Disentangling the *Peltigera polydactylon* Species Complex by Recognizing Two New Taxa, *P. polydactylon* subsp. *udeghe* and *P. seneca*

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Source: Herzogia, 29(2):514-528.
Published By: Bryological and Lichenological Association for Central Europe
DOI: http://dx.doi.org/10.13158/heia.29.2.2016.514
URL: http://www.bioone.org/doi/full/10.13158/heia.29.2.2016.514

Disentangling the *Peltigera polydactylon* species complex by recognizing two new taxa, *P. polydactylon* subsp. *udeghe* and *P. seneca*

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Abstract: Magain, N., Sérusiaux, E., Zhurbenko, M. P., Lutzoni, F. & Miadlikowska, J. 2016. Disentangling the *Peltigera polydactylon* species complex by recognizing two new taxa, *P. polydactylon* subsp. *udeghe* and *P. seneca*. – Herzogia **29**: 514–528.

A new species, *Peltigera seneca*, and two subspecies within *P. polydactylon* (s.str.), *P. polydactylon* subsp. *udeghe* and *P. polydactylon* subsp. *polydactylon*, were segregated from a broadly defined *P. polydactylon* s.lat., based mostly on molecular data and distinct geographical ranges. *Peltigera polydactylon* s.str. and *P. seneca* form well-supported monophyletic lineages that share a most recent common ancestor and were recognized as two distinct species by multiple species delimitation and validation methods. *Peltigera polydactylon* s. str. has a broad intercontinental distribution whereas the new species *P. seneca* is restricted to eastern North America where it seems to be rare. *Peltigera polydactylon* subsp. *udeghe* and *P. polydactylon* subsp. *polydactylon*, were defined to accommodate two monophyletic and strongly supported clades separated geographically (North America, eastern Northern Asia and Australasia versus Europe, Middle East and central Northern Asia, respectively). Despite the low genetic distance, especially for the ITS, between these two subspecies, they are well segregated genetically throughout their allopatric ranges. However, there seems to be an intermediary pattern of variation in the geographical area where both taxa are likely to co-occur (e.g., central and eastern Northern Asia). Phenotypic traits have limited value in distinguishing these three taxa. They are chemically (secondary metabolites) similar and share the same *Nostoc* (cyanobiont) phylogroup. Nevertheless, there are helpful phenotypic trends in addition to their diagnostic genotypes.

Zusammenfassung: Magain, N., Sérusiaux, E., Zhurbenko, M. P., Lutzoni, F. & Miadlikowska, J. 2016. Die Entwirrung des *Peltigera polydactylon*-Artenkomplexes durch die Erkennung zweier neuer Taxa, *P. polydactylon* subsp. *udeghe* und *P. seneca*. – Herzogia **29**: 514–528.

Eine neue Art, *Peltigera seneca*, und zwei Unterarten innerhalb von *P. polydactylon* (s.str.), *P. polydactylon* subsp. *udeghe* und *P. polydactylon* subsp. *polydactylon*, wurden von der breit definierten Art *P. polydactylon* s.lat. abgespalten, basierend vor allem auf molekularen Daten und unterschiedlicher geographischer Verbreitung. *Peltigera polydactylon* s.str. und *P. seneca* bilden gut unterstützte, monophyletische Abstammungslinien, die einen letzten gemeinsamen Vorfahren miteinander teilen, und sie wurden als zwei eigene Arten mit multipler Artendelimitierungs- und Validierungsmethoden abgegrenzt. *Peltigera polydactylon* s.str. hat eine weite interkontinentale Verbreitung, wohingegen die neue Art *P. seneca* auf das östliche Nordamerika begrenzt ist, wo sie selten vorzukommen scheint. *Peltigera polydactylon* subsp. *udeghe* und *P. polydactylon* subsp. *polydactylon* wurden definiert, um zwei monophyletische und stark unterstützte Clades abzugrenzen, die weitgehend getrennte geographische Verbreitungsmuster aufweisen (Nordamerika, östliches Nordasien und Australasien bzw. Europa, Naher Osten und zentrales Nordasien). Trotz der geringen genetischen Distanzen zwischen diesen beiden Unterarten, insbesondere für die ITS-Region, sind sie genetisch in den allopatrischen Verbreitungsgebieten überall gut abgegrenzt voneinander. Es scheint jedoch ein intermediäres Muster der Variation dort zu geben, wo beide Arten sympatrisch vorkommen (d.h. im zentralen und östlichen Nordasien). Anhand von phänotypischen Eigenschaften lassen sich die drei Taxa nur mit Einschränkungen

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voneinander abgrenzen. Sie sind chemisch ähnlich (anhand von Sekundärmetaboliten) und enthalten dieselbe *Nostoc* (Cyanobiont) Phylogruppe. Trotzdem gibt es zusätzlich zu den diagnostischen Genotypen hilfreiche phänotypische Trends.

Key words: Cyanolichens, lichen-forming fungi, *Nostoc*, phylogeny, species discovery and validation, taxonomy.

Introduction

Peltigera polydactylon (Neck.) Hoffm. sensu lato belongs to the polydactyloid clade (Magain et al. 2016) within section *Polydactylon* (one of eight sections recognized in the genus *Peltigera* by Miadlikowska & Lutzoni 2000), and includes bi-membered macrolichens with non-tomentose upper thallus surface that are associated with cyanobionts from the genus *Nostoc*. All putative species in the polydactyloid clade were reported exclusively from Asia and Australasia (Sérusiaux et al. 2009, Magain et al. 2016) with the exception of *P. polydactylon*, which is known to be broadly distributed in North America and Europe (Martinez et al. 2003). The most recent worldwide molecular phylogenetic study of section *Polydactylon* was based on five nuclear loci (Internal Transcribed Spacer region [ITS], nuclear ribosomal RNA-coding large subunit [nrLSU], β-tubulin, RNA polymerase II largest subunit [*RPB1*] and elongation factor 2 region 1 [*EFT2*.1]; Magain et al. 2016). It revealed the presence of at least 38 monophyletic putative species (20 of which are new) representing predominantly morphologically cryptic entities with restricted geographic distributions, many of which are known to associate with only one or two *Nostoc* phylogroups.

Magain et al. (2016) used a consensus approach to define species, based on the results of species discovery methods and phylogenetic affiliations of mycobionts supplemented with cyanobiont identity data (*Nostoc* phylogroups delimited using the *rbc*LX region) and geographic records for both symbionts. The authors demonstrated with high confidence that *P. polydactylon* represents a species complex consisting of three monophyletic groups sharing a most recent common ancestor (Fig. 3 in Magain et al. 2016). The first split supports a potentially new species (provisionally named *P*. sp. 10) sister to *P. polydactylon* s. str., which includes two strongly supported sister clades. Their Structurama analysis based on a multilocus dataset indicated that the two lineages within *P. polydactylon* s. str. represent distinct species whereas bGMYC analysis performed on the ITS alone considered them as a single species. A follow-up phylogenetic study by Magain et al. (unpubl.) based on additional markers (i.e., newly developed Collinear Orthologous Regions; COR markers), and more extensive species delimitation and validation analyses, as well as morphological examinations of a broader taxon sampling, showed that specimens from *P. polydactylon* s. str. in North America and Europe seem to be well isolated from each other and correspond to two distinct lineages, namely *P. polydactylon* 1 and *P. polydactylon* 2 (recognized as species by Structurama, bPTP, and bPP based on multilocus data) but no material from Northern Asia was included. Although Magain et al. (2016) used 54 collections from *P. polydactylon* s. str. for which the ITS was sequenced, multilocus sequencing was performed on a limited number of specimens (five specimens from Europe and four specimens from North America for *P. polydactylon* 1 and *P. polydactylon* 2, respectively). To better assess the taxonomic status of these two sister clades within *P. polydactylon* s. str. (i.e., two species versus one species with two intraspecific taxa), we increased the sampling, including new geographic areas (Australia, New Zealand, and Northern Asia), for a total of 16 specimens for which at least three loci were sequenced (Table 1).

Based on the newly acquired molecular data and performed phylogenetic analyses we concluded that specimens traditionally assigned to *P. polydactylon* s. lat. represent currently two

species: *P. polydactylon* s. str. and its sister newly recognized species, *P. seneca* (sp. nov. corresponding to *P.* sp. 10 in Magain et al. 2016), which is formally described here. Furthermore, two subspecies: *P. polydactylon* subsp. *polydactylon* [corresponding to *P. polydactylon* 1 in Magain et al. (unpubl.)] and *P. polydactylon* subsp. *udeghe* [subsp. nov. corresponding to *P. polydactylon* 2 in Magain et al. (unpubl.)] are proposed and formally introduced to accommodate the two mostly allopatric clades within *P. polydactylon* s. str.

Materials and methods

Sequences for seven loci (ITS, nrLSU, β-tubulin, *RPB1*, COR1b, COR3, and COR16) for nineteen representatives of *P. polydactylon* s. lat. were obtained (Table 1) and analyzed. A total of 34 new sequences from seven loci were added to the dataset assembled by Magain et al. (unpubl.; Table 1). For molecular data generation, alignments, phylogenetic and species delimitation analyses, see Magain et al. (2016). Pairwise distance matrices (uncorrected distances) were calculated using PAUP* v4.0a147 (Swofford 2003). Phylogenetic analyses on each locus separately and on a 7-locus concatenated dataset (ITS, nrLSU, *RPB1*, β-tubulin, and the three new COR markers; Magain et al. (unpubl.) were performed using RAxML v.7.4.2 with the GTRGAMMA model (Rodríguez et al. 1990) and 1000 bootstrap pseudoreplicates (Stamatakis et al. 2006) applied independently on four partition subsets estimated using PartitionFinder (greedy algorithm to explore all nucleotide substitution models under the BIC selection criterion; Lanfear et al. 2012) for the latter analysis. Thin Layer Chromatography (TLC) was performed on ten specimens from *P. polydactylon* s. lat. following Orange et al. (2010) and using solvents C and G. The distribution map was generated using R (R Development Core Team) and the package maps v. 3.1 (Becker & Wilks 1993, Brownrigg 2016).

Results

Phylogenetic analyses of the expanded dataset (Fig. 1) revealed *P. polydactylon* s. str. to consist of two previously recognized and strongly supported lineages corresponding to *P. polydactylon* 1 and 2 in Magain et al. (unpubl.). Both clades are geographically distinct and split specimens occurring in Europe and central Northern Asia (Krasnoyarsk Territory of Russia) from individuals collected in North America, Australia, and eastern Northern Asia (Khabarovsk Territory of Russia), supporting the existence of two species. However, in Northern Asia, P1541 representing *P. polydactylon* 1 from Krasnoyarsk Territory, and P3012 and P3033 representing *P. polydactylon* 2 from Khabarovsk Territory (Fig. 1) seem to harbor evidence of gene flow and show lower genetic distances among the putatively sympatric populations from the two clades. Pattern of variations in the *RPB1* sequences of these three specimens was intermediary between the European-Middle Eastern and North American populations, whereas the ribosomal genes (ITS and nrLSU) of all individuals examined, show four point mutations differences between the clades (Fig. 2). Based on this possible occurrence of gene flow between *P. polydactylon* clade 1 and 2, we propose to recognize the North American and Australasian populations of *P. polydactylon* s. str. as a new subspecies, *P. polydactylon* subsp. *udeghe*, separated from the European *P. polydactylon* subsp. *polydactylon*. We also provide a formal description of *P. seneca*, which represents the first divergence event within *P. polydactylon* s. lat. Overall thalli habits and selected morphological features of each taxon are shown in figure 3A–3H. Additional images are available online as part of the Lutzoni Lab website/*Peltigera* project/*P. neopolydactyla* complex (http://lutzonilab.org/peltigera/project/).

Table 1: Voucher information and GenBank accession numbers for sequences used in this study. In bold are shown newly added sequences not included in Magain et al. (2016). – Abbreviations: BC = British Columbia, MI = Michigan, MT = Montana, NC = North Carolina, NM = New Mexico, NS = Nova Scotia, PA = Pennsylvania.

ID	Species/ssp.	Voucher	ITS	nrLSU	b-tubulin	*RPB1*	COR1b	COR3	COR16	Number of loci
P3022	*Peltigera polydactylon* subsp. *udeghe*	Australia, H. Streimann 43811 (H)	**KX365427**	**KX365434**	**KX365421**	---	---	---	---	3
P1911	subsp. *udeghe*	Australia, K. & A. Kalb 21797 (DUKE)	**KX365428**	---	**KX365422**	---	**KX365480**	---	---	3
P3012	subsp. *udeghe*	Russia, Khabarovsk Territory, J. Miadlikowska & F. Lutzoni 07.30.2013-P3012 (DUKE)	**KX365429**	**KX365435**	**KX365423**	**KX365439**	**KX365481**	---	**KX373624**	6
P3033	subsp. *udeghe*	Russia, Khabarovsk Territory, J. Miadlikowska & F. Lutzoni 07.30.2013-P3033 (DUKE)	**KX365430**	**KX365436**	**KX365424**	**KX365440**	**KX365482**	---	---	5
P3015	subsp. *udeghe*	USA, MI, J. Miadlikowska & F. Lutzoni 06.27.2013-P3015 (DUKE)	KX365469	KX365473	---	KX365477	---	---	---	3
P71	subsp. *udeghe*	USA, NM, J. Hollinger 2462 (UBC)	KX365444	KX365452	KX365464	KX365459	KX365486	KX373618	KX373628	7
P1234	subsp. *udeghe*	USA, MT, B. McCune 29108 (OSU)	KX365445	KX365453	KX365465	KX365460	---	---	KX373629	5
P3052	subsp. *udeghe*	Canada, BC, J. Hollinger 1505 (UBC)	KX365470	KX365474	KX365476	KX365478	KX365487	KX373619	KX373630	7
P1541	subsp. *polydactylon*	Russia, Krasnoyarsk Territory, J. Miadlikowska & F. Lutzoni 06.26.2012-P1541 (DUKE)	**KX365431**	**KX365437**	---	**KX365441**	**KX365483**	**KX373617**	**KX373625**	6
P3018	subsp. *polydactylon*	Turkey, K. Yazici s.n. (H)	**KX365432**	---	**KX365425**	**KX365442**	**KX365484**	---	**KX373626**	5
P3021	subsp. *polydactylon*	Turkey, K. Yazici s.n. (H)	KX365471	**KX365438**	---	**KX365443**	KX365488	KX373620	KX373631	6
P856	subsp. *polydactylon*	Iran, A. A. Maassoumi 573 (B)	KX365446	KX365454	KX365466	---	---	---	---	3
N2069	subsp. *polydactylon*	Norway, J. Holtan-Hartwig 528 (O)	KX365447	KX365455	KX365467	---	---	---	---	3
P385	subsp. *polydactylon*	Norway, N. Magain s.n. (LG)	KX365448	KM005765	KM005820	KM005994	KX365489	KX373621	KX373632	7
P388	subsp. *polydactylon*	Norway, N. Magain s.n. (LG)	KX365449	KX365456	KX365468	KX365461	---	---	---	4
P849	subsp. *polydactylon*	Iceland, H. Kristinsson s.n. (AMNH)	**KX365433**	---	**KX365426**	---	**KX365485**	---	**KX373627**	4
P1652	*P. seneca*	Canada, NS	KX365450	KX365457	---	KX365462	---	---	---	3
P3050	*P. seneca*	USA, NC , J. Hollinger 670 (UBC)	KX365472	KX365475	---	KX365479	---	KX373622	KX373633	5
P450	*P. seneca*	USA, PA, J. C. Lendemer 16792 (NYBG)	KX365451	KX365458	---	KX365463	KX365490	KX373623	KX373634	6

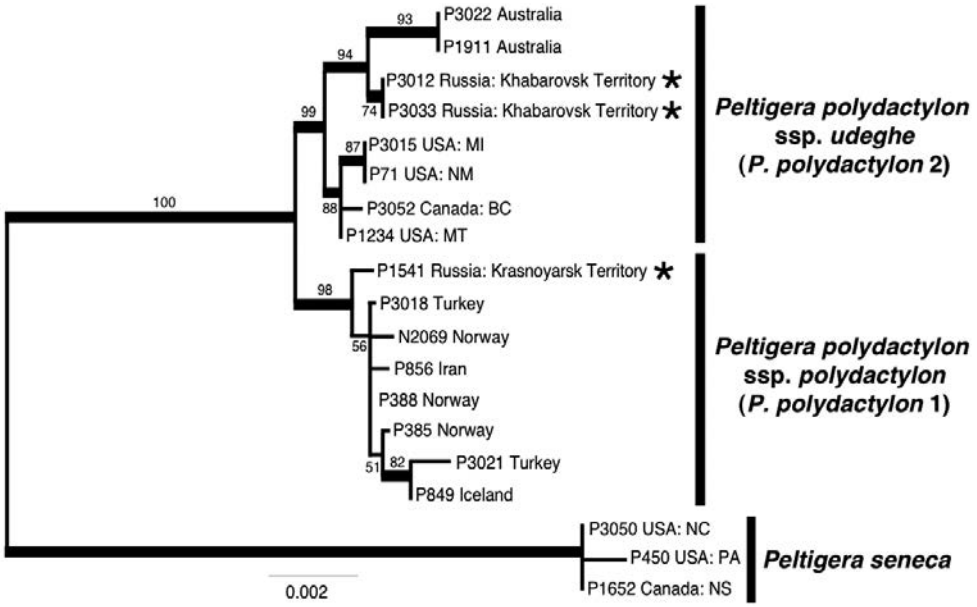

Fig. 1: Maximum likelihood phylogeny based on the concatenated 7-locus data set for 19 specimens from *P. polydactylon* s. str. and *Peltigera seneca.* The rooting of the tree is based on Magain et al. (2016). Thick branches represent bootstrap support above 70%. Bootstrap values above 50% are associated with internodes. *RPB1* sequences from specimens indicated by stars are more similar to each other than to the remaining representatives of their subspecies.

Peltigera polydactylon (Neck.) Hoffm. **subsp. *udeghe*** Magain, Miadl. & Sérus. **subsp. nov.** [MycoBank 817321] (Figs 3C–3F)

(= *Peltigera polydactylon* 2 in Magain et al. unpubl.)

Similar to *P. polydactylon* subsp. *polydactylon* and *P. seneca* sp. nov., but thallus always more or less phyllidiated and differs genetically and geographically.

Type: Russia. Khabarovsk Territory, Durminskoye forest-hunting area, ca. 200 km SSE of Khabarovsk, North of Durmin Mt., Rovnyi Creek valley, adjacent to foothills of the Sikhote-Alin' Range Mountains, 47.81666°N, 135.95673°E, elev. 328 m, stand of *Picea ajanensis* forest with *Pinus koraiensis* and *Abies nephrolepis* in otherwise broadleaf forest with extensive ground cover by ferns and mosses, over mosses and plant debris on ground, 30 July 2013, J. Miadlikowska & F. Lutzoni 07.30.2013-P3033 (holotype: DUKE [DNA-P3033]).

Description: Thallus forming rounded patches, up to 9 cm in diam., with rounded lobes of 1−3 cm long and c. 0.4−0.5 cm wide. Upper surface plane (or almost), smooth and rather shiny with frequent laminal cracks, margins typically raised and crisped, relatively fragile, pale brown to greyish brown when dry, often with greenish hue, becoming much darker with slate bluish tint when wet, upper surface of the same color as the thallus, lower surface with a distinct and regular network of pale orange to white, mostly elliptical (but sometimes more roundish) interstices (c. 1 × 0.5−0.7 mm) and flat or slightly raised veins, which are dark brown to black towards the center and pale orange brown at the margins; the network of interstices is well visible on the entire lower surface from the center to the lobe edges, which are covered by a minutely tomentose orange-brown hyphal layer. **Phyllidia** present on all examined thalli, but not on every lobe, often abundant, typically formed by torn crisped sections of the margin, rounded to irregular, sometimes lobulated, c. 2−3 × 1 mm. **Rhizines** sparse to abundant, scattered or rarely aggregated, dark brown, 1−3(−5) mm long, fasciculate to brush-like. **Apothecia** sometimes present, raised on elongated thallus lobes, and typically finger-shape (rarely saddle-shape),

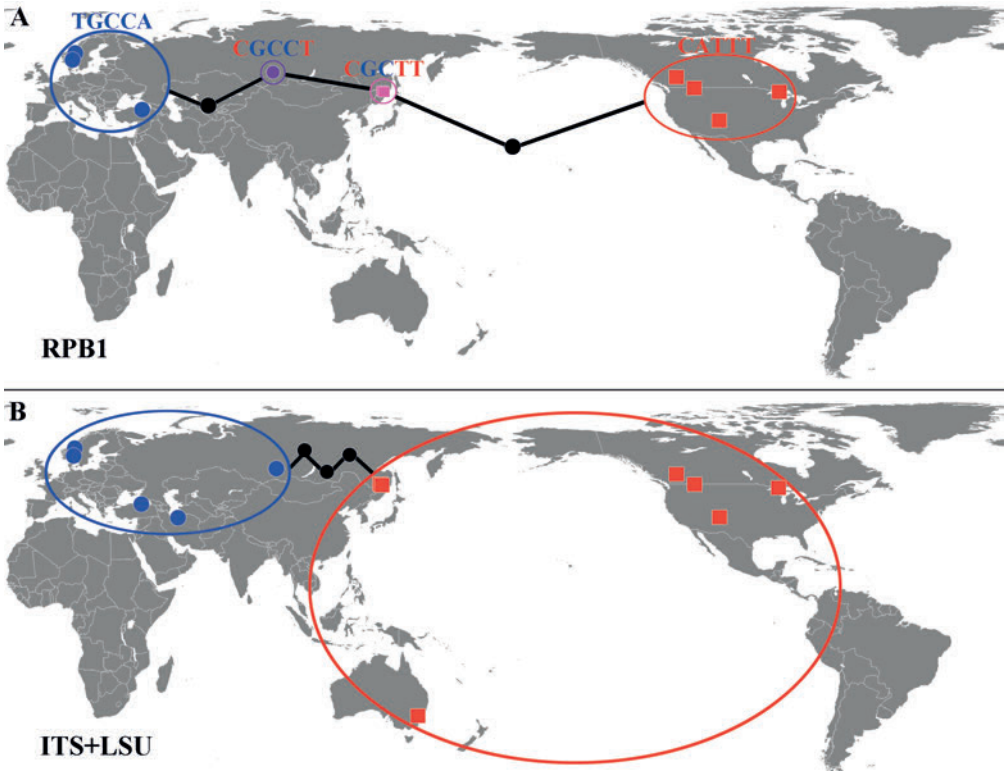

Fig. 2: Haplotype network, in the context of the geographic origin of sampled collections of *P. polydactylon* s. str. for the *RPB1* locus, showing intermediary variation (five variable sites across the entire sequence) between *P. polydactylon* subsp. *polydactylon* (in blue) and *P. polydactylon* subsp. *udeghe* (in red) found in three specimens in Russia (shown in pink and violet). Contrary to *RPB1*, ITS+nrLSU haplotypes are unique to each subspecies and differ by four point mutations. We could not obtain *RPB1* for specimens in Australia. Black dots represent unsampled single point mutations intermediates.

disc reddish brown to dark brown, c. 5 mm long. **Ascospores** narrowly fusiform with rounded ends, 3(−7)-septate, 39−78 × 1.8−4.0 µm (n=24). **Pycnidia** not seen.

Cyanobiont: *Nostoc* phylogroup V based on *rbc*LX phylogeny (Magain et al. 2016).

Secondary chemistry: by TLC, tenuiorin, methyl gyrophorate, peltidactylin, dolichorhizin, zeorin, Pnp-1 (sensu Vitikainen 1994); similar to *P. polydactylon* subsp. *polydactylon* and *P. seneca* (Magain et al. 2014; Fig. 4).

Etymology: This subspecies is named after the Udeghe people ("forest people" in the Udeghe language), the native population (less than 2000 people) from the Khabarovsk Territory where we came across the phyllidiated *P. polydactylon* s. str. Recently, the Bikin National Park was created to protect the largest remaining old-growth mixed forest in the Northern Hemisphere where some of the Udeghe people live.

Distribution: Australia (New South Wales), New Zealand, North America: Canada (Alberta, Ontario, Québec and British Columbia) and U.S.A. (Alaska, upper Michigan, Montana, New Mexico and South Dakota), eastern Northern Asia (Khabarovsk Territory of Russia) (Fig. 5).

Ecology: Collected in forested (subtropical, temperate and boreal) shady areas with relatively high humidity, over mosses and plant debris on ground, rocks, trees, and logs.

Notes: The new subspecies is morphologically and chemically similar to *P. polydactylon* subsp. *polydactylon* and *P. seneca* sp. nov., however, its thallus is always more or less phyllidiated and it is unique genetically (e.g., four and at least thirteen point mutations in the ITS and nrLSU in comparison with subsp. *polydactylon* [Fig. 2] and *P. seneca*, respectively), and distinct geographically (North America, eastern Northern Asia and Australasia).

Selected specimens examined and sequenced: **Australia**. **New South Wales,** Barrington Tops National Park, NE from Scone, 32˚05'S, 151˚30'E, elev. 500 m, in subtropical rainforest, 9 August 1988, K. & A. Kalb 22028 (DUKE; DNA-1912), 22029 (DUKE; DNA-P1910), 21797 (DUKE; DNA-P1911); Rutherford Creek, 11 km SE of Nimmitabel, 36°34' S, 149°36' E, temperate forest beside stream, on rock in stream, 14 Feb. 1990, H. Streimann 4381 (H; DNA-P3022). – **Canada**. **Alberta,** Edmonton, north bank of north Saskatchewan river, along western part of trail going west of Groat Bridge, 51°28'N, 112°42'E, moist east facing slope of densely forested hull dominated by *Picea mariana* and *Hylocomium splendens*, over mosses, edge of forest, sheltered, 8 April 1991, B. Goffinet 487 (Herb. B. Goffinet; DNA-N1885). **British Columbia,** Upper Clearwater Valley, Grouse Lake Trail, 51.859°N, 119.980°W, elev. 1000 m, mixed spruce-pine-aspen-birch forest, on ground, 19 October 2010, J. Hollinger 1505 (UBC; DNA-3052). **Ontario,** Bruce District, Fathom Five National Park, Flowerpot Island, along trail from Beachy Cove to Marl Bed, 45°17'50"N, 81°37'38"W, *Thuja*-dominated forest and calcareous fen (marl bed), 27 Sept. 2010, R. C. Harris 56572 (NYBG; DNA-P3150). **Québec,** Rivière-du-Loup county, Notre-Dame-des-Sept-Douleurs, Ile Verte, on the North-East face of the Gros Cap, 69°26'N, 48°01'W, vertical at the base of Gros Cap, 10 August 2009, C. Roy 09-5884-C (QFA; DNA-P3074). – **New Zealand. South Island,** Marlborough, 6 km NW of Kaikoura, Kowhai Bush, along entrance to Schoolhouse Road, 42.38333°S, 173.61667°E, in secondary forest dominated by *Leptospermum*, on the ground, 4 Feb. 1981, L. Tibell 10710 (UPS; DNA-P1749). – **Russia. Khabarovsk Territory,** Durminskoye forest-hunting area, c. 200 km SSE of Khabarovsk, N of Durmin Mt., Rovnyi Creek valley, adjacent to foothills of the Sikhote-Alin' Range Mts., 47.81876°N, 135.95750°E, elev. 326 m, along the creek, very humid forest dominated by *Alnus* sp., on mossy *Betula* log, 30 July 2013, J. Miadlikowska & F. Lutzoni 07.30.2013-P3012 (DUKE; DNA-P3012); Bol'shekhetzkhirskii State Reserve, Polovinka sector, c. 48 km SW of Khabarovsk, above the Polovinka Cabin at Polovinka Creek, 48.23929°N, 134.91116°E, elev. 357 m, broadleaf forest with occasional *Abies* and *Pinus*, dominated by *Tilia*, *Phellodendron*, and *Betula*, extremely rich understory, on mossy rock, 26 July 2013, J. Miadlikowska & F. Lutzoni 07.26.2013-P3047 (DUKE; DNA-P3047); Bol'shekhetzkhirskii State Reserve, Polovinka sector, c. 48 km SW of Khabarovsk, above the Polovinka Cabin at Polovinka Creek, 48.23853°N, 134.91013°E, elev. 377 m, broadleaf forest with occasional *Abies* and *Pinus*, dominated by *Tilia*, *Phellodendron*, and *Betula*, extremely rich understory, on mossy rock, 26 July 2013, J. Miadlikowska & F. Lutzoni 07.26.2013-P3042 (DUKE; DNA-P3042). – **U.S.A. Alaska,** Lake & Peninsula Co., Katmai National Park, NE side of Malone Lake, 58.4 0167°N, 156.1325°W, elev. 80 m, *Picea-Betula* forest and adjacent openings, 1 Aug. 2013, K. Spickerman 177 with B. McCune, L. Muggia, P. Nelson, T. Tønsberg & J. Walton (OSU; DNA-P4011); Seward Peninsula, Anvil Mt., 64°33.907'N, 165°22.23'W, elev. 335 m, *Salix* ticket by creeklet, 30 June 2002, B. McCune 36464 with P. Neitlich & E. Holt (OSU; DNA-P1235). **Michigan,** Upper Peninsula, Marquette Co., Huron Mountains, Huron Mountain Club area, along Lake Superior, Conway Bay trail, 46˚53.18'N, 87˚50.24'W, elev. 770 ft., hemlock forest with *Betula papyrifera* and sugar maple, on mossy trail, 27 June 2013, J. Miadlikowska & F. Lutzoni 06.27.2013-P3015 (DUKE; DNA-P3015). **Montana,** Flathead Co., Whitefish Spruce Swamp Preserve, Murdock property, S of Reservoir Road, E of Lakeshore Drive, 2.6 km N of Whitefish, 48.4352°N, 114.3380°W, elev. c. 927 m, *Picea-Equisetum* swamp forest, on *Betula* trunk, Sept. 2007, B. McCune 29108 (OSU; DNA-P1234). **New Mexico,** Santa Fe Co., Santa Fe National Forest, Chamisa Trail, 35.741°N, 105.858°W, elev. 2500 m, in north-facing valley with *Pseudotsuga*-aspen-birch forest, on mossy log, 24 March 2011, J. Hollinger 2462 (UBC; DNA-P71); Colfax Co., Carson National Forest, Elliott Barker Trail near Hwy 64, 36.408°N, 105.321°W, elev. 2750 m, on north to northeast-facing slope in spruce-fir-aspen forest, on mossy log, 26 March 2011, J. Hollinger 2399 (UBC; DNA-P3048); Oteri Co., c. 0.4 mi NE of the Cosmic Observatory, near 32°48'N, 105°47.3'W, elev. 9300 ft., on ground, 15 July 2007, R. D. Worthington 34885 (DUKE; DNA-P3163). **South Dakota,** Pennington County, Black Hills, Lost Cabin Trail, 43.874°N, 103.559°W, elev. 1825 m, in mixed forest on a north slope, on trailside soil, 7 July 2011, J. Hollinger 3463 (UBC; DNA-3117).

Peltigera polydactylon (Neck.) Hoffm. **subsp. *polydactylon*** (Figs 3A, 3B)

(= *Peltigera polydactylon* 1 in Magain et al. unpubl.)

For description see Vitikainen (1994).

Secondary chemistry: by TLC, tenuiorin, methyl gyrophorate, peltidactylin, dolichorhizin, zeorin, Pnp-1 (sensu Vitikainen 1994); similar to *P. polydactylon* subsp. *udeghe* and *P. seneca* (Magain et al. 2016b; Fig. 4).

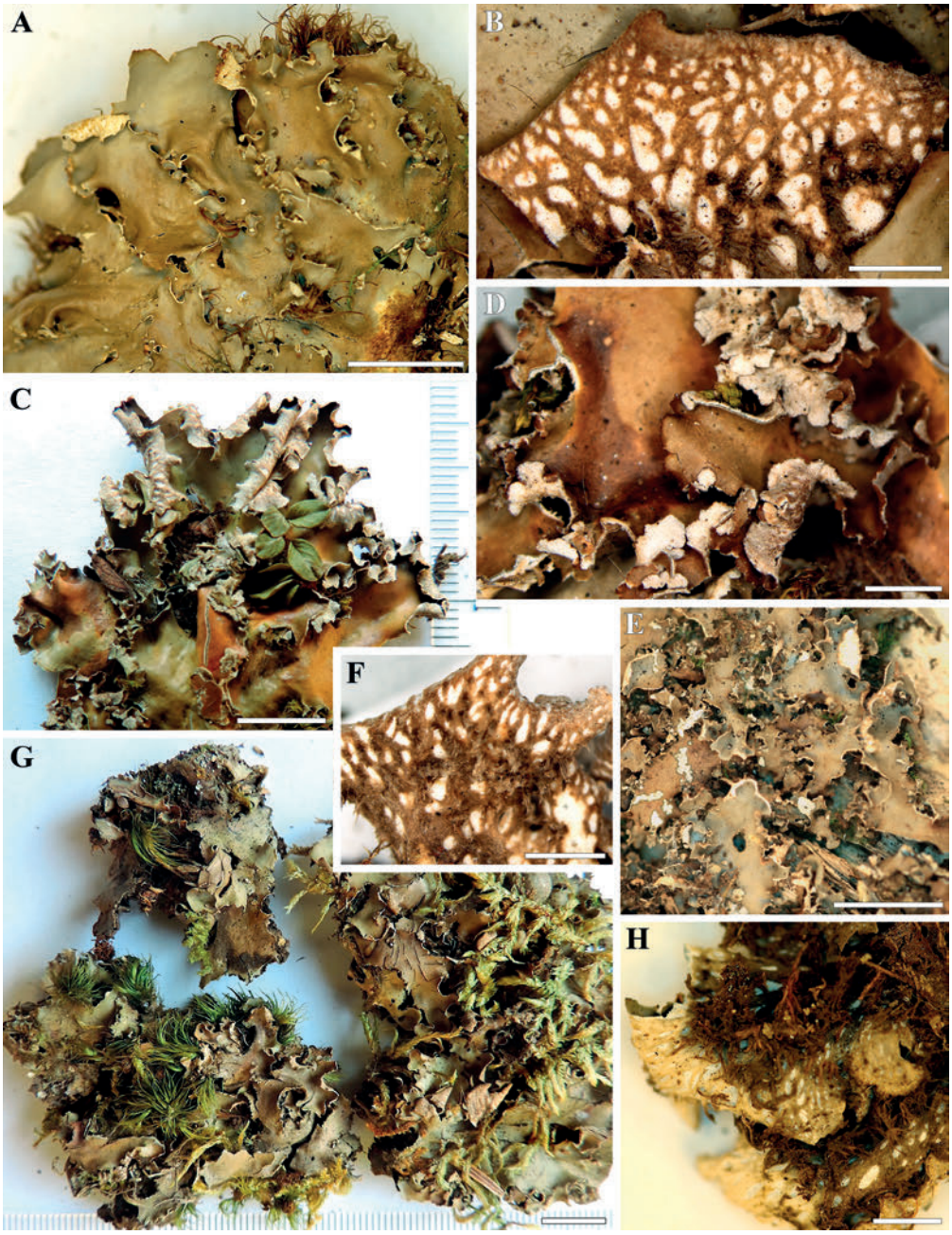

Fig. 3: Morphological features of *Peltigera polydactylon* s. lat. **A–B** – *Peltigera polydactylon* subsp. *polydactylon*. **A** – Thallus habit (P362, Norway). **B** – Lower side of thallus with distinct brown venation (Madeira). – **C–F** – *Peltigera polydactylon* subsp. *udeghe*. **C** – Thallus habit (P1235, U.S.A., Alaska). **D** – Raised, torned and phyllidiated margin (P1235, U.S.A., Alaska). **E** – Thallus (P3012, Russia, Khabarovsk Territory). **F** – Lower side of the thallus with distinct brown venation (P1235, U.S.A., Alaska). – **G–H** – *Peltigera seneca*. **G** – Thallus habit (P450, U.S.A., Pennsylvania). **H** – Lower side of the thallus with distinct brown venation toward the center and pale at the margin (P3050, U.S.A., North Carolina). – Scales: A,C,E,G = 1 cm; B,D,F,H = 2 mm [Photo credits: E. Sérusiaux and N. Magain].

Cyanobiont: *Nostoc* phylogroup V based on *rbc*LX phylogeny (Magain et al. 2016a).

Selected specimens examined and sequenced (Fig. 5)**: Iceland.** 65.56°N, 18°56W, elev. 350 m, 22 June 2005, H. Kristinsson s. n. (AMNH; DNA-P849). – **Iran. Mazandaran Province,** Ramsar, Javaherdeh, elev. 2000 m, 21 August 2002, A. A. Maassoumi 573 (B; DNA-P856). – **Norway**. **Akershus,** Siggerud, Vangenveien, 59°48.2'N, 10°59.1'E, 26 July 2011, N. Magain s. n. (LG; DNA-P388, P595). **Buskerud,** Lier, along the river Asdøla, 59°52.5'N, 10°18.3' E, 27 July 2011, N. Magain s. n. (LG; DNA-P369, P373, P374, P565); Krokkleiva, along a small river, 60°2.79'N, 10°19.1'E, 27 July 2011, N. Magain s. n. (LG; DNA-P573). **Hedmark,** Tynset, Kvikne Kobberverk, elev. 730-750 m, on mossy wall of old ruin, 18 July 2000, E. Timdal 9264 (O; DNA-N2007). **Møre og Romsdal,** Gjøra, Fjellgardsvegen, along the river Driva, 62°32'N, 9°05'E, 5 August 2011, N. Magain s. n. (LG; DNA-P3076). **Nord-Trøndelag,** N of Steinkjer, along a little river; 64°4.3'N, 11°35.16'E, 8 August 2011, N. Magain s. n. LG; DNA-P508). **Oppland,** Nord-Aurdal, Storebraten, N for Fagernes 17 June 1981, J. Holtan-Hartwig 528 (O; DNA-N2069); South of Grua, along the Hadelandsvegen, near a little river, 60°14.8'N, 10°41.3'E, 31 July 2011, N. Magain s. n. (LG; DNA-P616, P678); West of Aurdal, along the Fv220, 60°55.17'N, 9°22.8'E, 31 July 2011, N. Magain s. n. (LG; DNA-P685, P816); Ringebu, Nordåa-Søråa Nature Reserve, 61°33.51'N, 10°9.56'E, 1 August 2011, N. Magain s. n. (LG; DNA-P833). **Oslo,** Karlsrud, 29 July 2011, N. Magain s. n. (LG; DNA-P385). **Ostfold,** Romskog, Nybro, elev. 145 m, over moss, 23 Sept. 2000, B. P. Lofall bpl-L7635 (O; DNA-N2052). **Sogn og Fjordane,** Sogndal, Ylvesaker, elev. 1−5 m, on mossy boulder at sea level, 21 May 1998, J. E. Anonby 900 (BG; DNA-N1575). **Sør-Trøndelag,** along the E6 between Ulsberg and Berkak, 62°48.2'N, 10°1.91'E, 5 August 2011, N. Magain s. n. (LG; DNA-P522); Oppdal, near the Smegarden camping grounds, 62°32.03'N, 9°37.6'E, 4 August 2011, N. Magain s. n. (LG; DNA-P546, P547). **Telemark,** South of Amotsdal, along the Amotsdalvegen, 59°36.54'N, 8°25.3'E, 31 July 2011, N. Magain s. n. (LG; DNA-P673); Tinn municipality, near the intersection between the road Fv753 and the river Hellebekkae, 60°1.3'N, 8°50.7'E, 28 July 2011, N. Magain s. n. (LG; DNA-P682). – **Russia**. **Karachaevo-Cherkesiya Republic,** NW Caucasus, 6 km E of Teberda town, Dzhemagatskoe Canyon, right bank of Goralykol River, 43°27'13"N, 41°49'11"E, elev. 2130 m, mixed forest and subalpine meadows, 22 August 2012, M. P. Zhurbenko 2012.8.22.1 (DUKE; DNA-P3176, P3177). **Krasnoyarsk Territory,** Reserve "Stolby": near Fortress rocks, 55˚53.150'N, 92˚46.237'E, elev. 540 m, wet forest with *Abies sibirica*, *Picea obovata*, *Pinus siberica*, *Rubus nigra* and grasses, exposure SW, on mossy boulder and mossy slope along the trail, 24 June 2012, J. Miadlikowska & F. Lutzoni 06.24.2012-P1542 (DUKE; DNA-P1542); Kapella rock, 55°54.490'N, 92°43.427'E, elev. 712 m, mesic *Pinus sylvestris* forest with a few *Betula pubescens*, on mosses on exposed rocks, 26 June 2012, J. Miadlikowska & F. Lutzoni 06.26.2012-P1541 (DUKE; DNA-P1541). – **Sweden**. Åsele Lappmark, Vilhelmina: Saxnäs village, near Fjällgard (Stiftsgarden), elev. 560 m, path side, on soil, 8 August 1991, O. Vitikainen 12716a (H; DNA-P852). – **Switzerland**. [**Valais,**] Martigny, Gueuroz, June 2013, N. Magain s. n. (LG; DNA-N3199). – **Turkey**. **Trabzon,** Düzköy, Beypinari High Plateau, 40°47'19.98"N, 39°20'34.38"E, elev. 1575 m, June 2008, K. Yazıcı s.n. (H; DNA-P3018); Zigana Tatil Köyü, 40°40'17.42"N, 39°26'04.10"E, elev. 1713 m, 6 June 2008, K. Yazıcı s.n. (H; DNA-P3021).

Peltigera seneca Magain, Miadl. & Sérus. **sp. nov.** [MycoBank 817320] (Figs 3C–3F)

(= *Peltigera* sp. 10 in Magain et al. 2016, Magain et al. unpubl.)

Similar to *P. polydactylon* subsp. *udeghe* subsp. nov., but differs in having smaller thalli with narrower lobes and a broad, pale, marginal zone on the lower surface; distinct genetically, geographically and chemically (i.e., presence of an unidentified terpenoid detected by TLC methods; Fig. 5).

Type: U.S.A., Pennsylvania, Tioga Co., Tioga State Forest, Colton Road, c. 5 mi S of junction with US 6, c. 0.5 mi E of junction of Colton Road/Painter Leetonia Road, 41°42'30"N, 77°29'00"W, c. 580 m, seepy harwood forest with sparse *Tsuga*, on humus, 13 May 2009, J. C. Lendemer 16792 (holotype: NY [DNA-P450]).

Description: Thallus forming rounded patches, up to 5 cm diam., with lobes 1−2 cm long and c. 0.4−0.5 cm wide, with rounded ends, upper surface plane (or almost), smooth and rather shiny, margins slightly raised but never crisped or torn, rather fragile, greenish beige to brownish with greenish hue usually present when dry, becoming grey to dark grey when wet, lower surface with a dense network of pale brown to white, regular elliptical interstices (c. 1−2 × 0.5−0.8 mm) and slightly raised veins, which are dark brown to black towards the center and become pale to invisible at the margins (broad, pale, veinless zone). **Phyllidia** absent or present, but never as abundant as in *P. polydactylon* subsp. *udeghe*. **Rhizines** sparse to abundant, scattered or aggregated, dark brown, 1−3(−5) mm long, fasciculate to brush-like. **Apothecia** absent or rare (only few seen), on raised and narrow thallus lobes, saddle- to finger-shape, disc reddish brown to dark brown, c. 5 mm long. **Ascospores** narrowly fusiform with rounded ends, (5−)7-septate, 59−72 × 1.8−4.0 µm (n=7). **Pycnidia** not seen.

Cyanobiont: *Nostoc* phylogroup V based on *rbc*LX phylogeny (Magain et al. 2016a).

Secondary chemistry: by TLC, tenuiorin, methyl gyrophorate, peltidactylin, dolichorhizin, zeorin, Pnp-1 (*sensu* Vitikainen 1994); similar to *P. polydactylon* s. str. but with an additional unidentified terpenoid detected by TLC methods (Magain et al. unpubl.; Fig. 4).

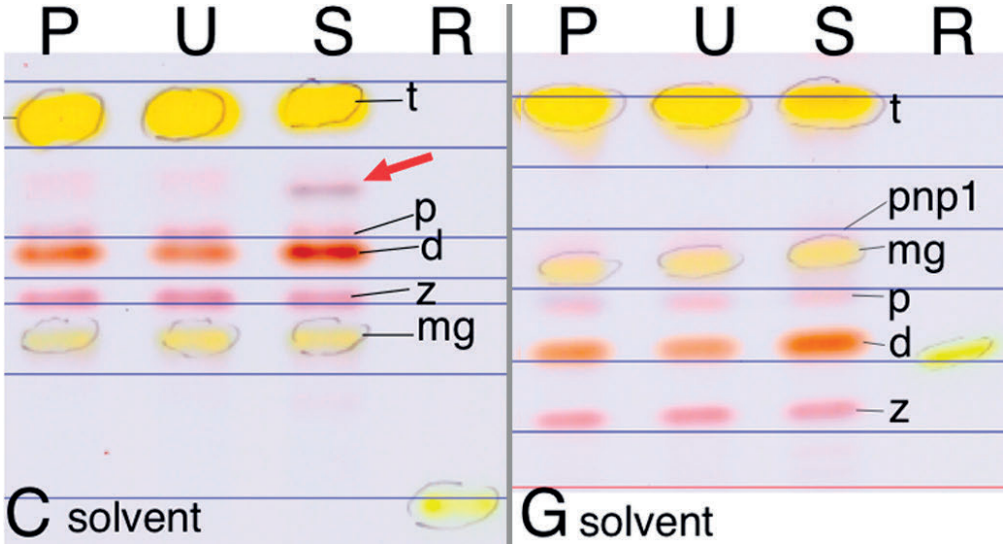

Fig. 4: Thin layer chromatograms in C and G solvents for *P. polydactylon* subsp. *polydactylon* (P), *P. polydactylon* subsp. *udeghe* (U), *P. seneca* (S), and a reference norstictic acid (R). Annotated lichen substances: dolichorhizin (d), methylgyrophorate (mg), peltidactylin (p), unknown terpenoid (pnp1 according to Vitikainen 1994), tenuiorin (t), and zeorin (z). Red arrow indicates an unidentified terpenoid detected by TLC methods in thalli of *P. seneca* only.

Etymology: This species is named after a tribe of Seneca Native Americans, the indigenous people who once inhabited the area of Tioga State Forest in Pennsylvania where the type specimen was collected.

Distribution: Rare and restricted to eastern North America: Canada (Nova Scotia), U.S.A. (Pennsylvania and North Carolina); known from three localities only (Fig. 5).

Ecology: Collected in temperate forested, shady areas with relatively high humidity; on humus and mossy rocks.

Notes: The new species is morphologically and chemically similar to *P. polydactylon* subsp. *udeghe* subsp. nov., however, it has smaller thalli with narrower lobes and veins on the marginal part of the lower surface become pale and less visible compared to the thallus center; it is also unique genetically (at least thirteen point mutations in the ITS and nrLSU), and distinct geographically (known from eastern North America only) and chemically (i.e., presence of an unidentified terpenoid detected by TLC methods, see Fig. 4).

Specimens examined and sequenced: Canada. Nova Scotia, (DNA-P1652) [further voucher data unavailable]. – **U.S.A. North Carolina,** Haywood Co., Great Smoky Mountains National Park, Cataloochee, gravel road between Cove Creek Gap and Sterling Gap, 35.645°N, 83.069°W, 900 m, on west-facing slope on mossy rock on road bank, 11 April 2010, J. Hollinger 670 (UBC; DNA-P3050).

Discussion

Although phenotypically difficult to distinguish (Figs 3A–3H), each taxon is well segregated molecularly (Fig. 1) based on a comparison of 33 specimens of *P. polydactylon* subsp. *poly-*

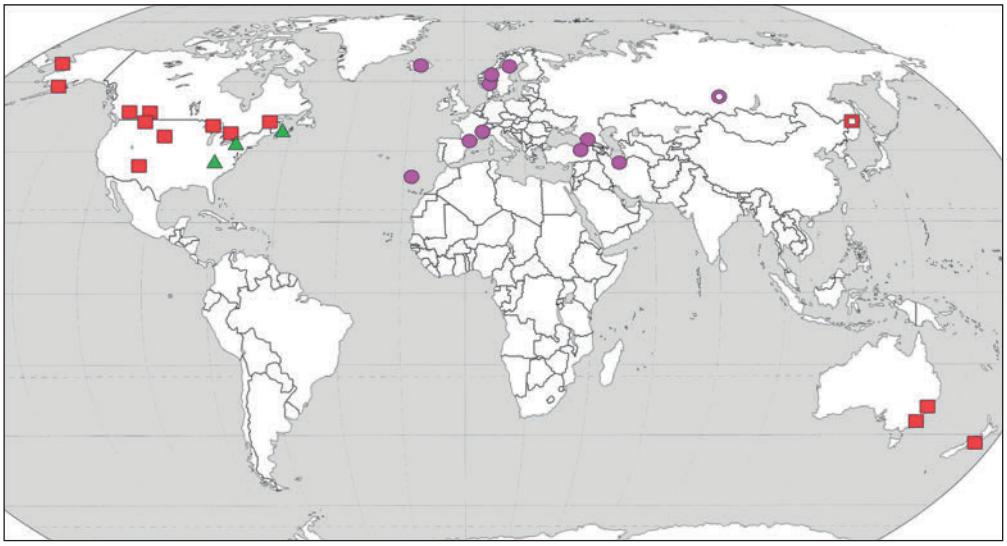

Fig. 5: Distribution map generated based on sequenced specimens (at least ITS) for *Peltigera polydactylon* subsp. *polydactylon* (purple circles), *P. polydactylon* subsp. *udeghe* (red squares), and *P. seneca* (green triangles). *RPB1* sequences from specimens indicated by a white centre are more similar to each other than to the remaining representatives of their subspecies (see Figs. 1 & 2).

dactylon with 24 specimens of *P. polydactylon* subsp. *udeghe*, and three of *P. seneca* for which we sequenced the ITS region (the DNA barcode marker for Fungi; Schoch et al. 2012) and additional loci for selected collections (Table 1; Magain et al. 2016). ITS sequences of *P. seneca* differ from *P. polydactylon* s. str. (i.e., subsp. *udeghe* and subsp. *polydactylon*) by four synapomorphic substitutions and two indels in the ITS1, and three substitutions and one indel in the ITS2. Only one substitution in ITS2 differentiates sequences of *P. polydactylon* subsp. *polydactylon* from subsp. *udeghe* (A versus C, respectively) and three substitutions in the nrLSU. While typical *RPB1* sequences from *P. polydactylon* subsp. *polydactylon* and *P. polydactylon* subsp. *udeghe* differ by five mutations (Fig. 2), a single specimen (DNA-P1541) collected in "Stolby" Reserve in Krasnoyarsk Territory in Russia, represents an intermediary sequence between the two subspecies. Its ITS sequence matches subsp. *polydactylon* (synapomorphic A in the segregating position), whereas its *RPB1* sequence shares three polymorphic sites with subsp. *polydactylon* while sharing the two other mutations, in this collinear series of five nucleotide substitutions, with subsp. *udeghe* (Fig. 2). This specimen was collected 4 km from a locality where another individual representing subsp. *polydactylon* (DNA-P1542) was sampled and for which no sign of mixing with subsp. *udeghe* was found. Similarly, specimens from the Khabarovsk Territory of Russia have the ITS haplotype of subsp. *udeghe* but their *RPB1* sequences contain three polymorphic sites shared with subsp. *udeghe* and two shared with subsp. *polydactylon* (Fig. 2). Therefore, *RPB1* sequences of *P. polydactylon* subsp. *polydactylon* (DNA-P1541) from the Krasnoyarsk Territory and of *P. polydactylon* subsp. *udeghe* (DNA-P3012 and DNA-P3033) from the Khabarovsk Territory of Russia differ by one substitution only. Similarly, the COR1b sequence from the *P. polydactylon* subsp. *polydactylon* (DNA-P1541) from the Krasnoyarsk Territory differs from typical *polydactylon* sequences by a single point mutation, which matches the haplotype of *P. polydactylon* subsp. *udeghe*. More collections from Northern Asia are needed to better delimit the geographic ranges of both subspecies in Asia and the extent of gene flow.

This pattern of genetic variation discovered in *P. polydactylon* s. str. corresponds to the definition of subspecies (Hawksworth 1974) "where two or more populations separated either geographically, ecologically or both throughout most of their range and distinguished by characters which might be used as criteria at the rank of species have intermediates where their distributions overlap (i.e., where they are sympatric) so that it is not possible to place some individuals in one subspecies or another." The rank of subspecies has not been commonly used in current taxonomy of lichen-forming fungi (but see e.g., Timdal 2002). Our data show that optimal taxon sampling and the use of multiple loci can influence species delimitation and suggest that the common use of species delimitation based on a single locus (mostly ITS for fungi) can result in inaccurate species delimitations. Several loci and different species delimitation and validation methods, as well as taxon sampling representing a broad geographical span of the studied species should be used. Currently, *P. polydactylon* s. lat. is represented in GenBank by six ITS sequences only. Three sequences from British Columbia, Canada (FJ709038, KC437643, KC437644; O'Brien et al. 2009, 2013) correspond to *P. polydactylon* subsp. *udeghe*, whereas the remaining three from Europe (JX195220, JX195229, JX195230; Kaasalainen et al. 2013) belong to subsp. *polydactylon*.

Overall *P. polydactylon* subsp. *udeghe* and *P. seneca* have relatively smaller thalli (up to 9 cm and 5 cm in diameter, respectively) than *P. polydactylon* subsp. *polydactylon* (reaching 10−20 cm in diameter in well-developed populations). The size and septation of the ascospores of *P. polydactylon* subsp. *udeghe* and *P. seneca* are similar to ascospores observed for *P. polydactylon* subsp. *polydactylon* in Europe (Holtan-Hartwig 1993, Vitikainen 1994). Phyllidia are usually present and abundant in *P. polydactylon* subsp. *udeghe* (all specimens from Australia are densely phyllidiated), whereas they are absent or rare in the other two taxa (Figs. 3A–3H). Vitikainen (1994) in his revision of the genus *Peltigera* in Europe stated that *P. polydactylon* is "often phyllidiated". Most specimens of *P. polydactylon* subsp. *polydactylon* we examined do not have phyllidia (Fig. 3). However, some thalli were found to be slightly phyllidiated, e.g., Belgium, J. Lambinon 63/371 (LG), but the material was too old for successful sequencing. It is very unlikely that the range of *P. polydactylon* subsp. *udeghe* extends to Europe because we did not detect any sign of recombination in the sequenced collections from this continent (multilocus data available for specimens from Norway, Iceland, Turkey and Iran). Phyllidia were also seen on thalli of *P. seneca.*

Based on other published records and morphological descriptions of *Peltigera* species in Alberta (Goffinet 1994) and British Columbia, Canada (Goward et al. 1995), the only two species with phyllidia and glabrous upper thallus in North America are *P. elisabethae* (section Horizontales*)* and *P. pacifica* (section *Polydactylon*). It is possible that *P. pacifica* was misidentified with phyllidiated forms of *P. polydactylon* subsp. *udeghe* in these regions. *Peltigera polydactylon* subsp. *polydactylon* was not found in North America and most specimens with relatively big, glabrous, and non-phyllidiated thalli traditionally identified as *P. polydactylon* belong to *P. neopolydactyla* s. lat. (representing *P. neopolydactyla* 1, a newly delimited species; Magain et al. 2016, Magain et al. unpubl.), which can resemble *P. polydactylon* s. str. especially when collected from soil. *Peltigera neopolydactyla* 1 is especially abundant in the Appalachian Mountains, where *P. seneca* is very rare (known from two localities only) and *P. polydactylon* subsp. *udeghe* was never found. The absence of phyllidia in *P. neopolydactyla* 1 and its relatively large thalli can help to distinguish this species from *P. polydactylon* subsp. *udeghe* and *P. seneca* in North America, in addition to the differences in the venation pattern (veins are rarely visible in the marginal parts of lobes in *P. neopolydactyla* 1 and *P. seneca* and are usually paler brown in *P. neopolydactyla* 1; whereas veins are dark brown and well vis-

ible toward the lobe margins of *P. polydactylon* subsp. *udeghe*). No record of glabrous species with phyllidia was reported from New Zealand (Galloway 2000), however, the description of *P. polydactylon* includes phyllidia according to the taxonomic treatment of *Peltigera* in Australia (Louwhoff 2009) where two morphotypes were recognized: phyllidiated morphotype 1, which very likely corresponds to *P. polydactylon* subsp. *udeghe*, and phyllidia lacking morphotype 2, which most likely represents *P.* sp. 3 from the dolichorhizoid clade (Magain et al. 2016).

Both species of the *P. polydactylon* complex share the same chemistry (Fig. 4): tenuiorin, methyl gyrophorate, peltidactylin, dolichorhizin, zeorin, Pnp-1 (Vitikainen 1994; referred to number 41 in Holtan-Hartwig 1993). Peltidactylin occurs usually in small quantities (weak as noted by Vitikainen 1994), as well as Pnp-1 (trace), whereas zeorin, and especially dolichorhizin, are abundant (the relative abundances of peltidactylin, dolichorhizin and zeorin seem to be an informative character for the chemical identification of species belonging to section *Polydactylon*). Holtan-Hartwig (1993) also identified traces of several other terpenoids (hopane-6 α,7ß,22-triol, number 20; hopane-15 α,22diol, number 35; and unidentified terpenoid 41) that were not detected in specimens we examined. *Peltigera seneca* contains an additional unidentified terpenoid that might not have been previously identified by Holtan-Hartwig (1993; Fig. 4). Three collections of *P. polydactylon* subsp. *udeghe* from Australia (from a single locality in New South Wales; DNA-P1911) examined by Kalb & Brandl (Anal. No. 486, 487, 484; Jan. 2001) contain tenuiorin, methyl gyrophorate, and dolichorhizin.

Mycobionts of all three taxa from the *P. polydactylon* species complex were consistently found to be associated with *Nostoc* phylogroup V (Magain et al. 2016), which is one of the most common cyanobionts associated with *Peltigera* species inside and outside section *Polydactylon* (unpublished data).

As currently defined, *P. polydactylon* subsp. *polydactylon* occurs in Europe (incl. Madeira in Macaronesia and North Caucasus in Russia), Middle East (Iran, Turkey) and central Northern Asia (confirmed record from the Krasnoyarsk Territory of Russia) whereas its sister subsp. *udeghe*, is restricted to eastern Northern Asia (Khabarovsk Territory of Russia), widespread across the Pacific in western North America extending north-east to Upper Michigan in the U.S.A., and Alberta, Ontario and Québec in Canada, and occurs also in Australia and New Zealand.

Based on the inferred phylogeny shown in Fig. 1, one possible diversification scenario within the *P. polydactylon* complex might be that the ancestor of *P. polydactylon* s. str., after splitting from the shared ancestor with *P. seneca*, initially spread in North America and dispersed eastward to Europe leading to the divergence between North American (subsp. *udeghe*) and European populations (subsp. *polydactylon*). North American populations also spread westward to Asia/Australasia through the Bering Strait. When the two well-isolated and diverged populations from Europe and North America met in Northern Asia, some degree of gene flow occurred. Another explanation could be that the diversification of the *P. polydactylon* complex originated in the most eastern parts of Russia, explaining the higher genetic diversity in central and eastern Northern Asia. Subsequent dispersal of the populations west, to Europe (*P. polydactylon* subsp. *polydactylon*) and east, to North America and Australasia (*P. polydactylon* subsp. *udeghe*), resulted in the isolation of the two taxa. This rather low level of intercontinental geographic isolation and differentiation seems to be an exception among species of *Peltigera* section *Polydactylon*. The geographical ranges of most species in this section (sensu Magain 2014, Magain et al. 2016) are usually much smaller and restricted to a single continent.

Peltigera seneca, which originated from the earliest divergence in the *polydactylon* s. lat. clade (Magain et al. 2016, Magain et al. unpubl.), seems to be rare and endemic to eastern North America, and the full extent of its distribution is unclear. As a result, the boundaries between geographic ranges of *P. seneca* and *P. polydactylon* subsp. *udeghe* in North America are not well delimited, likewise between the latter subspecies and *P. polydactylon* subsp. *polydactylon* in Northern Asia. Because the presence of phyllidia (which is often a reliable diagnostic character) is not restricted to *P. polydactylon* subsp. *udeghe*, and thallus size can vary depending on the age of the specimen, the only reliable feature to distinguish the three taxa within *P. polydactylon* s. lat., other than their geographical ranges, is their DNA sequences (e.g., ITS and nrLSU). It is possible that the lower side of the thallus (well visible venation extending to the lobe margin versus paler marginal zone) might be a consistent diagnostic feature allowing the distinction between *P. polydactylon* and *P. seneca*, however, more collections are necessary in order to confirm this observation.

Acknowledgements

We dedicate this paper to Prof. Josef Hafellner on the occasion of his 65th birthday in honour of his remarkable contribution to lichen systematics. A special appreciation is directed to people that assisted us during the *Peltigera* collecting trip in the Khabarovsk Territory: Rimma Andronova and staff from the Bol'shekhetzkhirskii State Reserve and Durminskoye forest-hunting area, as well as A. Elizabeth (Betsy) Arnold for their generous help in organizing the trip and for field assistance; and the collecting trip in Krasnoyarsk Territory: Anastasia Knorre, Altyna Dutbayeva and staff from "Stolby" National Wildlife Nature Reserve (Krasnoyarsk, Russia). The authors are thankful to all collaborators (especially B. Goffinet, T. Goward, J. Hollinger, J. Lendemer, B. McCune, and O. Vitikainen) and curators of several herbaria for providing material for this project, and L. Muggia for her editorial work. This study was supported by the National Science Foundation (NSF) REVSYS award on the genus *Peltigera* DEB-1025930 to J.M. and F.L., and NSF Dimensions of Biodiversity award DEB-1046065 to F.L.

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Manuscript accepted: 30 June 2016.

Communicated by: Lucia Muggia

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