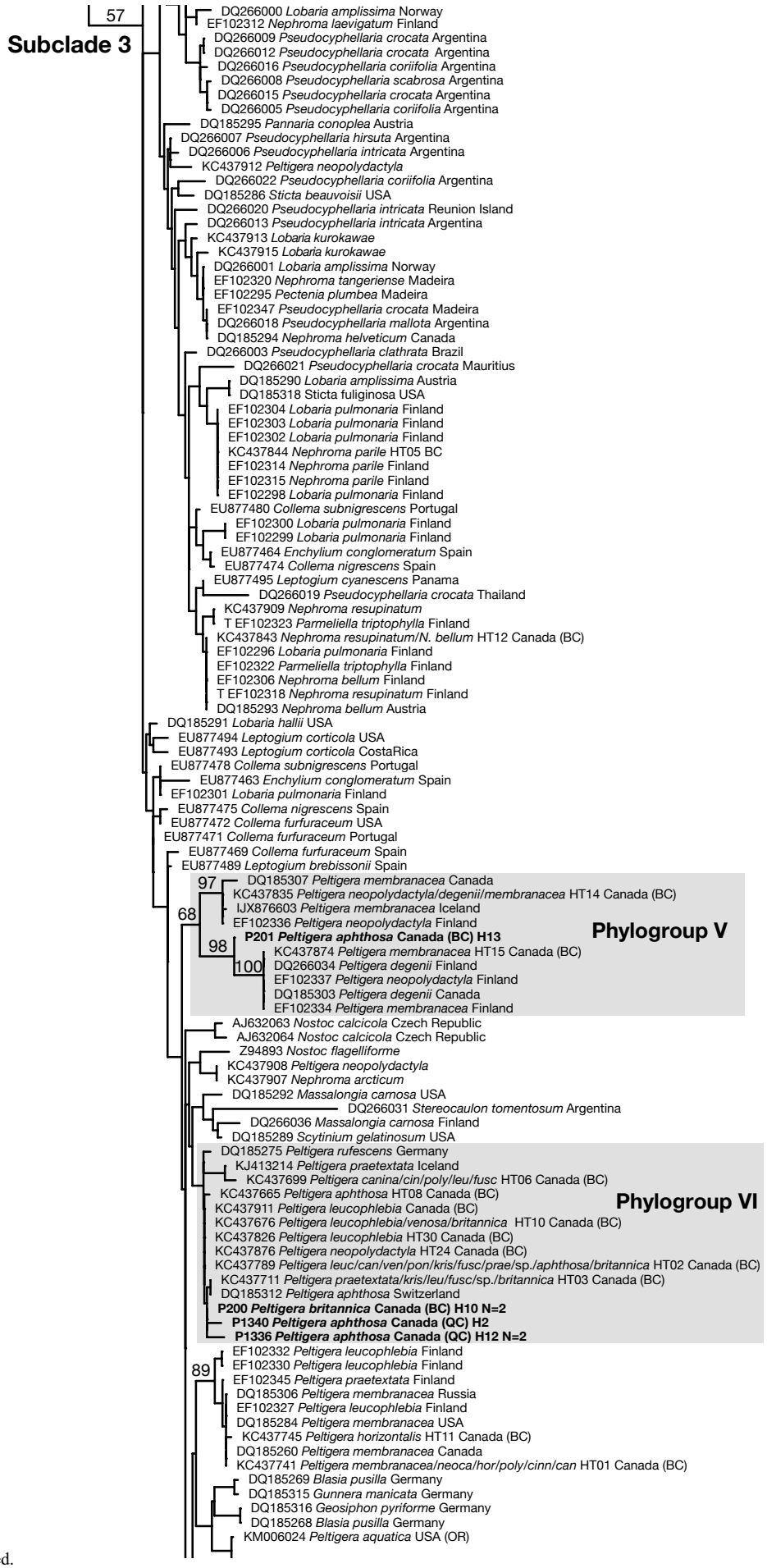
Molecularly defined species remain phenotypically inconsistent

This is the most comprehensive phylogenetic study of *Peltigera* section *Peltidea*, where all five recognized morphospecies are represented by multiple individuals from distinct geographic localities (Figs. 1 and 2). In all

Figure 3. Placement of 23 haplotypes of *Nostoc* (recognized among 51 newly sequenced cyanobionts) found in association with mycobionts from section *Peltidea* (plus one from section *Chloropeltigera*) within a broad phylogenetic context as revealed by maximum likelihood analysis of the *rbcLX* locus (ML3) for 318 OTUs. *Fischerella muscicola* (*Stigonematales*) was used to root the tree. Names for published sequences of free-living, non-lichen-associated, and lichen-associated symbionts are preceded by their GenBank accession numbers and followed by their geographic origin (when available). Names for new sequences (shown in bold) contain information about their geographic origin, haplotype designation (H), and number of individuals representing each haplotype (Appendix 1). Delimitation of clades, subclades, and phylogroups (gray boxes) follow Otálora et al. (2010), O'Brien et al. (2013), and Magain et al. (2017a). Bootstrap support values are provided above internodes.

previously published phylogenies (Miadlikowska & Lutzoni 2000, 2004; O'Brien et al. 2009; Magain et al. 2017a, b, 2018), section *Peltidea* has been poorly sampled owing in part to different taxonomic and geographical focusses. Similar to other lichen genera and species complexes (e.g., Leavitt et al. 2011c, 2016; Lücking et al. 2014), including *Peltigera* sections *Polydactylon* and *Peltigera*, where the proportion of undiscovered biodiversity was unexpectedly high (half or more of the recovered species were potentially new; Magain et al. 2017a, b, 2018), molecular data





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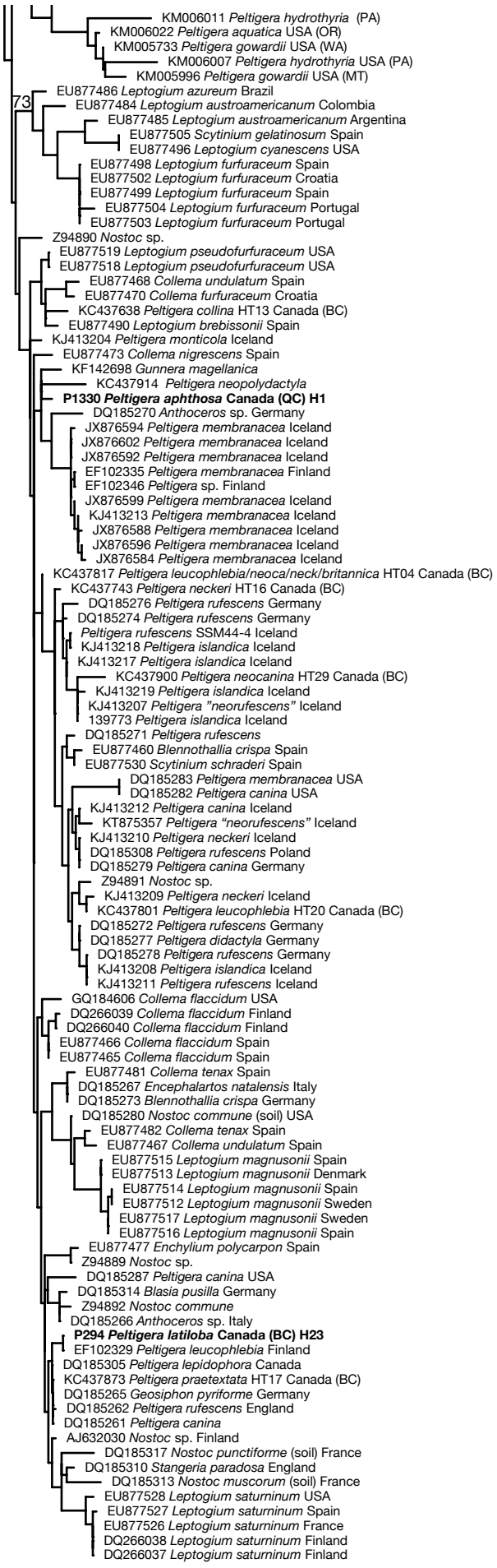

Figure 3. Continued.

support the recognition of 14 putative species for section *Peltidea*, of which nine are undescribed.

The impact of the incongruence among loci and implemented methods on currently recognized species has previously been reported across various taxonomic groups, including fungi and, among them, the genus *Peltigera* (e.g., Singh et al. 2014 and references therein; Wei et al. 2016; Magain et al. 2017a, b, 2018 and references therein; Da Silva et al. 2018). In this study we detected a relatively high level of discrepancy and uncertainty in the species number and species boundaries, including cases of paraphyletic/polyphyletic entities, as well as nested and singleton species, especially in *P. aphthosa* (Fig. 2). This high level of inconsistency among loci and analyses confirms that applying one species discovery method on a single locus, e.g., the ITS region does not provide a reliable species assignment scheme across different fungal groups (but see Lücking et al. 2014). Out of the five traditional morphospecies currently accepted in section *Peltidea*, only two, *P. chionophila* and *P. frippii*, were well circumscribed (with the exception of the ITS of P1140 in *P. chionophila*) and validated as such. The remaining three species – *P. aphthosa, P. britannica* and *P. malacea* – were split into multiple putative species corresponding to separate (with the exception of Clade 1 of *P. britannica*), sometimes highly supported lineages (Figs. 1 and 2). *Peltigera aphthosa* is the only morphospecies with uncertain monophyly unless we expand its

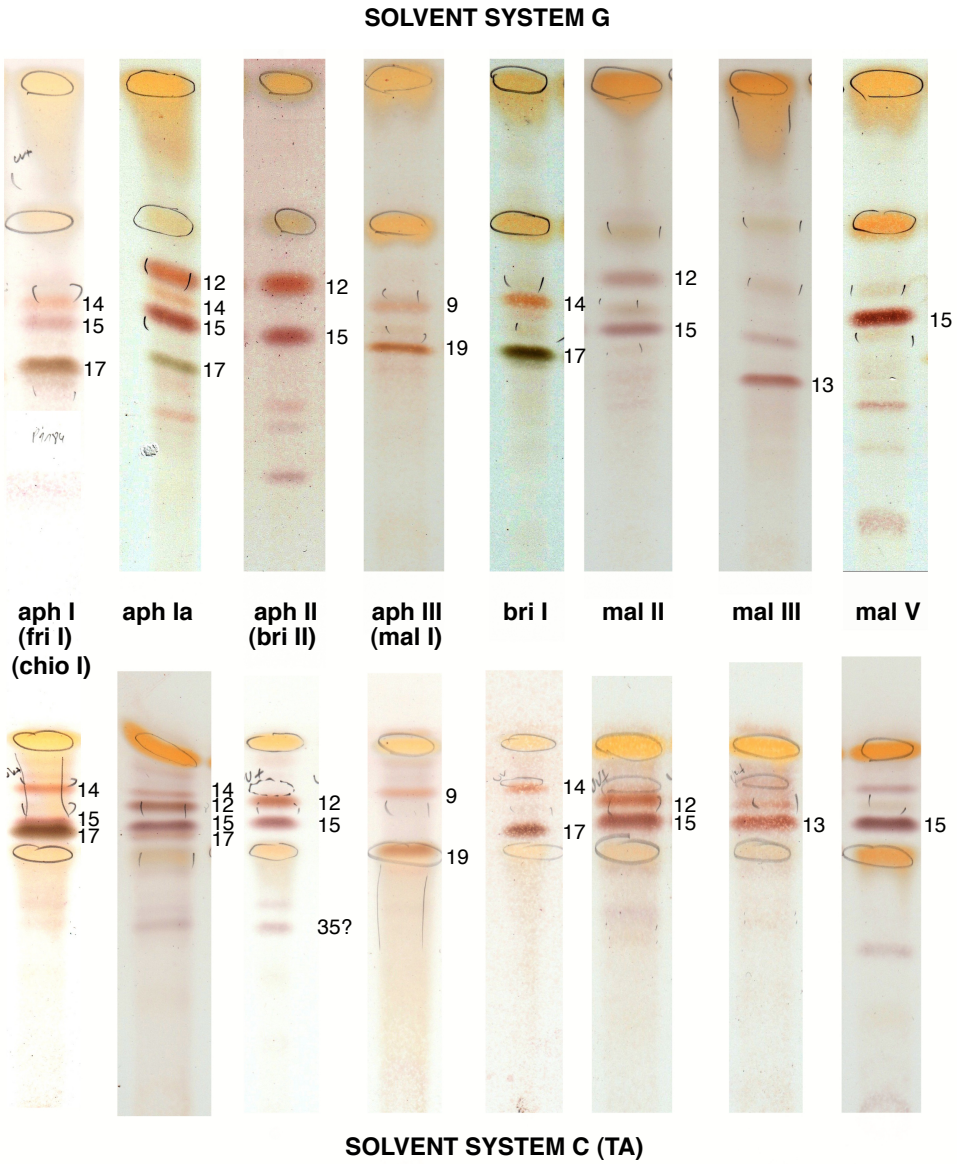

Figure 4. Chemotypes recognized based on Thin Layer Chromatography (TLC) analyses using the G solvent (top row) and C solvent (bottom row) among the examined specimens from section *Peltidea*. The chemotypes shown were part of different TLC plates, and therefore the placement of terpenoids cannot be directly compared. Chemotype abbreviations and terpenoid numbering follow Holtan-Hartwig (1993). Only conspicuous and previously recognized terpenoids are annotated. The top orange spots represent depside aggregates (mostly gyrophoric acid) and methyl gyrophorate. Abbreviations are as follow: aph = *P. aphthosa*, bri = *P. britannica*, chio = *P. chionophila*, fri = *P. frippii*, mal = *P. malacea*. Chemotypes: aph I/fri I/chio I (*P. aphthosa* I/P. *frippii* I/*P. chionophila* I): 15, 17, 14; aph Ia (*P. aphthosa* Ia/*P. britannica*): 15, 17, 14, 12; aph II/bri II (*P. aphthosa* II/P. *britannica* II): 15, 12 (other spots: 24, 35, 28 not visible); aph III/mal I (*P. aphthosa* III/P. *malacea* I): 19, 9; bri I (*P. britannica* I): 17, 14; mal II (*P. malacea* II): 15, 12 (14 not visible); mal III (*P. malacea* III): 13; mal V (*P. malacea* V): 15. 12 = dolichorrhizin, 15= zeorin, and 17 = phlebic acid B.

circumscription to include *P. britannica*, which is nested in *P. aphthosa* (but with low bootstrap support). The sister relationship of these two species cannot be ruled out because of the high instability of the internal branching within the *P. aphthosa* s.l. clade in our phylogenies (Figs. 1 and 2). "At its best *P. britannica* is very dissimilar to *P. aphthosa* in morphology (…)" (Vitikainen 1994) due to the presence of squamulose cephalodia and its wrinkled and pitted surface, however, these characters are not consistently present (e.g., specimens from Rocky Mts. of *P. britannica* 2) and therefore, it could also represent an intraspecific taxon (e.g., subspecies as suggested by Vitikainen 1994) or morphotype within a highly variable *P. aphthosa*. The status of *P. britannica* requires further investigation (more than four loci seem to be necessary). Both species need to be sampled extensively to test their monophyly and to evaluate possible interbreeding.

We were unsuccessful in establishing morphological, chemical or geographic markers for our nine putative species (Fig. 2). We compared the three distinct morphotypes (with corresponding chemotypes) recognized by Holtan-Hartwig (1993) in *P. aphthosa* and *P. malacea* from Norway with the morphological variation across the new species re-delimited in our geographically broader sampling. None of these phenotypic patterns were consistent with the new proposed species boundaries (Fig. 2). Most morphological features usually accepted as diagnostic in section *Peltidea* (e.g., Vitikainen 1994; Goward et al. 1995; Brodo et al. 2001) were observed across multiple species. For example, we observed a broad range of variation in several traits including thallus thickness, cephalodia morphology and vein distinctiveness across *P. aphthosa* s.l. (Fig. 5A–O). Although combinations of some features were predominantly associated with certain lineages, overall these morphological differences were not consistently correlated with the species delimitation scheme unveiled by our analyses.

Most specimens of *P. aphthosa* 1 are medium to small (up to 5 cm) with ascending, thick (1 mm), tomentous margin and boat shaped lobes (Fig. 5B), and have flat, adnate cephalodia (Fig. 5C). The lower surface is veinless or with pale veins in the margins abruptly becoming dark and fused towards the thallus center (Fig. 5A). This morphology is consistent with the description of European material by Vitikainen (1994) (potential *P. aphthosa* s.str. clade), however, it is also found in the remaining four lineages of *P. aphthosa* s.l. Specimens from *P. aphthosa* 2 have usually relatively thin thalli (0.3–0.6 mm) with a conspicuous dark venation in the lower surface extending all the way to the margin, and fasciculate rhizines arranged along the veins (Fig. 5D). Individuals with varying degree of conspicuous pale to dark veins in the lower surfaces, that become fused towards the center of the thallus, occur in addition to the distinct venation pattern in *P. aphthosa* 3 (Fig. 5G), *P. aphthosa* 4 (Fig. 5J) and *P. aphthosa* 5 (Fig. 5M). All lineages within *P. aphthosa* s.l. have flat, adnate cephalodia (Fig. 5C, F, H, K, L, O). However, smaller wart-shaped cephalodia were also observed in *P. aphthosa* 2 (Fig. 5E), *P. aphthosa* 4 (Fig. 5I) and *P. aphthosa* 5 (Fig. 5N). In *P. aphthosa* 5, cephalodia were usually warty near the margin of the thallus and become flat towards the thallus center (Fig. 5N, O).

The combination of almost veinless lower surface, flat, adnate cephalodia, and corticated underneath of apothecia was used to distinguish *P. aphthosa* from *P. leucophlebia* (Vitikainen 1994). However, sterile specimens often cannot be accurately assigned to either species because of the overlapping interspecies morphological variation, which seems to be a common phenomenon observed in the material collected in North America. Morphotypes of *P. leucophlebia* and *P. aphthosa* are more distant and therefore easily distinguished in Europe (Holtan-Hartwig 1993; Vitikainen 1994). Similar extensive morphological variation in North American collections (perhaps due to a wider range of available habitats) contrary to the European material has been reported for sections *Polydactylon* (e.g., *P. polydactylon* and *P. neopolydactyla*; Magain et al. 2017a, b) and *Peltigera* (e.g., *P. ponojensis/monticola* and *P. austroamericana* complexes; Magain et al. 2018) as well as other groups of lichens (Gueidan & Lendemer 2015). However, it is possible that in some cases it might be indicative of ongoing gene flow among lineages recognized as species based on selected molecular markers and analytical methods, similar to the potential gene flow signature discovered in Eurasia between two subspecies of *P. polydactylon*, which were initially considered as independent species (Magain et al. 2016).

Peltigera britannica is often distinguished from its close relatives (*P. aphthosa* and *P. chionophila*) by the presence of peltate (to squamulose), loosely attached cephalodia (Vitikainen 1994; Fig. 6A, G). However, we examined some specimens of *P. britannica* 1 with both adnate and peltate cephalodia (Fig. 6B) on the same thallus. Such variation might be indicative of a recent or incipient speciation event resulting in *P. britannica* s.l. and *P. aphthosa,* which could explain why their reciprocal monophyly was not recovered by phylogenetic inference based on four highly variable loci (Figs. 1 and 2). We also observed broadly overlapping morphological variation in *P. britannica* 1 and 2, in which the pilema ranges from dark and veinless in both lineages (Fig. 6C, H) to veinless with pale margin (Fig. 6C) or conspicuously veined (Fig. 6D) in *P. britannica* 1. Both lineages within *P. britannica* s.l. have warty to fully corticated apothecial reverses (Fig. 6E, F). *Peltigera chionophila* can be recognized by the presence of appressed, cerebriform cephalodia (Fig. 6I, J) and dark, well defined, rhizinate veins (Fig. 6K). It also has a narrow geographic distribution, being restricted to mountainous regions with heavy, prolonged snow cover (Goward & Goffinet 2000).

Holtan-Hartwig (1993) reported from Norway three morphotypes in *P. malacea*, which differed mainly in thallus size and thickness, as well as abundance of hairs on the upper surface. While the morphological variation captured in these morphotypes was observed in our material (Fig. 7A–J), some features were not consistently associated with the phylogenetic lineages revealed by our analyses (Figs. 1 and 2). Nevertheless, we did detect some

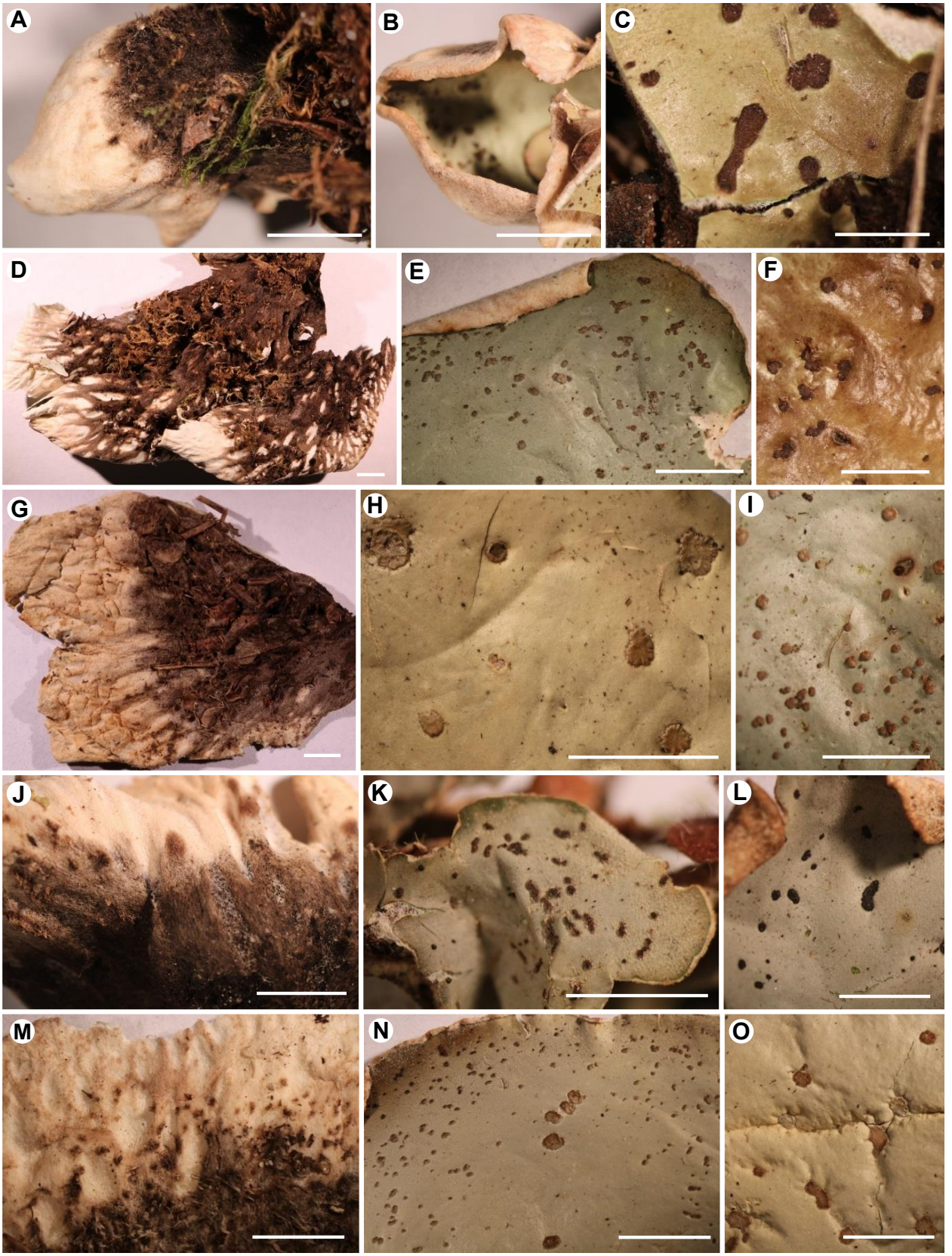

Figure 5. Morphological variation in *P. aphthosa* s.l. A – veinless margin on the lower surface of *P. aphthosa* 1; B – boat shaped lobe of *P. aphthosa* 1; C – adnate cephalodia of *P. aphthosa* 1; D – well-defined, narrow veins in lower surface of *P. aphthosa* 2; E – warty cephalodia on the upper surface of *P. aphthosa* 2; F – adnate cephalodia on the upper surface of *P. aphthosa* 2; G – pale veins on the margin of the lower surface of *P. aphthosa* 3; H – adnate cephalodia on the upper surface of *P. aphthosa* 3; I – warty cephalodia on the upper surface of *P. aphthosa* 4; J – veins on the lower surface of *P. aphthosa* 4; K, L – elongated, adnate cephalodia on the upper surface of *P. aphthosa* 4; M – pale veins on the margin of the lower surface of *P. aphthosa* 5; N – warty cephalodia on the margin of the upper surface of *P. aphthosa* 5; O – adnate cephalodia in the center of the upper surface of the thallus in *P. aphthosa* 5. Scale bars = 5 mm.

general trends. Most specimens of *P. malacea* 2 matched morphotype B *sensu* Holtan-Hatwig (1993), with thick (1 mm) and exceptionally long (up to 10 cm) lobes (Fig. 7C) and hairs towards the lobe margin (Fig. 7D). *Peltigera malacea* 5 was the only clade where almost every examined specimen had a white pruina near the lobe margin (Fig. 7H). Less morphological consistency was present in the remaining lineages, where for example the lobes of *P. malacea* 4 are furnished with a dense erect tomentum inward almost to the thallus center in some specimens while in others they are almost entirely glabrous throughout (Fig. 7F, G). Although most specimens of *P. frippii* have a shiny and glabrous upper cortex (Fig. 7I), some specimens have distinct marginal hairs (Fig. 7J). Marginally tomentose specimens of *P. frippii* can be distinguished from *P. malacea* s.l. by the presence of well-defined, rather narrow veins below, i.e., in contrast to the lower surface of *P. malacea* s.l., which is either veinless or furnished with a few diffuse veins. The observed morphological variation among and within the putative species of section *Peltidea* has limited taxonomic significance.

With the exception of aph Ia (from *P. aphthosa*, *P. britannica*, and *P. malacea*), all chemotypes noted by us have previously been reported from *Peltigera* in Norway (Holtan-Hartwig 1993), Europe in general (Vitikainen 1994), and Québec, Canada (Kotarska 1999) (Fig. 4). We did not observe any apparent association between chemotypic variation and 1) species identity (e.g., chemotypes were shared by *P. aphthosa*, *P. frippi* and *P. chionophila*, and by *P. aphthosa* and *P. malacea*), 2) geography (e.g., mal I was reported from *P. malacea* specimens collected in Europe and North America), and 3) *Nostoc* phylogroups (e.g., thalli of *P. britannica* containing *Nostoc* phylogroup IV displayed two different chemotypes) (Figs. 2 and 4). Contrary to some lichen groups where secondary metabolites continue to play a primary or secondary diagnostic role in species recognition (e.g., in *Cladonia, Graphidaceae*, *Haematomma*, *Heterodermia*, *Lecanora*, *Parmeliopsis, Pertusariaceae, Rhizoplaca,* and *Xanthoparmelia*; Tehler & Källersjö 2001; Staiger 2002; Nash & Elix 2004; Ryan et al. 2004; Smith & Lumbsch 2004; Lumbsch et al. 2008; Lücking et al. 2008; Leavitt et al. 2011c; Papong & Lumbsch 2011), including rare cases in the genus *Peltigera* (e.g., presence of unique chemotypes in *P. venosa* and selected newly delimited species in section *Polydactylon*), chemotypic variation (presence/absence and the quantity of terpenoids) across section *Peltidea* seems to be driven by other factors (environment, habitat, thallus developmental stage, lichen microbiome; e.g., Culberson et al. 1977; Bialonska & Dayan 2005; Toni & Piercey-Normore 2013; Calcott et al. 2018) than phylogenetic relatedness of mycobionts. Although within a broader phylogenetic context certain degree of similarity in triterpenoids composition occurs within selected sections (e.g., *Horizontales*, *Polydactylon*), shared chemotypes are reported from distantly related lineages of the genus (e.g., chemotype with only

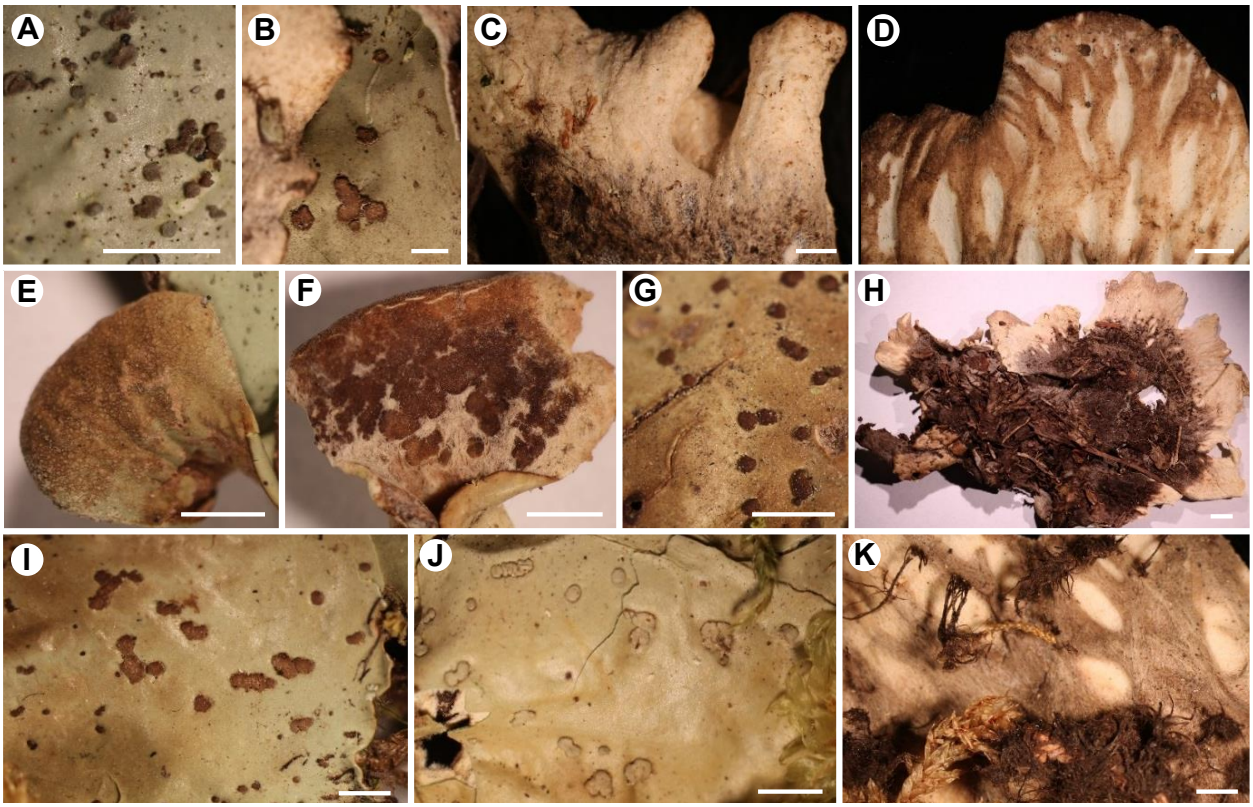

Figure 6. Morphological variation in *P. britannica* s.l. and *P. chionophila.* A, B – cephalodia on the upper surface of *P. britannica* 1; C, D – lower surface of *P. britannica* 1; E – underneath of apothecia of *P. britannica* 1; F – underneath of apothecia of *P. britannica* 2; G – cephalodia on the upper surface of *P. britannica* 2; H – lower surface of *P. britannica* 2; I, J – appressed cephalodia on the upper surface of *P. chionophila*; K – veins and rhizines of *P. chionophila.* Scale bars; A–G, I–K = 2 mm; H = 5 mm.

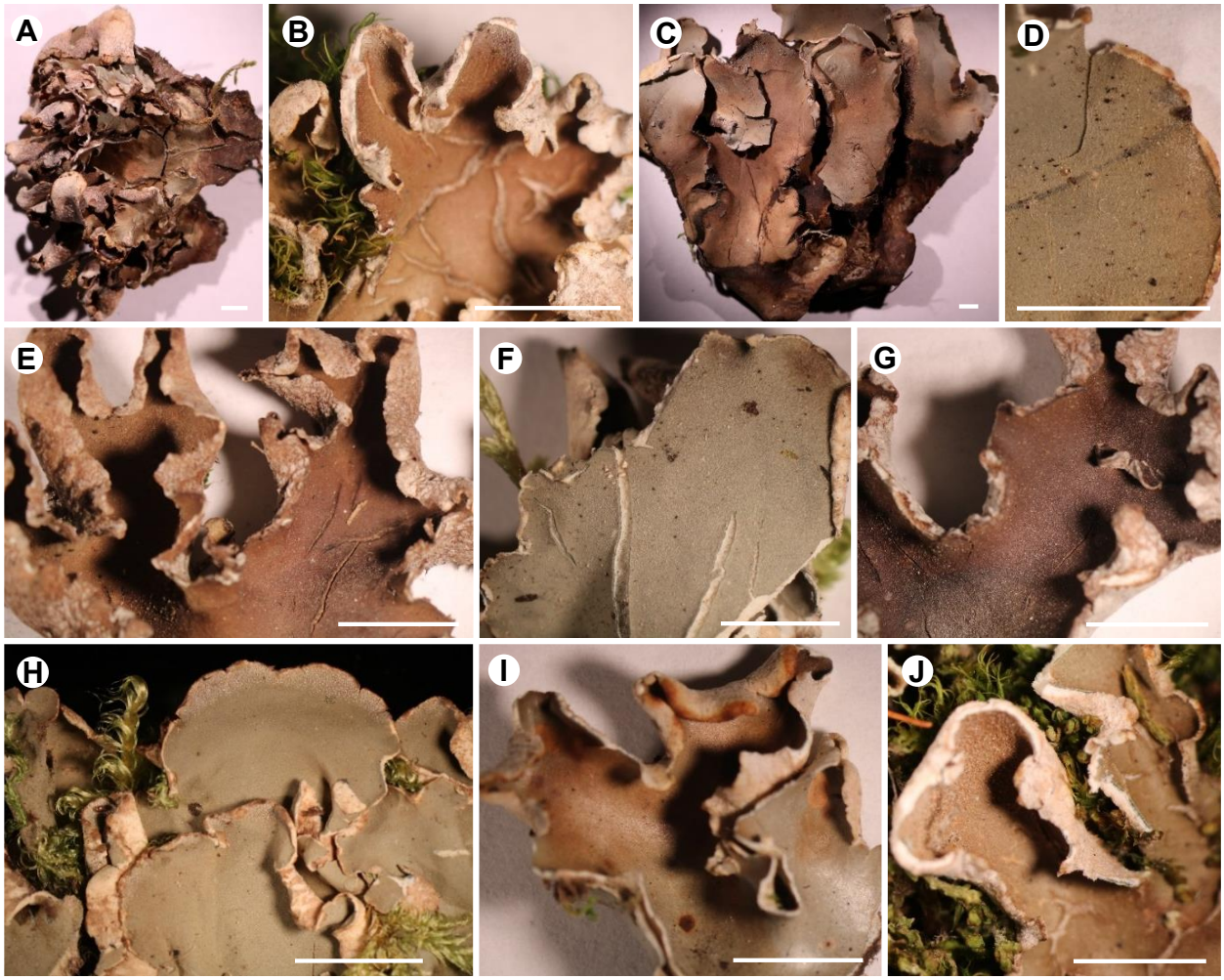

Figure 7. Morphological variation in *P. malacea* s.l. A, B – upper surface of *P. malacea* 1; C – lobes of *P. malacea*; D – hairs on the margin of the upper surface of *P. malacea* 2; E – lobes of *P. malacea* 3; F – upper surface margin with dense tomentum in *P. malacea* 4; G – lobes of *P. malacea* 4; H – pruina on the margin of the upper surface of *P. aphthosa* 5; I – glabrescent lobes of *P. frippii*; J – hairs on the margin of the upper surface of *P. frippii.* Scale bars = 5 mm.

zeorin visible was detected in *P. aphthosa*, *P. phyllidiosa*, *P. scabrosa*, *P. horizontalis*, and *P. neopolydactyla* s.l.; Holtan-Hartwig 1993; Kotarska 1999).

High specificity of both symbionts in section *Peltidea*

All cyanobionts found in lichen thalli from section *Peltidea* belong to *Nostoc* Clade II, mostly subclade 2 (phylogroups III and IV); only a few cyanobionts represented subclade 3 (phylogroups V and VI; Figs. 2 and 3). Based on a broad sampling (Europe, North America, and Far East Russia), our results confirmed a high and nearly exclusive specificity of *Nostoc* phylogroup III with *P. malacea* s.l. (*P. malacea* 1, 3, 4, and 5; Figs. 2 and 3) reported earlier by O'Brien et al. (2013) for specimens collected in Canada (British Columbia) and Europe, though it should be noted that *P. malacea* s.l. can also associate with *Nostoc* phylogroup IV (*P. malacea* 2). Such a degree of specificity is highly unusual; in general, *Peltigera* species are more specialized than their *Nostoc* partners (Magain et al. 2017a). Possibly this asymmetric specialization helps to maintain the lichen symbiosis through time, especially in a symbiotic system where the photobiont is transmitted predominantly horizontally from one generation to the next (Chagnon et al. 2018). One-to-one reciprocal specificity is extremely rare in sections *Polydactylon* (phylogroups IX and XIb paired with *P.* sp. 11 and *P. neopolydactyla* 5, respectively; Magain et al. 2017a) and *Peltigera* (two symbiont pairs, *P. patagonica,* and *P. vainioi* pairing with *Nostoc* phylogroup XXVIII and XXVIa, respectively; Magain et al. 2018), and a rare phenomenon in cyanolichens in general at a global scale (Otálora et al. 2010). This is consistent with the hypothesis that "…some form of genetically determined specificity is operating…" (O'Brien et al. 2013) and warrants further study. Mycobionts from the remaining species of section *Peltidea* were found mostly in association with *Nostoc* phylogroup IV (in subclade 2), which is closely related to phylogroup III (part of the same highly supported clade; Fig. 3) and somewhat also selective in *Peltigera,* interacting almost exclusively with members of a single section (*Peltidea*), i.e., with the exception of *P. neopolydactyla* 4. Interestingly, subclade 2 contains another *Nostoc* lineage, which is involved in an exclusive, reciprocally specific interaction with *Scytinium lichenoides*, one of five *Collemataceae* species which co-speciated with their cyanobacterial partner (Otálora et al. 2010).

A few representatives of *P. britannica* s.l. and *P. aphthosa* s.l. are associated with *Nostoc* phylogroups from subclade 3, which includes other sections of the genus *Peltigera* and other genera within *Peltigerales*. Contrary to local specialists (species associating with distinct *Nostoc* phylogroups in different geographic regions) reported from section *Polydactylon* (e.g., *P. occidentalis*; Magain et al. 2017a) and species from other genera (e.g., *Nephroma parile*; Fedorowitz et al. 2012), we did not find any geographic evidence explaining the alternative pairings of *P. aphthosa* and *P. britannica* with their *Nostoc* partners (phylogroup IV versus V and VI).

Considering that the first split in the phylogeny of section *Peltidea* divides bimembered and trimembered lichens (Figs. 1 and 2), it is interesting that most cyanobionts in both types of symbioses (subclade 2) are phylogenetically closely related. One possible interpretation of this pattern is that mycobiont switches to alternative cyanobacterial partners have been evolutionarily infrequent in section *Peltidea*, in which partnerships formed instead from a pool of closely related *Nostoc* clades. Alternatively, the low level of genetic variation in their cyanobacterial symbionts may simply reflect a case of coevolution. There are also instances where the same *Nostoc* (phylogroup IV) occurs across broad geographic distances in both types of thalli: bimembered *P. malacea* and *P. frippii* from Norway and trimembered *P. aphthosa* in Alaska. This contrasts with phylogenetic relationships within the genus *Nephroma* (trimembered species are polyphyletic), where Fedorowitz et al. (2012) suggested that repeated evolutionary transitions between the two types of symbioses involved a concurrent switch from one lineage of *Nostoc* symbionts to another.

Considering that apothecia have not been frequently observed in section *Peltidea* (however, they are locally common, e.g., in coastal populations of *P. britannica* and *P. chionophila* in British Columbia, Canada), and vegetative propagules (i.e., isidia and soredia) are absent, it is very likely that these species disperse mostly via thallus fragmentation (enabling vertical transmission of the photobiont). However, rare events of sexual reproduction (horizontal transmission of the photobiont) might have led to the acquisition of new *Nostoc* lineages from subclade 3 by trimembered species (Fig. 3). Three findings support the scenario that trimembered species in this section are derived from bimembered associations by the acquisition of a green alga (*Coccomyxa* sp.), which became the dominant photobiont, while *Nostoc* became restricted to external cephalodia: 1) the most recent common ancestor of *Peltigera* was reconstructed as a bimembered cyanolichen (Miadlikowska & Lutzoni 2004), 2) the observed close relationships between *Nostoc* from bi- and trimembered species in *Peltidea,* and 3) the ontogenic similarity in both symbiotic forms (Miadlikowska & Lutzoni 2004 and references therein). This could have been followed by a transition from a trimembered to a bimembered symbiotic state according to O'Brien et al. (2013), through a mechanism where cephalodia develop into independent cyanomorphs that led eventually to the speciation of bimembered *P. frippi* and *P. malacea*. Magain and Serusiaux (2014) proposed the same evolutionary scenario for the family *Pannariaceae* where subsequent divergence events resulted from a potential emancipation of cephalodia.

Conclusions

We refrain from proposing formal changes to the current taxonomy of *Peltigera* section *Peltidea* owing, first, to the lack of reliable diagnostic (morphological, chemical and geographic) characters to circumscribe its molecular diversity, and second, to a lack of convergence among loci and analytical methods among most currently recognized species. In the case of *P. malacea,* all newly recognized putative species represent lineages within a broadly circumscribed, yet monophyletic morphospecies, which is widely accepted and easily recognized. Trimembered species from sections *Peltidea* and *Chloropeltigera* (e.g., *P. aphthosa*, *P. leucophlebia*, *P. britannica*, *P. latiloba*, and *P. chionophila*) are often difficult to distinguish, especially in areas where they co-occur and because they frequently display overlapping phenotypes. Unless shown to be reproductively isolated, a formal infraspecific status (e.g., variety or subspecies) might be biologically more appropriate for accommodating the uncovered phylogenetic structure within the current, monophyletic species within section *Peltidea*. The possible broader delimitation of *P. aphthosa* (to include *P. britannica*) and the questionable phylogenetic status of *P. britannica* as a separate species corroborated by morphological observations (specimens from North America lacking its signature characteristic – the shape of cephalodia) should be confirmed based on additional collections and loci before redefining their taxonomic boundaries. Careful reevaluation of morphological traits and discovery of novel diagnostic characters, as well as population genetic analyses to investigate gene flow among putative species, should facilitate future taxonomic conclusions. Furthermore, the exclusive nature of *Nostoc* phylogroup III and selected clades of *P. malacea* s.l. deserves further attention.

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Supplementary electronic material

Appendix 1. Specimens used in this study with their reference numbers (DNA extraction number), species assignment and clade number (based on BPP validation; Fig. 2 and Appendix 2), voucher information, mycobiont and cyanobiont (*Nostoc*) sequence type/haplotype assignment, *Nostoc* phylogroup (based on Magain et al. 2017a and this study; Fig. 3), chemotype (based on TLC analyses; Fig. 4), and GenBank accession number (sequences obtained as part of this study are in bold). Download file

Appendix 2. Putative species delimited within section *Peltidea* with bGMYC on each locus separately (β-tubulin, COR1b, COR3, and ITS) and Structurama (gamma shape of 1 and 3 for *P. malacea* and *P. aphthosa* clades, respectively) on the concatenated 4-locus data set (numbers represent distinct haplotypes included) and validated by the BPP analysis. Download file

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