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PELTIGERA PHYLLIDIOSA (*PELTIGERACEAE*, ASCOMYCOTINA), A NEW SPECIES FROM THE SOUTHERN APPALACHIANS CORROBORATED BY ITS SEQUENCES

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Abstract: The new species *Peltigera phyllidiosa* Goffinet & Miądlikowska from the Southern Appalachians, eastern U.S.A., is closely related to *P. collina* and *P. neckeri*. Like these species it has a glabrous upper cortex and black fingernail- or saddle-shaped apothecial discs, but differs in its laminal phyllidia. Variation in nucleotide sequences of the Internal Transcribed Spacer (ITS) of the nrDNA repeat region correlates with the presence or absence of phyllidia, supporting the distinction of *P. phyllidiosa* from *P. collina* and *P. neckeri*.

Introduction

The genus *Peltigera* is represented by 29 taxa in North America (Thomson 1950; Goward *et al.* 1995). Among glabrous species, three are characterized by the presence of phyllidia, namely *P. degenii* Gyeln., *P. elisabethae* Gyeln. and *P. pacifica* Vitik., which are easily distinguished on morphological and chemical grounds (Vitikainen 1985, 1994). While examining the collections of *Peltigera* at DUKE, the senior author found three sterile, glabrous, phyllidiate specimens identified as either *P. polydactylon* (Necker) Hoffm. or *P. praetextata* (Sommerf.) Zopf. Phyllidia are *a priori* incongruent with *P. polydactylon*, and although glabrous specimens of *P. praetextata* are occasionally found, the above specimens differ from the latter species, by the presence of terpenoids and tridepsides (Vitikainen 1994; Goward *et al.* 1995). The chemistry of these phyllidiate samples is identical, among sympatric *Peltigerae*, to that of *P. horizontalis* (Huds.) Baumg. and *P. elisabethae*, which can both be distinguished, among other traits, by their fasciculate rhizines that are arranged in concentric rows.

Subsequent field explorations in Alabama, Georgia, North Carolina and Missouri led to the collection of additional glabrous phyllidiate specimens, including fertile thalli. The black and fingernail- or saddle-shaped apothecia suggest a relationship to the allopatric *P. collina* (Ach.) Schrad. and *P. neckeri* Müll. Arg., both of which lack phyllidia. Except for the vegetative propagules, the phyllidiate thalli are morphologically identical to *P. collina*. Correlations

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TABLE 1. *Voucher information for collections included in the molecular analysis, and GenBank accession number for the ITS sequences obtained for* Peltigera *samples*

Taxon	Voucher*	GenBank accession number
P. phyllidiosa 1	Georgia: *Goffinet* 4561	AF074975
P. phyllidiosa 2	North Carolina: *Goffinet* 4629	idem *P. phyllidiosa* 1
P. phyllidiosa 3	Missouri: *Reeb* 6/97	idem *P. phyllidiosa* 1
P. collina 4	British Columbia: *Goward* 91–2074	AF074976
P. collina 5	British Columbia: *Goward* 96–317a	idem *P. collina* 4
P. collina 6	Greenland: *Hansen* (Lichenes Groenlandici Exsiccati 493)	idem *P. collina* 4
P. collina 7	Austria: *Hafellner & Hafellner* (Vezda Lichenes Selecti Exsiccati 2439)	AF108142
P. collina 8	Washington State: *Davis* 2278 (UBC)	AF074977
P. collina 9	South Korea: *Park* 2581	AF074978
P. neckeri 10	Poland: *Miądlikowska* UGDA-L 5240	AF075725

*Specimens in DUKE, unless otherwise stated.

between the presence of phyllidia, variation in secondary metabolites and nucleotide sequences in the Internal Transcribed Spacer (ITS) region of the nuclear repeat coding for ribosomal RNA genes are examined here.

Materials and Methods

This study incorporates material deposited at DUKE, F, UBC, UGDA-L and the personal herbarium of the senior author.

Chemical analyses

Terpenoids and tridepsides were extracted in warm acetone and loaded on glass plates coated with silica gel (Merck F_{256}). Thin-layer chromatography (TLC) was performed in solvents A, B′, and C following Culberson & Ammann (1979) and Culberson & Johnson (1982).

DNA extraction, amplification and sequencing

Thallus fragments (restricted to lobe margins) were removed from fresh or dried herbarium collections of the phyllidiate thalli, as well as of *P. collina* and *P. neckeri* (Table 1). DNA extraction followed a modification of Doyle and Doyle's (1987) method (Goffinet *et al.* 1998). The ITS region was amplified using the universal primer ITS1 and ITS4 (White *et al.* 1990), and the Polymerase chain reaction (PCR) protocol followed Goffinet & Goward (1998). The double-stranded templates were sequenced using the Dye Terminator Cycle Sequencing Kit (Perkin Elmer), and the resulting fragments were separated on polyacrylamide gels (Long Ranger Singel™ FMC BioProducts) using a ABI Prism™ 377 DNA Sequencer (Perkin Elmer). Sequences obtained were edited using Sequencher 3·0 (Gene Codes Corporation), and manually aligned in PAUP 3·1 (Swofford 1993). The fungal nature of the amplified locus was checked by comparing the sequences of the 5·8S gene obtained to those gathered previously by using fungal-specific primers (see Goffinet & Bayer 1997).

Delimitation of the spacer and coding regions was achieved by comparing the obtained sequences with fungal sequences available from GenBank and by comparisons with secondary structure models for the genes coding for the small and large subunit of the ribosomal RNA. Representative sequences of individual genotypes were submitted to GenBank (see Table 1).

Results

Morphology

Glabrous *Peltigerae* are represented in the Southern Appalachians by *P. elisabethae* (defined by a schizidiate upper surface, a lower surface lacking distinct veins and with rhizines arranged in concentric lines), *P. horizontalis* (defined by plane apothecial disc, distinct veins, and rhizines arranged in concentric lines), *P. neopolydactyla* (Gyeln). Gyeln. s. lat. (typically defined by saddle-shaped apothecia, broad lobes, broad veins, long simple rhizines), *P. polydactylon* (defined by saddle-shaped apothecia, rather narrow lobes with a crisped margin, and distinct narrow veins) and a group of samples with randomly arranged short rhizines and laminal or marginal phyllidia. *Peltigera evansiana* Gyeln., which has a scanty tomentum restricted to the margin and may thus appear glabrous, is characterized by its granular to dorsiventral laminal isidia and lack of terpenoids and tridepsides.

Chemistry

All specimens bearing phyllidia share the following array of terpenoids and tridepsides: tenuiorin, methyl gyrophorate and gyrophoric acid (both generally in trace amounts), zeorin and one unidentified compound turning yellow after acid treatment and being heated. Sympatric *P. horizontalis* and *P. elisabethae* are characterized by the same set of chemicals, except for one population of the former, which has one additional unidentified compound (*Goffinet* 4566, DUKE). By contrast, randomly selected populations of sympatric *P. neopolydactyla* s. lat (DUKE) of the Southern Appalachians have either dolichorrhizin and/or peltidactylin in addition to zeorin and tenuiorin. The chemistry of *P. collina* among North American, European and Asian specimens varied in the presence or absence of various terpenoids, particularly peltidactylin. The latter was absent in at least two North American collections (*Culberson* 16476 from Arizona, and *Weber* 9796 from Colorado, DUKE), as well as from the single Asian population studied (*Park* 2581, DUKE).

DNA analysis

The amplification using universal primers yield a single product per reaction, of about 600 nucleotides (nts). Sequencing reactions using these terminal primers yielded a clear chromatogram with low background signal for all samples analysed. The size of the ITS region varied between 579 nts (*P. phyllidiosa*) and 588 nts (*P. neckeri*), and alignment of the sequences required the insertion of 14 gaps. The sequences of the 5·8 S gene were identical in size and completely alignable with sequences of other *Peltigerae* obtained using fungal specific primers (Goffinet & Bayer 1997). All but one point mutations and all insertions or deletions were confined to the spacer regions (Tables 2 and 3). The ITS sequences are identical among phyllidiate samples but vary between these and *P. neckeri* and *P. collina*, as well as within the latter species (Table 3). European and North American individuals of *P. collina* show no or little differentiation among them, but deviate by 12 to 13 base changes from the South Korean sample. This Asian specimen was furthermore more similar

TABLE 2. *Length (in nucleotides) of the ITS1, 5·8S, and ITS2 in all sequenced populations of* Peltigera phyllidiosa, P. collina *and* P. neckeri

Taxon	ITS1	5·8S	ITS2	Total
P. phyllidiosa 1/2/3	185	157	237	579
P. collina 4/5/6/7	188	157	237	582
P. collina 8	188	157	239	584
P. collina 9	191	157	237	585
P. neckeri 10	192	157	239	588

TABLE 3. *Distribution of variable sites and gaps within the alignment of the ITS region among all sampled populations of* Peltigera phyllidiosa, P. collina *and* P. neckeri

	ITS1	5·8S	ITS2	Total
Variable	19	1	15	35
Gaps	9*	0	8‡	17

*including one site where the insertion has a point mutation between *P. collina* and *P. neckeri* and is thus counted once as a site with a gap, as well as a site with character-state variation.
‡contiguous gaps at sites 355 & 356 are counted as one.

TABLE 4. *Pairwise comparisons of ITS sequences between all populations of* Peltigera phyllidiosa, P. collina, *and* P. neckeri*

Taxon	1/2/3	4/5/6	7	8	9	10
P. phyllidiosa 1/2/3	–	6	6	6	12	10
P. collina 4/5/6	25	–	0	1	7	7
P. collina 7	26	1	–	1	7	7
P. collina 8	25	0	1	–	8	9
P. collina 9	26	12	13	12	–	5
P. neckeri 10	22	10	11	10	8	–

*Above diagonal: absolute distance when gaps only are considered (contiguous gaps are counted as single events). Below diagonal: absolute distances for alignable sequences with gaps excluded.

in its nucleotide sequence to *P. neckeri*, represented here by a Polish collection. Absolute distances between these samples vary between 0 and 26 point mutations, with an additional 0 to 12 differences due to insertions or deletions (Table 4).

In view of the distinctions described above, the Appalachians specimens are recognized as:

FIG. 1. *Peltigera phyllidiosa* (Isotypus, *Goffinet* 4561). Habit (× 3·8). Note the laminal and marginal phyllidia, and the upturned elongated lobe with a black apothecial disc (only recurved margin seen).

Peltigera phyllidiosa Goffinet & Miądlikowska sp. nov.

Peltigerae collinae affinis. Thallus etomentosus, glaber; lobi 0·5–2·0 cm lati, isidiato-phyllidiati. Apothecia revoluta nigra; Ascosporae aciculares, triseptatae. Pycnidia ignota.

Typus: United States of America, Georgia, Rabun County, Southern Appalachians, southeast escarpment of the Southern Blue Ridge, Chattahoochie National Forest, 34°58′N, 83°18′W, lower portion of trail from parking area to top of mountain, mountain slope with mixed hardwood forest with *Betula lenta*, *Quercus rubra* and *Q. montana*, and *Magnolia*, base of *Quercus*, 4 October 1997, *Goffinet* 4561 (DUKE—holotypus; herb. Goffinet, NY, H—isotypi).

(Fig. 1)

Thallus 5–8 cm in diameter, grey to greyish-brown when dry. Lobes 0·5–2·0 cm broad, plane to concave with reflexed or rarely plane or erect margins. *Phyllidia* present along margin or laminal cracks, branched, becoming dorsiventral, spreading laterally, forming small 'cushions' of squamules. *Soredia* absent. *Upper surface* smooth, shiny, pruinose near tip of lobes, rarely maculate toward apex, glabrous. *Lower surface* reticulate, veins distinct, flat to bulging, slightly raised above the interstices, dark brown to pale toward margin; interstices elliptic, white; rhizines simple, rarely fasciculate, up to 5 mm long, concolourous to veins. *Cortex* paraplenchymatous, up to 45 μm thick, photobiontic layer containing cyanobacteria (*Nostoc*) up to 75 μm thick, medulla up to 120 μm thick, and veins up to 150 μm thick. *Pycnidia* not seen.

Apothecia on short elongate lobes, disc black, scabrose, with either strongly recurved margins or saddle-shaped, up to 7 mm long. *Paraphyses* linear

simple, with swollen apical cells. *Asci* clavate, 8-spored. *Spores* acicular, triseptate 40–71 × 2·0–3·5 μm wide.

Chemistry. Tenuiorin, methyl gyrophorate and gyrophoric acid (both often in trace amounts), zeorin, and one unidentified compound turning yellow after treatment with 10% sulphuric acid and being heated (R_f classes 5, 5, 5–6 in solvents A, B′, C).

Ecology. Peltigera phyllidiosa is currently known only from mesophytic forests, where it grows over mosses at the base of deciduous trees or on boulders. It extends from upper montane (*Dey* 5313, DUKE) to Piedmont forests (*Robinson* s.n., DUKE) in North Carolina, northern Georgia and adjacent northern Alabama.

Distribution. Known only from eastern North America, where it is restricted to the Southern Appalachians (Alabama, Georgia, Missouri and North Carolina).

Remarks. Phyllidia are characteristic of various *Peltigerae* such as *P. andensis* Vitik., *P. continentalis* Vitik., *P. degenii*, *P. elisabethae*, *P. pacifica*, or *P. praetextata*. *Peltigera phyllidiosa* differs from these by its glabrous upper cortex (versus *P. continentalis* and *P. praetextata*), the presence of terpenoids and tridepsides (versus *P. degenii*), the shape and colour of the apothecia, as well as the lack of peltidactylin and dolichorrhizin (versus *P. pacifica*) and the distinctly veined lower surface and the shape of the apothecia (versus *P. andensis* and *P. elisabethae*). Using Gyelnik's key to the isidiate *Peltigerae* (Gyelnik 1931), phyllidiate specimens from the Southern Appalachians would key out as *P. microphylla* (Anders) Gyeln., now considered conspecific with *P. elisabethae* (Vitikainen 1994). Sterile specimens of *P. phyllidiosa* are reminiscent of lobulate forms of *P. horizontalis* (Goffinet *et al.* 1995). Such forms of the latter were recognized by Gyelnik (1927) as a distinct species, *P. zopfii* Gyeln., a concept that has not withstood recent critical studies (Vitikainen 1994). The type of *P. zopfii* (W!) has rhizines arranged along concentric lines and, furthermore, bears apothecia that are horizontal, indicating that this taxon is thus clearly distinct from *P. phyllidiosa*.

Peltigera phyllidiosa is easily distinguished among eastern North American *Peltigerae* by its glabrous upper cortex, which is lined along its margins and cracks by phyllidia. The sympatric *P. evansiana* (*Goffinet* 5179, DUKE) differs by the terete to somewhat dorsi-ventral and branched laminal isidia (Goffinet & Hastings 1994). At maturity the black apothecia of *P. phyllidiosa* terminate in short elongate lobes, a characteristic of *P. collina* and *P. neckeri*. Distinctive features of the latter include a nearly continuous black lower surface (confluent or diffuse veins) with few interstices, and a chemical profile that includes dolichorrhizin, zeorin as well as several other terpenoids (Holtan-Hartwig 1993; Goffinet *et al.* 1995). *Peltigera collina* is morphologically (except for the vegetative propagules) as well as chemically nearly identical with *P. phyllidiosa*. In both species the lobes are often pruinose, but unlike in *P. collina*, where they

can also be scabrose (Holtan-Hartwig 1993; Vitikainen 1994), the laminal apices are always smooth in *P. phyllidiosa*. One collection of *P. collina* from the Pacific coast of Washington (*Davis* 2278, UBC) has a seemingly dentate margin. These mostly unbranched 'isidia' appear dorsiventral at first, but soon become terete and knobby, with only few being somewhat branched. Whether these would ultimately develop into soredia or represent developing regeneration or stress lobules is not clear. The terpenoids and tridepsides in this thallus compose a chemotype similar to other western North American populations (i.e., tenuiorin, methyl gyrophorate, gyrophoric acid [both in trace amounts], zeorin, peltidactylin [in trace amounts] and an unidentified compound reacting yellow to consecutive acid and heat treatments). Finally, the ITS sequence of the lichenized fungus of this collection is identical to that of other *P. collina* samples included here, suggesting that this population should be retained in *P. collina*.

Chemically, *P. phyllidiosa* differs from known chemotypes of *P. collina* by invariably lacking peltidactylin and dolichorrhizin. Either or both of these accompany zeorin in *P. collina* (White & James 1987; Vitikainen 1994; Martínez *et al.* 1997; Miądlikowska & Holtan-Hartwig 1997). Even Norwegian specimens of *P. collina*, for which zeorin was the only reported triterpenoid (Holtan-Hartwig 1993), are now known to produce peltidactylin also, either as a major compound or in trace amounts (Miądlikowska & Holtan-Hartwig, unpubl.). A preliminary survey of chemical profiles for a set of North American, European and Asian samples of *P. collina* (DUKE) confirmed that peltidactylin is present, albeit often in trace amounts, in most of these (Goffinet, unpubl.), but also revealed that some populations lack peltidactylin and also dolichorrhizin (e.g., *Culberson* 16476, *Weber* 9796, and *Hansen* Exs. 493, DUKE), and are thus chemically identical with *P. phyllidiosa*, from which they differ by the marginal soredia.

The three samples of *P. phyllidiosa*, representing three geographically distinct populations, show no differences in their ITS sequences, but significant differentiation from *P. collina* samples from North America and Europe, suggesting that the groups of phyllidiate or sorediate populations should be considered distinct taxonomic entities. This genetic pattern is indeed consistent with that observed for two putatively related species of *Ramalina*, *R. panizzei* and *R. fastigiata* (Groner & LaGreca 1997). The only Asian collection of *P. collina* included here (*Park* 2581, DUKE; Park 1990) differs from conspecific individuals by as many as 13 point mutations and 12 indels in the spacer sequences. Since the coding sequence, the 5·8 S gene, is identical with all other populations of *P. collina*, contamination of the DNA of the South Korean sample could be excluded. Bearing soredia, this individual is *a priori* identical in morphology to typical *P. collina*, while its chemistry is identical to that of one chemotype of *P. collina* and to that of *P. phyllidiosa*. Whether the Asian specimen is indicative of another genotypic lineage of *P. collina*, a lineage marking the transition between *P. phyllidiosa* and the *P. collina* clade, needs to be examined further by more extensive sampling of populations of *P. collina* and *P. neckeri*.

Holtan-Hartwig (1993) tentatively included *P. collina* in the *P. scabrosa* group, on the basis of the often scabrose lobe tips. Inferring evolutionary

relationships from reproductive structures (i.e., apothecia) may instead support close affinities of *P. collina* to *P. neckeri*, as both taxa share a typically black, fingernail- or saddle-shaped disc produced on short lobes. Features of the apothecia are *a priori* not less prone to homoplasy (Vitikainen 1995), and thus not necessarily more informative than vegetative (including chemical) traits. Phylogenetic reconstruction of the *Peltigerae* based on rDNA sequences supports, however, close affinities between *P. phyllidiosa*, *P. collina* and *P. neckeri* (Miądlikowska & Lutzoni, unpubl.), as indicated by apothecial features. Based on comparisons of ITS sequences among these taxa (Table 4), *P. collina* appears more similar to *P. neckeri* than to *P. phyllidiosa*, a hypothesis also confirmed by an analysis of nuclear ribosomal RNA encoding genes for a broad sample of *Peltigerae* (Miądlikowska & Lutzoni, unpubl.).

Correlations between morphological and genetic characters support the hypothesis that the phyllidiate specimens belong to a distinct taxon, here considered a species. Differing only in the nature of their vegetative propagules, *P. phyllidiosa* and *P. collina* may represent sibling species (Culberson 1986). As any biological unit, sibling species should not be considered *a priori* static evolutionary entities, and may thus undergo subsequent cladogenesis. A morphological and chemical identity of *P. phyllidisa* and *P. collina*, except for the presence of phyllidia versus soredia, does therefore not imply that these are sister species but merely that they share—albeit not exclusively—a common ancestor. Tehler (1982) argued that recognizing taxa on the sole basis of their reproductive mode (i.e., asexual versus sexual) could obscure evolutionary relationships, an argument supported recently by ITS sequence data analyses of sterile and fertile specimens of *Dendrographa leucophaea* (Lohtander *et al.* 1998). Alternatively Goffinet and Goward (1998) argued in favor of sibling speciation in the case of *Lobaria silvae-veteris* (Goward & Goffinet) Goward & Goffinet and *L. oregana* (Tuck.) Müll. Arg. on the basis of morphological difference, and despite these taxa sharing identical chemistries and ITS sequences. Clearly the basis for a species pair hypothesis may vary between taxa, and the concept as a whole should not be accepted or rejected universally. Indeed, if the phylogenetic hypothesis of *P. phyllidiosa* being sister to the *P. collina-P. neckeri* pair is correct (see Miądikowska & Lutzoni, unpubl.), then a failure to recognize *P. phyllidiosa* on the basis of it being identical to *P. collina* except for its phyllidia would lead to a paraphyletic taxon as suggested by nucleotide sequences, and thus obscure character transformations within this group.

Dey (1978) reported eight lichens as strictly endemic to the Appalachians; one of these, namely *Xanthoparmelia monticola* (J. P. Dey) Hale has recently been reported from Mexico (Hale 1990). The total distribution of these taxa, of which most appear more commonly at higher elevation in the Southern Appalachians, extends further north than the current limit of *P. phyllidiosa*. The presence of a population of *P. phyllidiosa* at higher elevation in the Southern Appalachians may suggest that this species has a broader distribution in the Appalachians, which may perhaps be revealed upon careful examination of eastern North American populations of *P. polydactylon*, particularly.

Other specimens examined (all at DUKE unless otherwise indicated): U.S.A.: *Alabama*: Dekalb Couny, Buck's Pocket State Park, primitive campground trail to Point Rock, mixedwood forest with sandstone boulders, along South Santy Creek, 180–250 m alt., 1998, *Goffinet* 5207, 5221 (DUKE, hb. Goffinet). *Missouri*: Oregon County, Mark Twain National Forest, McCormack Lake Recreation Area, in lower part of McCormack Hollow and adjacent Gasconade Dolomite bluff along N side of Eleven Point River E of McCormack Hollow, S of McCormack Lake, 36°48–49′N, 91°21′W, 180–280 m elev. (Greer USGS 7·5′ quad), 1997, *Reeb* 6/97 (F). *North Carolina*: Aveny County, E of Linville, Blue ridge Parkway, S of crossing of US 221, trail going N of Linn Cove viaduc information center, W of Parkway, mixedwood forest with *Rhododendron*, base of *Quercus* beginning of trail, 1998, *Bernard Goffinet* 5186, *François* and *Louis Goffinet* (hb. B. Goffinet); Macon County, Southern Appalachians, along Buck Creek Rd, 2·85 miles from the intersection with US64, which is ± 10·1 miles from the intersection of NC106 and US64 at Highlands. Serpentine barren, steep ravine along Buck Creek with mixed mesophytic forest, 1997, *Goffinet* 4621, 4629, 4630 (LG), 4631 (hb. Goffinet), 4636; Durham County, Farrington Road, base of tree, 1958, *Robinson* s.n.; Haywood County, Stair Mountain in the Balsam Mountains, Fire Cherry Community, ± 1930 m, on *Quercus*, 1972, *Dey* 5313; Stanly County, near Stamfield, at the base of *Quercus alba*, 1958, *Culberson* 7116; Yancey County, Blue Ridge Parkway, mile post 340, Crabtree Meadows, trail to Crabtree falls, 35°38′15″N, 81°22′25″W, ± 1000 m alt., base of tree, 1998, *B. Goffinet* 5197, *François*, & *Louis Goffinet* (hb. B. Goffinet) and idem, on rock *Bernard Goffinet* 5198 & *François* and *Louis Goffinet* (DUKE, hb. B. Goffinet, UBC).

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