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ASSESSING REPRODUCTIVE ISOLATION IN HIGHLY DIVERSE COMMUNITIES OF THE LICHEN-FORMING FUNGAL GENUS *PELTIGERA*

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The lichen-forming fungal genus *Peltigera* includes a number of species that are extremely widespread, both geographically and ecologically. However, morphological variability has lead to doubts about the distinctness of some species, and it has been suggested that hybridization is common in nature. We examined species boundaries by looking for evidence of hybridization and gene flow among seven described species collected at five sites in British Columbia, Canada. We found no evidence of gene flow or hybridization between described species, with fixed differences between species for two or more of the three loci examined. Reproductive isolation did not reflect a solely clonal mode of reproduction as there was evidence of ongoing gene flow within species. In addition, we found five undescribed species that were reproductively isolated, although there was evidence of ongoing or historical gene flow between two of the new species. These results indicate 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.

KEY WORDS: **β-tubulin, cyanolichens, gene flow, ITS, reproductive isolation, *RPB*1.**

Lichens are among nature's most elegantly coevolved symbiotic associations involving filamentous fungi and microalgae or cyanobacteria organized into complex tissues that bear little resemblance to the growth patterns of the individual partners (Hawksworth 1988). One-fifth of all described fungi are obligate lichen-formers (Kirk et al. 2001), reflecting the evolutionary success of this nutritional strategy. Lichenization has also enabled fungi to become the ecologically dominant ground cover in many terrestrial ecosystems, particularly in polar and alpine regions (Kappen 1973; Longton 1997; Cornelissen et al. 2001).

Long-range dispersal is thought to be challenging for lichen fungi because spore-mediated (sexual) reproduction requires de novo acquisition of a compatible photosynthetic partner upon germination. This requirement can be circumvented by vegetative dispersal involving specialized propagules that package fungal tissue with the cells of the photosynthetic partner (e.g., soredia, isidia), or by unspecialized thallus fragmentation. However, these types of diaspores tend to be large and have much shorter dispersal kernels than sexual spores (Walser et al. 2005). Nevertheless, many lichen species are extremely broadly distributed, often across all major landmasses on the earth. In addition to broad geographic distributions, several groups of lichens are characterized by the formation of dense mats comprising large numbers of congeneric species. The processes underlying the generation and maintenance of this diversity in the face of homogenizing gene flow and competitive exclusion by closely related species are unknown, and it has been suggested that the species richness of lichens has been overestimated (Hawksworth 2001).

One group of lichen forming fungi that exemplifies these features is the genus *Peltigera*. These terricolous (ground inhabiting)

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and muscicolous (moss inhabiting) foliose (leaf-like thalli) macrolichens form two kinds of symbiotic associations, depending on the species: bimembered symbioses involving a cyanobacterium (*Nostoc*) and a fungus; and trimembered symbioses involving a green alga (*Coccomyxa*) as the main photosynthetic partner distributed throughout the thallus, and *Nostoc* present in external structures on the upper or lower surface of the thallus. The genus *Peltigera* includes 66 accepted species (Martínez et al. 2003), most of which are distributed across multiple continents. However, many of the species descriptions that were originally developed for European specimens are difficult to apply to material from other continents, leading to taxonomic confusion and uncertainty as to the true geographic range of many species (e. g.; Holtan-Hartwig 1993; Goward et al. 1995; Miadlikowska et al. 2003). Particularly problematic are specimens that exhibit diagnostic characters from two or more different species, which has lead to the suggestion that many *Peltigera* specimens represent either genetic hybrid individuals or mechanical hybrids resulting from the fusion of independent thalli of different species (Goffinet and Hastings 1995).

For *Peltigera* and many other taxonomically problematic groups of lichen fungi, species delimitation is increasingly being based on the phylogenetic relationships inferred from DNA sequence data (Grube and Kroken 2000). Two general approaches have been taken to incorporate phylogenetic data into species recognition. The first, known as the phylogenetic species concept (PSC), requires that members of a population form a monophyletic group in gene trees and share diagnosable morphological characters to qualify for recognition as a species (McKitrick and Zink 1988). This approach has been applied to define new species in a variety of fungal lineages, including the genus *Peltigera* (Goffinet and Miadlikowska 1999; Goward and Goffinet 2000; Goffinet et al. 2003). In the case of the *Peltigera canina* complex (*Peltigera* section *Peltigera*), species recognition has also been based on a "molecular synapomorphy": a hypervariable region in the nuclear ribosomal internal transcribed spacer (ITS) 1 region that varies in length from eight to 126 base pairs across the section (Miadlikowska et al. 2003). This repeat-rich region is highly similar within monophyletic groups corresponding to recognized species, but the repeat motifs and their organization differ dramatically among species, making it unalignable across the group. By incorporating the phylogenetic signal contained within this region and other unalignable portions of the ITS region and nuclear ribosomal large subunit (LSU), Miadlikowska et al. (2003) resolved four putative species as monophyletic: *P.* "*fuscopraetextata,*" *P.* "*neocanina,*" *P.* "*neorufescens,*" and *P.* "*pallidorufescens*."

The second approach that has been used to infer species boundaries from molecular phylogenetic data, the genealogical species concept (GSC), is based on comparisons of gene trees from multiple unlinked loci. The presence of the same groups in different gene trees suggests that reproductive barriers exist between members of different groups (Avise and Ball 1990; Dykhuizen and Green 1991). This concept and its associated recognition criteria have been used extensively to diagnose cryptic speciation in fungi (Geiser et al. 1998; Taylor et al. 2000; Dettman et al. 2003; Pringle et al. 2005), but has been applied in only a few cases for lichen-forming fungi (Kroken and Taylor 2001a). Although the GSC is successful in sexually reproducing groups, it cannot be applied to asexual fungi because clonal propagation will result in genealogies that are concordant throughout the evolutionary history of lineages. Thus, strict application of the GSC would result in every genet being diagnosed as a unique species (Coyne and Orr 2004).

For these reasons, it is useful to base species diagnosis on comparisons of the amount of genetic exchange occurring among individuals within species to the amount of exchange between putative species. Unfortunately, few studies have examined population structures of lichen-forming fungi. Microsatellite-based studies have found evidence for extensive clonal reproduction in the endangered Florida endemic *Cladonia perforata* (Yahr 2004) whereas the circumboreal species *Lobaria pulmonaria* was found to be primarily outcrossing (Walser et al. 2004). However, both species exhibited mixed mating systems combining both outcrossing and clonal propagation. In the genus *Letharia*, genealogical incongruence among loci has been taken as evidence of outcrossing in apotheciate (sexual) species and for sorediate species that rarely produce apothecia (Kroken and Taylor 2001b).

In this study, a multilocus approach to delimit species of *Peltigera* in biotic sympatry was implemented. Sequence data from the ITS, β-tubulin, and RNA polymerase II largest subunit (*RPB*1) were analyzed in a phylogenetic framework to detect monophyletic groups consistent with the PSC and GSC. The data were also analyzed in a population genetic framework to measure outcrossing and polymorphism within and between putative phylogenetic species. The use of multigene data and the combination of analytical methods allows high-resolution detection of recent speciation events.

Materials and Methods

TAXON SAMPLING

Sampling was conducted at five sites in British Columbia, Canada: Spahats Creek (51°44′N, 120°01′′W), Battle Creek (51°53′N, 120°01′W), Ghost Lake (52°92′N, 120°88′W), Cameron Ridge (52°79′N, 120°01′W), and Barkersville (53°05′N, 120°51′W). The Battle Creek site is located approximately 20 km north of Spahats Creek. Ghost Lake and Cameron Ridge are approximately 100 km north of Battle Creek, and Barkersville is approximately

50 km west of Ghost Lake. At each of these sites, mats of bryophytes covered large areas of the ground with a diverse assemblage of *Peltigera* species growing throughout the mats. Circular sample plots (diameter = 1 m) were established in areas that had the highest abundance of *Peltigera* thalli to maximize the probability that lichen thalli were located close enough to each other to exchange gametes. At two of the sites, plots were arranged in 3 × 3 grids with 5 m spacing between plots. Three such grids were established at Spahats Creek and one was established at Battle Creek. At the other three sites, *Peltigera* thalli were patchily distributed, so plots were established to maximize *Peltigera* density within a 100 m^2 area (eight plots at Ghost Lake, four at Barkersville, one at Cameron Ridge). Lobes were taken from all *Peltigera* thalli in each plot and air dried before processing for molecular work.

DATA COLLECTION

DNA was extracted from lichen specimens using the PUREGENE Ultrapure DNA extraction kit (Gentra Systems, Minneapolis, MN). The ITS, *RPB*1, and β-tubulin genes were amplified via polymerase chain reaction (PCR) using the following primers: ITS1 F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) for ITS, AF (Stiller and Hall 1997) and CR (Matheny et al. 2002) for *RPB*1, T1 (O'Donnell and Cigelnik 1997) and bt2b (Glass and Donaldson 1995) for β-tubulin. PCR reactions (35 μL) consisted of: 35 μg BSA, 1 U *Taq* DNA polymerase (ABgene), dNTPs (0.2 mM each), primers (0.5 μM each), and PCR buffer. For ITS reactions, 1.5 μM $MgCl_2$ were used whereas 2.5 μM $MgCl_2$ were used for β-tubulin and *RPB*1 reactions. The following thermal cycler profile was employed for all PCR reactions: an initial denaturation of 95°C for 5 min, followed by 35 cycles of 95°C for 45 sec, 52°C for 90 sec, and 72°C for 90 sec, with a final extension of 72°C for 10 min. PCR products were purified using Qiaquick PCR purification columns (Qiagen, Valencia, CA) and sequenced using Big Dye chemistry with an ABI 3730 automated sequencer (PE Applied Biosystems, Foster City, CA).

In some cases, the β-tubulin primers produced heterogeneous PCR products due to preferential amplification of endolichenic fungi, which are asymptomatic and commonly present within lichen thalli (Hofstetter et al. 2006; Arnold et al. 2009). Individual fragments were isolated by cloning using the Topo-TA 5-min PCR cloning kit (Invitrogen, Carlsbad, CA) and *Peltigera* sequences were identified by homology to sequences from homogeneous PCR products and published *Peltigera* sequences (Buschbom and Mueller 2004). These sequences were used to design a *Peltigera*-specific primer (TTCTCCCCACGCGTCTAC) that consistently amplified homogeneous PCR products. We are confident that this primer targets orthologous *Peltigera* genes because it yields sequences that are phylogenetically congruent with the other loci examined here and to published *Peltigera* phylogenies (Miadlikowska and Lutzoni 2000; Miadlikowska et al. 2003).

Sequences were assembled using Sequencher 4.2 (Gene Codes, Ann Arbor, MI) and manually aligned using MacClade 4.0 (Maddison and Maddison 2000). Protein-coding genes were aligned with reference to the amino acid translations. Intron positions in β-tubulin were identified by examining the orthologous *ben*A sequence of *Aspergillus nidulans* (GenBank Accession no. M17519). Species identification of specimens was confirmed by comparing ITS sequences to the GenBank database using BLAST (Altschul et al. 1997) and the ITS sequences were aligned to the corresponding GenBank sequences. Separate ITS alignments were made for *Peltigera* section *Peltidea (P. aphthosa, P. britannica, P. chionophila,* and *P. malacea*) and *Peltigera* section *Chloropeltigera* (*P. leucophlebia*), as well as for two of the subclades of *Peltigera* section *Peltigera* identified by Miadlikowska et al. (2003): group C (*P. canina*, *P. evansiana, P. praetextata*, and the putative new species *P.* "*fuscopraetextata*" and *P.* "*pallidorufescens*") and group D (*P. cinnamomea* and *P.* "*neocanina*"). For *RPB*1 and β-tubulin, sequences were aligned across the genus and *Nephroma* sequences from specimens collected in the sample plots were included as outgroups (*N. arcticum*, *N. bellum,* and *N. parile* for *RPB*1 and *N. bellum* for β-tubulin). Regions judged to be ambiguously aligned (40 and 107 ITS characters for section *Peltigera*, groups C and D respectively, 24 *RPB*1 characters and 134 β-tubulin characters) were excluded from phylogenetic analyses. When outgroups were excluded, all *RPB*1 characters and all but seven β-tubulin characters could be included.

PHYLOGENETIC ANALYSES

Phylogenetic analyses were conducted on a single representative of each unique sequence type using unweighted parsimony as implemented in PAUP* 4.0b10 (Swofford 2003) with gaps coded as a fifth character state. Branch support was assessed using parsimony bootstrapping with 100 replicates and values ≥70% were considered significant (Hillis and Bull 1993). All searches were conducted with 10 random addition replicates and all equally parsimonious trees were saved. For *RPB*1 and β-tubulin, analyses were conducted on the complete datasets including outgroups to determine the rooting of the ingroup. The outgroups were then removed and the alignment was refined, allowing additional characters from the introns to be included in the analysis.

For ITS sequences from species in *Peltigera* section *Peltigera*, most of the intraspecific variation was contained within a hypervariable region in the ITS1 (ITS1-HR) that could not be unambiguously aligned among species (see Miadlikowska et al. 2003). To capture the phylogenetic signal contained within this region without violating assumptions about positional homology, this entire region was treated as a single sequence feature in phylogenetic analyses by recoding it as a series of discrete characters.

This was done in two ways. First, the entire region was treated as a single character (or two characters in the case of clade D where the ITS1-HR was divided by a 33 bp region that could be aligned across the group). Step matrices were then applied to the characters based on pairwise sequence differences as calculated using the program INAASE (Lutzoni et al. 2000). Second, the region was recoded using 23 characters to describe the nucleotide composition of the region, including sequence length, the frequency of each nucleotide and each dinucleotide pair, the proportion of each nucleotide in mononucleotide repeats, and the average spacing between identical nucleotides. This analysis was conducted using the program ARC as previously described (Miadlikowska et al. 2003). To account for dependence among these ARC characters, they were down-weighted following Reeb et al. (2004): 1.0 for sequence length, 0.25 for nucleotide frequency, 0.1 for dinucleotide frequency, 0.5 for mononucleotide repeat frequency and average spacing between identical nucleotides. Both INAASE and ARC can be obtained from http://www.lutzonilab.net/downloads.

NUCLEOTIDE POLYMORPHISM ANALYSES AND GENE-FLOW ESTIMATION

For each species, including putative ITS phylogenetic species (see results), basic nucleotide polymorphism statistics, including number of polymorphic sites and estimates of 4 $N\mu$ based on the number of segregating sites (θ; Watterson 1975) and average pairwise distances (π; Nei 1987) were calculated using SITES (Hey and Wakeley 1997). SITES was also used to detect recombination events between segregating sites within loci using the four-gamete test (Hudson and Kaplan 1985). Intergenic recombination was detected using the four-gamete test, treating each haplotype as a single discrete character using the program Multilocus 1.2 (Agapow and Burt 2001). Linkage disequilibrium was detected by calculating the index of association (Brown et al. 1980; Maynard Smith et al. 1993), using a modified standardization of variances (r_D), which is not sensitive to differences in sample size (Agapow and Burt 2001). Because specimens collected from the same 1-m sample plot were frequently identical at all three loci and were likely to be separate fragments of the same individual, r_D calculations were repeated with multiple representatives of the same species from the same plot randomly excluded. Significance of deviations from panmixia was assessed by shuffling combinations of alleles 10,000 times.

Genetic differentiation between putative species was assessed by counting the numbers of shared polymorphisms and fixed differences and calculating F_{ST} values using SITES (Hey and Wakeley 1997). Calculations were done for comparisons of sister species and for all pairwise combinations of species in which phylogenetic relationships were not well resolved. The combined data from each species pair were also used to calculate r_D, using a single specimen per species per plot as above.

Results

ITS PHYLOGENIES

A total of 221 specimens were collected. Based on morphology and ITS BLAST results, the most common *Peltigera* sections encountered were *Chloropeltigera* (76 sequences), *Peltidea* (53 sequences), *Peltigera* clades C (38 sequences), and D (12 sequences) sensu Miadlikowska et al. (2003). The remaining 42 ITS sequences were not analyzed further because sample sizes per species were too low to assess gene flow. Sequences have been deposited in GenBank (FJ708820–FJ709040).

For section *Peltidea*, four main clades were resolved, each containing GenBank reference sequences from a different species: *P. aphthosa*, *P. britannica*, *P. malacea*, and *P. chionophila* (Fig. 1A). Bootstrap support was $\geq$ 89% for the latter three clades, but was only 68% for *P. aphthosa*. There was no support for relationships among clades. Within *P. aphthosa*, six different haplotypes were recovered from our sampling, with the most common haplotype (20 out of 26 sequences) being identical to the *P. aphthosa* sequence from GenBank. Four of the other haplotypes differed from this sequence by one substitution each, whereas the fifth differed by a two bp indel. There was more genetic variation within *P. britannica*, with 13 sequences distributed fairly evenly among seven haplotypes (1–3 sequences each). One *P. britannica* sequence from GenBank grouped within this clade but it was distinct from these sequences. Haplotypes differed by one to six substitutions and/or indels. *Peltigera malacea* was also dominated by a single haplotype (12 out of 14 sequences), with two other singletons that differed from the common haplotype by one or two substitutions. Two additional *P. malacea* sequences from GenBank were both unique as well. A fourth species in the section, *P. chionophila*, was only represented by three GenBank sequences.

Sequences from section *Chloropeltigera* comprised 15 different haplotypes that formed three well-supported monophyletic groups in phylogenetic analyses (Fig. 1B), suggesting the presence of cryptic species within this group. The largest and most diverse clade (clade I) contained 44 sequences and nine haplotypes, whereas the other two clades each included three haplotypes (clade II with six sequences and clade III with 26). Clade I included haplotypes that differed by one to six substitutions and/or indels, whereas the other clades each comprised a single numerically dominant haplotype with two additional haplotypes that differed by one or two substitutions. This section is represented by ITS sequences for a single described species in GenBank: *P. leucophlebia*. Both GenBank sequences fell within clade I.

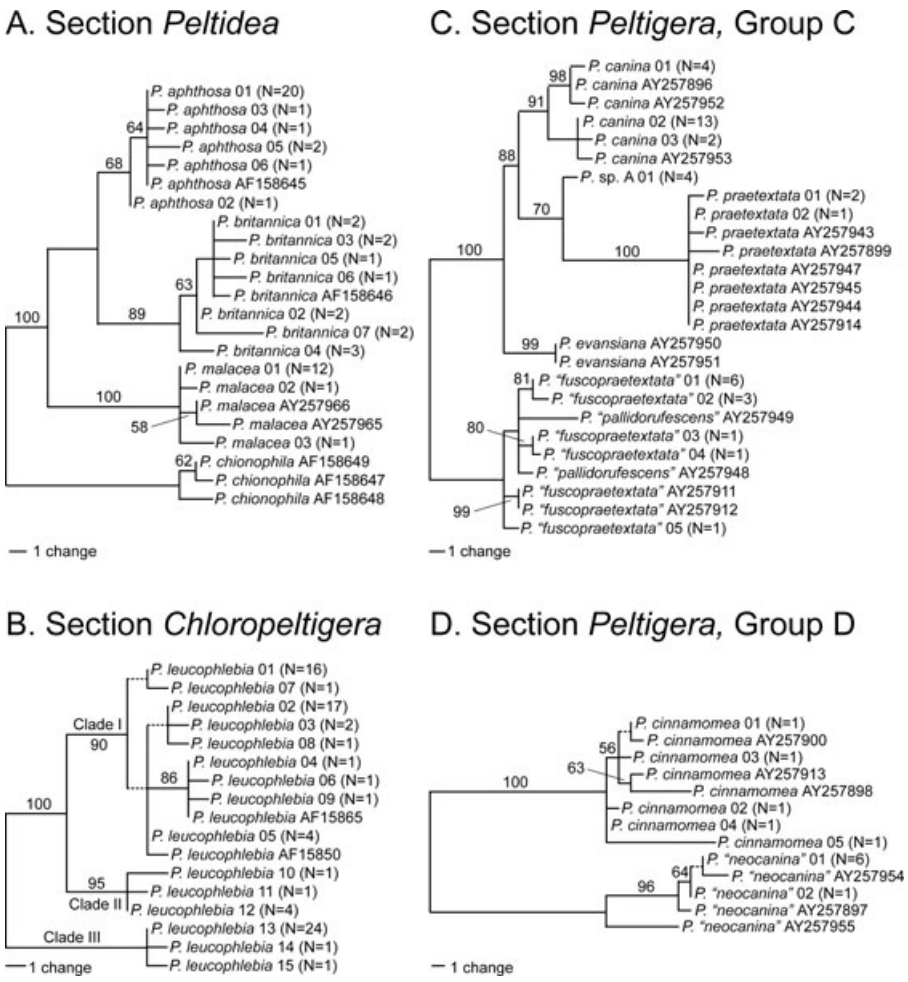

Figure 1. ITS phylogenies of *Peltigera* sections. Numbers above branches represent parsimony bootstrap values ≥ 50%. *N* indicates the number of individuals for each haplotype. The same tree(s) were obtained on each of 10 random-addition replicates. Rooting is arbitrary. Dashed lines indicate nodes that were not present in the strict consensus. (A) Section *Peltidea.* Single most parsimonious trees (tree length = 63 steps, CI = 0.905). Dataset included 44 variable characters, 35 of which were parsimony informative (570 characters total). (B) Section *Chloropeltigera*. One of seven most parsimonious trees (tree length = 32 steps, CI = 0.938). Dataset included 30 variable characters, 19 of which were parsimony informative (557 characters total). Distinct clades are named for comparison to other gene trees. (C) Section *Peltigera* clade C sensu Miadlikowska et al. (2003). Single most parsimonious tree derived from analysis of 510 unambiguously aligned nucleotide positions, including 28 variable characters, 21 of which were parsimony informative (tree length = 32 steps, CI = 0.906), and 23 ARC characters from the hypervariable region ITS1-HR (all parsimony informative, length = 64 steps, CI = 0.906). One haplotype that did not group with sequences from any named species is provisionally named *P.* sp A (see text). (D) Section *Peltigera* clade D sensu Miadlikowska et al. (2003). One of 27 most parsimonious trees derived from analysis of 472 unambiguously aligned nucleotide positions (26 variable characters, 21 parsimony informative, tree length = 27, CI = 1.00), and two INAASE characters from the hypervariable region (tree length = 41, CI = 1.00).

In the first group from section *Peltigera*, corresponding to group C in Miadlikowska et al. (2003), the ITS1-HR contained most of the intraspecific variation but because the length varied between 1 and 40 bp across species, it could not be unambiguously aligned and INAASE failed to capture the phylogenetic signal in the region. However, analysis with the characters from ARC produced a tree with a similar topology to the trees obtained when the region was excluded from the analysis, but with all unique haplotypes resolved. The phylogeny comprised four strongly supported (bootstrap >90%) clades and one additional haplotype that was distinct from all others (*P.* sp. A; Fig. 1C). *Peltigera canina* included three GenBank sequences and 19 sequences distributed among three haplotypes, none of which matched the GenBank sequences. Haplotypes differed by one to six substitutions and/or indels. Four *P. praetextata* sequences from GenBank were identical to one sequence from this dataset, whereas one other haplotype (comprising two sequences) and two other unique GenBank sequences differed from this haplotype by one or two substitutions

each. The third clade consisted of two *P. evansiana* sequences from GenBank. GenBank sequences corresponding to the provisional species *P.* "*fuscopraetextata*" (two identical sequences) and *P.* "*pallidorufescens*" (two unique sequences) formed a clade with 12 sequences distributed across five haplotypes, none of them identical to any of the GenBank sequences. These sequences included four substitutions and one indel in the sequence flanking the ITS1-HR, but most of the variation was contained within this region, which consisted of 3–6 "A"s followed by 10–17 "T"s (see Miadlikowska et al. 2003).

In the case of group D from Miadlikowska et al. (2003), there was an additional variable region separated from the ITS1-HR by a conserved block of 20 bp. The variation in sequence length of the ambiguously aligned regions flanking this conserved block (15–54 bp and 24–49 bp) was small enough that each could be recoded as a single character and step matrices could be applied to the characters based on pairwise sequence differences using INAASE. The resulting phylogeny (Fig. 1D) resolved two distinct clades. One included three GenBank sequences from *P. cinnamomea*, and five additional sequences. All sequences were unique in the variable regions and they also differed by up to seven substitutions/indels elsewhere in the ITS. The other clade included six identical sequences and a seventh that had one "AT" repeat fewer in the microsatellite of the ITS1-HR. Three GenBank sequences assigned to the provisional species *P.* "*neocanina*" differed by up to eight substitutions/indels in the flanking regions and each also had a different number of AT repeats in the ITS1-HR.

PROTEIN-CODING GENE PHYLOGENIES

*RPB*1 was obtained for 163 of the 179 specimens that were analyzed for ITS and β-tubulin was obtained for 164. Because these genes could be aligned unambiguously across the genus, a single analysis was conducted for each. Nine additional *Peltigera* species that were collected in the sample plots were included to confirm the phylogenetic placement of these species within the genus. Sequences have been deposited in GenBank (*RPB*1*:* FJ709041–FJ709249, β-tubulin: FJ709250–FJ709456). Both genes resolved all included sections as delimited by Miadlikowska and Lutzoni (2000) as monophyletic with high ($\geq$ 97%) support in all cases except for section *Horizontales* (64% β-tubulin, 83% *RPB*1; Fig. 2A, B). Within sections, protein-coding genes provided less resolution than ITS. As a result, many species that were well-supported as being monophyletic in the ITS phylogenies were unresolved. In the case of *P. leucophlebia*, all clade III specimens had the same *RPB*1 haplotype and two β-tubulin haplotypes that formed a distinct clade (94% bootstrap support). Specimens of *P. leucophlebia* clades I and II shared derived haplotypes for both protein-coding genes, indicating ongoing or historical gene flow. For all other species, the data are consistent with reproductive isolation because there were no other cases in which haplotypes from different species were more closely related to each other than they were to conspecifics.

POLYMORPHISM STATISTICS AND LINKAGE DISEQUILIBRIUM

For ITS, values of θ varied from 0.0000 (for *Peltigera* sp. A, for which all sequences were identical and for *P.* "*fuscopraetextata*" and *P.* "*neocanina*," where the haplotypes differed only by indels) to 0.0056 for *P. leucophlebia* clade III (Table 1). Values of π were similar except for *P. aphthosa*, *P. leucophlebia* clade I, and *P. malacea*, which were all dominated by a single haplotype, resulting in lower values for π. For the other two loci, haplotype distributions tended to be more balanced, so π was often larger. No evidence was found for intragenic recombination at any of the loci, but intergenic recombination could be inferred for *P. britannica*, *P. canina,* and *P. leucophlebia* clade I by the presence of all four possible combinations of alleles at two loci. Although panmixia could not be rejected for most species, *Peltigera canina*, *P. leucophlebia* clade I, and *P. malacea* had significant linkage disequilibrium (Table 1). In the case of *P. leucophlebia* clade I, this appeared to be due to sampling different fragments from the same individual thallus because r_D was not significant when a single sample was included per plot. The ability of the index of association to reject panmixia appeared to be highly dependent on sample size as two of the species with the highest r_D values (*P.* "*neocanina*" and *P. leucophlebia* clade III) were nonsignificant whereas *P. canina* and *P. leucophlebia* had lower r_D values that were significant.

For each pairwise comparison of related species, except *P. leucophlebia* clades I and II, there were fixed differences between the species in two or three loci (Table 2). There was one shared polymorphism in the ITS between *P. leucophlebia* clades I and III and one shared polymorphism between *P. leucophlebia* clades I and II in both β-tubulin and *RPB*1. There was a high level of genetic differentiation between all pairs of species at all three loci as measured by F_{ST}. When sequences from two species were combined together, linkage disequilibrium values were higher than for either species on its own, except for when *P. leucophlebia* clade I was analyzed with one of the other *P. leucophlebia* clades (0.117 vs. 0.442 for clade II on it own, 0.302 vs. 0.663 for clade III on its own) suggesting reduced intergenic recombination between species.

Discussion

These results provide support for two of the putative species (i.e., *P.* "*fuscopraetextata*" [including *P.* "*pallidorufescens*"] and *P.* "*neocanina*") proposed by Miadlikowska et al. (2003) and a third new species (*P.* sp. A) in *Peltigera* section *Peltigera*. There

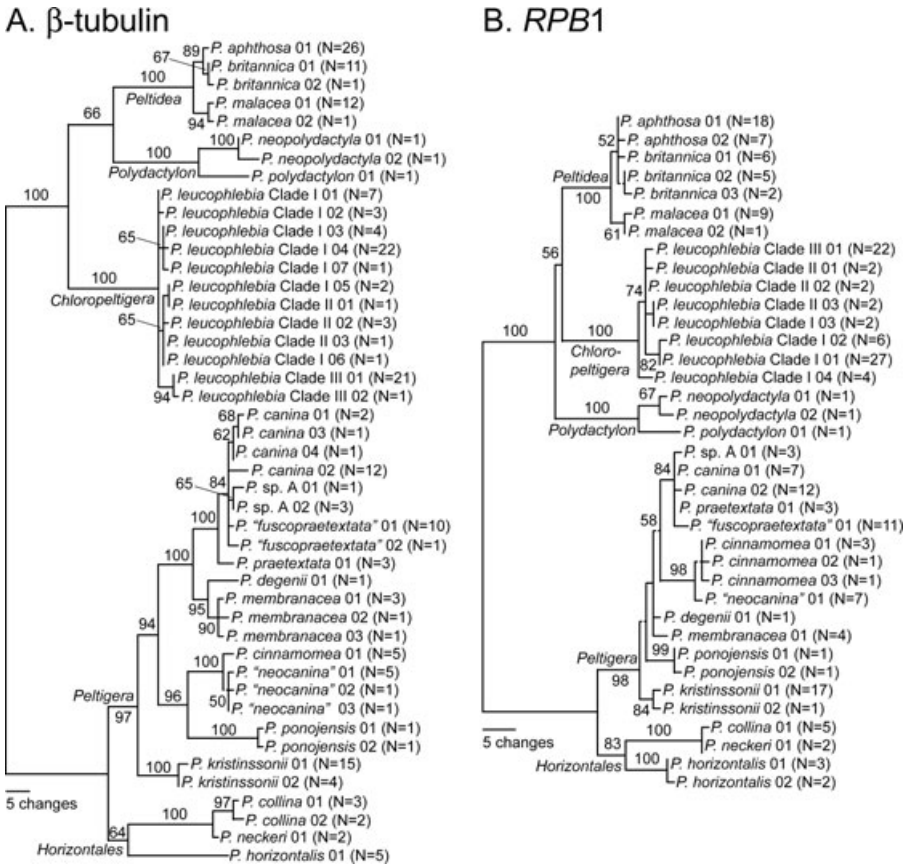

Figure 2. Protein-coding gene phylogenies of *Peltigera*. Numbers above branches represent parsimony bootstrap values ≥50%. *N* indicates the number of individuals for each haplotype. The same trees were obtained on each of 10 random-addition replicates. Dashed lines indicate nodes that were not present in the strict consensus. Names on branches correspond to the sectional designations of Miadlikowska and Lutzoni (2000). Rooting is based on a separate analysis that included *Nephroma* spp. as an outgroup (results not shown). *Peltigera leucophlebia* sequence names correspond to the clades in the ITS phylogeny (Fig. 1B). (A) β-tubulin phylogeny. One of four most parsimonious trees (tree length = 284 steps, CI = 0.803). Dataset included 183 variable characters, 153 of which were parsimony informative (533 characters total). (B) *RPB*1 phylogeny. One of 96 most parsimonious trees (tree length = 146 steps, CI = 0.856). Dataset included 109 variable characters, 89 of which were parsimony informative (667 characters total).

is also evidence for one, and possibly two, new species in section *Chloropeltigera.* They also confirm the status of controversial species, such as *P. britannica* and *P. praetextata*.

Peltigera "*fuscopraetextata*" (including *P.* "*pallidorufescens*") and *P.* "*neocanina*" are genetically distinct from their described relatives, and there is no evidence of genetic exchange between them when they occur in sympatry. These species were proposed based on morphological criteria (T. Goward, pers. comm.), and they were found to form monophyletic groups in ITS and LSU phylogenies (Miadlikowska et al. 2003), although identification of specimens remains a challenge. *Peltigera* "*fuscopraetextata*" is distinguished from other members of section *Peltigera* by its narrow, distinctly elevated and densely arranged veins with deep and elongated interstices. Rhizines are sometimes similar to *P. canina* but often less divided and more isolated (not fused). *Peltigera neocanina* is morphologically less distinct, having characters that are intermediate between *P. canina* and *P. cinnamomea*.

In the phylogeny of Miadlikowska et al. (2003), *P.* "*fuscopraetextata*" was found to form a distinct sister group to another proposed species, *P.* "*pallidorufescens*," although only two specimens of each were included. The branches separating the groups were extremely short and one of them did not receive significant support. One of the new sequences collected here comes out as sister to a clade containing both *P.* "*fuscopraetextata*" and *P.* "*pallidorufescens,*" although with weak support. This sequence either represents a third undescribed species in this group, or else these two names are synonymous. Synonymy is supported by the fact that relationships within this group are not well resolved, that almost all of the variation within this group was in the ITS1-HR, and that their ITS-HR shared the same pattern of nucleotide composition (see Miadlikowska et al. 2003). Furthermore, the specimen

Table 1. Polymorphism statistics for *Peltigera* species examined. *N*, number of individuals sampled; N_{poly}, number of polymorphic sites; *H*, number of unique haplotypes; θ, estimate of 4 $N\mu$ per base pair using the number of polymorphic sites; π, estimate of 4 $N\mu$ per base pair using the average pairwise differences; r_D, multilocus linkage disequilibrium (index of association); *rD* (≥ 5 m), index of association calculated with no more than one individual from each plot.

Species	*N*	ITS				β-tubulin				*RPB*1				r_D[2]	r_D (≥ 5 m)[2]
		N_{poly}	*H*	θ	π	N_{poly}	*H*	θ	π	N_{poly}	*H*	θ	π		
P. aphthosa	26	6	6	0.0026	0.0008	0	1	0.0000	0.0000	1	2	0.0004	0.0006	0.180	0.012
P. britannica	13	8	7	0.0042	0.0035	1	2	0.0006	0.0003	3	3	0.0015	0.0020	0.043[1]	0.047
P. canina	19	4	3	0.0021	0.0026	7	4	0.0040	0.0048	1	2	0.0004	0.0007	**0.444**[1]	**0.230**[1]
P. cinnamomea	5	2	4	0.0017	0.0018	0	1	0.0000	0.0000	2	3	0.0014	0.0012	∞	∞
P. "*fuscopraetextata*"	12	0	5[3]	0.0000	0.0000	3	2	0.0019	0.0010	0	1	0.0000	0.0000	0.259	0.333
P. malacea	14	3	3	0.0016	0.0007	2	2	0.0012	0.0006	1	2	0.0005	0.0003	**0.629**	**0.614**
P. "*neocanina*"	7	0	4[3]	0.0000	0.0000	2	3	0.0016	0.0015	0	1	0.0000	0.0000	0.603	undif.[4]
P. praetextata	3	1	2	0.0012	0.0012	0	1	0.0000	0.0000	0	1	0.0000	0.0000	undif.[4]	undif.[4]
P. sp. A	4	0	1	0.0000	0.0000	2	2	0.0021	0.0019	0	1	0.0000	0.0000	undif.[4]	undif.[4]
P. leucophlebia:															
Clade I	44	7	9	0.0027	0.0009	6	7	0.0027	0.0026	7	4	0.0025	0.0022	**0.114**[1]	0.042[1]
Clade II	6	3	3	0.0022	0.0017	2	3	0.0018	0.0019	2	3	0.0013	0.0016	0.232	0.447
Clade III	26	13	3	0.0056	0.0052	1	2	0.0005	0.0002	0	1	0.0000	0.0000	0.624	0.663

[1]Evidence for interlocus recombination by four gamete test.

[2]Values in bold were significantly higher than values from 10,000 permuted datasets ($P \leq 0.05$).

[3]In these species, several haplotypes were distinguished based on indel polymorphisms that were not included in calculations of polymorphism statistics.

[4]Values could not be calculated because all variances in the denominator were zero.

with the most distinct ITS sequence (*P.* "*fuscopraetextata*" 05) was identical to all other *P.* "*fuscopraetextata*" sequences in the *RPB*1 phylogeny and 9 of 10 other sequences in the β-tubulin phylogeny.

The number of species in the *P. leucophlebia* complex has not been fully resolved by this study. There is strong evidence that Clade III is reproductively isolated from the other two and that representatives of this clade are morphologically distinct (see below). The deep divergence in the ITS phylogeny between clades I and II and the high F_{ST} values between them suggest that they also represent distinct species, but the presence of shared derived haplotypes between clades I and II in the *RPB*1 and β-tubulin

Table 2. Numbers of fixed differences, numbers of shared polymorphisms, fixation indices (F_{ST}), and multilocus linkage disequilibrium for comparisons of closely related *Peltigera* species.

Comparison	ITS			β-tubulin			RPB1			r_D[1,2]
	Shared	Fixed	F_{ST}	Shared	Fixed	F_{ST}	Shared	Fixed	F_{ST}	
P. canina–*P.* sp. A	0	1	0.683	0	1	0.649	0	1	0.849	**0.335**
P. *canina*–P. "*fuscopraetextata*"	0	11	0.944	0	0	0.667	0	2	0.907	**0.365**
P. canina–*P. praetextata*	0	12	0.927	0	5	0.851	0	0	0.611	**0.283**
P. sp. A–*P.* "*fuscopraetextata*"	0	9	1.000	0	1	0.702	0	3	1.000	**0.430**
P. sp. A–*P. praetextata*	0	6	0.950	0	6	0.923	0	1	1.000	**0.538**
P. "*fuscopraetextata*"–*P. praetextata*	0	18	0.982	0	5	0.955	0	2	1.000	**0.518**
P. cinnamomea–*P.* "*neocanina*"	0	24	0.980	0	3	0.901	0	2	0.833	**0.510**
P. aphthosa–*P. britannica*	0	4	0.786	0	2	0.960	0	0	0.380	**0.438**
P. leucophlebia:										
Clade I–Clade II	0	4	0.864	1	0	0.612	1	0	0.553	**0.117**
Clade I–Clade III	1	2	0.808	0	3	0.835	0	1	0.771	**0.302**
Clade II–Clade III	0	0	0.791	0	4	0.887	0	1	0.680	**0.679**

[1]All comparisons used datasets with no more than one representative of each species from a single plot, except for *P.* sp. A, *P. praetextata*, *P.* "*neocanina*," and *P. cinnamomea* for which sample sizes were small.

[2]Values in bold were significantly higher than values from 10,000 permuted datasets ($P \leq 0.05$).

phylogenies indicates that they either have not yet reached full reproductive isolation, or that not enough time has passed since genetic exchange ceased for these loci to achieve reciprocal monophyly at either locus. Resolution of this issue will require examination of many more specimens and additional loci.

The discovery that *P. leucophlebia* comprises multiple species in North America sheds some light on disagreements in the literature about the distinctness of *P. aphthosa* and *P. leucophlebia*. Most taxonomists working in Europe have maintained that these two species are distinct based on the discontinuous cortex under the apothecia and the lack of distinct veination on the lower surface of the latter species (e.g.; Vitikainen 1994). However, Thomson (1979), working on North American material, insisted that there was no correlation between vein type and apothecial cortex, and treated the two as varieties of a single species. Examination of the *P. leucophlebia* specimens collected for this study indicates that, although all fertile specimens have a discontinuous cortex under the apothecia, the venation patterns differ. Members of clades I and II have *P. aphthosa*-type venation, whereas members of clade III have venation typical of *P. leucophlebia* in Europe. This suggests that clade III represents the true *P. leucophlebia*, which was originally described from Europe (Vitikainen 1994), whereas clades I and II represent new species unknown in Europe. However, it will be necessary to include specimens from other geographic locations to confirm that clade III is conspecific with *P. leucophlebia*.

Peltigera britannica was elevated to the species rank by Tønsberg and Holtan-Hartwig (1983), and distinguished from *P. aphthosa* by its peltate cephalodia and wrinkled upper surface. These characters are very variable in the material from British Columbia (see also Goward et al. 1995), but our ITS phylogenetic evidence supports the distinction of *P. britannica* from morphologically similar *P. aphthosa* and *P. leucophlebia* (Fig. 1A, B). It appears that the color of the veinless portion of the lower surface (white in *P. britannica*; gray to brown in *P. aphthosa*) may be a good diagnostic character to distinguish these species in the field. *Peltigera praetextata* is distinguished from *P. canina* primarily by the presence of phyllidia on the thallus margins, although the consistency of this character has been debated in the literature (Thomson 1950; Lindahl 1953). *Peltigera praetextata* can also be distinguished based on thallus color (brownish tint), and the shape and color of the veins (smooth and narrow, becoming brown toward thallus center) and rhizines (mostly simple, not confluent; Vitikainen 1994), although these characters are also variable in the material from British Columbia. Despite the morphological overlap between these species, the genetic data unambiguously support their status as distinct species.

Several operational criteria have been proposed in the literature to apply the GSC to sequence data (Dykhuizen and Green 1991; Geiser et al. 1998; Dettman et al. 2003). In fungi, the most common criteria have involved comparisons of single-gene phylogenies from different loci. If the same clades are present in the majority of single-locus genealogies, this is taken as evidence that the clades represent reproductively isolated lineages (Dettman et al. 2003; Pringle et al. 2005). In addition to the somewhat arbitrary "50% plus one" cutoff, this method requires rapidly evolving loci to produce a sufficiently resolved phylogeny to apply it. In this study, sister species rarely formed distinct clades in the protein-coding gene phylogenies, but application of population genetic criteria revealed evidence for reproductive isolation. This method therefore provided greater sensitivity to detect recently isolated lineages.

Previous studies that have used DNA sequence data for species recognition in *Peltigera* have applied a traditional PSC, requiring monophyly in single-locus (ribosomal DNA) phylogenies and diagnosable morphological differences (Goffinet and Miadlikowska 1999; Goward and Goffinet 2000; Goffinet et al. 2003; Miadlikowska et al. 2003). In all cases examined here, deeply divergent lineages in the ITS phylogenies that received high bootstrap support were found to be reproductively isolated. Pairwise ITS sequence similarity values were $> 99\%$ within species in all cases except *P.* "*fuscopraetextata*" ($>98\%$) and *P. cinnamomea* and *P.* "*neocanina*" ($>97\%$), whereas pairwise similarities between members of different species were $< 97\%$ except among the different *P. leucophlebia* clades. These results indicate that clustering ITS sequences with $> 97\%$, as is commonly done in environmental sampling of fungi (e.g.; O'Brien et al. 2005a), provides a reasonable proxy for species identification in this case. However, ITS sequence similarity would have underestimated the species richness of the *P. leucophlebia* lineage (at least two putative species; Fig. 1B), therefore, a multilocus approach is needed for accurate species delimitation in any particular case.

Our results confirm that the genus *Peltigera* forms species-rich assemblages in British Columbia, Canada. In addition to the 12 species examined here, 11 additional co-occurring named species were collected at lower frequencies. On the basis of ITS divergence, there appear to be at least three additional cryptic species in this material (results not shown), indicating that up to 12 congeneric species were collected from each 100 m^2 area, with a total of at least 26 species collected from the five sites. The processes responsible for the maintenance of this diversity are unknown, but competition for symbiotic partners may be a major driver of diversity in mutualisms (Bruns 1995; van der Heijden et al. 1998). There is extensive sharing of genetically identical photosynthetic partners among *Peltigera* species (Rikkinen et al. 2002; O'Brien et al. 2005b), hence, the potential for symbiont competition exists. It has been suggested that lichens that produce specialized asexual codispersal propagules can facilitate the dispersal of sexually reproducing fungal species that must reconstitute the symbiosis after dispersal (Rikkinen 2003). Dependence

of sexually reproducing lichen forming fungal species on photosynthetic partners from asexually reproducing species with limited dispersal ability could result in localized hot spots of diversity (Thompson 1994).

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