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Phylogenetic placement, species delimitation, and cyanobiont identity of endangered aquatic *Peltigera* species (lichen-forming Ascomycota, Lecanoromycetes)[1]

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- *Premise of this study:* Aquatic cyanolichens from the genus *Peltigera* section *Hydrothyriae* are subject to anthropogenic threats and, therefore, are considered endangered. In this study we addressed the phylogenetic placement of section *Hydrothyriae* within *Peltigera*. We delimited species within the section and identified their symbiotic cyanobacteria.
- *Methods:* Species delimitation and population structure were explored using monophyly as a grouping criterion (RAxML) and Structurama based on three protein-coding genes in combination with two nuclear ribosomal loci. The 16S and *rbcLX* sequences for the cyanobionts were analyzed in the broad phylogenetic context of free-living and symbiotic cyanobacteria.
- *Key results:* We confirm with high confidence the placement of section *Hydrothyriae* within the monophyletic genus *Peltigera*; however, its phylogenetic position within the genus remains unsettled. We recovered three distinct monophyletic groups corresponding to three species: *P. hydrothyria*, *P. gowardii* s.s., and *P. aquatica* Miadl. & Lendemer, the latter being formally introduced here. Each species was associated with an exclusive set of *Nostoc* haplotypes.
- *Conclusions:* The ITS region alone provides sufficient genetic information to distinguish the three morphologically cryptic species within section *Hydrothyriae*. Section *Hydrothyriae* seems to be associated with a monophyletic lineage of *Nostoc*, that has not been found in symbiotic association with other members of *Peltigera*. *Capsosira lowei* should be transferred to the genus *Nostoc*. Potential threats to *P. aquatica* should be re-examined based on the recognition of two aquatic species in western North America.

Key words: aquatic lichens; *Capsosira*; cyanobiont; elongation factor 2; fungal systematics; multilocus phylogenetics; mycobiont; *Nostoc*; section *Hydrothyriae*; species delimitation.

Lichens represent one of the most successful and widespread types of symbiosis (Nash, 2008). Although lichens are well known for their ability to survive in extreme environments, their biodiversity, distribution, and population structure can be seriously affected by anthropogenic environmental disturbances. The main threats to fauna and flora in general also affect lichens and include local and global scale changes (e.g., habitat degradation, loss, and fragmentation) resulting from urbanization, agriculture, pollution, and climate change (Nash, 2008; Scheidegger and Werth, 2009). The specific habitat requirements of lichens are hardly ever considered when establishing protected areas. Understanding species boundaries, especially in genera such as *Peltigera* Willd. that are suspected to include many cryptic species (O'Brien et al., 2009), is crucial

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for developing successful conservation strategies and management practices (Scheidegger and Werth, 2009).

The cosmopolitan genus *Peltigera* (Peltigerales, Lecanoromycetidae) includes predominantly muscicolous and terricolous foliose lichen-forming fungi. These lichens are associated with the cyanobacterium *Nostoc* Vaucher ex Bornet & Flahault and form bimembered thalli. A few species are also associated with the green alga *Coccomyxa* Léger & Hesse in addition to the cyanobiont *Nostoc* and thus form trimembered thalli. Although *Peltigera* is one of the earliest described lichen-forming fungi (Willdenow, 1787), this genus is still poorly known compared with other macrolichens. Variations in its morphological traits have been difficult to interpret by taxonomists using traditional taxonomic practices, which were unable to distinguish among phenotypic plasticity, genetically based phenotypic variation and cryptic speciation, all suspected to occur in *Peltigera*. Progress in understanding the biodiversity of this genus was thus greatly impeded even though molecular techniques have been available to lichenologists for more than 25 yr. Moreover, hybridization may also be a factor in this genus according to Goffinet and Hastings (1995), a process that has rarely been addressed by evolutionary studies of fungi in general. Many unknown morphologically cryptic species are probably hidden under what are thought to be common *Peltigera* species (e.g., O'Brien et al., 2009; Lendemer and O'Brien, 2011) such that some species are likely to be threatened by extinction, before they are recognized as distinct.

Molecular phylogenetic analyses coupled with traditional systematic studies, e.g., morphology, chemistry, biogeography, and ecology, have been useful in resolving some of these issues (e.g., Goffinet and Miadlikowska, 1999; Miadlikowska et al., 2003; Sérusiaux et al., 2009). In addition to the ITS region (Goffinet and Miadlikowska, 1999; Goffinet et al., 2003), selected molecular markers, e.g., *RPB1* and β-tubulin, showed intra- and interspecies variation that allowed the recognition of biologically meaningful species within species complexes of *Peltigera* in North America (O'Brien et al., 2009). Using these three nuclear markers, these authors concluded that the genus *Peltigera* is more diverse in western North America than originally perceived and that morphological variability is due largely to the presence of undescribed species rather than hybridization or intraspecific variation. Currently, more than 90 species of *Peltigera* are recognized worldwide (Goffinet et al., 2003; Martínez et al., 2003; Vitikainen, 2006; Kirk et al., 2008; Sérusiaux et al., 2009; Han et al., 2013), and 37 of them occur in North America (Esslinger, 2010). To date, seven species (*P. castanea* Goward, Goffinet & Miadl., *P. cinnamomea* Goward, *P. chionophila* Goward & Goffinet, *P. gowardii* Lendemer & H. O'Brien, *P. hydrothyria* Miadlikowska & Lutzoni, *P. pacifica* Vitik., and *P. phyllidiosa* Goffinet & Miadlikowska) have been reported as endemic to North America (Goffinet and Miadlikowska, 1999; Goward and Goffinet, 2000; Miadlikowska and Lutzoni, 2000; Goffinet et al., 2003; Martínez et al., 2003; Lendemer and O'Brien, 2011). Prior to this study, only two *Peltigera* species were known to be aquatic (*P. gowardii* and *P. hydrothyria*).

In 2000, Miadlikowska and Lutzoni published the first phylogeny for the genus *Peltigera*. They proposed a new infrageneric classification consisting of eight monophyletic sections, one of which, section *Hydrothyriae* Miadlikowska & Lutzoni, was created for the aquatic monospecific genus *Hydrothyria* J. L. Russell. This genus was subsumed within *Peltigera* (*H. venosa* J. L. Russell = *Peltigera hydrothyria*) based on a single aquatic representative included in the phylogenetic analyses. Although the affiliation of *Hydrothyria* with *Peltigera* received significant support (100% bootstrap), its accurate placement within *Peltigera* was not settled by this phylogenetic study.

In the past, *Hydrothyria* was considered a member of the Collemataceae Zenker (Russell, 1856) because of its unique morphology (cyanolichen with an unstratified gelatinous thallus when wet and lacking rhizines) and its ecology (found in streams attached to rocks at or below water level). Due to its aquatic habitat and narrow ecological amplitude, *Peltigera hydrothyria* s.l. was the subject of various studies focusing mainly on potential anthropogenic threats to its populations and on developing potential conservation strategies (see summary by Poulsen and Carlberg, 2007).

Despite the disjunct distribution (eastern and western areas of North America) and chemical variation (populations with and without detectable secondary compounds) of *P. hydrothyria* s.l., its circumscription was never questioned until the recent systematic revision and molecular phylogenetic analyses (based on ITS) of material collected in the United States (Lendemer and O'Brien, 2011). The study revealed that *P. hydrothyria* represents a species complex consisting of three strongly supported monophyletic groups: the eastern *P. hydrothyria* and two allopatric western clades recognized within a single newly described species, *P. gowardii* s.s. and *P. gowardii* s.l. (Lendemer and O'Brien, 2011). The two western clades are partly sympatric, overlapping only in Washington State, with the range of *P. gowardii* s.s. extending northward and that of *P. gowardii* s.l. extending southward. Although morphologically cryptic, the western populations of *P. gowardii* are chemically distinct (lacking methylgyrophorate) from the eastern *P. hydrothyria*. The authors concluded that the recognition of *P. gowardii* s.l. as a third species within the section *Hydrothyriae* should be reexamined based on additional sampling and multiple unlinked molecular markers.

The cyanobiont of *P. hydrothyria* was identified as *Capsosira lowei* Casamatta, S. R. Gomez & J. R. Johansen, a new species of the family Capsosiraceae (A. Borzì) L. Geitler (Stigonematales), based on phenotypic characters of the cyanobacterium isolated from a lichen specimen collected in the southern Appalachian Mountains of North Carolina (Casamatta et al., 2006). Although morphologically similar to members of the Stigonematales Geitler (e.g., cell division in two planes), molecular evidence (16S phylogeny and the similarity in the ITS domains structure) supported close affiliation of *C. lowei* with *Nostoc commune* Vaucher ex Bornet & Flahault, a member of the Nostocales Cavalier-Smith (Casamatta et al., 2006). This finding raised questions about the validity of morphological synapomorphies used to circumscribe the genus *Nostoc* (Casamatta et al., 2006; Korelusová, 2008). Lendemer and O'Brien (2011) reported that *Nostoc* sp. was the cyanobiont associated with *P. gowardii*; however, no molecular data were included to support their statement.

The main objectives of the current study were to (1) revisit the phylogenetic placement of section *Hydrothyriae* within the genus *Peltigera*; (2) reevaluate species delimitations within this section, especially the potential for the presence of two species within what is currently recognized as *P. gowardii*; (3) assess the identity of the cyanobionts associated with members of the section *Hydrothyriae* and their affiliation with free-living and other symbiotic cyanobacteria; and (4) provide an overview of the distribution, ecology, and threat factors to the conservation of aquatic *Peltigera* species.

To reach these goals, we sequenced three nuclear protein-coding genes and two nuclear ribosomal RNA loci for the mycobiont representing all sections of *Peltigera*, as well as the *rbcLX* region and 16S ribosomal RNA of the cyanobiont found in members of section *Hydrothyriae*. We also incorporated new data on distribution, habitat requirements, and potential endangerment factors based on a recent inventory of aquatic *Peltigera* species in Canada.

MATERIALS AND METHODS

Taxon sampling and data acquisition—We selected 18 individuals of *P. gowardii* and 17 specimens of *P. hydrothyria* to address species delimitation and phylogenetic relationships among members of section *Hydrothyriae*, as well as to reveal the identity of their cyanobionts. Of these, 10 specimens of *P. gowardii* and nine of *P. hydrothyria* (collections from the USA only) were previously included in the systematic study by Lendemer and O'Brien (2011), where the ITS region was analyzed under maximum parsimony. We expanded the geographical range of the sampling by adding material collected mainly in western Canada (British Columbia) for *P. gowardii* (six individuals, plus two collections from the USA) and in three eastern provinces of Canada (Nova Scotia, New Brunswick, and Québec) for *P. hydrothyria* (eight individuals). All new collections of aquatic *Peltigera* from Canada were obtained as part of the Canadian inventory of both species performed by DR, FA, and RC. To revisit the phylogenetic position of section *Hydrothyriae* within the genus *Peltigera*, 45 individuals from 30 species were selected worldwide to represent the remaining seven sections (Appendix 1).

For the mycobiont, we targeted five molecular markers, including two nuclear ribosomal loci: ca. 0.6 kb of the internal transcribed spacer (ITS) region and ca.1.2 kb of the large subunit (nrLSU), which are commonly used in molecular systematics of lichen-forming fungi including the genus *Peltigera* (e.g., Miadlikowska and Lutzoni, 2000; Goffinet et al., 2003; Miadlikowska et al., 2003; Miadlikowska and Lutzoni, 2004; Lendemer and O'Brien, 2011). The three single-copy protein-coding genes we sequenced for the mycobiont are: ca. 0.7 kb of β-tubulin and ca. 0.8 kb of the RNA polymerase II largest subunit (*RPB1*), which were shown to be valuable markers for species delimitation within the genus *Peltigera* (O'Brien et al., 2009), and ca. 0.8 kb of the first region of the elongation factor 2 (*EFT2*-1), a new molecular marker developed as part of the Assembling the Fungal Tree of Life project (AFToL 2).

For the cyanobiont, we sequenced ca. 1.0 kb of the *rbcLX* region (which includes the last 82 amino acids of the RUBISCO large subunit [*rbcL*], a putative chaperone gene [*rbcX*] and two intergenic spacers; Li and Tabita, 1997), and the ribosomal RNA small subunit (16S). We generated three new 16S and 33 new *rbcLX* sequences for cyanobionts associated with aquatic *Peltigera* species (Appendix 1). To these data sets we added 16S ribosomal RNA and *rbcLX* sequences from GenBank to represent the biodiversity of free-living and symbiotic cyanobacteria mainly from the Nostocales and Stigonematales.

We obtained a total of 296 new sequences: 260 for the mycobionts (16 ITS, 38 nrLSU, 70 β-tubulin, 63 *EFT2*-1, and 73 *RPB1*) and 36 for their cyanobionts (33 of *rbcLX* and three of 16S). Twenty four sequences, mostly ITS, were downloaded from GenBank (19 of which were generated by Lendemer and O'Brien, 2011), and 46 sequences were missing (Appendix 1). All new sequences generated for this study were derived from DNA extracted directly from a single lichen thallus at a time.

Most of the new sequences were generated using the Sigma REDExtract-N-Amp Plant PCR Kit (St. Louis, Missouri, USA) for DNA isolation and R4775 Sigma REDExtract-N-Amp PCR ReadyMix for the PCR reaction (for detailed information, see Rivas Plata et al., 2013). Alternatively, a standard DNA isolation procedure employing 2% SDS lysis buffer (Zolan and Pukkila, 1986) was used. Sources for laboratory protocols and primers used for generating the new sequences of nrLSU can be found in the report of Lutzoni et al. (2004) and Hofstetter et al. (2007); ITS, *RPB1* and β-tubulin in O'Brien et al. (2009); 16S and *rbcLX* in Elvebakk et al. (2008). PCR amplification of the *EFT2*-1 was performed using the following designed primers: *EFT2*-1F (5′-AAYATGWS-BGTBATYGC-3′) and *EFT2*-4R (5′-GGVACCATYTTVGARAC-3′). Conditions for the touchdown PCR for *EFT2*-1 were as follows: 94°C for 30 s, 55°C for 30 s (–0.4°/cycle), 72°C for 1 min (+2 s/cycle) for 24 cycles; 94°C for 30 s, 45°C for 30 s, 72°C for 2 min (+3 s/cycle) for 12 cycles; 72°C for 10 min, followed by storage at 4°C. All PCR amplicons were cleaned with ExoSAP (Affymetrix, Santa Clara, California, USA) following the manufacturer's protocol. Sequencing was carried out in 10 μL reactions using: 1 μL primer (10 μmol/L), 1 μL purified PCR product, 0.75 μL Big Dye (Big Dye Terminator Cycle sequencing kit, ABIPRISM version 3.1; Perkin-Elmer, Applied Biosystems, Foster City, California, USA), 3.25 μL Big Dye buffer, and 4 μL double-distilled water. Automated reaction clean-up and visualization was performed at the Duke Genome Sequencing and Analysis Core Facility of the Institute for Genome Sciences and Policies (for details see Gaya et al., 2012).

All newly acquired sequences were subjected to BLAST searches to confirm the fungal or cyanobacterial origin of each sequence fragment. They were assembled and edited using the software package Sequencher 4.1 (Gene Codes Corp., Ann Arbor, Michigan, USA) and aligned manually with the program MacClade 4.07 (Maddison and Maddison, 2003). The "Nucleotide with AA color" option was used for guiding (delimiting exons and introns) all alignments for protein-coding genes. Ambiguously aligned regions sensu Lutzoni et al. (2000) were delimited manually and excluded from subsequent analyses.

Data sets and analyses—We assembled and analyzed four data sets (Appendix 1), two for the mycobiont (ML1 and ML2) and two for the cyanobiont (ML3 and ML4). To address the first objective (i.e., the phylogenetic placement of the section *Hydrothyriae* within the genus *Peltigera*), we assembled a 4-locus (β-tubulin + *RPB1* + *EFT2*-1 + nrLSU) data set for 48 OTUs selected across the genus *Peltigera*, including three representatives of *P. hydrothyria* and two of *P. gowardii*, as well as two members of *Solorina* Ach. as the outgroup (ML1 in Appendix 1). To address the second objective (i.e., species delimitations within section *Hydrothyriae*), we assembled a 4-locus (β-tubulin + *RPB1* + *EFT2*-1 + ITS) data set for 52 OTUs, including 35 members of the section *Hydrothyriae* (18 individuals of *P. gowardii* and 17 individuals of *P. hydrothyria*) and 17 representatives from four other sections [*Peltidea* (Ach.) Vain., *Chloropeltigera* Gyeln., *Phlebia* Wallr., and *Polydactylon* Miadlikowska and Lutzoni] serving as outgroups (ML2 in Appendix 1). Compared with the sampling by Lendemer and O'Brien (2011), we increased the taxon sampling (from 21 to 35 individuals) by including mainly Canadian populations that were not previously sampled. Furthermore, we expanded molecular data by adding three single-copy protein-coding genes to the ITS region, the sole marker analyzed in that previous study (692 vs. 3231 characters) under the maximum parsimony optimization criterion. Due to the rapid sequence divergence of the ITS region resulting in a high proportion of ambiguously aligned regions (almost half of the ITS alignment; see also Lendemer and O'Brien [2011]), ITS was not included for the ougroup taxa in ML2 analyses.

To address the third objective (i.e., to unveil the identity and phylogenetic affiliation of cyanobionts associated with *P. gowardii* and *P. hydrothyria*), we assembled two data sets: the 16S and the *rbcLX* data set for the ML3 and ML4 phylogenetic analyses, respectively. The ML3 data set incorporated 110 mostly published 16S sequences representing free-living and symbiotic cyanobacteria from the order Nostocales (families Nostocaceae Eichler, Rivulariaceae Frank, Microchaetaceae Lemmermann, and Scytonemataceae Frank) and selected taxa from the orders Stigonematales, Pleurocapsales Cavalier-Smith, and Gloeobacterales Cavalier-Smith. The latter was used to root this 16S-based tree. This data set contained 24 sequences used by Casamatta et al. (2006), including *Capsosira lowei*, a cyanobiont isolated from *P. hydrothyria*. We added two 16S sequences of the cyanobiont from *P. gowardii* collected in the state of Washington and one from *P. hydrothyria* collected in Québec, Canada (Appendix 1).

ML4 consisted of 275 mostly published *rbcLX* sequences of free-living and symbiotic *Nostoc* spp. selected from the *Nostoc* clades I and II (Otálora et al., 2010; O'Brien et al., 2013) and 17 newly sequenced individuals of *P. gowardii* and 16 individuals of *P. hydrothyria* from several localities mainly in Canada (Appendix 1). Identical haplotypes were collapsed to one representative using the program Map (Aylor et al., 2006); however, we reincorporated some of the initial sequences that had been erroneously collapsed because the program excluded variable sites if they contained missing data.

Maximum likelihood analyses using the program RAxMLHPC-MPI-SSE3 (Stamatakis, 2006) were performed on all data sets (ML1–4) at the nucleotide level. 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 approximated with four categories in all analyses. Partitions for the ML1 and ML2 analyses were estimated with the program PartitionFinder v.1.1.0 (Lanfear et al., 2012) using greedy search and the Bayesian information criterion (BIC) model selection. For the ML1 data set, two partitions were defined. The first partition consisted of all introns, β-tubulin third codon position, and *RPB1* third codon position; the second partition incorporated the remaining sites of β-tubulin, *RPB1*, the entire *EFT2*-1 and nrLSU. For the ML2 data set, four partitions were defined. The first partition consisted of ITS and all introns; the second partition consisted of β-tubulin first codon position, *RPB1*

first codon position and *EFT2*-1 first codon position; the third partition consisted of β-tubulin third codon position, *RPB1* second codon position, and part of the *EFT2*-1 second codon position; and the fourth partition incorporated the remaining sites of β-tubulin, *RPB1*, and *EFT2*-1. For the ML3 analysis, a single partition corresponding to the 16S was used, whereas for the ML4 analysis, three partitions corresponding to the first, second, and third codon position of the *rbcLX* were defined. To detect topological incongruence among single-locus data sets, we implemented a reciprocal 70% ML bootstrap support criterion (Mason-Gamer and Kellogg, 1996; Reeb et al., 2004). A conflict was assumed to be significant if a group of taxa was supported as monophyletic at ≥70% with one locus but supported as nonmonophyletic, using the same bootstrap threshold, by another locus. No conflict was detected among the single-locus data sets part of the ML1 and ML2 concatenated data sets. Map and RAxML analyses were completed through 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 and Carbone, 2014). The ML2 concatenated data set and the resultant most likely RAxML tree were deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S15894).

To infer population structure from genetic data for the aquatic *Peltigera* (ML2 data set restricted to 35 individuals from the section *Hydrothyriae*; Appendix 1), we used Structurama (Huelsenbeck et al., 2011). The program assumes that the sampled loci are in linkage equilibrium and implements a Markov chain Monte Carlo (MCMC) sampling strategy to approximate the posterior probability that individuals are assigned to specific populations. We ran the Markov chain for 1 million cycles sampling every 1000th cycle. We allowed the number of populations to be a random variable (following a Dirichlet process prior) with a gamma probability distribution (hyperprior).

***Alignments*—**Ambiguously aligned sites, which were excluded from phylogenetic analyses (Table 1), were localized in introns of the protein-coding genes (especially β-tubulin with 40 excluded sites from ML2 analyses) and nrLSU (with 83 excluded sites from ML1 analyses). From the total of 3340 characters, 109 where ambiguously aligned and excluded, whereas the remaining 3231 characters were included in ML1 analyses. ITS and *RPB1* alignments did not contain any ambiguously aligned regions, and both loci were entirely included in the ML2 analyses (a total of 2578 characters, 50 of which were ambiguously aligned and excluded from ML2; Table 1). Among the five fungal loci analyzed in this study, nrLSU was the longest (1282 characters), while the other loci provided comparable numbers of unambiguously aligned characters (from 501 characters for β-tubulin to 783 characters for *EFT2*-1; Table 1). From a total of 979 cyanobacterial 16S sites, 32 sites were excluded (ambiguously aligned) and the remaining 947 characters were analyzed (ML3). Both spacers in *rbcLX* were too variable to be unambiguously aligned across the Nostocales, or even within the section *Hydrothyriae* alone, and were removed from subsequent phylogenetic analyses (ML4; 633 characters).

RESULTS AND DISCUSSION

***Phylogenetic placement of the section* Hydrothyriae—**Miadlikowska and Lutzoni (2000) reported a monophyletic delimitation of the genus *Peltigera* including *P. hydrothyria* (*Hydrothyria venosa*) and introduced section *Hydrothyriae* (one of the eight sections circumscribed within the genus), to accommodate this aquatic member of *Peltigera*. However, the phylogenetic placement of *P. hydrothyria* (only one specimen was included in that study), which represented the first divergence event within the genus, as well as the remaining deep relationships among sections, were poorly supported (bootstrap values below 50%) based on the combined nrLSU and morphological and chemical data analyzed under a maximum parsimony optimization criterion (fig. 8 of Miadlikowska and Lutzoni, 2000).

In this study, we replaced phenotypic characters with data from three single-copy protein-coding genes (β-tubulin, *RPB1*, and *EFT2*-1) concatenated with the previously used nuclear ribosomal LSU locus for a total of 3231 characters (898 variable characters) compared with 1209 characters in the previous study (Miadlikowska and Lutzoni, 2000). We extended the taxon sampling within section *Hydrothyriae* from one specimen to five individuals of *P. hydrothyria* (three collections) and *P. gowardii* (two collections) for a total of 46 operational taxonomic units (OTUs) comprising the ingroup (Fig. 1). They represented 31 of more than 90 currently recognized species (e.g., Vitikainen, 2006; Kirk et al., 2008; Sérusiaux et al., 2009; Han et al., 2013) classified in all remaining sections of the genus *Peltigera* (Miadlikowska and Lutzoni, 2000). To minimize the number and size of ambiguously aligned regions, we restricted the outgroup to include only the genus *Solorina* (family Peltigeraceae), which was consistently shown to be the sister group to *Peltigera* in previous phylogenetic studies (e.g., Miadlikowska and Lutzoni, 2000, 2004; Muggia et al., 2011; Spribille and Muggia, 2012).

In the resulting most likely phylogeny (Fig. 1), all aquatic individuals of *P. hydrothyria* and *P. gowardii* are grouped together to form the monophyletic, highly supported, section *Hydrothyriae*. Its placement as the first evolutionary split within *Peltigera* (Miadlikowska and Lutzoni, 2000) was reconstructed but at a low level of confidence (BS = 40%). Our phylogeny confirms monophyletic delimitations of each section introduced by Miadlikowska and Lutzoni (2000) for which we have multiple species, as well as the relationships among sections that received high bootstrap support in 2000, e.g., sister group relationship between sections *Peltigera* and *Retifoveatae,* and their close affiliation with section *Horizontales* (clade III; Fig. 1). For the first time, sections *Peltidea*, *Chloropeltigera*, and *Phlebia*, which include all known trimembered *Peltigera* lichens,

TABLE 1. Contribution (number of sites) of each locus to the combined data sets (ML1 and ML2) and genetic variation within and among species of section *Hydrothyriae*. Numbers in parenthesis include indels. Ambiguously aligned sites excluded from phylogenetic analyses follow the plus sign.

Statistic	ITS/nrLSU	β-tubulin	*RPB1*	*EFT2*-1	Combined
Total no. of sites included in ML1	1282+83*	504+19	661+6	783+1	3231+109
Total no. of sites included in ML2	592+0	501+40	661+0	774+10	2528+50
No. of haplotypes in *Hydrothyriae*	4	3	3	4	N/A
No. of segregating sites in *Hydrothyriae*	16 (23)	20	11	18	65 (72)
No. of polymorphic sites with exclusively shared states for:					
P. gowardii s.s. + *P. gowardii* s.l.	7 (9)	5	3	5	20 (22)
P. gowardii s.s. + *P. hydrothyria*	3 (4)	2	3	3	11 (12)
P. hydrothyria + *P. gowardii* s.l.	5 (7)	12	5	10	32 (34)
No. of sites with uniquely different states for each aquatic species	1 (2)	1	0	0	2 (3)
No. of sites polymorphic within each aquatic species	(1)	0	0	1	1 (2)

Note: * ITS was replaced by nrLSU in the ML1 analysis, otherwise, all numbers in the ITS/nrLSU column refer to ITS, not nrLSU.

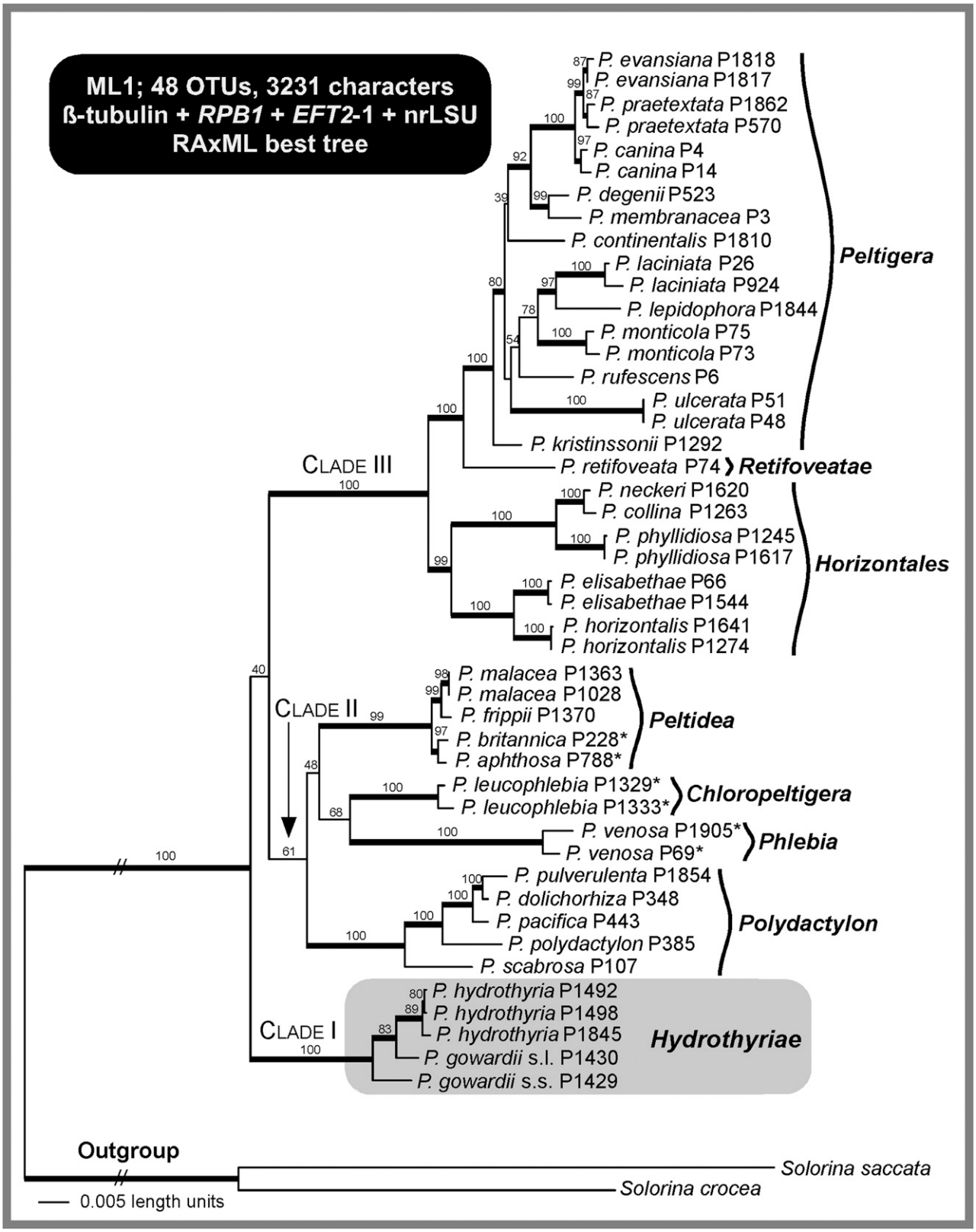

Fig. 1. Placement of aquatic *Peltigera hydrothyria* and *P. gowardii* from section *Hydrothyriae* in the phylogenetic context of the genus *Peltigera* (represented by 46 members from all known sections) as revealed by maximum likelihood analysis based on combined β-tubulin, *RPB1*, *EFT*2-1, and nrLSU loci (ML1). Two individuals from the genus *Solorina*, the closest relative of *Peltigera* (e.g., Miadlikowska and Lutzoni, 2000; Muggia et al., 2011), were used as outgroup to root the *Peltigera* tree. Values associated with internodes represent bootstrap support (BS). Thicker internodes indicate strongly supported (BS ≥ 80%) relationships. Stars indicate trimembered species of *Peltigera*.

were grouped together within a single clade (BS = 48%), sister to section *Polydactylon* to form clade II (Fig. 1). However, these two sets of relationships were not strongly supported. The phylogenetic uncertainty associated with the deepest splits in the *Peltigera* phylogeny may have resulted from a rapid early radiation event, as illustrated by very short internodes holding longer branches representing most sections and clade III (Fig. 1). For determining the accurate placement of section *Hydrothyriae*, more loci and a more extensive taxon sampling are needed. The support of a single origin of the trimembered symbiotic state within *Peltigera* in future studies would also confirm a single acquisition of a green alga (*Coccomyxa* W. Schmidle) to form trimembered thalli within that genus. Consequently, the potential monophyly of the trimembered species would also support the hypothesis of a reversal to a bimembered symbiotic state during the evolution of the lineage leading to the speciation of *P. malacea* (Ach.) Funck and *P. frippii* Holt.-Hartw., which are two bimembered lichen species in the section *Peltidea* (Fig. 1) and might explain, in part, the high level of reciprocal (nearly one-to-one) specificity observed for symbionts of *P. malacea*, which is rarely encountered in lichen symbioses (Otálora et al., 2010; O'Brien et al., 2013).

Species delimitations within section **Hydrothyriae—**Lendemer and O'Brien (2011) introduced the possibility of recognizing three aquatic *Peltigera* species: two cryptic taxa in western North America, *P. gowardii* s.s. and *P. gowardii* s.l. (highlighting that the later might be a new unnamed species), and *P. hydrothyria*, an eastern North American species. Our most likely tree for section *Hydrothyriae* (Fig. 2) fully agrees with the three main clades of the ITS phylogeny inferred with maximum parsimony by Lendemer and O'Brien (2011). Together the results of ML1 and ML2 revealed a strongly supported monophyletic section *Hydrothyriae*, with three distinct lineages corresponding to *P. hydrothyria*, its sister group *P. gowardii* s.l. (bootstrap value of 97%), and *P. gowardii* s.s.

Each locus (Tables 1, 2) provided a similar level of genetic information among populations within and among each of the three monophyletic groups (*P. gowardii* s.s., *P. gowardii* s.l., and *P. hydrothyria*; Fig. 2). No intraspecific variation in all analyzed loci was found among the sampled individuals of each potential species except for ITS and *EFT2*-1 of *P. hydrothyria* (Table 2). A single-site polymorphism within *P. hydrothyria* occurred in the ITS (two haplotypes differed by a single indel) and in *EFT2*-1 (two haplotypes differed by single nucleotide substitution present in one individual). However, each of the three monophyletic groups was uniquely distinct (Table 2). For the 72 polymorphic sites (including indels in the ITS) found across the concatenated loci (Table 1), the lowest level of genetic variation among all sampled individuals was found in *RPB1* (11), whereas the ITS, β-tubulin, and *EFT2*-1 provided a similar degree of polymorphism (23, 20, and 18 sites, respectively). The highest number of exclusively shared nucleotides at polymorphic sites was between *P. hydrothyria* and *P. gowardii* s.l. for all loci except for ITS where *P. gowardii* s.s. and *P. gowardii* s.l. shared more unique nucleotides at polymorphic sites. However, the difference was by a slight margin compared with β-tubulin and *EFT2*-1 where the number of sites segregating in favor of *P. hydrothyria* with *P. gowardii* s.l. (12/20 and 10/18, respectively) was at least twice as high as for *P. gowardii* s.l. with *P. gowardii* s.s. (5/20 and 5/18, respectively; Table 1). Overall, *P. hydrothyria* exclusively shared the same nucleotide with *P. gowardii* s.l. at 34 polymorphic sites, whereas *P. hydrothyria* shared exclusively the same nucleotide with *P. gowardii* s.s. at only 12 polymorphic sites, and *P. gowardii* s.l. and *P. gowardii* s.s. shared exclusively the same nucleotide at 22 polymorphic sites (Fig. 2, Table 1). Fixed polymorphic sites unique to each of the three species were extremely rare across all loci (two in ITS and one in β-tubulin).

The Structurama analysis grouped all sequences into three distinct populations at the highest probability of 0.81. The presence of two *EFT2*-1 alleles in *P. hydrothyria* (a single point mutation shared with *P. gowardii* s.s.) enforced the alternative four-population scenario but with a low probability of 0.16. Notably, each of the 35 individuals was correctly allocated to the population ($p > 0.95$) corroborating the monophyletic groups revealed by phylogenetic analyses (Fig. 2).

As pointed out earlier by Lendemer and O'Brien (2011), *P. gowardii* s.l. is morphologically indistinguishable from both *P. hydrothyria* and *P. gowardii* s.s. and chemically and geographically more similar to *P. gowardii* s.s. Both *P. gowardii* s.s. and s.l. lack any detectable secondary compounds, and their ranges overlap in western North America (Washington state). However, all records, confirmed by molecular data (this study), for *P. gowardii* s.s. are from more northern localities (spreading up to Alaska) and a single occurrence in Montana, whereas *P. gowardii* s.l. has been reported from more southern states (e.g., Oregon and California) (Fig. 2, Appendix 1). The first diversification event within the section *Hydrothyriae* is represented by *P. gowardii* s.s. and therefore indicates that the aquatic *Peltigera* probably originated in the western area of North America, followed by a subsequent split into two lineages, *P. gowardii* s.l. (spreading south to California) and *P. hydrothyria* (isolated in the Appalachian mountain of eastern North America). Detailed morphological studies on fresh living specimens are needed to determine whether there are subtle differences that could distinguish the species and which might correlate with the genetic differentiation found in this section.

Cyanobiont identity within the section **Hydrothyriae—**Casamatta et al. (2006) circumscribed a new *Capsosira* species (*C. lowei*; Capsosiraceae, Stigonematales) to accommodate the cyanobiont isolated from the thallus of *P. hydrothyria* collected in North Carolina, which was reported as a filamentous cyanobacterium with true branching. However, the authors stated that phylogenetic analyses of the 16S, and structural similarity of the ribosomal ITS region supported this new species as being affiliated with *Nostoc* (Nostocales), i.e., sister to *N. commune* UTEX584 with high bootstrap support (Casamatta et al., 2006). As expected, based on previous phylogenetic studies on cyanobacteria (e.g., Turner et al., 1999; O'Brien et al., 2005; Korelusová, 2008), most relationships in our 16S phylogeny were poorly supported (Fig. 3), but the overall topology and delimitation of major clades were in agreement with the existing 16S and *rbcLX* phylogenies (e.g., *Nostoc* clade I and II, see Otálora et al., 2010 and O'Brien et al., 2009; H5 and H3, see Korelusová, 2008).

The three cyanobacteria from the aquatic *Peltigera* thalli and *C. lowei* were nested within one of the few well-supported monophyletic groups (72% bootstrap support) within the *Nostoc* clade II, which contains the majority of symbiotic *Nostoc* associated with plants and lichens, as well as many free-living taxa of

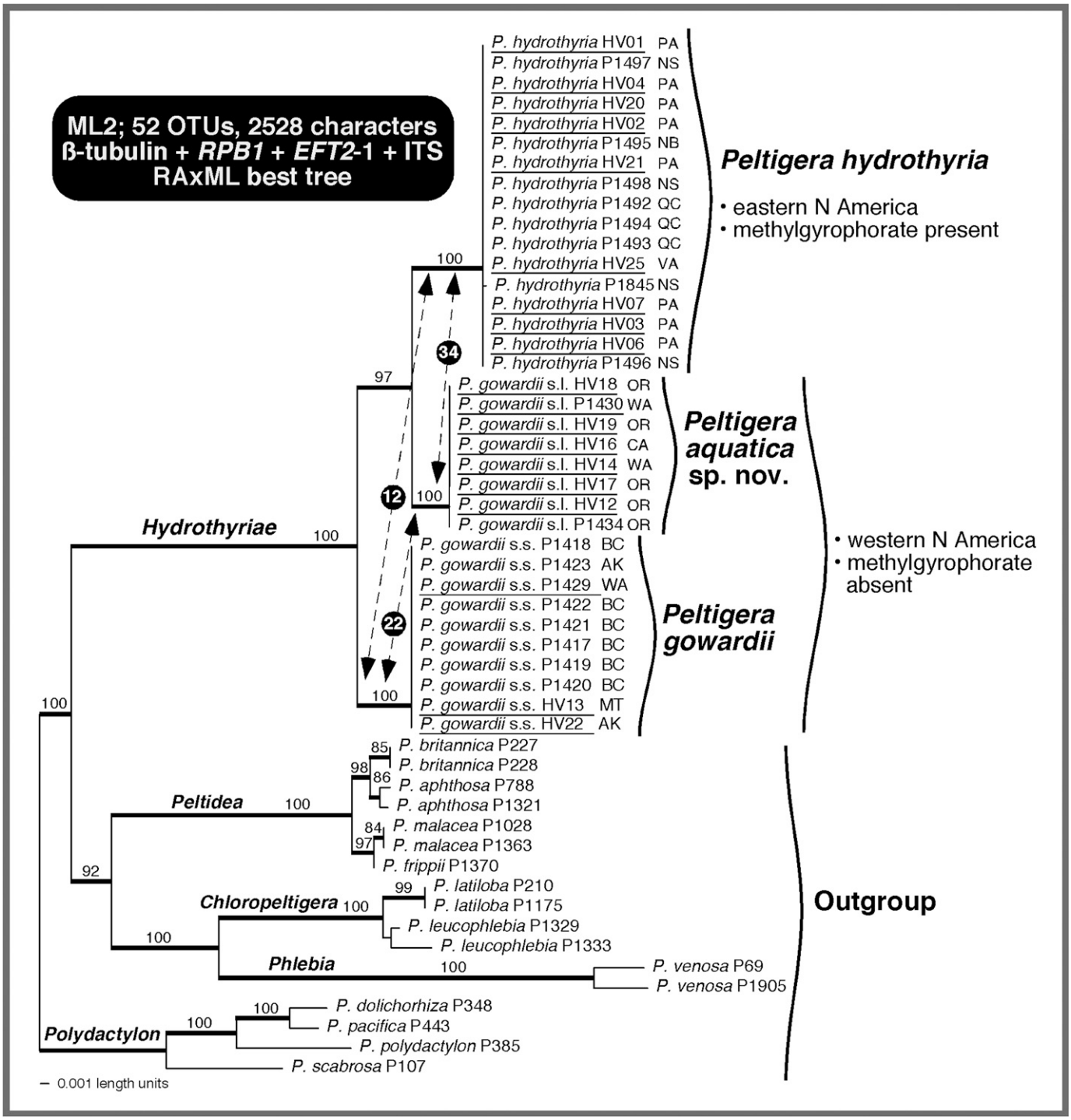

Fig. 2. Phylogenetic delimitation of *P. hydrothyria*, *P. gowardii*, and the newly proposed *P. aquatica* (*P. gowardii* s.l.) as revealed by a maximum likelihood analysis of 35 individuals from section *Hydrothyriae* based on combined β-tubulin, *RPB1*, *EFT2*-1, and ITS loci (ML2). A total of 11 species from sections *Chloropeltigera*, *Peltidea*, *Phlebia*, and *Polydactylon* formed the outgroup. Values above internodes represent bootstrap support (BS). Thicker internodes indicate strongly supported (BS ≥ 80%) relationships. Abbreviations after taxon names within section *Hydrothyriae* indicate the geographical origin of sequenced individuals (states in the USA, Canadian provinces). Underlined names indicate specimens included in the study by Lendemer and O'Brien (2011). Numbers of polymorphic sites with shared nucleotides between pairs of species (dotted arrows) are shown in black circles (see Table 2).

Nostoc, including *Nostoc commune* Vaucher ex Bornet & Flahault UTEX584 (Fig. 3). Phylogenetic relationships within the *Nostoc* clade II are mostly uncertain based on the 16S. The phylogenetic study of heterocystous cyanobacteria by Korelusová (2008) showed similar placement of *C. lowei* (in Nostocales; clade H5) among lichen cyanobionts of *Nostoc*. Our results suggest that cyanobacteria associated with the aquatic *Peltigera*, including *C. lowei*, represent *Nostoc* s.l., however, its closest relatives

TABLE 2. Haplotypes (H) sampled within each *Peltigera* species from the section *Hydrothyriae*, and their geographic origins. For the r*bcLX* locus, five spacer types are reported after the slash.

DNA extract no.	Taxon	Voucher	ITS 4H	β-tubulin 4H	*RPB1* 3H	*EFT2*-1 4H	*rbcLX* 14H/5 spacers
P1430	*P. aquatica* (*P. gowardii* s.l.)	USA, WA	H4	H3	H2	H3	H14/S5
HV18	*P. aquatica* (*P. gowardii* s.l.)	USA, OR	H4	H3	H2	H3	H13/S5
HV19	*P. aquatica* (*P. gowardii* s.l.)	USA, OR	H4	H3	H2	H3	H11/S5
HV12	*P. aquatica* (*P. gowardii* s.l.)	USA, OR	H4	H3	H2	N/A	H12/S5
HV14	*P. aquatica* (*P. gowardii* s.l.)	USA, WA	H4	H3	H2	N/A	N/A
HV16	*P. aquatica* (*P. gowardii* s.l.)	USA, CA	H4	H3	H2	N/A	H11/S5
HV17	*P. aquatica* (*P. gowardii* s.l.)	USA, OR	H4	H3	H2	N/A	H14/S5
P1434	*P. aquatica* (*P. gowardii* s.l.)	USA, OR	H4	N/A	H2	N/A	H14/S5
All	*P. aquatica* (*P. gowardii* s.l.)	Western	1H	1H	1H	1H	4H/1
P1417	*P. gowardii* s.s.	Canada, BC	H1	H1	H1	H1	H6/S2
P1418	*P. gowardii* s.s.	Canada, BC	H1	H1	N/A	H1	H5/S2
P1419	*P. gowardii* s.s.	Canada, BC	H1	H1	H1	H1	H5/S2
P1420	*P. gowardii* s.s.	Canada, BC	H1	H1	H1	H1	H7/S2
P1421	*P. gowardii* s.s.	Canada, BC	H1	H1	H1	H1	H5/S2
P1422	*P. gowardii* s.s.	Canada, BC	H1	H1	H1	H1	H5/S2
P1423	*P. gowardii* s.s.	USA, AK	H1	H1	N/A	H1	H10/S4
P1429	*P. gowardii* s.s.	USA, WA	H1	H1	N/A	H1	H10/S4
HV13	*P. gowardii* s.s.	USA, MT	H1	H1	H1	H1	H5/S2
HV22	*P. gowardii* s.s.	USA, AK	H1	H1	H1	N/A	H5/S2
All	*P. gowardii* s.s.	Western	1H	1H	1H	1H	4H/2
P1492	*P. hydrothyria*	Canada, QC	H2	H2	H3	H2	H3/S1
P1493	*P. hydrothyria*	Canada, QC	H2	H2	H3	H2	H8/S3
P1494	*P. hydrothyria*	Canada, QC	H2	H2	H3	H2	H9/S3
P1495	*P. hydrothyria*	Canada, NB	H3	H2	H3	H2	H2/S1
P1496	*P. hydrothyria*	Canada, NS	H2	H2	H3	H2	H9/S3
P1497	*P. hydrothyria*	Canada, NS	H3	H2	H3	H2	H2/S1
P1498	*P. hydrothyria*	Canada, NS	H3	H2	H3	H2	H9/S3
P1845	*P. hydrothyria*	Canada, NS	H3	H2	H3	H4	H9/S3
HV25	*P. hydrothyria*	USA, VA	H3	N/A	N/A	N/A	H9/S3
HV07	*P. hydrothyria*	USA, PA	H3	H2	H3	N/A	H9/S3
HV06	*P. hydrothyria*	USA, PA	H3	H2	H3	N/A	H9/S3
HV04	*P. hydrothyria*	USA, PA	H3	H2	H3	H2	H9/S3
HV03	*P. hydrothyria*	USA, PA	H3	H2	H3	N/A	H9/S3
HV21	*P. hydrothyria*	USA, PA	H3	H2	N/A	H2	H1/S1
HV20	*P. hydrothyria*	USA, PA	H3	H2	H3	H2	H3/S1
HV02	*P. hydrothyria*	USA, PA	H3	H2	H3	H2	H4/S1
HV01	*P. hydrothyria*	USA, PA	H3	H2	H3	H2	N/A
All	*P. hydrothyria*	Eastern	2H	1H	1H	2H	6H/2

Notes: AK, Alaska; BC, British Columbia; CA, California; MT, Montana; NB, New Brunswick; NS, Nova Scotia; OR, Oregon; PA, Pennsylvania; QC, Québec; VA, Virginia; WA, Washington State.

could not be established with high confidence. We could not evaluate the phylogenetic placement of the whole genus *Capsosira* (Capsosiraceae, Stigonematales) because the cultures for the remaining two species (*C. brebissonii* Kützing ex Bornet & Flahault—type species and *C. brasiliensis* C. L. Sant'Anna & S. M. F. Silva) were not available in public depositories.

The genus *Nostoc* is shown to be a nonmonophyletic assemblage (Fig. 3), that is in need of a comprehensive molecular systematic treatment. Recently, an attempt toward disentangling this complex taxon was made by introducing a new genus, *Desmonostoc* Hrouzek & Ventura, to accommodate *Nostoc muscorum* Agardh ex Bornet & Flahault and related unnamed strains (Hrouzek et al., 2013). However, this change was based on a very restricted data set lacking completely lichen-associated strains and other commonly used reference cultures. Our phylogenetic results do not support a monophyletic genus *Desmonostoc* because *D. muscorum* (Agardh ex Bornet & Flahault) Hrouzek & Ventura (SAG 57.79) is nested in the *Nostoc* clade II (with strong support), whereas the phylogenetic placement of *D. linckia* (IAM M-251) falls outside clade II (Fig. 3).

Based on the *rbcLX* analysis, all *Hydrothyriae* cyanobionts were placed in subclade 3, extended cluster 1 (Fig. 4; Appendix S1, see Supplemental Data with the online version of this article), as a monophyletic group closely related to other symbiotic *Nostoc* strains associated with *Gunnera* L., *Blasia* L. and *Geosiphon* F. Wettst. As for previous *rbcLX* phylogenies of that scale, most relationships are not well supported (see Otálora et al., 2010 and O'Brien et al., 2013). Because of its unusual habitat, it is possible that a unique lineage of *Nostoc* s.l. is associated with aquatic *Peltigera* and other co-occurring aquatic cyanolichens [i.e., *Leptogium rivulare* (Ach.) Mont. from Collemataceae] in western North America, but no data are available for the cyanobiont from aquatic members of the Collemataceae.

Four distinct *Nostoc* haplotypes were detected in each of the two western species (*P. gowardii* s.l. and *P. gowardii* s.s.), and six in the eastern species (*P. hydrothyria*). Phylogenetic relationships among these cyanobionts did not reflect monophyletic circumscription of the corresponding mycobiont species (Table 2; Fig. 2 vs. Fig. 4A). Two types of *rbcLX* spacers were present

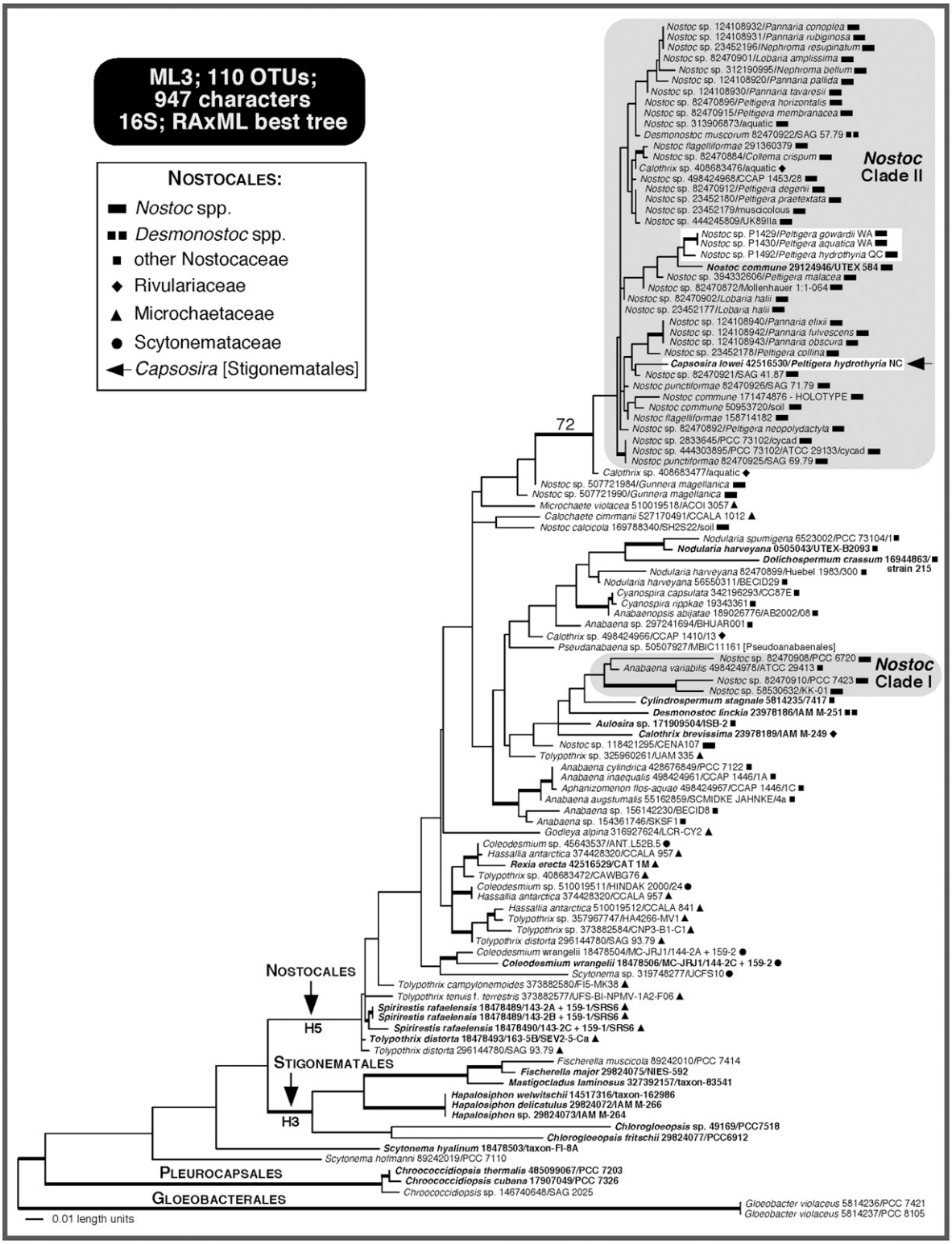

Fig. 3. Placement of four cyanobionts (*Nostoc* sp. and *Capsosira lowei*) associated with section *Hydrothyriae* (white boxes) in the phylogenetic context of cyanobacteria (108 representatives) mostly from the Nostocales (families Nostocaceae, Microchaetaceae, Scytonemataceae, and Rivulariaceae), and related orders Stigonematales and Pleurocapsales, revealed by maximum likelihood analysis of the16S rRNA gene (ML3). Two representatives of Gloeobacterales (*Gloeobacter violaceus*) were used to root the tree. Thicker internodes indicate relationships with BS ≥ 70%. Taxon names in boldface indicate sequences included in the phylogeny presented in fig. 3 of Casamatta et al. (2006). *Nostoc* clade I and clade II correspond to clades delimited by O'Brien et al. (2005) in their fig. 2, whereas H3 (Stigonematales) and H5 (Nostocales) correspond to clades delimited by Korelusová (2008) in their fig. 1. GenBank identification numbers for all published sequences included are shown after each terminal name.

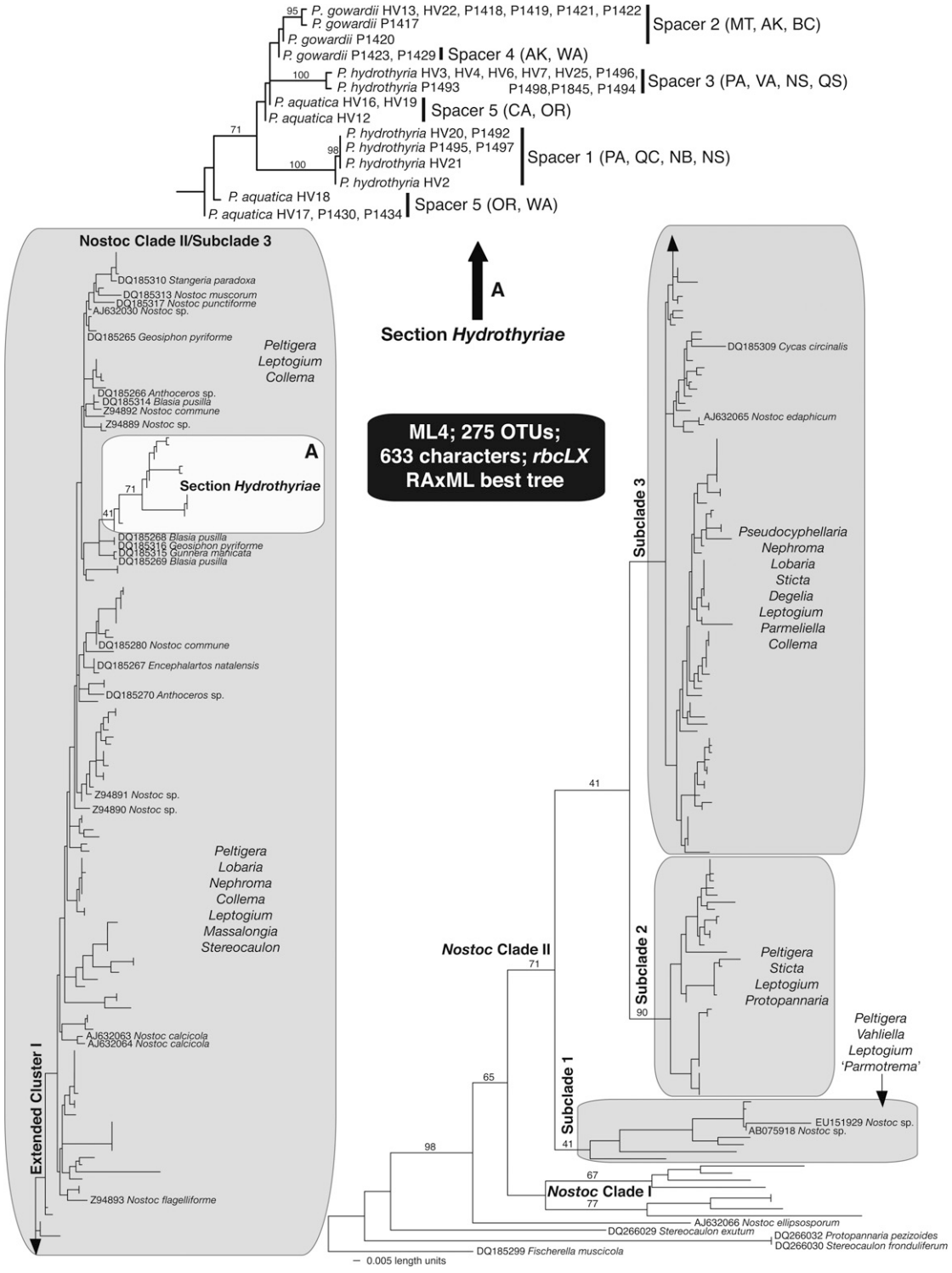

Fig. 4. Monophyly of 33 newly sequenced cyanobionts (representing 14 haplotypes) associated with *Peltigera* section *Hydrothyriae* (white box) and their close affiliation with members of *Nostoc* clade II (subclade 3) revealed by maximum likelihood analysis of 271 individuals representing putative *Nostoc* spp. based on the *rbcLX* locus (ML4). *Fischerella muscicola* (Stigonematales) was used to root the tree. Names representing GenBank sequences of free-living and nonlichen-associated symbionts are shown. The remaining terminal branches correspond to GenBank sequences of cyanobionts (Appendix S1) from various lichen genera listed within each subclade. Delimitation of clades, subclades, and cluster I follows Otálora et al. (2010) and O'Brien et al. (2013). Bootstrap support values ≥ 70%, as well as support values for important internodes that are < 70%, are shown above internodes. A: Phylogenetic relationships among 14 haplotypes representing cyanobionts found in the section *Hydrothyriae* (*P. gowardii* s.s., *P. aquatica* [*P. gowardii* s.l.] and *P. hydrothyria*) part of the ML4 phylogeny (white box). Cyanobionts with identical sequences are listed after species names of their fungal partner. Unique types of spacers within the *rbcLX* locus (part of the ambiguously aligned region that were excluded from the ML4 analysis) and geographical provenance of the lichen thalli are listed after the black vertical bars (for details see Table 2 and Appendix 1). Bootstrap support values > 70% are shown above internodes.

among the haplotypes associated with *P. gowardii* s.s. and *P. hydrothyria*, and a single spacer was found in the cyanobionts of *P. gowardii* s.l. (Table 2). Although the spacers were not alignable across all 14 haplotypes and therefore had to be excluded from the subsequent phylogenetic analysis, their sequences were unique for each mycobiont species. The presence of species-specific *rbcLX* sequences would suggest that each of the three aquatic species evolved in association with different strains of *Nostoc* (Table 2, Fig. 4A), which may be one of the factors shaping speciation in the section *Hydrothyriae* as it has been proposed for other lichen-forming fungi (e.g., Kroken and Taylor, 2000; Elvebakk et al., 2008; Fernández-Mendoza et al., 2011; O'Brien et al., 2013; Magain and Sérusiaux, 2014).

***Recognition of a new species within section* Hydrothyriae—**A clear segregation of all polymorphic sites for multiple loci, the lack of overlap between cyanobiont strains, and potentially distinct geographical patterns, strongly support the recognition of the three monophyletic groups within section *Hydrothyriae* as representing three distinct species (Fig. 2). Therefore, previously recognized as *P. gowardii* s.l. (Lendemer and O'Brien, 2011) is described here as a new species: *P. aquatica*.

Peltigera aquatica Miadl. & Lendemer sp. nov. (MycoBank # MB 809067)—Differs from two other recognized aquatic species (*P. gowardii*, GenBank JF837364; *P. hydrothyria*, JF837365) by having six molecular synapomorphies (including indels) within the nuclear ribosomal internal transcribed spacers (ITS1 and ITS2) at the following positions: (1) 88–97: a chain of 10 adenines vs. 7 for *P. gowardii* and 9 for *P. hydrothyria*; (2) 107: cytosine vs. thymine for *P. gowardii* and adenine for *P. hydrothyria*; (3) 116: adenine vs. cytosine for *P. gowardii* and *P. hydrothyria*; (4) 120–128: a chain of 9 thymines vs. 7 for *P. gowardii* and *P. hydrothyria*; (5) 175: thymine vs. guanine for *P. gowardii* and *P. hydrothyria*; (6) 386: guanine vs. adenine for *P. gowardii* and *P. hydrothyria* (Appendix S2, see online Supplemental Data).

Type—USA, Oregon: Lane County, Ridge Creek, Cougar Reservoir, 44.058N 122.22W, *D. Glavitch s.n.* (with L. Geiser), 17 February 2007 (NY-01117843, holotype).

Morphology—Similar to *P. hydrothyria* and *P. gowardii* (Fig. 5C, D). Detailed description is provided in Lendemer and O'Brien (2011; Taxonomic section, *P. gowardii*).

Chemistry—Similar to *P. gowardii* in that no substances were detected by thin layer chromatography (TLC) and standard spot tests (see Lendemer and O'Brien [2011]). *Peltigera hydrothyria* contains methylgyrophorate and methyllecanorate and sometimes traces of gyrophoric or lecanoric acid (which give a C+ pink reaction to acetone extracts; Lendemer and O'Brien, 2011).

Etymology—The name of this new species reflects its aquatic habit.

Ecology and distribution—As circumscribed here, the distribution of *P. aquatica* is restricted to the mountains of western United States, extending from central/northern California (Sierra ranges) northward to Oregon and Washington (Cascade ranges) where it co-occurs with *P. gowardii*. The latter species is found in the Northern Cordillera, but has a more maritime tendency and a more northern distribution. The geographical range of *P. gowardii* spans an area from Montana (Rocky Mountains) to British Columbia (Columbia Mountains) in Canada and Alaska.The actual geographical range of *P. aquatica* has to be verified by sequencing the ITS region of the existing collections of *P. gowardii* and populations that were never previously sampled in western North America (Fig. 5A). *Peltigera hydrothyria* is restricted to the eastern part of North America, extending from the southern Appalachian mountains of the USA (Georgia) to Nova Scotia (Canada) (Fig. 5B). The conservation and management status of the newly circumscribed species, *P. aquatica*, needs to be reevaluated in the western United States.

Currently based on the anticipated geographic distribution, its status varies from unranked to imperiled and vulnerable depending on the state (Peterson, 2010). More information about the geographical ranges, ecology, and potential threats to species within section *Hydrothyriae* is provided in online Appendix S3.

Specimens examined—See records of *P. aquatica* in Appendix 1 and Fig. 5A.

Conclusions—Although we expanded the taxon sampling and the number of molecular markers, this phylogenetic study demonstrated that four loci (three protein-coding genes and nrLSU) are not sufficient for reconstructing with high confidence the relationships among sections in the genus *Peltigera*, including the placement of the section *Hydrothyriae*. More characters (preferably single-copy protein-coding genes) are needed to capture deep evolutionary splits within the genus. However, the ITS region alone provided sufficient genetic information to distinguish three morphologically cryptic species within section *Hydrothyriae*. The addition of three protein-coding genes confirmed the ITS-based phylogenetic results from Lendemer and O'Brien (2011) and supported the recognition of a new species, *Peltigera aquatica*. Our phylogenies for nostocalean cyanobacteria indicate that molecular revisions of the genus *Nostoc* (known to be nonmonophyletic) and reliable identification of cyanobionts associated with lichens are needed. Future studies should be based on more complete sampling, with symbiotic strains and reference taxa well represented in the analyzed data and using more variable, but alignable, markers. Switches to different *Nostoc* strains might be associated with the cospeciation events within section *Hydrothyriae*. Future detailed morphological, anatomical, and chemical revision of aquatic *Peltigera* based on freshly collected material may reveal phenotypic features correlated with the molecular data.

All collections of *P. gowardii* should be verified molecularly (based on the ITS region) to tease apart the actual geographical ranges of *P. aquatica* and *P. gowardii*, especially in the areas where both species co-occur. The molecular approach may not be possible for the old herbarium specimens especially if the material was collected in the last century. Potential threats to the populations of morphologically cryptic *P. aquatica* and *P. gowardii* should be then re-evaluated to assure survival of both taxa. Because of the aquatic habit, unique ecology (certain level of year-round humidity, stream-water flow, a generally low water temperature, pH close to neutral, and a lack of silt), and restricted geographical ranges, species from section *Hydrothyriae*, and very likely other co-occurring macrolichens (e.g., members of the Collemataceae and Verrucariaceae), are endangered as a result of anthropogenic activities negatively affecting lichen thalli and their habitat (e.g., through human recreation and communication infrastructure, forest management, global climate change, and pollution). Populations of aquatic *Peltigera* should continue to be monitored and protected.

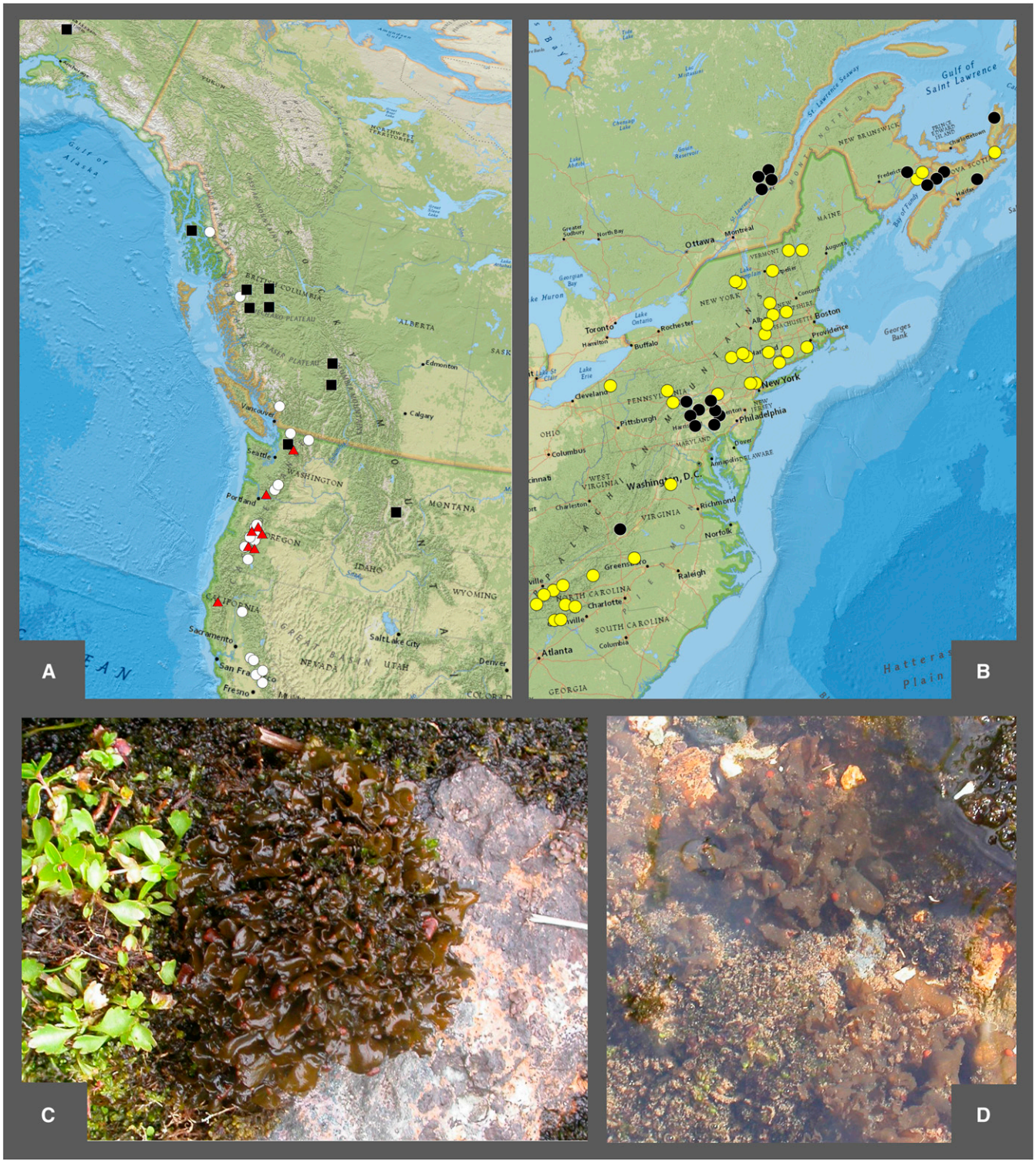

Fig. 5. Geographic distribution and habit of aquatic *Peltigera*. The top left panel (A) shows the occurrences of *P. aquatica* (A; red triangles) and *P. gowardii* (A; black squares) confirmed by molecular data. The white circles represent other sites where aquatic *Peltigera* occur but from which specimens have not been examined using molecular techniques. The top right panel (B) shows the occurrences of *P. hydrothyria* (B; black circles) confirmed by molecular data along with the overall distribution of this species (yellow circles) in eastern North America. Note the four black circles shown in the province of Québec represent specimens from different parts of the same stream. The distribution maps were prepared using records from the NYBG herbarium, the Duke herbarium, the COSEWIC (2013a, b) reports, as well as the papers by Lendemer and O'Brien (2011), and Lendemer and Anderson (2012). The bottom panels show thalli of *P. gowardii*. One is colonizing a rock close to water level by a stream on Hudson Bay Mountain, Smithers, British Columbia, Canada (C) and a second is growing submerged in the stream (D). All three aquatic *Peltigera* species are morphologically very similar to one another and have not, to date, been differentiated on this basis. *Photo credit:* David Richardson.

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APPENDIX 1. Taxon sampling (*Peltigera* and *Solorina*) with associated voucher information and corresponding sequences used in this study. GenBank accession numbers indicate newly generated sequences, whereas GB identification numbers refer to published sequences. Missing sequences in the ML1 and ML2 combined data sets are indicated by dashes (—). Non-targeted sequences are shown as not applicable (N/A). Taxon names in boldface belong to section *Hydrothyriae*.

DNA extract no.	Taxon	Voucher information	ITS	nrLSU	β-tubulin	*RPB1*	*EFT2*-1	*rbcLX*
P788[1, 2]	*Peltigera aphthosa*	Norway; Magain s.n.; LG	N/A	14422959	KM005826	KM005941	KM005880	N/A
P1321[2]	*P. aphthosa*	Norway; Magain s.n.; LG	N/A	N/A	KM005859	KM005948	—	N/A
P1430[1, 2, 3]* (SLJ3839)	***P. aquatica* (*P. gowardii* s.l.)**	USA, Washington; Joneson 3839; NY-01118503	334089940	KM005736	KM005832	KM005992	KM005922	KM006027
HV18[2, 3]	***P. aquatica* (*P. gowardii* s.l.)**	USA, Oregon; Glavich (with Geiser) s.n.; NY-01117843	334089946	N/A	KM005843	KM005990	KM005920	KM006025
HV19[2, 3]	***P. aquatica* (*P. gowardii* s.l.)**	USA, Oregon; Glavich s.n.; NY-01117842	334089945	N/A	KM005844	KM005991	KM005921	KM006026
HV12[2, 3]	***P. aquatica* (*P. gowardii* s.l.)**	USA, Oregon; Nadel s.n.; NY-01117826	334089944	N/A	KM005839	KM005986	—	KM006022
HV14[2]	***P. aquatica* (*P. gowardii* s.l.)**	USA, Washington; Nadel s.n.; NY-01117828	334089943	N/A	KM005840	KM005987	—	—
HV16[2, 3]	***P. aquatica* (*P. gowardii* s.l.)**	USA, California; Wischart s.n.; NY-01117823	334089942	N/A	KM005841	KM005988	—	KM006023
HV17[2, 3]	***P. aquatica* (*P. gowardii* s.l.)**	USA, Oregon; Glavich s.n.; NY-01117827	334089941	N/A	KM005842	KM005989	—	KM006024
P1434[2, 3]	***P. aquatica* (*P. gowardii* s.l.)**	USA, Oregon; Haesborg RRN301; UPS-L 1725114	KM005774	N/A	—	KM005993	—	KM006028
P227[2]	*P. britannica*	Canada, British Columbia; Goward 09-436; UBC	N/A	N/A	KM005817	KM005935	—	N/A
P228[1, 2]	*P. britannica*	Canada, British Columbia; Goward 09-120; UBC	N/A	KM005739	KM005818	KM005936	KM005874	N/A
P4[1]	*P. canina*	Iceland; Miadlikowska et al. s.n.; DUKE	N/A	KM005740	KM005821	KM005924	KM005861	N/A
P14[1]	*P. canina*	Iceland; Miadlikowska et al. s.n.; DUKE	N/A	KM005741	KM005803	KM005925	KM005862	N/A
P1263[1]	*P. collina*	Chile; Hollinger 1929; UBC	N/A	KM005742	KM005794	KM005945	KM005884	N/A
P1810[1]	*P. continentalis*	Russia, Siberia; Miadlikowska et al. s.n.; DUKE	N/A	KM005743	KM005807	KM005957	KM005891	N/A
P523[1]	*P. degenii*	Norway; Magain s.n.; LG	N/A	KM005744	KM005828	KM005939	KM005878	N/A
P348[1, 2]	*P. dolichorhiza*	Mexico, Hidalgo; Herrera-Campos 13382; MEXU	N/A	KM005745	KM005819	KM005937	KM005875	N/A
P66[1]	*P. elisabethae*	USA, New Mexico; Hollinger 2397; DUKE	N/A	KM005746	—	KM005929	KM005867	N/A
P1544[1]	*P. elisabethae*	Russia, Siberia; Miadlikowska et al. s.n.; DUKE	N/A	KM005747	KM005802	KM005953	KM005887	N/A
P1817[1]	*P. evansiana*	USA, Pennsylvania; Lendemer (with Harris) 17422; NY-01105603	N/A	KM005748	KM005808	KM005958	KM005892	N/A

APPENDIX 1. Continued.

DNA extract no.	Taxon	Voucher information	ITS	nrLSU	β-tubulin	*RPB1*	*EFT2*-1	*rbcLX*
P1818[1]	*P. evansiana*	USA, Pennsylvania; Lendemer (with Harris) 17753; NY-01103610	N/A	KM005737	KM005809	KM005959	KM005893	N/A
P1370[1, 2]	*P. frippii*	Russia, Siberia; Andreyev s.n.; LE-91762 p.p.	N/A	14422955	KM005800	KM005952	KM005886	N/A
P1417[2, 3]	***P. gowardii* s.s.**	Canada, British Columbia; Richardson s.n.; CANL-125686	KM005775	N/A	KM005847	KM005966	KM005899	KM005998
P1418[2, 3]	***P. gowardii* s.s.**	Canada, British Columbia; Richardson s.n.; CANL-125691	KM005776	N/A	KM005848	—	KM005900	KM005999
P1419[2, 3]	***P. gowardii* s.s.**	Canada, British Columbia; J and R Pojar s.n.; CANL-125693	KM005777	N/A	KM005849	KM005967	KM005901	KM006000
P1420[2, 3]	***P. gowardii* s.s.**	Canada, British Columbia; Richardson s.n.; CANL-125692	KM005778	N/A	KM005850	KM005968	KM005902	KM006001
P1421[2, 3]	***P. gowardii* s.s.**	Canada, British Columbia; Richardson s.n.; CANL-125689	KM005779	N/A	KM005851	KM005969	KM005903	KM006002
P1422[2, 3]	***P. gowardii* s.s.**	Canada, British Columbia; Richardson s.n.; CANL-125690	KM005780	N/A	KM005852	KM005970	KM005904	KM006003
P1423[2, 3]	***P. gowardii* s.s.**	USA, Alaska; Dillman and Geiser s.n.; CANL-125694	KM005781	N/A	KM005853	—	KM005905	KM006004
P1429[1, 2, 3] * (SLJ3840)	***P. gowardii* s.s.**	USA, Washington; Joneson 3840; NY-01118502	334089949	—	KM005830	—	KM005906	KM006005
HV13[2, 3]	***P. gowardii* s.s.**	USA, Montana; Wheeler 877; NY-01117824	334089948	N/A	—	KM005964	KM005898	KM005996
HV22[2, 3]	***P. gowardii* s.s.**	USA, Alaska; Walton 10362; NY-01117829	334089947	N/A	—	KM005965	—	KM005997
P1274[1]	*P. horizontalis*	China, Jilin; Sohrabi (with Ghobad-Nehjad) 16639; DUKE	N/A	KM005749	KM005795	KM005946	KM005885	N/A
P1641[1]	*P. horizontalis*	USA, North Carolina; Lendemer (with Tripp) 8136; NY	N/A	KM005750	KM005806	KM005956	KM005890	N/A
P1492[1, 2, 3] *	***P. hydrothyria***	Canada, Québec; Richardson s.n.; CANL-125687	KM005782	KM005751	KM005831	KM005978	KM005912	KM006014
P1493[2, 3]	***P. hydrothyria***	Canada, Québec; Richardson s.n.; CANL-125688	KM005783	N/A	KM005854	KM005979	KM005913	KM006015
P1494[2, 3]	***P. hydrothyria***	Canada, Québec; Anderson 151060; NSPM	KM005784	N/A	KM005855	KM005980	KM005914	KM006016
P1495[2, 3]	***P. hydrothyria***	Canada, New Brunswick; Anderson 151066; NSPM	KM005786	N/A	KM005856	KM005981	KM005915	KM006017
P1496[2, 3]	***P. hydrothyria***	Canada, Nova Scotia; Anderson 1510479; NSPM	KM005785	N/A	KM005857	KM005982	KM005916	KM006018
P1497[2, 3]	***P. hydrothyria***	Canada, Nova Scotia; Anderson 151675; NSPM	KM005787	N/A	KM005801	KM005983	KM005917	KM006019
P1498[1, 2, 3]	***P. hydrothyria***	Canada, Nova Scotia; Anderson 151055; NSPM	KM005788	KM005753	KM005858	KM005984	KM005918	KM006020
P1845[1, 2, 3]	***P. hydrothyria***	Canada, Nova Scotia; Anderson 159031; NSPM	KM005789	KM005752	KM005811	KM005985	KM005919	KM006021
HV25[2, 3]	***P. hydrothyria***	USA, Virginia; Huber s.n.; NY-01117830	334089960	N/A	—	—	—	KM006013
HV07[2, 3]	***P. hydrothyria***	USA, Pennsylvania; Munch s.n.; NY-01117835	334089958	N/A	KM005838	KM005976	—	KM006010
HV06[2, 3]	***P. hydrothyria***	USA, Pennsylvania; Munch s.n.; NY-01117839	334089957	N/A	KM005837	KM005975	—	KM006009
HV04[2, 3]	***P. hydrothyria***	USA, Pennsylvania; Munch s.n.; NY-01117837	334089956	N/A	KM005836	KM005974	KM005909	KM006008
HV03[2, 3]	***P. hydrothyria***	USA, Pennsylvania; Munch s.n.; NY-01117836	334089955	N/A	KM005835	KM005973	—	KM006007
HV21[2, 3]	***P. hydrothyria***	USA, Pennsylvania; Lendemer 10-02-07-02; NY-01117841	334089954	N/A	KM005846	—	KM005911	KM006012
HV20[2, 3]	***P. hydrothyria***	USA, Pennsylvania; Lendemer 10-02-07-01; NY-01117840	334089953	N/A	KM005845	KM005977	KM005910	KM006011
HV02[2, 3]	***P. hydrothyria***	USA, Pennsylvania; Stabley 09-23-05-02; NY-01117834	334089952	N/A	KM005834	KM005972	KM005908	KM006006
HV01[2]	***P. hydrothyria***	USA, Pennsylvania; Stabley 09-23-05-01; NY-01117833	334089951	N/A	KM005833	KM005971	KM005907	—
P1292[1]	*P. kristinssonii*	Canada, Québec; Gagnon s.n.; QFA-0594989	N/A	KM005754	KM005796	KM005947	—	N/A
P26[1]	*P. laciniata*	Costa Rica; Miadlikowska et al. s.n.; DUKE	N/A	KM005756	KM005815	KM005926	KM005863	N/A
P924[1]	*P. laciniata*	Colombia; Lücking 33692; UDBC	N/A	KM005755	KM005827	—	KM005864	N/A
P1175[2]	*P. latiloba*	Russia, Sakha Rep.; Ahti 64755; H	N/A	N/A	KM005792	KM005943	KM005882	N/A
P210[2]	*P. latiloba*	Locality; Nelson 11-034; UBC	N/A	N/A	KM005816	KM005934	KM005873	N/A
P1844[1]	*P. lepidophora*	Canada, British Columbia; Goward s.n.; UBC	N/A	KM005738	KM005810	KM005960	KM005894	N/A
P1329[1, 2]	*P. leucophlebia*	Canada, Québec; Gagnon s.n.; QFA-0594936	N/A	—	KM005797	KM005949	—	N/A

APPENDIX 1. Continued.

DNA extract no.	Taxon	Voucher information	ITS	nrLSU	β-tubulin	*RPB1*	*EFT2*-1	*rbcLX*
P1333[1, 2]	*P. leucophlebia*	Canada, Québec; Gagnon s.n.; QFA-0594851	N/A	—	KM005798	KM005950	—	N/A
P1028[1, 2]	*P. malacea*	Norway; Magain s.n.; LG	N/A	KM005757	KM005790	KM005942	KM005881	N/A
P1363[1, 2]	*P. malacea*	Russia, Karelia; Uotila 44104; H	N/A	—	KM005799	KM005951	—	N/A
P3[1]	*P. membranacea*	Iceland; Miadlikowska et al. s.n.; DUKE	N/A	KM005758	KM005814	KM005923	KM005860	N/A
P73[1]	*P. monticola*	Austria; Türk 37593; H	N/A	KM005759	KM005824	—	KM005869	N/A
P75[1]	*P. monticola*	Norway; Ahti 65831; H	N/A	KM005760	KM005825	KM005932	KM005871	N/A
P1620[1]	*P. neckeri*	USA, Arkansas; Harris 45472; NY-00660022	N/A	KM005761	KM005805	KM005955	KM005889	N/A
P443[1, 2]	*P. pacifica*	Canada, British Columbia; Vitikainen 13080; H	N/A	KM005762	KM005822	KM005938	KM005877	N/A
P1245[1]	*P. phyllidiosa*	USA, North Carolina; Hollinger 1097; UBC	N/A	KM005763	KM005793	KM005944	KM005883	N/A
P1617[1]	*P. phyllidiosa*	USA, Tennessee; Buck 56402; NY-01195226	N/A	KM005764	KM005804	KM005954	KM005888	N/A
P385[1, 2]	*P. polydactylon*	Norway; Magain s.n.; LG	N/A	KM005765	KM005820	KM005994	KM005876	N/A
P570[1]	*P. praetextata*	Norway; Magain s.n.; LG	N/A	KM005766	KM005829	KM005940	KM005879	N/A
P1862[1]	*P. praetextata*	Reunion Island; Kalb (with Kalb) s.n.; DUKE	N/A	—	KM005813	KM005961	KM005896	N/A
P1854[1]	*P. pulverulenta*	Ecuador; Kalb (with Kalb) s.n.; DUKE	N/A	—	KM005812	KM005995	KM005895	N/A
P74[1]	*P. retifoveata*	Russia, Sakha Republic; Ahti 61821; H	N/A	KM005767	—	KM005931	KM005870	N/A
P6[1]	*P. rufescens*	Iceland; Miadlikowska et al. s.n.; DUKE	N/A	KM005768	KM005823	—	—	N/A
P107[1, 2]	*P. scabrosa*	Canada, Québec; Miadlikowska et al. s.n.; DUKE	N/A	KM005769	KM005791	KM005933	KM005872	N/A
P48[1]	*P. ulcerata*	Costa Rica; Miadlikowska et al. s.n.; DUKE	N/A	KM005770	—	KM005927	KM005865	N/A
P51[1]	*P. ulcerata*	Costa Rica; Miadlikowska et al. s.n.; DUKE	N/A	KM005771	—	KM005928	KM005866	N/A
P1905[1, 2]	*P. venosa*	Norway; Magain s.n.; LG	N/A	KM005772	—	KM005962	KM005897	N/A
P69[1, 2]	*P. venosa*	USA, New Mexico; Hollinger 2475; DUKE	N/A	KM005773	—	KM005930	KM005868	N/A
N/A[1]	*Solorina crocea*	Russia, Karelia; Ahti (with Fadayeva) s.n.; DUKE	N/A	123979314	—	123979355	—	N/A

Notes: *16S newly obtained sequences included in the ML3 analysis: P1429 – KM005733, P1430 – KM005734, P1492 – KM005735; the remaining 16S sequences were downloaded from GenBank (see Fig. 3); [1]ML1 analysis (48 mycobiont OTUs, 4-locus data set; Fig. 1); [2]ML2 analysis (52 mycobiont OTUs, 4-locus data set; Fig. 2); [3]ML4 analysis (275 cyanobacteria OTUs, *rbcLX* data set; Fig. 4, Appendix S1).