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New approach to an old problem: Incorporating signal from gap-rich regions of ITS and rDNA large subunit into phylogenetic analyses to resolve the *Peltigera canina* species complex

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Abstract: The *Peltigera canina* species complex consists of foliose lichenized bitunicate ascohymenial discomycetes forming section *Peltigera* within the genus *Peltigera* (Lecanoromycetes, lichen-forming Ascomycetes). To test the circumscription of highly polymorphic species and to resolve relationships among putative members of the *P. canina* complex, part of the nuclear ribosomal DNA large subunit (LSU rDNA) and the entire internal-transcribed spacer (ITS rDNA) were sequenced for 84 individuals representing 33 putative *Peltigera* taxa. Seventeen of the 25 taxa from the *P. canina* complex are well established and widely accepted. The remaining eight taxa have been proposed recently but are undescribed. A hypervariable region in ITS1 (ITS1-HR, sites 111–237 in our alignment) showed remarkable variation in length, especially in the *P. canina* complex, ranging from 8 to 126 bp, and contained several microsatellites. We describe here an alignment-free method to code such large gap-rich hypervariable regions for phylogenetic analyses. Variation among ITS1-HR sequences greatly contributed to species delimitation and species identification and can be a major asset to future population studies for specific species within section *Peltigera*. Sequences of ITS1-HR alone were sufficient to identify all existing species of *Peltigera* from the *P. canina* species complex and related sections *Retifoveatae* and *Horizontales* included in this study. However, only when INAASE (for short ambiguously aligned regions) and ITS1-HR coded characters were added to the combined analysis of nonambiguous LSU and ITS sites was it possible to reach the level of phylogenetic resolution and support necessary to disentangle the *P. canina* complex. We report here complete concordance between phylogenetically based and morphologically based species delimitation for 15 of the 17 species from the *P. canina* complex (*P. canina*, *P. cinnamomea*, *P. degenii*, *P. evansiana*, *P. frigida*, *P. kristinssonii*, *P. laciniata*, *P. lambinonii*, *P. lepidophora*, *P. membranacea*, *P. monticola*, *P. ponojensis*, *P. praetextata*, *P. rufescens* and *P. ulcerata*). Four of the eight newly proposed but undescribed taxa most likely represent new species (*P.* ''*fuscopraetextata*'', *P.* ''*neocanina*'', *P.* ''*neorufescens*'' and *P.* ''*scotteri*'') within the *P. canina* complex. We found that morphologically and chemically distinct *P. didactyla* s. str. and *P. didactyla* var. *extenuata* form two non-sister monophyletic entities, therefore the latter taxon should be recognized at the species level (*P. extenuata*). The North American and European populations of the morphologically uniform *P. degenii* might represent two sibling species because they were found to be genetically distinct and monophyletic. Two major monophyletic groups within the *P. canina* complex (CICADE = CInnamomea + CAnina + DEgenii group and PORUDI = POnojensis + RUfescens + DIdactyla group) seem to be correlated with different humidity preferences. Although some authors previously have suggested interspecies recombination within the *P. canina* complex, we did not find statistically significant evidence for this phenomenon based on LSU and ITS sequences.

Key words: Bayesian inference, gap-rich alignments, INAASE characters, internal transcribed spacer (ITS1, 5.8S, ITS2), lichenized Ascomycota, maximum likelihood, maximum parsimony, microsatellites, morphospecies, nuclear large-subunit ribosomal DNA (LSU rDNA), *Peltigera canina* species complex, Peltigerales, phylogenetics, section *Peltigera*

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INTRODUCTION

Species complexes are abundant among lichen-forming fungi. The *Peltigera canina* species group comprises many worldwide taxa that often are difficult to identify. These primarily terricolous and muscicolous foliose macrolichens inhabit a wide range of environments, from somewhat xeric, exposed, often unstable localities (e.g., *P. didactyla* var. *didactyla*, *P. ponojensis* and *P. rufescens*) to humid, sheltered, usually wooded sites (e.g., *P. praetextata*, *P. degenii* and *P. cinnamomea*).

From the broadly defined type species for the genus *Peltigera*, *Lichen caninus* L. (Linnaeus 1753, *Peltigera canina*), several taxa have been segregated, most of which were members of the *P. canina* group. According to Gyelnik (1927 and 1933), *P. canina* and allied species are defined by the ''caninaeform'' type of venation, i.e., veins that are relatively thin, elevated, with rather wide interspaces on the lower surface of the thallus and by an arachnoid tomentum (with hyphal tips appressed or entangled) developed on the upper surface of the thallus (Figs. 1–5 in Vitikainen 1994). In 1993, Holtan-Hartwig, based on Norwegian collections, proposed a more precise circumscription of the *P. canina* group. According to this author, members of the *P. canina* group (eight species) are well characterized by: 1) the internal and external structure of veins on the lower side of the thallus ''. . . cylindrical veins with the central parts composed of parallel hyphae in which the walls are conglutinated. The outer parts of the veins are composed of branched hyphae or hyphae branching in right angles from the veins''; 2) the mostly tomentose upper cortex of the thallus ''. . . appressed, thin-walled and branched hairs on the upper side . . . ''; and 3) the lack of secondary metabolites detectable by thin layer chromatography (TLC), except in *P. didactyla*. This morphological and chemical circumscription of the *P. canina* group subsequently has been widely accepted since then (e.g., Goffinet and Hastings 1994, Martínez et al 1997, Martínez Moreno 1999, Vitikainen 1994). However, Vitikainen (1994) suggested that *P. retifoveata* (placed by Holtan-Hartwig [1993] in a group of its own), as well as some other non-European tomentose species with secondary metabolites (e.g., *P. laciniata*) and glabrous secondary metabolite-deficient species (e.g., *P. austroamericana* and *P. subhorizontalis*), also should be included in the *P. canina* group.

Based on a broad phylogenetic study of taxa mainly from North America and Europe, Miadlikowska and Lutzoni (2000) redefined the *P. canina* group using monophyly as the grouping criterion and recognized this group as one of eight sections (*Peltigera* section *Peltigera*) within the genus *Peltigera*. A total of 16 species were included in this section. Eight species (*P. cinnamomea* Goward, *P. continentalis* Vitik., *P. evansiana* Gyeln., *P. frigida* R. Sant., *P. laciniata* (G. Merr. ex Riddle) Gyeln., *P. lambinonii* Goffinet, *P. monticola* Vitik. and *P. ponojensis* Gyeln.) were added to the set of taxa selected by Holtan-Hartwig (1993; *P. canina* [L.] Willd., *P. degenii* Gyeln., *P. didactyla* [With.] J. R. Laundon, *P. kristinssonii* Vitik., *P. lepidophora* [Vain.] Bitter, *P. membranacea* [Ach.] Nyl., *P. praetextata* [Sommerf.] Zopf, and *P. rufescens* [Weiss] Humb.). Most of these species are widely distributed in Europe and North America, with the exception of *P. lambinonii* (Africa and Australia), *P. continentalis* (Asia), *P. frigida* and *P. laciniata* (Central and South America).

Morphological and chemical attributes of the newly circumscribed *P. canina* complex remain essentially unchanged compared to Holtan-Hartwig's delimitation of this species group. The triterpenoid zeorin, present in *P. laciniata*, and the glabrous upper cortex of the thallus and horizontal apothecia of *P. frigida* are new features expanding the morphological and chemical delimitation for this species group *sensu* Holtan-Hartwig. Three nonmolecular synapomorphic character states define this section: 1) the absence of depsides and terpenoids (followed by two reversals in lineages leading to *P. laciniata* and *P. didactyla* var. *extenuata*); 2) the frequent observation of pycnidia on specimens from this section; and 3) the veins composed of hyphae, which are mostly to completely parallel and conglutinated. The characters complete the description of section *Peltigera*: exclusively bimembered, with cyanobacteria (*Nostoc*) as the photobiont; rhizines simple or branched (fibrillose, flocculent or penicillate); apothecia pale brown to dark brown, usually vertical and saddle-shaped; vegetative propagules (isidia, phyllidia, and soredia) frequent; if present, terpenoids do not co-occur with depsides within the same individual. A large number of lichenicolous fungi that are associated with the genus *Peltigera* (peltigericolous fungi) are found predominantly on members of the *P. canina* complex (Miadlikowska 1999).

Comparatively few studies have been directed toward members of the *P. canina* complex; most have examined structure and development of vegetative propagules in selected species (Darbishire 1926; Lindahl 1953, 1960; Strato 1921; Thomson 1948) to elucidate their diagnostic use for species delimitation. However, some of the conclusions from these studies were conflicting, e.g., using phyllidia as a valid character to separate *P. praetextata* from the morphologically similar *P. canina*. A summary of the features of pycnidia and conidia observed in some *Peltigera* species, including three members of section *Peltigera* (*P.*

canina, *P. rufescens* and *P. laciniata*), was presented by Lindahl (1959).

Many of the phenotypic characters traditionally used to resolve taxonomic units within the *P. canina* complex are unreliable, owing to a high degree of morphological variability among the species (Goffinet and Hastings 1994, Goffinet et al 1994, Goward et al 1995, Miadlikowska and Lutzoni 2000, Vitikainen 1994). This has resulted in the description of many species and intraspecific taxa that, upon closer re-examination, proved to be variants of previously described species (Vitikainen 1994). In addition, intermediate pheno- and chemotypes frequently are identified within a population or an individual thallus of species from the *P. canina* complex. Goffinet and Hastings (1995) hypothesized such patterns of variation observed in *P. didactyla* var. *extenuata* and *P. didactyla* s. str. by an introgressive hybridization or thallus fusion. Morphometric work on selected traits (spore size, thallus thickness, and vein width and height) was conducted on *P. canina*, *P. membranacea* and *P. praetextata* populations using statistical methods to better delimitate these species (Martínez and Burgaz 1996).

Despite successful in vitro resynthesis of *Peltigera praetextata* and *P. didactyla* thalli (Stocker-Wörgötter and Türk 1988, 1990, 1991), experimental studies (including mating tests) are not practical or feasible for resolving taxonomic questions within the *P. canina* complex and lichen-forming fungi in general. Therefore, morphological continuity within species and morphological discontinuity among species has been used as the main criteria for delimitating lichen-forming fungal species. Taxonomy at the species level within the *P. canina* complex relies mainly on vegetative features of the thallus, e.g., tomentum type and pattern, vein and rhizine morphology and arrangement and vegetative propagule presence (type and shape)/absence.

For the past 10 years, a phylogenetic species concept based on molecular characters (ITS sequences alone and LSU + ITS combined; except for Kroken and Taylor [2001] who used multiple loci) was employed to circumscribe species within lichen species complexes and to evaluate a posteriori which phenotypic characters are associated with each distinct monophyletic species (*Pseudocyphellaria crocata* complex, Miadlikowska et al 2002; *Peltigera* section *Horizontales*, Goffinet and Miadlikowska 1999; *Lasallia*, Niu and Wei 1993; *Ramalina*, Groner and LaGreca 1997; and *Xanthoria*, Franc and Kärnefelt 1998). In all these cases, monophyletic groups of individuals were supported by distinct morphological traits. Grube and Kroken (2000) reviewed the implication of molecular phylogenetic approaches to resolve species complexes in lichenized fungi.

When a large fraction of the variable sites of an alignment is ambiguous due to length variation among sequences, recovering phylogenetic signal from these regions can greatly contribute toward increasing phylogenetic resolution and confidence. In 2000, Lutzoni et al described a new method (INAASE) to integrate signal from ambiguously aligned regions of DNA sequences into phylogenetic analyses without violating positional homology. Because this method relies in part on pairwise alignments for all pairs of sequences within an ambiguous region and involves step matrices, INAASE can be implemented only on relatively small ambiguous regions (rarely up to 50 sites long). A method is needed that would allow the incorporation of large ambiguous regions into phylogenetic analyses without requiring alignments or imposing that all sites (ambiguously and nonambiguously aligned sites) be optimized directly (e.g., POY, Wheeler 1996) when reconstructing phylogenies.

Our detailed reassessment of morphological, chemical and molecular data for the genus *Peltigera* (Miadlikowska and Lutzoni 2000) was designed mainly to address questions at the intrageneric level. Although the *P. canina* complex was well sampled, our previous study did not focus on this section and included only one molecular dataset—the nuclear LSU rDNA. The aims of this molecular phylogenetic study were: 1) to test the monophyletic recognition of the *Peltigera canina* complex (section *Peltigera*) *sensu* Miadlikowska and Lutzoni (2000) and its sister relationship to section *Retifoveatae*; 2) to evaluate the delimitation of existing morphospecies within the *P. canina* complex; 3) to assess the validity of newly proposed putative morphospecies; 4) to detect recombination events among members of the *P. canina* complex; and 5) to evaluate the potential contribution of INAASE characters (Lutzoni et al 2000), and a new method developed here that generates coded characters from a large hypervariable region of 126 sites long within ITS1 (ITS1-HR), toward resolving this species complex.

To achieve these goals, a 1.4-kb fragment at the 5′ end of the nuclear large-subunit rDNA (LSU rDNA) and the entire internal transcribed spacer (ITS1, 5.8S and ITS2) were sequenced for 25 putative species (17 described, including *P. ulcerata*, and eight undescribed species) selected from the *Peltigera canina* complex and eight additional species from sections *Horizontales*, *Phlebia* and *Retifoveatae*. Each data partition was analyzed separately and together using neighbor joining, maximum likelihood and maximum parsimony. Coded characters derived from am-

biguously aligned parts of the alignments (including a hypervariable region within the ITS1 [ITS1-HR]) were added to unambiguously aligned sites for maximum-parsimony searches. Bootstrap support (BS, Felsenstein 1985) and Bayesian Metropolis coupled Markov chain Monte Carlo tree sampling (BMCMCMC, Huelsenbeck 2000, Larget and Simon 1999) were used to estimate levels of confidence associated with phylogenetic relationships revealed by this study. The contribution of INAASE and recoded ITS1-HR characters was evaluated in light of results from the BS and BMCMCMC analyses without these characters.

MATERIALS AND METHODS

Taxon sampling.—A total of 84 specimens representing 33 putative taxa were sampled. Of these, 25 taxa (71 specimens) belong to the *P. canina* complex and eight (13 specimens) are members of other sections within the genus *Peltigera* (TABLE I). Seventeen of the 25 taxa from the *P. canina* complex are well established and widely accepted. Affiliation of *P. ulcerata* (occurring outside Europe and North America) to section *Peltigera* was anticipated, based solely on morphological features, and therefore was tested in this study. The remaining eight taxa from the *P. canina* complex, represented here by 17 specimens, are potential species currently under study by T. Goward. Taxa selected for this study cover a wide spectrum of morphological and chemical variation within the *P. canina* complex. They represent all known members of the complex in Europe and North America and selected members from other continents. Several *Peltigera* species with tomentose thalli (e.g., *P. dilacerata* [Gyeln.] Gyeln., *P. erioderma* Vain., *P. fibrilloides* [Gyeln.] Vitik., *P. pindarensis* Awashthi & M. Joshi, *P. rufescentiformis* [Gyeln.] Dodge, *P. patagonica* Räsänen., *P. soredians* Vitik., and *P. spuriella* Vain.) or with glabrous but acid-deficient thalli (e.g., *P. austroamericana* Zahlbr. and *P. subhorizontalis* Gyeln.) occurring outside Europe and North America were not included in this study. Based on those morphological traits, these species very likely are to be part of section *Peltigera*, however their putative inclusion within section *Peltigera* remains unconfirmed.

With the exception of *P. continentalis* and the putative species *P.* "*fuscoponojensis*", all *Peltigera* taxa in this study were represented by at least two specimens. Both LSU and ITS were sequenced for single specimens of *P. ulcerata* and *P. lambinonii*. An additional three ITS sequences were obtained for *P. lambinonii* and one extra LSU sequence was obtained for *P. ulcerata* (see TABLE I). Thirteen specimens representing eight species from three out of the seven remaining sections in the genus *Peltigera* also were included (sections *Horizontales*, *Phlebia* and *Retifoveatae*). This selection of taxa and the rooting of the trees using *P. malacea* were based on a comprehensive phylogenetic study of the genus *Peltigera* by Miadlikowska and Lutzoni (2000).

DNA isolation, amplification and sequencing.—Well-preserved lichen thalli lacking any visible symptoms of fungal infection were selected for DNA isolation. All external pieces of plant material attached to the targeted part of the thallus were removed under a dissecting microscope. Small thallus fragments from terminal parts of lobes from freshly collected or herbarium specimens were sampled for DNA isolation. The oldest specimen from which DNA was successfully isolated was collected in 1984. Total DNA was isolated with the Purgene Kit (GENTRA Systems) from fragments containing both cyano- and mycobionts, following the manufacturer's protocol for filamentous fungi. DNA concentration was estimated by spectrophotometry or by visual comparison with a positive control (λ 100 ladder, concentration 10, 20, 40 Ng) on an ethidium-bromide-stained 1.5% TBE agarose gel and accordingly was diluted with distilled water (usually 10 times) before amplification.

Symmetric polymerase chain reactions (PCR) amplified a 1.4-kb fragment at the 5′ end of the nuclear LSU rDNA and the entire ITS region (ITS1, 5.8S and ITS2). The LSU rDNA PCR amplification and both the LSU and the ITS sequencing was done as outlined in Miadlikowska and Lutzoni (2000) and Miadlikowska et al (2002). The amplification reaction for ITS was prepared for a final 50-μL volume containing 32.7 μL of sterile double-distilled water, 5.0 μL of 10× Taq polymerase reaction buffer (Boehringer-Mannheim), 5.0 μL dNTP, 1 μL of 100× Bovine Serum Albumin (BSA; BioLabs), 0.3 μL Taq DNA polymerase (Boehringer-Mannheim), 2.5 μL for each of the 10 μM primers ITS1F or NS24R (5′-TAAAAGTCGTAACAAGGTTT-3′) and ITS4 (Gardes and Bruns 1993, White et al 1990), and 1.0 μL of template genomic DNA. PCR was performed on Peltier Thermal Cyclers PTC-200 (MJ Research) under these conditions: denaturation at 95 C for 1 min; 25 cycles at 95 C for 1 min, annelation at 53 C for 45 s, extension at 72 C for 2 min; 14 cycles at the same temperature conditions as above but with time extension of 5 s per cycle. Samples then were held for an additional 10 min at 72 C to complete primer extensions, after which they were kept at 4 C until electrophoresis on 1.5% TBE agarose gel was performed. Sequence fragments were subjected to BLAST searches to verify their identities.

A total of 82 sequences for the ITS and 79 sequences for the LSU were gathered for this study (TABLE I). Sequences for each molecule were assembled and aligned by using Sequencher 3.0 (Gene Codes). Alignments were optimized by eye and corrected manually when necessary. The secondary structure of the LSU rDNA of *Saccharomyces cerevisiae* (Larsen et al 1993) was used to verify the LSU alignment and to provide an additional criterion to delimit ambiguously aligned regions (Kjer 1995, Lutzoni et al 2000).

Phylogenetic analyses.—All phylogenetic analyses were performed using PAUP* 4.0b 8a (Swofford 2001). Because we were unable to complete LSU and ITS sequences for the exact same set of specimens, these datasets with different numbers of specimens were analyzed: LSU, 79 and 77 specimens; ITS, 82 and 77 specimens; and combined LSU + ITS, 77 specimens.

Datasets were subjected to weighted maximum-parsimony (MP), maximum-likelihood (ML) and neighbor-joining (NJ) searches (TABLE II). The MP and NJ analyses were

TABLE I. Voucher information for 84 *Peltigera* specimens, and GenBank accession numbers for 79 nuclear LSU rDNA and 82 ITS sequences representing the 33 putative taxa included in this study

Taxon[a]	Voucher	GenBank Accession Number[b]	
		LSU	ITS
Peltigera "*boreorufescens*" 1	Canada, *Goward* & *Ceska 82-1084* (UBC)	(AF286820)	AY257896
P. "*boreorufescens*" 2	Canada, *Goward* & *Ceska 82-562* (UBC)	(AF286781)	AY257897
P. canina (L.) Willd. 1	Canada, *Wong* & *Wong 4297* (CANL)	(AF286821)	AY257952
P. canina 2	Poland, *Czyzewska* 009205 (LOD-L)	(AF286822)	AY257953
P. cinnamomea Goward 1	Canada, *Brodo* & *Hamilton 21921* (CANL)	(AF286787)	AY257898
P. cinnamomea 2	Canada, *Goward*, *Miller* & *Nelson 85-305* (UBC)	(AF286784)	AY257899
P. cinnamomea 3	Canada, *Goward 82-1416* (UBC)	(AF286811)	AY257900
P. continentalis Vitik.	Mongolia, *Huneck 88-151* (H)	(AF286777)	AY257890
P. degenii Gyeln. 1	Poland, *Ahti* & *Drozdowicz 45565* (UGDA-L)	(AF286794)	AY257901
P. degenii 2	Canada, *Gowan 2582* (CANL)	(AF286789)	AY257902
P. degenii 3	Finland, *Vitikainen 10836* (H)	(AF286793)	AY257903
P. degenii 4	Canada, *LaFarge-E. 8773* (DUKE)	AY257905	AY257904
P. didactyla var. *didactyla* (With.) J. R. Laundon 1	Poland, *Miadlikowska 5233* (UGDA-L)	(AF286806)	AY257929
P. didactyla var. *didactyla* 2	Poland, *Lesiak* & *Czyzewska 009202* (LOD-L)	(AF286804)	AY257930
P. didactyla var. *didactyla* 3	Brazil, *Marcelli*, *Ahti* & *Yano 28333* (H)	AY257932	AY257931
P. didactyla var. *extenuata* (Nyl. ex Vainio) Goffinet & Hastings 1	Poland, *Faltynowicz* & *Miadlikowska 5235* (UGDA-L)	(AF286809)	AY257937
P. didactyla var. *extenuata* 2	Poland, *Cieslinski 1296* (KTC)	(AF286808)	AY257938
P. didactyla var. *extenuata* 3	Poland, *Butkus 5236* (UGDA-L)	(AF286810)	AY257939
P. didactyla var. *extenuata* 4	Canada, *Goffinet* & *Goward 97-289* (UBC)	AY257941	AY257940
P. didactyla var. *extenuata* 5	Canada, *Pelletier* & *Gauthier 445434* (QFA)	—	AY257942
P. evansiana Gyeln. 1	Canada, *Goward 94-972* (UBC)	(AF286819)	AY257950
P. evansiana 2	Canada, *Goward 89-145* (UBC)	(AF286818)	AY257951
P. frigida R. Sant. 1	Argentina, *Stenroos 2158* (H)	(AF286780)	AY257893
P. frigida 2	Argentina, *Stenroos 2187* (H)	AY257895	AY257894
P. "*fuscoponojensis*"	Canada, *Goward* & *Bringhurst 94-1017* (UBC)	(AF286795)	AY257910
P. "*fuscopraetextata*" 1	Canada, *Goward 93-434* (UBC)	(AF286817)	AY257911
P. "*fuscopraetextata*" 2	USA, *Goward* & *Knight 90-154* (UBC)	(AF286816)	AY257912
P. kristinssonii Vitik. 1	Canada, *Goward 81-1718* (UBC)	(AF286779)	AY257891
P. kristinssonii 2	Canada, *Goward* & *Findlay 83-506* (UBC)	(AF286778)	AY257892
P. laciniata (G. Merr. ex Riddle) Gyeln. 1	Ecuador, *Kalb 511* (H)	(AF286799)	AY257922
P. laciniata 2	Ecuador, *Kalb* & *Kalb 437* Lich. Neotrop. (H)	AY257924	AY257923
P. lambinonii Goffinet 1	Australia, *Tibell 12401* (H)	(AF286803)	AY257933
P. lambinonii 2	Zaire, *Lambinon 72/Z/102* (LG)	—	AY257934
P. lambinonii 3	Rwanda, *Lambinon 74/788* (LG)	—	AY257935
P. lambinonii 4	Zaire, *Lambinon 71/Z/1111* (LG)	—	AY257936
P. "*latopraetextata*" 1	Canada, *Goward* & *Lea 90-876* (UBC)	(AF286785)	AY257913
P. "*latopraetextata*" 2	Canada, *Goward* & *Clark 81-1576* (UBC)	(AF286786)	—
P. "*latopraetextata*" 3	Canada, *Goward 94-691* (UBC)	AY257915	AY257914
P. lepidophora (Vain.) Bitter 1	Canada, *Nuyt 10083-L26* (QFA)	(AF286798)	AY257920
P. lepidophora 2	Canada, *Goward 97-293* (UBC)	(AF286797)	AY257921
P. membranacea (Ach.) Nyl. 1	Canada, *Gowan 2134* (CANL)	(AF286792)	AY257906
P. membranacea 2	Poland, *Olszewski 5238* (UGDA-L)	(AF286791)	AY257907
P. membranacea 3	Russia, *McCune et al. 24874* (OSU)	AY257909	AY257908
P. monticola Vitik. 1	Poland, *Faltynowicz 5239* (UGDA-L)	(AF286768)	AY257872
P. monticola 2	Yugoslavia, *Vitikainen 7196* (H)	(AF286770)	AY257873

TABLE I. Continued

Taxon[a]	Voucher	GenBank Accession Number[b] LSU	ITS
P. monticola 3	Poland, *Toborowicz 13.08.1976* (KTC)	(AF286769)	AY257874
P. monticola 4	Switzerland, *Vust 452186* (G)	AY257876	AY257875
P. monticola 5	Switzerland, *Vust 452188* (G)	AY257878	AY257877
P. monticola 6	Switzerland, *Vust 452189* (G)	AY257880	AY257879
P. monticola 7	Switzerland, *Vust 452187* (G)	AY257882	AY257881
P. "*neocanina*" 1	Canada, *Goward 95-689* (UBC)	(AF286782)	AY257954
P. "*neocanina*" 2	Canada, *Goward* & *Clement 94-233* (UBC)	(AF286783)	AY257955
P. "*neorufescens*" 1	Canada, *Goward 95-688* (UBC)	(AF286796)	AY257916
P. "*neorufescens*" 2	Canada, *Goward* & *Clark 81-1591* (UBC)	(AF286801)	AY257917
P. "*neorufescens*" 3	Canada, *Goward* & *Knight 91-1458* (UBC)	AY257919	AY257918
P. "*pallidorufescens*" 1	Canada, *Goward* & *Clement 94-232* (UBC)	(AF286815)	AY257948
P. "*pallidorufescens*" 2	Canada, *Goward* & *Lea 92-214* (UBC)	(AF286812)	AY257949
P. ponojensis Gyeln. 1	Canada, *Goward 82-1233* (CANL)	(AF286773)	AY257883
P. ponojensis 2	Poland, *Bielczyk 42116* (KRAM-L)	(AF286771)	AY257884
P. ponojensis 3	Poland, *Kiszka 2.09.1988* (KRAP-L)	(AF286772)	AY257885
P. praetextata (Sommerf.) Zopf 1	Poland, *Cieslinski 1208* (KTC)	(AF286813)	AY257943
P. praetextata 2	Poland, *Gos 5242* (UGDA-L)	(AF286814)	AY257944
P. praetextata 3	France, *Reeb VR 97* (F)	AY257946	AY257945
P. praetextata 4	Canada, *Reeb VR 9-VIII-97* (F)	—	AY257947
P. rufescens (Weiss) Humb. 1	Canada, *Wong 4067* (CANL)	(AF286802)	AY257925
P. rufescens 2	Poland, *Faltynowicz 5243* (UGDA-L)	(AF286800)	AY257926
P. rufescens 3	Canada, *Lutzoni 99.07.18-24* (F)	AY257928	AY257927
P. "*scotteri*" 1	Canada, *Goward 81-1289a* (UBC)	(AF286774)	AY257886
P. "*scotteri*" 2	Canada, *Goward* & *Miege* 95-1153 (UBC)	(AF286788)	AY257887
P. ulcerata Müll. Arg. 1	Brazil, *Marcelli, Ahti* & *Yano 28234* (H)	AY257956	—
P. ulcerata 2	Brazil, *Marcelli, Ahti* & *Yano* 28385 (H)	AY257958	AY257957
P. collina (Ach.) Schrad.*	Norway, *Vitikainen 11485* (H)	(AF286765)	AY257969
P. elisabethae Gyeln. 1*	Poland, *Bielczyk 42135* (KRAM-L)	(AF286762)	AY257961
P. elisabethae 2*	Italy, *Vitikainen 10292* (H)	(AF286763)	AY257962
P. horizontalis (Huds.) Baumg. 1*	Canada, *Goward 81-1663* (CANL)	(AF286760)	AY257959
P. horizontalis 2*	Canada, *Zoladecki* & *Lutzoni 11336-L7* (QFA)	(AF286761)	AY257960
P. malacea (Ach.) Funck 1*	Poland, *Faltynowicz 5237* (UGDA-L)	(AF286756)	AY257965
P. malacea 2*	Poland, *Cieslinski* (KTC)	(AF286757)	AY257966
P. neckeri Hepp. ex Müll. Arg. 1*	Poland, *Miadlikowska 5240* (UGDA-L)	(AF286766)	(AF075725)
P. neckeri 2*	Canada, *Goward 97-291* (UBC)	AY257964	AY257963
P. phyllidiosa Goffinet & Miadlikowska*	USA, *Reeb VR 10-X-97/6* (F)	(AF286764)	AY257968
P. polydactyloides Nyl.*	Tanzania, *Koponen 44127* (H)	(AF286767)	AY257967
P. retifoveata Vitik. 1*	Canada, *Goward* & *Burger 94-1004* (UBC)	(AF286776)	AY257888
P. retifoveata 2*	Canada, *Goward* & *Goward 83-514* (UBC)	(AF286775)	AY257889

[a] Specimen names followed by an asterisk represent sequences used as outgroup, i.e., outside section *Peltigera* (Miadlikowska and Lutzoni 2000). Names of taxa in quotation marks are newly recognized by T. Goward, but not formally published.

[b] Accession numbers in parentheses were obtained from GenBank and were part of a previous phylogenetic study of the genus *Peltigera* by Miadlikowska and Lutzoni (2000) based on morphological, chemical and molecular data; all other sequences were generated by J.M. for this study. Abbreviations of herbaria follow Index Herbariorum available at http://www.nybg.org/bsci/ih/ih.html.

carried out on data matrices containing only variable characters. Symmetric step matrices were created for unambiguous portions of the LSU and ITS alignments using the computer program STMatrix 2.1 (written by S. Zoller and available upon request to S.Z. or F.L.), as outlined in Miadlikowska et al (2002). ITS1, ITS2 and 5.8S each were subjected to a specific symmetric step matrix. Gaps were used as a fifth character state for unambiguous portions of the alignment. All ambiguously aligned regions were excluded. However, these regions were recoded with the program INAASE (Lutzoni et al 2000) and then re-integrated into the dataset for neighbor-joining (NJ1) and maximum-par-

TABLE II. Summary of datasets and results from phylogenetic analyses (NJ, ML, and MP).

Phylogenetic analyses	Total no. of sites[a]	No. of variable sites[a]	No. of constant sites	No. of ambiguous sites	Total no. of characters analyzed[b]	No. of trees obtained
NJ1: LSU 79 OTUs	1352 (5)	116 (5)	1153	83	116	1
NJ2: ITS 82 OTUs	889	74	195	620	74	1
MP1: LSU 77 OTUs	1352 (5)	113 (5)	1157	82	113 [70]	NA
MP2: ITS 77 OTUs	889 (15)	84 (15)	185	620	84 [75]	NA
MP3: LSU+ITS 77 OTUs	2221	182	1336	703	182 [130]	NA
MP4: LSU+ITS 77 OTUs	2241 (20)	202 (20)	1336	703	202 [150]	15 441
MP5: LSU+ITS 77 OTUs	2265 (20+24)	226 (20+24)	1336	703	226 [174]	1620
ML1: LSU 77 OTUs	1347	107	1158	82	107	14
ML2: ITS 77 OTUs	874	59	195	620	59	8
ML3: LSU+ITS 77 OTUs	2221	166	1352	703	166	8

[a] Number of coded characters are found in parentheses; the number after "+" refers to the number of characters associated with the ITS1 hypervariable region. All other numbers in parentheses refer to the number of INAASE characters. [b] Number of parsimony informative sites are found in square brackets. "NA" = not applicable due to the enormous number of equally most parsimonious trees obtained during the search.

simony analyses (MP1, MP2, MP4 and MP5). Sixteen of these 20 INAASE-coded characters were subjected to a specific symmetric step matrix implemented during maximum-parsimony searches.

A high level of length variation among ITS sequences, especially within ITS1, required the exclusion of 70% of the total 889 sites in the alignment. Site positions 111–237 of the ITS1 alignment were extremely variable across species but conserved within each putative species. In some individuals, microsatellites with a single nucleotide and short fragment repeats were present within the ITS1-HR region. This ambiguously aligned part of the ITS alignment was excluded from all phylogenetic analyses. Because this region was more than 125 sites long, we were unable to recode it with INAASE. Instead, we established a set of 24 characters to capture features of this hypervariable region (APPENDIX 1). When characters consisted of continuous variables, each different value was treated as a separate character state. All 24 ITS1-HR characters were tested for independence with a "tree-free" method by O'Keefe and Wagner (2001) to detect mutual compatibilities among characters. ITS1-HR characters 1–24 refer to: 1, the sequence length (total number of nucleotides); 2, adenine frequency (the number of "A"s divided by the total number of nucleotides); 3, thymine frequency; 4, guanine frequency; 5, cytosine frequency; 6, "AT" pair frequency (the number of "AT" pairs encountered using a sliding window of two nucleotides, divided by the total number of nucleotide pairs); 7, "AC" pair frequency; 8, "AG" pair frequency; 9, "CG" pair frequency; 10, "CT" pair frequency; 11, "GT" pair frequency; 12, "AA" pair frequency; 13, "GG" pair frequency; 14, "TT" pair frequency; 15, "CC" pair frequency; 16, "AAAAGTTCT" motif: absent = 0, present = 1; 17, contiguous "AA" pair frequency (the number of contiguous pairs of "A"s encountered using a sliding window of two nucleotides, divided by the total number of "A"s); 18, contiguous "TT" pair frequency; 19, contiguous "GG" pair frequency; 20, contiguous "CC" pair frequency; 21, adenine distribution (the number of discontinuities among "A"s divided by the total number of "A"s); 22, thymine distribution; 23, guanine distribution; 24, cytosine distribution.

To explore the impact of INAASE characters (five in the LSU and 15 in the ITS datasets) and 24 noncorrelated characters derived from the hypervariable ITS1 region (ITS1-HR) in recovering phylogenetic relationships within the *P. canina* group, phylogenetic analyses (NJ, MP and ML) were carried out on LSU and ITS datasets, separately and combined, with and without coded characters (TABLE II). The LSU 79 OTU dataset (NJ1) incorporated an additional sequence for *P. ulcerata* and *P.* "*latopraetextata*", whereas the ITS 82 OTU dataset (NJ2) contained four additional sequences (three for *P. lambinonii* and one for *P. praetextata*). The purpose of the NJ1 and NJ2 analyses was to confirm the validity of taxa represented by only one LSU or ITS sequence in the 77 OTU combined dataset. The goal of the separate MP and ML analyses was to test for topological congruence between the two data partitions. PAUP* settings for all weighted maximum-parsimony analyses were the same as in Miadlikowska and Lutzoni (2000). A hierarchical likelihood ratio test (Modeltest 3.04PPC, Posada and Crandall 1998) was used to select models and parameters for NJ2 and ML searches. Each type of datasets containing 77 OTUs was subjected to Modeltest. All maximum-likelihood analyses were done with heuristic searches of 100 random-addition sequences (RAS); 20 000 TBR rearrangements per RAS; MulTrees option in effect; reconnection limit equal 8; and all branches of effectively zero length were collapsed. Constant sites were excluded from all ML analyses.

Branch support, congruence test, sequence comparison and recombination analyses.—Branch support for NJ, MP and ML trees was estimated by bootstrap analyses (Felsenstein 1985), using the same parameter settings as for the initial search. To evaluate the robustness of the MP and NJ tree bipartitions, 1000 bootstrap replicates with 2 RAS per boot-

strap replicate were performed. For ML1–ML3 analyses, 500 bootstrap replicates were conducted with 2 RAS per bootstrap replicate.

For ML1, ML2, and ML3 datasets, in addition to bootstrapping, posterior probabilities (PP) were calculated as implemented in MrBayes 2.01 (Huelsenbeck 2000). All BMCMCMC analyses were run with four chains simultaneously, each initiated with a random tree, and flat prior; one out of every 20 trees was sampled for 1 000 000 generations with substitution parameters estimated during the search. The first 20 000 sampled trees from each run were discarded before calculating the majority-rule consensus tree to ensure that all chains had converged at the same level. Majority-rule consensus trees were assembled with PAUP* for the remaining 30 000 BMCMCMC sampled trees. Posterior probabilities for topological bipartitions were considered statistically significant when $P \geq 0.95$.

Within an MP framework, congruence between LSU and ITS data partitions was assessed by inspecting bootstrap scores ≥70% resulting from the separate MP analyses (Mason-Gamer and Kellogg 1996), as outlined in Miadlikowska and Lutzoni (2000). Conflict between ML trees derived from LSU and ITS, when analyzed separately, was detected when two different relationships (one monophyletic and the other nonmonophyletic) for the same set of taxa were supported by posterior probabilities ≥0.95.

To detect recombination, four methods, based on patterns of substitution, discordance in phylogenies and phylogenetic incongruence on a site-by-site basis (Posada and Crandall 2001) were used: PLATO (Grassly and Holmes 1997), ''Reticulate'' (Jakobsen and Easteal 1996), RDP (Martin and Rybicki 2000) and GENECONV 1.81 (Sawyer 1989). Each heterogeneous region of the alignments detected by PLATO was treated as a separate data partition, and conflict between these partitions and the remaining part of the LSU or ITS was tested with a BMCMCMC approach as for the combinability test used for ML trees (see above).

RESULTS

DNA sequence variation and alignments.—LSU sequences were similar in length (1316–1334 nucleotides), whereas the ITS sequences varied remarkably in length, from 503 to 660 nucleotides (ITS1: 158–294; 5.8S: 158–161; ITS2: 181–263). The hypervariable ITS1 region alone, located between sites 111 and 237 of our ITS alignment, showed differences in length ranging from 8 bp for *Peltigera lepidophora* to 126 bp for one *P. membranacea* individual.

Separate phylogenetic analyses on LSU and ITS data (NJ1 and NJ2; ML1 and ML2).—For the NJ1 analysis, mean character differences among sequences were used to allow the inclusion of five INAASE characters for a grand total of 116 variable characters (TABLE II). *Peltigera ulcerata* 1, included exclusively in the NJ1 analysis, confirmed the validity of LSU sequence for *P. ulcerata* 2; they form a highly supported monophyletic group (BS = 100%; tree not shown). The relationships among *P.* ''*latopraetextata*'' specimens, including *P.* ''*latopraetextata*'' 1, which was present only in the NJ1 dataset, were unresolved; therefore we were unable to validate sequences for this putative species. NJ2 analysis was performed using the K80 substitution model (Kimura 1980) with these parameters: ti/tv ratio = 2.6996, and equal rates for all sites. Three additional specimens of *P. lambinonii* (specimens 2–4), included exclusively in the NJ2 search, formed a monophyletic assemblage with *P. lambinonii* 1. The relationships among *P. praetextata* specimens, including *P. praetextata* 4 present only in the NJ2 dataset, were unresolved; therefore we were unable to validate sequences for this species.

The ML1 analysis was performed using the TrN substitution model (Tamura and Nei 1993) with this rate matrix: [A–C] = 1.0000, [A–G] = 3.1119, [A–T] = 1.0000, [C–G] = 1.0000, [C–T] = 5.1928, and [G–T] = 1.0000. Fourteen equally most-likely trees resulted from the ML1 search (ln likelihood = −1010.68249; result not shown). In the ML1 tree, the *Peltigera canina* complex is defined as a monophyletic and significantly supported group (PP = 1.00; BS = 78%). Most of the resolution and high support values were found at the base and along the ''backbone'' of the tree, including a sister relationship between the *P. canina* complex and section *Retifoveatae*.

The ML2 analysis was performed using the K80 substitution model (Kimura 1980) with ti/tv ratio = 2.6712. Eight equally most-likely trees resulted from this analysis (ln likelihood = −672.00570; tree not shown). Monophyly of the *Peltigera canina* complex did not receive significant support (PP = 0.78 and BS = 50%). None of the discrepancies between the ML2 analysis of the ITS were in conflict with the ML1 analysis of the LSU. In general, ML analysis of the ITS dataset resulted in a slightly more resolved tree (eight equally most-likely trees for ML2 versus 14 trees for ML1) but with far less support (especially for the ''backbone'') than the ML analysis of the LSU dataset.

Maximum-likelihood analyses on LSU and ITS combined dataset (ML3).—Because no conflict was detected when examining ML1 and ML2 topological bipartitions that received significant posterior probabilities (≥0.95), the LSU and ITS rDNA datasets were analyzed together. ML3 analysis was performed using the K80 substitution model (Kimura 1980) with ti/tv ratio = 2.1604. Eight equally most-likely trees part of four islands hit 92 out of 100 RAS were revealed by the ML3 search (ln likelihood = −1790.54212; FIG. 1).

The *Peltigera canina* complex (section *Peltigera*) is well defined as a monophyletic entity and is highly supported (PP = 1.00, BS = 84%) on the ML3 tree. Seven major monophyletic groups are recognized on the ML3 tree (A–G; FIG. 1); four of them obtained significant posterior probabilities: the *P. ponojensis* (A) group, *P. canina* s. str. (C) group, *P. cinnamomea* (D) group and *P. rufescens* (E) group. Phylogenetic relationships among groups within the *P. canina* complex remain questionable, except for well-supported sister relationships between the *P. canina* (C) group and the *P. degenii* (B) group and between the latter C + B group with the *P. cinnamomea* (D) group. Section *Retifoveatae* is the closest relative to the *Peltigera canina* complex, with PP = 0.98 and 76% bootstrap support. Section *Horizontales* does not represent a monophyletic entity, based on this combined dataset; instead, two distinct monophyletic groups, the *P. horizontalis* + *P. elisabethae* group and the *P. neckeri* group, both with high BS and PP values, were recognized.

Maximum-parsimony analysis on LSU and ITS combined dataset (MP3–5).—Because no conflict was detected when examining MP1 and MP2 topological bipartitions that received BS support ≥70%, the LSU and ITS rDNA datasets were analyzed together. MP3 and MP4 analyses were carried out mainly to compare their respective bootstrap support with support values associated with MP5 and ML3 (TABLE III). We were not able to complete the 1000 replicates for MP3 without limiting the number of TBR swapping. When the re-arrangement limit was set to 50 000 and the reconnection limit equal to eight, the MP3 analysis resulted in 10 509 most-parsimonious trees (tree length = 596.79 steps; CI [excluding uninformative characters] = 0.5957, RI = 0.8840; results not shown). From MP4 analysis, 15 441 most-parsimonious trees (tree length = 936.25 steps; CI [excluding uninformative characters] = 0.6512, RI = 0.8932; results not shown) were obtained. Because we could not reject independence for any of the 24 ITS1-HR characters, all were included in the MP5 search. The MP5 search resulted in 1620 most-parsimonious trees, part of a single island hit 935 out of 1000 RAS (tree length = 1538.91 steps; CI [excluding uninformative characters] = 0.6474, RI = 0.8589; FIG. 2). Topologies from these three MP searches (MP3–5) generally were similar. When different, competing pairwise discrepancies among MP3–5 and ML3 trees never were supported by bootstrap values ≥70%.

The MP5 analysis with INAASE and ITS1-HR characters generated the highest number of highly supported internodes, even when compared to ML3 (TABLE III). On the MP5 tree, monophyly of the *Peltigera canina* complex was highly supported (BS = 99%; FIG. 2). Six major monophyletic groups (A–E and G) were recognized within section *Peltigera*; all were highly supported by bootstrap values ≥70%, except for the *P. rufescens* (E) group. This tree was similar to the ML3 topology (FIG. 1), except that the *P. continentalis* (F) group was not present on the MP5 tree (FIG. 2). Bootstrap support was significantly higher for groups B and G (<50% in ML3 versus >70% in MP5) and slightly higher for group E compared to ML3. Sister relationships between groups B and C (BS = 79%) and group D (BS = 72%) are the only well-supported relationships among the six main groups. This group now will be referred to as the CICADE group (standing for CInnamomea + CAnina + DEgenii).

MP5 provided a high level of resolution and bootstrap support within species groups and, therefore, was the most useful in recognizing monophyletic groups (FIG. 1 versus FIG. 2; ML3 versus MP5; TABLE III). The *P. ponojensis* (A) group incorporated specimens representing three taxa: *P. monticola*, *P. ponojensis* and the putative *P.* "*scotteri*". *Peltigera monticola* and *P. ponojensis* each form a monophyletic group (BS = 100%, BS = 71%, respectively). *Peltigera* "*scotteri*" 1 might represent a lineage distinct from *P. monticola* and *P. ponojensis*.

The *P. rufescens* (E) group included specimens representing five taxa: three widely accepted species (*P. laciniata*, *P. lepidophora* and *P. rufescens*) and two potentially new taxa (*P.* "*fuscoponojensis*" and *P.* "*neorufescens*"). *Peltigera laciniata*, *P. lepidophora* and *P. rufescens* (including two specimens initially identified as *P.* "*neorufescens*") were recognized as monophyletic entities (BS ≥ 98%). Specimens of *P.* "*neorufescens*" were found in two different groups; specimens 2 and 3 are closely related to members of *P. rufescens*, and specimen 1 is sister to *P.* "*fuscoponojensis*" (BS = 99%). The phylogenetic affiliation of the latter two specimens to the *P. rufescens* group is uncertain, while the remaining taxa within this group form a monophyletic entity (BS = 76%).

The *Peltigera didactyla* (G) group included specimens representing three known species (*P. didactyla*, *P. lambinonii* and *P. ulcerata*) and one intraspecific taxon (*P. didactyla* var. *extenuata*). *Peltigera didactyla* var. *extenuata* was delimited as a robust monophyletic entity (BS = 100%), sister to other sorediate species that were part of a single highly supported group (BS = 84%). Two of the three specimens of *P. didactyla* var. *didactyla* are grouped together, while the third specimen (No. 2) was quite different; its relationship with specimens 1 and 3, as well as with *P. ulcerata* and *P. lambinonii*, is uncertain.

The *Peltigera degenii* (B) group incorporated spec-

TABLE III. Variation of bootstrap support values (BS) at selected internodes when comparing MP3-5 to ML3 (bold; FIG. 1). Only internodes with BS or posterior probabilities ≥50% were compared. Groups and node numbers are shown in FIGS. 1 and 2.

Group	Node No.	LSU+ITS (ML3) PP	**LSU+ITS (ML3) BS**	LSU+ITS (MP5) BS	LSU+ITS (MP4) BS	LSU+ITS (MP3) BS
A	1	0.67	<**50**	+>44↑	+>32↑	0
A	36	NA	**NA**	+81⇑	+61	NA
A	37	NA	**NA**	+83⇑	+63	+64
A	2	1.00	**89**	+11	+11	−3
A	38	NA	**NA**	+71⇑	NA	NA
A	3	1.00	**81**	−14↓	−15↓	−15↓
A	4	1.00	**77**	+23	+19	+10
A	5	1.00	**98**	+2	+2	+1
B	6	0.94	**54**	+44↑	+43↑	−>4
B	7	1.00	**82**	+17	+16	+3
B	39	NA	**NA**	+92⇑	+93⇑	NA
B	8	1.00	**94**	+6	+6	+3
B	9	0.69	<**50**	+>32↑	+>27↑	NA
C	40	NA	**NA**	+97⇑	+53	NA
C	41	NA	**NA**	+92⇑	NA	NA
C	42	NA	**NA**	+100⇑	+57	NA
C	43	NA	**NA**	+76⇑	NA	NA
C	10	0.96	**62**	+26↑	−3	−5
C	44	NA	**NA**	+81⇑	NA	NA
C	45	NA	**NA**	+99⇑	+52	+<50
C	46	NA	**NA**	+94⇑	+79⇑	NA
C	11	0.99	**63**	NA	−>13	+3
C	12	1.00	**99**	+1	+1	−1
B+C	13	0.98	**70**	+9	+16	−8↓
D	47	NA	**NA**	+88⇑	+82⇑	NA
D	48	NA	**NA**	+76⇑	+54	NA
D	14	1.00	**85**	+15	+15	+11
D	15	1.00	**84**	−10	+13	−1
B+C+D	16	0.95	**54**	+18↑	+10	−1
E	17	1.00	**95**	+4	+5	−4
E	18	1.00	**96**	+4	+4	+4
E	19	1.00	**98**	+2	+2	+2
E	20	0.90	**69**	+29↑	−5	−4
E	21	0.89	<**50**	+>29↑	+>28↑	NA
E	22	0.99	**58**	+8	+7	−4
F	23	0.99	**83**	+17	+16	+12
F	24	1.00	**100**	0	0	0
G	25	1.00	**91**	+7	+8	−1
G	27	0.99	**77**	+7	+15	−16↓
G	26	1.00	**100**	0	0	−39↓
G	28	0.53	<**50**	+>33↑	+>44↑	0
Sect. *Peltigera*	29	1.00	**84**	+15	+15	+10
Sects. *Pelt.* + *Ret.*	31	0.98	**76**	−7↓	−17↓	−23↓
Outgroup	30	1.00	**100**	0	0	0
Outgroup	34	1.00	**92**	+8	+8	+6
Outgroup	49	NA	**NA**	+77⇑	+<50	NA
Outgroup	35	1.00	**100**	0	0	0
Outgroup	50	NA	**NA**	+100⇑	+60	NA
Outgroup	51	NA	**NA**	+100⇑	+88⇑	NA
Outgroup	32	1.00	**95**	+5	+5	+3
Outgroup	33	1.00	**100**	0	0	0
Outgroup	52	NA	**NA**	+91⇑	+97⇑	+75⇑

NA = node does not exit; "↓" = bootstrap value became <70%; "↑" = bootstrap value became ≥70%; "⇑" = bootstrap value became ≥70% for a node that did not exist on ML3 tree.

imens representing two well-established species (*P. degenii* and *P. membranacea*) and the putative *P.* "*scotteri*". All specimens of *P. membranacea* belonged to a single group (BS = 100%). *Peltigera degenii* was partitioned into two distinct monophyletic groups: a North American *P. degenii* I group (specimens 2 and 4, including *P.* "*scotteri*" 2), and a European *P. degenii* II group (specimens 1 and 3), both with surprisingly high support (BS = 98% and BS = 99%, respectively).

The *Peltigera canina* s. str. (C) group incorporated individuals belonging to eight taxa: four known species (*P. canina*, *P. cinnamomea*, *P. evansiana* and *P. praetextata*) and four potential taxa (*P.* "*boreorufescens*", *P.* "*fuscopraetextata*", *P.* "*latopraetextata*" and *P.* "*pallidorufescens*"). When compared to ML3 (FIG. 1), MP3 and MP4, adding 24 coded characters from the ITS1-HR greatly improved the resolution and reduced phylogenetic uncertainty at the interspecific level within this most complex group of species. The monophyletic and highly supported *Peltigera canina* species (including *P.* "*boreorufescens*" 1) and *P. praetextata* species (including *P. cinnamomea* 3 and *P.* "*latopraetextata*" 3) were sister to each other (BS = 76%). They shared a common ancestor with *P. evansiana* (BS = 81%). The newly proposed *P.* "*pallidorufescens*" and *P.* "*fuscopraetextata*" were defined as monophyletic sister taxa (BS = 94%). Their common ancestor was part of the earliest divergence event within the *P. canina* s. str. group.

The *Peltigera cinnamomea* (D) group incorporated specimens from four taxa: one known species (*P. cinnamomea*) and three potential taxa (*P.* "*boreorufescens*", *P.* "*latopraetextata*" and *P.* "*neocanina*"). Two robust monophyletic groups were reconstructed with MP5. One group represents *P. cinnamomea*, including *P.* "*latopraetextata*" 1 (BS = 100%), and the second group represents a putative taxon composed of *P. neocanina* (specimens 1 and 2) and *P.* "*boreorufescens*" 2.

Relationships among *Peltigera continentalis*, *P. frigida* and *P. kristinssonii* are uncertain in MP5. Each seems to represent independent lineages. With MP5, the distinct *P. horizontalis* + *P. elisabethae* and *P. neckeri* groups formed a well-supported monophyletic section *Horizontales* (BS = 91%).

ITS1-HR sequence variation within and among monophyletic Peltigera *taxa.*—A total of 51 different sequences of the ITS1 hypervariable region (ITS1-HR) were present among 77 *Peltigera* individuals in this study. Eighteen of these sequences were found in at least two *Peltigera* individuals, and 33 were unique. The 24 characters we used to characterize these sequences captured the diversity and pattern of variation among the ITS1-HR sequences within the *P. canina* complex and outgroup taxa included here (FIG. 3).

Sequences of the ITS1-HR were identical or highly similar in length and nucleotide composition within each monophyletic taxon recognized at or below species level with MP5 (FIGS. 2 and 3). The ITS1-HR sequences were identical within each of these eight taxa from the *P. canina* complex: *P. didactyla* var. *extenuata*, *P. evansiana*, *P. frigida*, *P. kristinssonii*, *P. lepidophora*, *P. ponojensis*, *P. praetextata* and the putative *P.* "*fuscopraetextata*". The same was true for four species outside section *Peltigera* (*P. horizontalis*, *P. neckeri*, *P. malacea* and *P. retifoveata*). The ITS1-HR sequences were highly specific to each of the monophyletic entities, i.e., identical sequences never were detected in two different monophyletic taxa. Therefore, when new and highly distinct ITS1-HR sequences are found within the *P. canina* complex (e.g., *P.* "*neocanina*"), they are very likely to represent new taxa.

Contribution of INAASE and ITS1-HR characters to phylogenetic confidence.—The addition of 20 INAASE characters brought the number of internodes with bootstrap values ≥70% from 21 in MP3 to 33 in MP4 (TABLE III). When 24 ITS1-HR characters were added to the 20 INAASE characters (MP5), the total number of internodes with BS values ≥70% increased to 48. The number of internodes that received BS values ≥70% with ML3 bootstrap was 25, compared to a total of 29 internodes obtaining posterior probabilities ≥0.95. All internodes that received a BS value ≥70% in MP3 and MP4 also were supported highly in MP5 or ML3. Only MP5 revealed nodes (a total of four) that were exclusive to this analysis and highly supported. There were two nodes that received bootstrap values ≥70% only in the ML3 analysis, compared to 10 nodes with high BS values exclusively in MP5. Only four nodes with BS values ≤70% in the MP5 analyses received a BS value ≥70% in ML3 or a posterior probability ≥0.95. This is a very small number of nodes compared to ML3 bootstrap with 26

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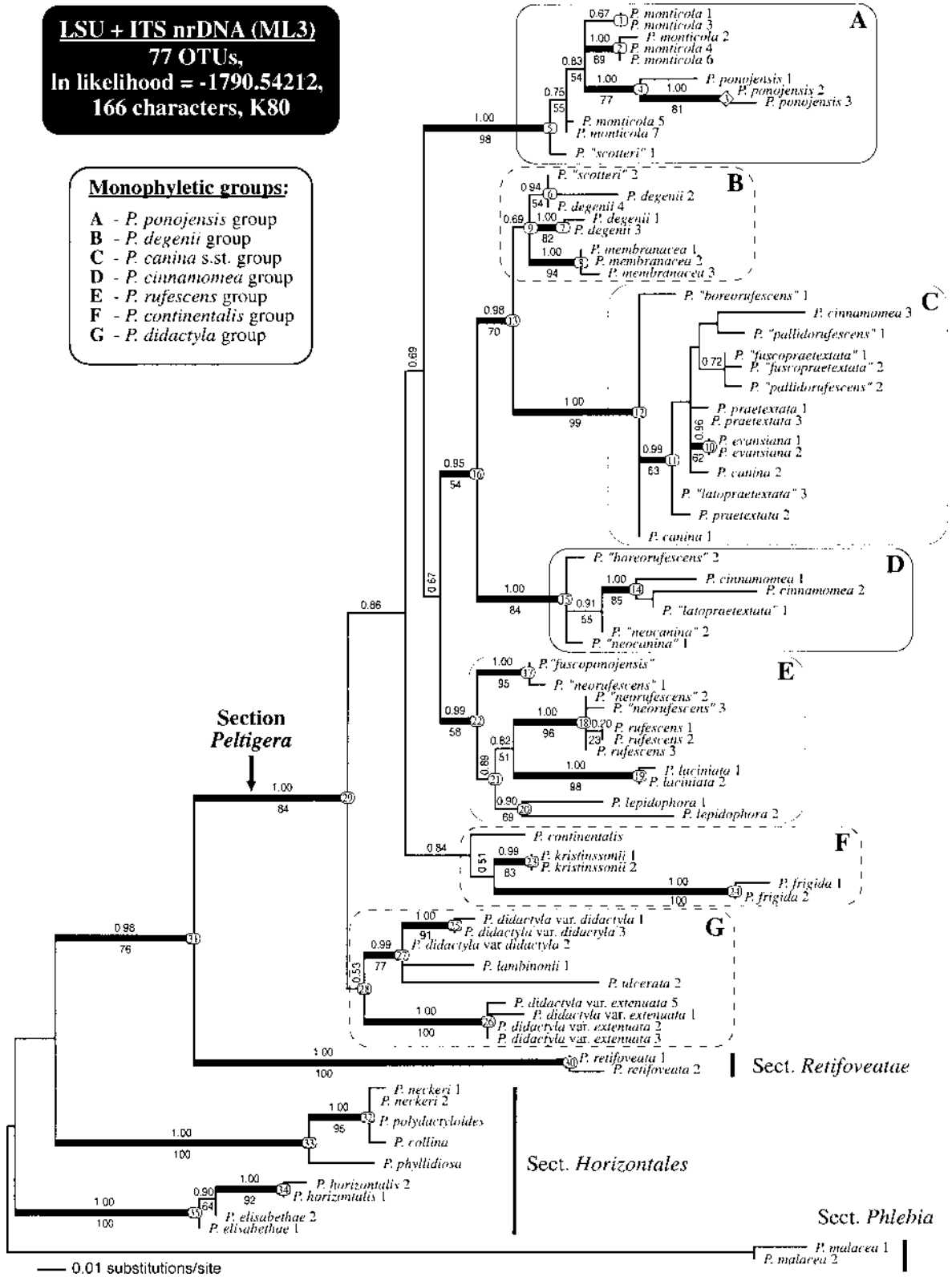
FIG. 1. ML3 analysis. One of eight most-likely trees for 64 individuals representing 25 *Peltigera* taxa from the *P. canina* species complex (section *Peltigera*) based on nuclear LSU and ITS rDNA combined dataset (TABLE II). A total of 13 specimens were selected from eight outgroup species. Bootstrap support values BS ≥ 50% are provided below each internode. Posterior probabilities ≥0.50 are provided above each internode. Internodes with PP ≥ 0.95 are shown as thicker lines. Boxes A–G



represent main monophyletic groups (containing at least two species) within the *P. canina* complex. Groups that are circumscribed by continuous lines (boxes A, C, D and E) have posterior probabilities ≥0.95. Poorly supported groups are delimited with dashed lines. Numbers in small boxes refer to node numbers in TABLE III. These nodes were selected because they received BS ≥70% or a posterior probability ≥0.95 in ML3 or MP5 (FIG. 2). Names in quotation marks indicate putative undescribed species within the *P. canina* complex.

nodes ≤70% BS that were highly supported by BMCMCMC or other MP bootstrap analyses. Bayesian BMCMCMC was not much better than ML3 bootstrap, with 22 nodes that obtained posterior probabilities ≤0.95 and were supported with bootstrap values ≥70% in at least one ML or MP bootstrap analysis.

Testing for recombination in the P. canina *species complex.*—Our results were highly dependent on the method used. RDP and Reticulate did not detect any clusters of incompatible sites within LSU and ITS alignments, suggesting that there was no recombination event among the included sequences. GENECONV showed some evidence for gene conversion and recombination. However, because of the different amount of variation among sequences (some sequences were very similar whereas others were quite distinct), none of the patterns were found to be significant. PLATO detected heterogeneity for two regions within the LSU and ITS alignments. However, no conflict was detected, when comparing topological bipartitions that received significant posterior probabilities (≥0.95) in ML1 and ML2 with topologies that resulted from the respective ML analyses on the LSU and ITS data partitions defined by PLATO.

DISCUSSION

Morphological versus phylogenetic species recognition in the Peltigera canina *species complex.*—We evaluated the morphological delimitation of 17 widely recognized species and eight additional putative species from the *Peltigera canina* complex (section *Peltigera*) using a combined LSU and ITS rDNA phylogeny. We found a complete concordance between phylogenetically based and morphologically based species circumscriptions for 15 of the 17 accepted species (*P. canina*, *P. cinnamomea*, *P. degenii* [monophyly not supported], *P. evansiana*, *P. frigida*, *P. kristinssonii*, *P. laciniata*, *P. lambinonii*, *P. lepidophora*, *P. membranacea*, *P. monticola*, *P. ponojensis*, *P. praetextata*, *P. rufescens* and *P. ulcerata*; FIG. 2). *Peltigera lambinonii* and *P. ulcerata*, each represented in the combined analyses by a single individual, were shown to be monophyletic based on more sequences included in the separate analyses of ITS and LSU datasets, respectively. Although seemingly morphologically homogenous, populations of *P. degenii* from North America and Europe are genetically and biogeographically divided into two putative cryptic species (TABLE I, FIGS. 2 and 3). Because no type material for *P. degenii* was included in this study (collected in Japan, Vitikainen 1994) and because of our limited sampling of *P. degenii* s. l., we cannot assign names to these two putative cryptic species. Results of further systematic studies on *P. degenii* s. l. will be presented in a separate publication. We found higher genetic variation among species than among individuals within each of these monophyletic groups.

Peltigera praetextata includes morphotypes without phyllidia (*P. praetextata* 6), as well as morphotypes with phyllidia (specimens 2 and 3). When present, phyllidia are taxonomically significant and a valuable diagnostic character for *P. praetextata* (see Lindahl 1960), however, this is not the only diagnostic character distinguishing *P. praetextata* from its sister species *P. canina* (see Goffinet and Hastings 1994, Vitikainen 1994).

The phenotypic delimitation of *P. didactyla* conflicts with its phylogenetic circumscription. The commonly accepted delimitation of *P. didactyla* encompasses *P. didactyla* var. *didactyla* and *P. didactyla* var. *extenuata*, which differ both on morphological and chemical grounds (Goffinet and Hastings 1995). *Peltigera didactyla* var. *extenuata* represents a monophyletic lineage, nonsister to *P. didactyla* s. str., and therefore should be recognized at the species level. Because of specimen *P. didactyla* var. *didactyla* 2, the taxonomic delimitation of *P. didactyla* s. str. is uncertain. This specimen is anomalous in containing secondary metabolites (gyrophoric acid and methyl gyrophorate) usually present only in members of *P. didactyla* var. *extenuata*. A detailed systematic treatment of all taxa belonging to the *P. didactyla* (G) group, as delimited here, are addressed in a separate publication (Goffinet et al 2003).

Status of the putative new species.—As reported by Miadlikowska and Lutzoni (2000) and confirmed by this study, the eight newly proposed *Peltigera* taxa belong to the *P. canina* species complex (section *Peltigera*). Four taxa, *P.* "*fuscopraetextata*" (part of the *P. canina* s. str. group), *P.* "*neocanina*" (part of the *P. cinnamomea* group), *P.* "*neorufescens*" (part of the *P.*

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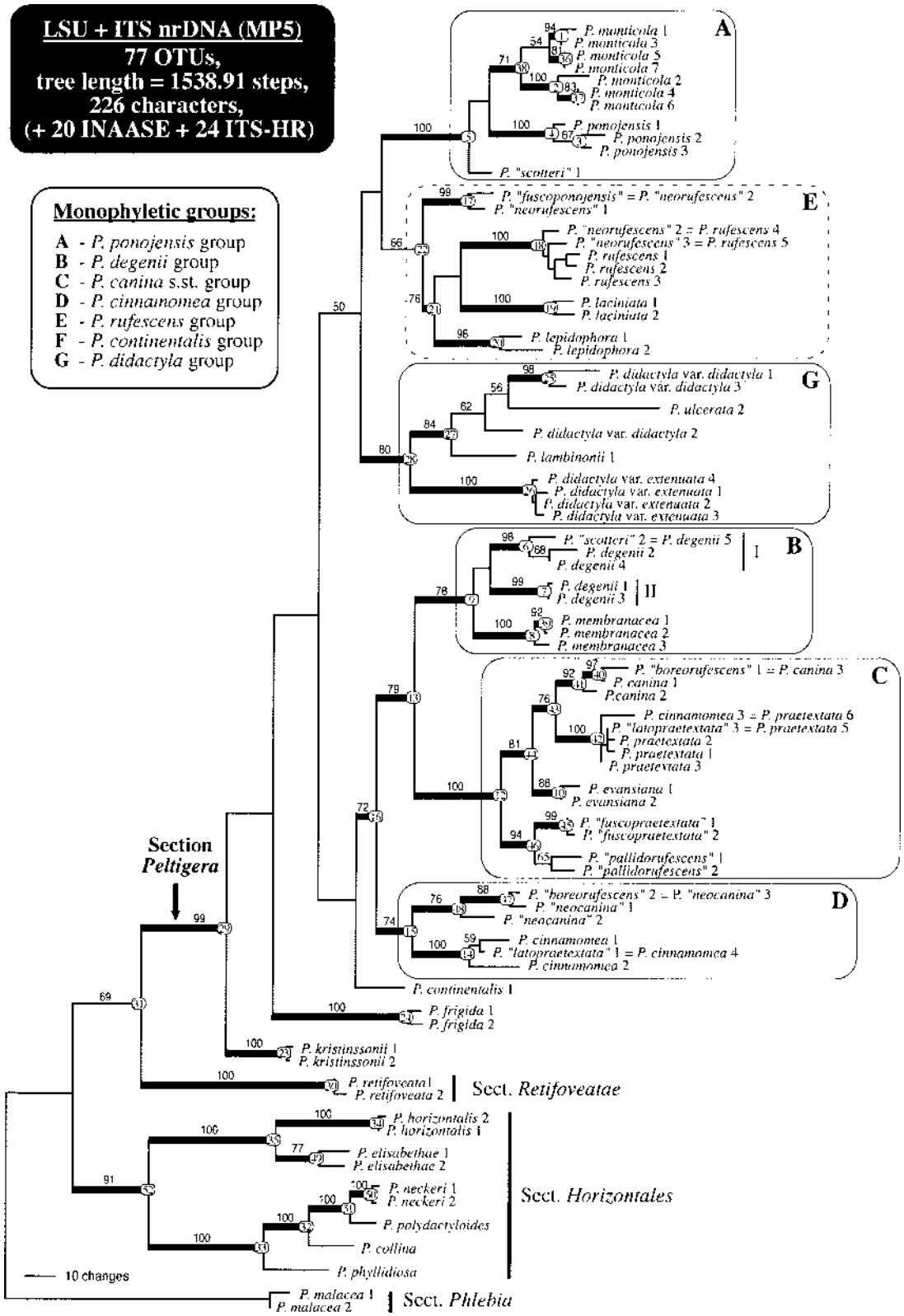
FIG. 2. MP5 analysis. One of 1620 most-parsimonious trees for 64 individuals representing 25 *Peltigera* taxa from the *P. canina* species complex (section *Peltigera*) based on nuclear LSU and ITS rDNA combined dataset, including 20 INAASE and 24 ITS1-HR characters (TABLE III and APPENDIX 1). A total of 13 specimens were selected from eight outgroup species. Bootstrap support values ≥50% are provided above each internode, which are shown as thicker lines. Boxes A–E and G represent the same monophyletic groups within the *P. canina* complex as recognized in ML3 (FIG. 1). Groups that are



circumscribed by continuous lines (boxes A–D and G) have BS ≥ 70%. Poorly supported Group E is delimited with a dashed line. Numbers in small boxes refer to node numbers in TABLE III. These nodes were selected because they received BS ≥ 70% or a posterior probability ≥0.95 in MP5 or ML3 (FIG. 1). Names in quotation marks indicate putative undescribed species within the *P. canina* complex. In the *P. degenii* (B) group, subgroups I and II correspond to a North American and a European clade, respectively.

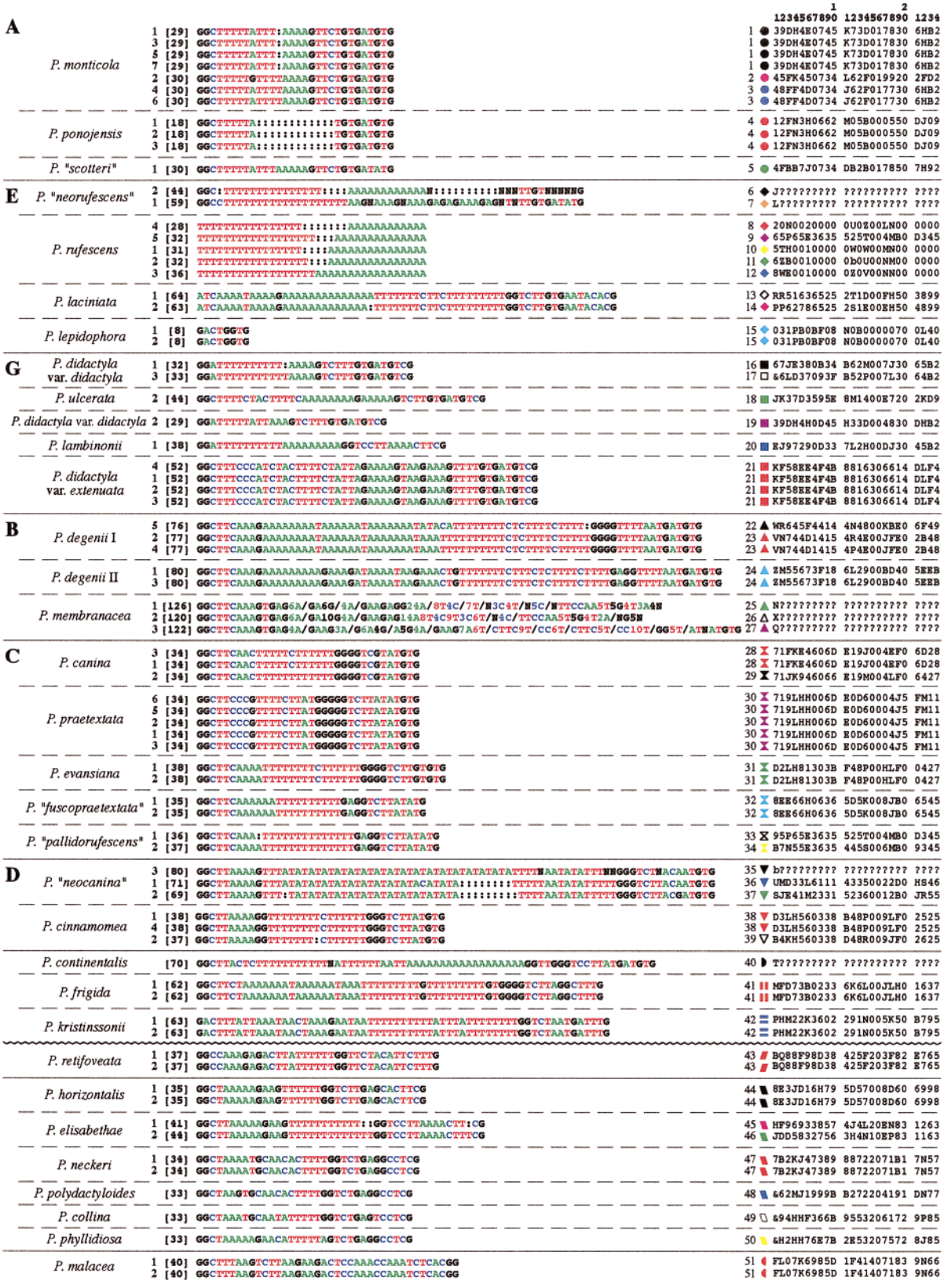


FIG. 3. Sequences of the ITS1 hypervariable region (ITS1-HR) for 64 individuals representing 25 *Peltigera* taxa from the *P. canina* species complex (above wavy line) and 13 individuals from eight outgroup species (below wavy line). When specimens initially were misidentified, only the revised identifications (name after "=" in FIG. 2) and associated specimen numbers were included here. Continuous lines separate major monophyletic groups (A–E, G) within the *P. canina* and

rufescens group) and *P.* "*scotteri*" (part of the *P. ponojensis* group) most likely represent four new species that need to be described, based on an extensive sampling of populations (FIG. 2). Detailed field studies on these species already were initiated. Unlike *P.* "*neocanina*" and *P.* "*fuscopratextata*", not all specimens initially identified as *P.* "*neorufescens*" and *P.* "*scotteri*" were part of a single well-supported monophyletic group with highly distinct ITS sequences. Despite these misidentifications before this study, these four newly circumscribed phylogenetic species are distinguishable morphologically. Formal descriptions of these new *Peltigera* species will be the subject of a separate publication in collaboration with T. Goward. The epithets appearing in this paper in quotations and originally published by Miadlikowska and Lutzoni (2000) are herbarium names not intended for eventual effective and valid publication.

The taxonomic status of one of the other four putative taxa (*P.* "*pallidorufescens*") is uncertain. *Peltigera* "*pallidorufescens*" is sister to *P.* "*fuscopraetextata*" but is not well supported. For this reason we prefer to await further data before drawing any taxonomic conclusion. The other three putative taxa (*P.* "*boreorufescens*", *P.* "*latopraetextata*" and *P.* "*fuscoponojensis*") are clearly part of other species within *Peltigera* and should not be recognized as new species. Some individuals were part of the newly defined species *P.* "*neocanina*" and *P.* "*neorufescens*", whereas the other specimens were nested in the well-established *P. canina*, *P. cinnamomea*, *P. degenii* I, *P. rufescens* and *P. praetextata* species (FIG. 2). The differences among these putative taxa can be subtle, apparently owing to phenotypic plasticity.

Contribution of the ITS1 hypervariable region (ITS1-HR) and INAASE characters to evolutionary studies of the Peltigera canina *complex.*—Variation among ITS1-HR sequences greatly contributed to species delimitation and hence species identification and can be a major asset to future population studies for specific species within section *Peltigera*. Sequences of ITS1-HR alone were sufficient to identify all existing species of *Peltigera* currently placed in the *P. canina* complex, as well as related sections *Retifoveatae* and *Horizontales* (FIG. 3). This molecular marker thus provides an exceptional tool for reliable identification of a lichen assemblage with few, or subtle, diagnostic morphological and chemical characters. Given that the amplification and sequencing of ITS1 alone would be sufficient for any studies requiring reliable identifications of members of this complex, it is likely that a simple RFLP analysis of the ITS1 would provide the same level of accuracy as sequences for species identification.

By using the ITS1-HR marker, it also was possible to recognize new undescribed species within the *P. canina* complex. Variation among these sequences was in agreement with each of the monophyletic morphospecies and permitted the recognition of distinct species even when represented by only one specimen in the combined dataset (e.g., *P. continentalis*, *P. lambinonii* and *P. ulcerata*; FIGS. 2 and 3). In one case (*P. degenii* I and II), ITS1-HR sequences were sufficient to detect individuals belonging to putative cryptic species. Because most individuals of *Peltigera* in this study were collected in the Northern Hemisphere (North America and Europe), the complete intra- and interspecific ITS1-HR variation within the *P. canina* complex is probably higher than we have documented. The LSU and ITS nrDNA datasets analyzed separately (including INAASE characters and 24 coded characters from ITS1-HR) could not phylogenetically delimit all morphospecies evaluated here (see Kroken and Taylor 2001). Only when INAASE and ITS1-HR characters were added to the combined analysis (MP5) were we able to reach the level of resolution, support and disentanglement of the *P. canina* complex shown here (FIGS. 1 versus 2; TABLE III). For example, *Peltigera monticola* (*P. ponojensis* group), *P. canina*, *P. praetextata*, *P.* "*fuscopraetextata*" (*P. canina* s. str. group) and *P.* "*neocanina*" (*P. cinnamomea* group) were circumscribed and highly supported (BS $\geq$ 70%) as monophyletic species only with the MP5 analysis. These ITS1-HR characters greatly contributed to the determination of interspecific relationships, especially among species in the *P. canina* s. str. group (e.g., the sister relationships between *P. canina* and *P. praetextata* and between *P. canina* + *P. praetextata* and *P. evansiana*). These relationships were corroborated partly by the

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sections (*Retifoveatae*, *Horizontales* and *Phlebia*) within outgroup, as revealed by MP5 (FIG. 2). Dashed lines separate putative *Peltigera* species, as shown in MP5 tree (FIG. 2). Sequences were aligned within each putative species category, except for *P. membranacea* (where the alignment was not possible) and for group A (where sequences were aligned among putative species). Sequence length (number of nucleotides) is provided in square brackets to the left of each sequence. Each different ITS1-HR sequence received a different colored symbol and number found in the right side of each sequence. Twenty-four coded characters describing features of these sequences are shown to the right of this figure (see APPENDIX 1 and "*Phylogenetic analyses*" in Materials and Methods for a description of these characters).

high degree of similarity between *P. praetextata* and *P. canina*, based on selected anatomical and morphological features, such as spore size, thallus thickness, width and height of veins. Only differences in vein height were statistically significant between these two species (Martínez and Burgaz 1996).

The hypervariability of the ITS1-HR was due largely to the presence of microsatellites. Sixteen of the 33 taxa recognized here included at least one microsatellite, 15 of which were found in the *P. canina* complex. Some of these microsatellites were highly variable within species and will be ideal markers for population studies. *Peltigera membranacea*, *P.* "*neocanina*" and *P. rufescens* are good examples, because none of the specimens sampled had an identical microsatellite length, even when collected from the same area (e.g., *P.* "*neocanina*"; TABLE I, FIG. 3).

Adding only the 20 INAASE characters to the combined dataset (i.e., without ITS1-HR characters) in the MP4 analyses was sufficient for the total number of well-supported internodes (BS ≥ 70%) to be greater than the number of internodes with BS ≥ 70% and posterior probabilities ≥0.95 from ML3 analyses. According to a recent simulation study comparing the statistical performance of ML and MP bootstrapping to a Bayesian MCMCMC approach (Alfaro et al 2003), MP bootstrapping often supports fewer internodes (BS ≥ 70%) than ML bootstrap and BMCMCMC. Because at the time we conducted this study we could not accommodate multiple models simultaneously within an ML or BMCMCMC framework, including models that can accommodate INAASE and ITS1-HR coded characters, our MP bootstrap provided a higher level of phylogenetic confidence than ML bootstrap and Bayesian approach. Therefore, the addition of INAASE and ITS1-HR characters to our MP analyses had a greater positive impact on the level of phylogenetic confidence than using more powerful methods (i.e., requiring less data to converge on the correct topology; Huelsenbeck and Hillis 1993, Yang 1996) such as ML and BMCMCMC, but with fewer characters (i.e., without INAASE and ITS1-HR characters).

Ecological preferences and diversification within the P. canina *complex.*—None of the phylogenetic analyses provided strong support for deep relationships within the *P. canina* complex. With MP5, two main monophyletic groups were reconstructed: the B+C+D (CICADE) and A+E+G (PORUDI) groups. The well-supported CICADE group includes closely related (BS = 79%) *P. degenii* (B) + *P. canina* s. str. (C) groups and *P. cinnamomea* (D) group (BS = 72%), whereas the weakly supported PORUDI group, consists of *P. ponojensis* (A), *P. rufescens* (E), and *P. didactyla* (G) groups (BS = 50%). This major division within the *canina* complex seems to be correlated with humidity preferences. Species from the CICADE group are mesophytic and subhygrophytic (common in woodlands), and rarely occur in xeric sites (*P. canina* sometimes grows in subxeric conditions); whereas species from the PORUDI group are xerophytic and, to much lesser extent, mesophytic (see also character 69 in Miadlikowska and Lutzoni 2000). Species from the latter group mostly are heliophilous and common on periodically disturbed soils. Three species with uncertain affiliations within section *Peltigera* (*P. continentalis*, *P. frigida* and *P. kristinssonii*) and the outgroup species selected for this study are common in mesic habitats. Section *Peltigera*, which seems to be one of the two most-derived sections within this genus (Miadlikowska and Lutzoni 2000), includes most of the known xeric and heliophilous *Peltigera* species. Therefore, it would be interesting to determine if they all are derived from a single evolutionary event, from multiple independent origins at various times during the evolution of *Peltigera* or from multiple independent origins at one specific time during the evolution of this genus.

In general, phenotypic similarity seems to be positively correlated with degree of relatedness among species and species groups within the *P. canina* complex (e.g., close phylogenetic relationships of *P. degenii* s. l. with *P. membranacea*, *P. canina* with *P. praetextata*, and *P. monticola* with *P. ponojensis*; FIG. 2). Such a correlation, however, does not apply in all cases. For example, *P. cinamomea* (group D) can be very similar to nonphyllidiate, broad lobed, morphotypes of *P. praetextata* (group C; FIG. 2).

Our reconstructed phylogeny (MP5 tree) provides a framework for further systematic, biogeographical and evolutionary studies on the *Peltigera canina* species complex. To gain confidence in the deep relationships among major groups in this section, inclusion of additional molecular characters is needed.

Relationships outside section Peltigera.—Phylogenies resulting from this study (FIGS. 1 and 2) are the first to be reconstructed for this section, based on two molecular markers. Despite the removal of chemical and morphological characters and the addition of the ITS to the LSU dataset, relationships outside the *P. canina* complex are in agreement with our previous results (Miadlikowska and Lutzoni 2000). This study significantly confirms the monophyletic delimitation of the section *Peltigera* (including *P. ulcerata*; PP ≥ 0.95 and BS ≥ 70%) and its sister relationship with the monospecific section *Retifoveatae* (FIGS. 1 and 2). By incorporating ITS1-HR and INAASE characters in MP5, the bootstrap support for section *Hor-*

izontales increased from 68% (in Miadlikowska and Lutzoni 2000) to 91% in the MP5 analysis (FIG. 2).

The recombination process within the Peltigera canina *complex.*—The remarkably high intraspecific and low interspecific levels of morphological/chemical variation, as well as the frequent occurrence of pycnidia in the *Peltigera canina* complex, compared to other sections, has led to the assumption that interspecific recombination is frequent in this group (e.g., Goffinet and Hastings 1995). Despite this belief, evidence of recombination never was found to be statistically significant with ITS and LSU sequences. Sampling strategies for populations, species and loci designed specifically to detect recombination are needed to establish the importance of this process in the evolution of this complex group of species.

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APPENDIX 1. Data matrix for 24 coded characters derived from the hypervariable region of ITS1 (ITS1-HR; sites 111–237) for the 77 *Peltigera* individuals included in the combined MP5 analyses. These characters are described under ''Materials and Methods—*Phylogenetic analyses*.'' Character states for each of the 24 characters are shown in FIG. 3. Different values were coded as different character states

Taxon[a]	1	2	3	4	5	6	7	8	9	10	11
P. ''*boreorufescens*'' 1	34	0.09	0.50	0.27	0.15	0.06	0.06	0.00	0.06	0.18	0.18
P. ''*boreorufescens*'' 2	79	?	?	?	?	?	?	?	?	?	?
P. canina 1	34	0.09	0.50	0.27	0.15	0.06	0.06	0.00	0.06	0.18	0.18
P. canina 2	34	0.09	0.53	0.27	0.12	0.06	0.06	0.00	0.06	0.12	0.18
P. cinnamomea 1	38	0.13	0.55	0.24	0.08	0.08	0.00	0.03	0.03	0.14	0.16
P. cinnamomea 2	37	0.14	0.54	0.24	0.08	0.08	0.00	0.03	0.03	0.14	0.17
P. cinnamomea 3	34	0.09	0.44	0.29	0.18	0.18	0.00	0.00	0.06	0.18	0.18
P. continentalis	70	?	?	?	?	?	?	?	?	?	?
P. degenii 1	80	0.36	0.39	0.16	0.09	0.09	0.03	0.14	0.01	0.14	0.10
P. degenii 2	77	0.39	0.42	0.13	0.07	0.13	0.01	0.04	0.01	0.11	0.08
P. degenii 3	80	0.36	0.39	0.16	0.09	0.09	0.03	0.14	0.01	0.14	0.10
P. degenii 4	77	0.39	0.42	0.13	0.07	0.13	0.01	0.04	0.01	0.11	0.08
P. didactyla var. *didactyla* 1	32	0.19	0.53	0.22	0.06	0.10	0.00	0.10	0.03	0.10	0.16
P. didactyla var. *didactyla* 2	39	0.33	0.44	0.18	0.05	0.11	0.00	0.11	0.03	0.08	0.11
P. didactyla var. *didactyla* 3	33	0.18	0.55	0.21	0.06	0.09	0.00	0.09	0.03	0.09	0.16
P. didactyla var. *extenuata* 1	52	0.27	0.39	0.19	0.15	0.14	0.04	0.14	0.04	0.16	0.12
P. didactyla var. *extenuata* 2	52	0.27	0.39	0.19	0.15	0.14	0.04	0.14	0.04	0.16	0.12
P. didactyla var. *extenuata* 3	52	0.27	0.39	0.19	0.15	0.14	0.04	0.14	0.04	0.16	0.12
P. didactyla var. *extenuata* 4	52	0.27	0.39	0.19	0.15	0.14	0.04	0.14	0.04	0.16	0.12
P. evansiana 1	38	0.11	0.55	0.24	0.11	0.03	0.03	0.00	0.03	0.16	0.19
P. evansiana 2	38	0.11	0.55	0.24	0.11	0.03	0.03	0.00	0.03	0.16	0.19
P. frigida 1	62	0.27	0.48	0.18	0.06	0.12	0.00	0.02	0.03	0.08	0.10
P. frigida 2	62	0.27	0.48	0.18	0.06	0.12	0.00	0.02	0.03	0.08	0.10
P. ''*fuscoponojensis*''	44	?	?	?	?	?	?	?	?	?	?
P. ''*fuscopraetextata*'' 1	35	0.26	0.49	0.17	0.09	0.18	0.00	0.06	0.03	0.12	0.09
P. ''*fuscopraetextata*'' 2	35	0.26	0.49	0.17	0.09	0.18	0.00	0.06	0.03	0.12	0.09
P. kristinssonii 1	63	0.30	0.56	0.10	0.05	0.26	0.03	0.06	0.00	0.06	0.06
P. kristinssonii 2	63	0.30	0.56	0.10	0.05	0.26	0.03	0.06	0.00	0.06	0.06
P. laciniata 1	64	0.42	0.39	0.09	0.09	0.05	0.06	0.05	0.02	0.11	0.06
P. laciniata 2	63	0.41	0.40	0.10	0.10	0.10	0.06	0.05	0.02	0.11	0.06
P. lambinonii	29	0.21	0.48	0.24	0.07	0.18	0.00	0.11	0.04	0.11	0.21
P. ''*latopraetextata*'' 1	38	0.13	0.55	0.24	0.08	0.08	0.00	0.03	0.03	0.14	0.16
P. ''*latopraetextata*'' 3	34	0.09	0.44	0.29	0.18	0.18	0.00	0.00	0.06	0.18	0.18
P. lepidophora 1	8	0.13	0.25	0.50	0.13	0.00	0.14	0.14	0.00	0.14	0.43
P. lepidophora 2	8	0.13	0.25	0.50	0.13	0.00	0.14	0.14	0.00	0.14	0.43
P. membranacea 1	126	?	?	?	?	?	?	?	?	?	?
P. membranacea 2	120	?	?	?	?	?	?	?	?	?	?
P. membranacea 3	122	?	?	?	?	?	?	?	?	?	?
P. monticola 1	29	0.21	0.48	0.24	0.07	0.14	0.00	0.07	0.04	0.11	0.25
P. monticola 2	30	0.17	0.50	0.27	0.07	0.07	0.00	0.07	0.03	0.10	0.31
P. monticola 3	29	0.21	0.48	0.24	0.07	0.14	0.00	0.07	0.04	0.11	0.25
P. monticola 4	30	0.20	0.50	0.23	0.07	0.13	0.00	0.07	0.03	0.10	0.24
P. monticola 5	29	0.21	0.48	0.24	0.07	0.14	0.00	0.07	0.04	0.11	0.25
P. monticola 6	30	0.20	0.50	0.23	0.07	0.13	0.00	0.07	0.03	0.10	0.24
P. monticola 7	29	0.21	0.48	0.24	0.07	0.14	0.00	0.07	0.04	0.11	0.25
P. ''*neocanina*'' 1	73	0.36	0.48	0.11	0.06	0.47	0.06	0.01	0.01	0.04	0.08
P. ''*neocanina*'' 2	69	0.33	0.49	0.13	0.04	0.52	0.02	0.03	0.03	0.04	0.09
P. ''*neorufescens*'' 1	59	?	?	?	?	?	?	?	?	?	?
P. ''*neorufescens*'' 2	28	0.04	0.57	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00
P. ''*neorufescens*'' 3	32	0.44	0.56	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00
P. ''*pallidorufescens*'' 1	36	0.17	0.58	0.17	0.08	0.14	0.03	0.06	0.03	0.11	0.09

APPENDIX 1. Extended

12	13	14	15	16	17	18	19	20	21	22	23	24
0.03	0.12	0.30	0.00	0	0.33	0.59	0.44	0.00	0.33	0.35	0.44	0.80
?	?	?	?	0	?	?	?	?	?	?	?	?
0.03	0.12	0.30	0.00	0	0.33	0.59	0.44	0.00	0.33	0.35	0.44	0.80
0.03	0.12	0.36	0.00	0	0.33	0.67	0.44	0.00	0.33	0.28	0.44	0.75
0.08	0.11	0.38	0.00	0	0.60	0.67	0.44	0.00	0.20	0.29	0.44	0.67
0.08	0.11	0.39	0.00	0	0.60	0.65	0.44	0.00	0.20	0.30	0.44	0.67
0.00	0.15	0.18	0.00	0	0.00	0.40	0.50	0.33	0.67	0.53	0.40	0.33
?	?	?	?	0	?	?	?	?	?	?	?	?
0.24	0.03	0.23	0.00	0	0.66	0.58	0.15	0.00	0.31	0.39	0.77	0.86
0.30	0.05	0.26	0.00	0	0.77	0.63	0.40	0.00	0.20	0.34	0.50	0.80
0.24	0.03	0.23	0.00	0	0.66	0.58	0.15	0.00	0.31	0.39	0.77	0.86
0.29	0.05	0.26	0.00	0	0.77	0.63	0.40	0.00	0.20	0.34	0.50	0.80
0.10	0.03	0.36	0.00	0	0.50	0.65	0.14	0.00	0.33	0.29	0.71	0.50
0.24	0.03	0.29	0.00	0	0.69	0.65	0.14	0.00	0.23	0.29	0.71	0.50
0.09	0.03	0.38	0.00	0	0.50	0.67	0.14	0.00	0.33	0.28	0.71	0.50
0.12	0.02	0.18	0.04	0	0.43	0.45	0.10	0.25	0.50	0.50	0.80	0.63
0.12	0.02	0.18	0.04	0	0.43	0.45	0.10	0.25	0.50	0.50	0.80	0.63
0.12	0.02	0.18	0.04	0	0.43	0.45	0.10	0.25	0.50	0.50	0.80	0.63
0.12	0.02	0.18	0.04	0	0.43	0.45	0.10	0.25	0.50	0.50	0.80	0.63
0.08	0.11	0.38	0.00	0	0.75	0.67	0.44	0.00	0.00	0.28	0.44	0.75
0.08	0.11	0.38	0.00	0	0.75	0.67	0.44	0.00	0.00	0.28	0.44	0.75
0.21	0.08	0.33	0.00	0	0.77	0.67	0.45	0.00	0.18	0.30	0.45	0.75
0.21	0.08	0.33	0.00	0	0.77	0.67	0.45	0.00	0.18	0.30	0.45	0.75
?	?	?	?	0	?	?	?	?	?	?	?	?
0.15	0.06	0.32	0.00	0	0.56	0.65	0.33	0.00	0.33	0.29	0.50	0.67
0.15	0.06	0.32	0.00	0	0.56	0.65	0.33	0.00	0.33	0.29	0.50	0.67
0.13	0.02	0.37	0.00	0	0.42	0.66	0.17	0.00	0.47	0.31	0.67	0.67
0.13	0.02	0.37	0.00	0	0.42	0.66	0.17	0.00	0.47	0.31	0.67	0.67
0.32	0.02	0.25	0.00	0	0.74	0.64	0.17	0.00	0.22	0.32	0.67	0.83
0.31	0.02	0.26	0.00	0	0.73	0.64	0.17	0.00	0.23	0.32	0.67	0.83
0.07	0.04	0.25	0.00	0	0.33	0.50	0.14	0.00	0.50	0.43	0.71	0.50
0.08	0.11	0.38	0.00	0	0.60	0.67	0.44	0.00	0.20	0.29	0.44	0.67
0.00	0.15	0.18	0.00	0	0.00	0.40	0.50	0.33	0.67	0.53	0.40	0.33
0.00	0.14	0.00	0.00	0	0.00	0.00	0.25	0.00	0.00	0.50	0.50	0.00
0.00	0.14	0.00	0.00	0	0.00	0.00	0.25	0.00	0.00	0.50	0.50	0.00
?	?	?	?	0	?	?	?	?	?	?	?	?
?	?	?	?	0	?	?	?	?	?	?	?	?
?	?	?	?	0	?	?	?	?	?	?	?	?
0.11	0.04	0.25	0.00	1	0.50	0.50	0.14	0.00	0.33	0.43	0.71	0.50
0.10	0.03	0.28	0.00	1	0.60	0.53	0.13	0.00	0.20	0.40	0.75	0.50
0.11	0.04	0.25	0.00	1	0.50	0.50	0.14	0.00	0.33	0.43	0.71	0.50
0.10	0.03	0.28	0.00	1	0.50	0.47	0.14	0.00	0.33	0.43	0.71	0.50
0.11	0.04	0.25	0.00	1	0.50	0.50	0.14	0.00	0.33	0.43	0.71	0.50
0.10	0.03	0.28	0.00	1	0.50	0.47	0.14	0.00	0.33	0.43	0.71	0.50
0.11	0.04	0.25	0.00	1	0.50	0.50	0.14	0.00	0.33	0.43	0.71	0.50
0.07	0.04	0.17	0.00	0	0.19	0.34	0.38	0.00	0.77	0.63	0.50	0.70
0.06	0.04	0.18	0.00	0	0.17	0.35	0.33	0.00	0.78	0.62	0.56	0.67
?	?	?	?	0	?	?	?	?	?	?	?	?
0.41	0.00	0.56	0.00	0	0.92	0.94	0.00	0.00	0.00	0.00	0.00	0.00
0.42	0.00	0.55	0.00	0	0.93	0.94	0.00	0.00	0.00	0.00	0.00	0.00
0.06	0.06	0.43	0.00	0	0.33	0.71	0.33	0.00	0.50	0.24	0.50	0.67

APPENDIX 1. Continued

Taxon[a]	1	2	3	4	5	6	7	8	9	10	11
P. "*pallidorufescens*" 2	37	0.19	0.57	0.16	0.08	0.14	0.03	0.06	0.03	0.11	0.08
P. ponojenisis 1	18	0.11	0.50	0.33	0.06	0.18	0.00	0.06	0.06	0.06	0.35
P. ponojenisis 2	18	0.11	0.50	0.33	0.06	0.18	0.00	0.06	0.06	0.06	0.35
P. ponojenisis 3	18	0.11	0.50	0.33	0.06	0.18	0.00	0.06	0.06	0.06	0.35
P. praetextata 1	34	0.09	0.44	0.29	0.18	0.18	0.00	0.00	0.06	0.18	0.18
P. praetextata 2	34	0.09	0.44	0.29	0.18	0.18	0.00	0.00	0.06	0.18	0.18
P. praetextata 3	34	0.09	0.44	0.29	0.18	0.18	0.00	0.00	0.06	0.18	0.18
P. rufescens 1	31	0.48	0.52	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00
P. rufescens 2	32	0.53	0.47	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00
P. rufescens 3	35	0.51	0.49	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00
P. "*scotteri*" 1	30	0.27	0.47	0.20	0.10	0.21	0.00	0.07	0.03	0.10	0.17
P. "*scotteri*" 2	76	0.42	0.40	0.13	0.08	0.16	0.04	0.04	0.01	0.10	0.08
P. ulcerata 2	44	0.34	0.34	0.18	0.14	0.05	0.05	0.09	0.05	0.19	0.12
*P. collina**	33	0.21	0.36	0.24	0.18	0.16	0.03	0.06	0.06	0.16	0.13
P. elisabethae 1*	41	0.27	0.44	0.17	0.12	0.05	0.03	0.08	0.05	0.13	0.08
P. elisabethae 2*	44	0.25	0.48	0.16	0.11	0.05	0.02	0.07	0.05	0.12	0.07
P. horizontalis 1*	35	0.26	0.34	0.26	0.14	0.03	0.06	0.15	0.09	0.15	0.09
P. horizontalis 2*	35	0.26	0.34	0.26	0.14	0.03	0.06	0.15	0.09	0.15	0.09
P. malacea 1*	40	0.35	0.23	0.18	0.25	0.08	0.13	0.08	0.05	0.18	0.03
P. malacea 2*	40	0.35	0.23	0.18	0.25	0.08	0.13	0.08	0.05	0.18	0.03
P. neckeri 1*	34	0.24	0.27	0.27	0.24	0.06	0.09	0.03	0.12	0.15	0.12
P. neckeri 2*	34	0.24	0.27	0.27	0.24	0.06	0.09	0.03	0.12	0.15	0.12
*P. phyllidiosa**	33	0.30	0.27	0.24	0.18	0.09	0.06	0.13	0.09	0.16	0.06
*P. polydactyloides**	33	0.18	0.27	0.30	0.24	0.03	0.13	0.09	0.13	0.16	0.16
P. retifoveata 1*	37	0.22	0.43	0.19	0.16	0.11	0.11	0.11	0.03	0.14	0.08
P. retifoveata 2*	37	0.22	0.43	0.19	0.16	0.11	0.11	0.11	0.03	0.14	0.08

[a] Specimen names followed by an asterisk represent sequences used as outgroup, i.e., outside of the section *Peltigera* (Miadlikowska and Lutzoni 2000). Names of taxa in quotation marks are newly recognized by T. Goward, but not formally published.

"?" = missing character state due to the presence of unknown nucleotides in the ITS1-HR sequence.

APPENDIX 1. Extended, Continued

12	13	14	15	16	17	18	19	20	21	22	23	24
0.08	0.06	0.42	0.00	0	0.43	0.71	0.33	0.00	0.43	0.24	0.50	0.67
0.00	0.06	0.24	0.00	0	0.00	0.44	0.17	0.00	0.50	0.44	0.67	0.00
0.00	0.06	0.24	0.00	0	0.00	0.44	0.17	0.00	0.50	0.44	0.67	0.00
0.00	0.06	0.24	0.00	0	0.00	0.44	0.17	0.00	0.50	0.44	0.67	0.00
0.00	0.15	0.18	0.00	0	0.00	0.40	0.50	0.33	0.67	0.53	0.40	0.33
0.00	0.15	0.18	0.00	0	0.00	0.40	0.50	0.33	0.67	0.53	0.40	0.33
0.00	0.15	0.18	0.00	0	0.00	0.40	0.50	0.33	0.67	0.53	0.40	0.33
0.47	0.00	0.50	0.00	0	0.93	0.94	0.00	0.00	0.00	0.00	0.00	0.00
0.52	0.00	0.45	0.00	0	0.94	0.93	0.00	0.00	0.00	0.00	0.00	0.00
0.50	0.00	0.47	0.00	0	0.94	0.94	0.00	0.00	0.00	0.00	0.00	0.00
0.14	0.03	0.24	0.00	1	0.50	0.50	0.17	0.00	0.38	0.43	0.67	0.50
0.27	0.05	0.22	0.00	0	0.78	0.57	0.40	0.00	0.33	0.40	0.50	0.83
0.26	0.02	0.16	0.00	0	0.73	0.47	0.13	0.00	0.20	0.47	0.75	0.83
0.09	0.06	0.13	0.03	0	0.43	0.33	0.25	0.17	0.43	0.58	0.63	0.67
0.20	0.05	0.33	0.03	0	0.73	0.72	0.29	0.20	0.18	0.22	0.57	0.60
0.19	0.05	0.37	0.02	0	0.73	0.76	0.29	0.20	0.18	0.19	0.57	0.60
0.15	0.06	0.21	0.00	0	0.56	0.58	0.22	0.00	0.33	0.33	0.67	0.80
0.15	0.06	0.21	0.00	0	0.56	0.58	0.22	0.00	0.33	0.33	0.67	0.80
0.18	0.05	0.08	0.05	0	0.50	0.33	0.29	0.20	0.43	0.56	0.57	0.70
0.18	0.05	0.08	0.05	0	0.50	0.33	0.29	0.20	0.43	0.56	0.57	0.70
0.12	0.09	0.09	0.03	0	0.50	0.33	0.33	0.13	0.38	0.56	0.56	0.75
0.12	0.09	0.09	0.03	0	0.50	0.33	0.33	0.13	0.38	0.56	0.56	0.75
0.16	0.06	0.13	0.03	0	0.50	0.44	0.25	0.17	0.40	0.44	0.63	0.67
0.06	0.09	0.09	0.03	0	0.33	0.33	0.30	0.13	0.50	0.56	0.60	0.75
0.06	0.06	0.28	0.03	0	0.25	0.63	0.29	0.17	0.63	0.31	0.57	0.67
0.06	0.06	0.28	0.03	0	0.25	0.63	0.29	0.17	0.63	0.31	0.57	0.67