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Standard Paper

Characterization of simple sequence repeat loci for *Peltigera membranacea* (lichenized *Ascomycota*) and its *Nostoc* photobiont

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Abstract

To facilitate population-genetic studies, we developed simple sequence repeat (SSR) markers and a molecular species identification assay for *Peltigera membranacea* (*Ascomycota*, *Peltigerales*), a common ground-dwelling lichen of forest and tundra ecosystems. Additional markers were developed for its *Nostoc* photobiont. Twenty-one fungal markers for *P. membranacea* were found to be polymorphic, with the number of alleles ranging from 3–21. Nei's unbiased gene diversity ranged from 0.588 to 0.640 in four significantly structured ($F_{ST} = 0.059$) mycobiont populations. For the *Nostoc* photobiont, 14 polymorphic SSR were developed, yielding 4–14 alleles each, with gene diversity ranging from 0.062 to 0.771 in four populations showing substantial population structure ($F_{ST} = 0.278$). The new markers developed are suitable for population genetic studies of *Peltigera membranacea* and of its cyanobiont, and at the same time allowed us to distinguish 98.5% of *P. membranacea* specimens from morphologically similar species of *Peltigera*.

Key words: ascomycetes, genetic diversity, microsatellites, molecular species identification, *Peltigerales*, simple sequence repeats (SSR)

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Introduction

Peltigera membranacea (Ach.) Nyl. is a cyanolichen distributed on the western side of continents of the Northern Hemisphere. In the boreal ecozone, the species is common and locally abundant. It usually grows on the ground among bryophytes and graminoids in sites with some woody vegetation or in grasslands, on the base of tree trunks or on moss-covered rocks. *Peltigera membranacea* differs morphologically from other species of *Peltigera* by its downy, raised veins on the lower surface, its papery-thin thallus, its long, isolated, squarrosely branched rhizines, and by the size of its ascospores (Vitikainen 1994; Martinez & Burgaz 1996).

The haploid mycobiont (i.e. the lichen-forming fungus *P. membranacea*) forms a mutualistic symbiosis with cyanobacterial *Nostoc* photobionts. Its photobiont strains are closely related to (but not identical with) *N. punctiforme* (Miao *et al.* 1997; Gagunashvili & Andrésson 2018). By channeling nitrogen fixed by its cyanobacterial photobiont, *P. membranacea* is important for ecosystem function in northern ecosystems, where locally it grows in comparatively high abundance.

While the phylogeny and taxonomy of the genus *Peltigera* is well studied (Miadlikowska & Lutzoni 2000, 2004; Miadlikowska *et al.* 2003; O'Brien *et al.* 2009; Wedin *et al.* 2009; Jüriado *et al.* 2017, 2019; Magain *et al.* 2018), its population subdivision has received little attention to date, partly because of a lack of suitable markers. So far, the development of microsatellites has been reported for 18 species of lichen-forming fungi (Walser *et al.* 2003; Magain *et al.* 2010; Widmer *et al.* 2010; Mansournia *et al.* 2011; Jones *et al.* 2012; Guzow-Krzeminska & Stocker-Wörgötter 2013; Werth *et al.* 2013; Alors *et al.* 2014; Belinchón *et al.* 2014; Devkota *et al.* 2014; Nadyeina *et al.* 2014; Törra *et al.* 2014; Prieto *et al.* 2015; Lutsak *et al.* 2016; Lagostina *et al.* 2017).

Peltigera membranacea lacks specialized small vegetative propagules and, in our study area, thalli with apothecia do occur but are uncommon. Clonal spread through thallus fragments should thus be its predominant dispersal mode, and it has been shown that thallus fragments disperse over very short distances (Dettki 1998; Heinken 1999), relative to other propagule types. If dispersal by short-reaching thallus fragments predominated in *P. membranacea*, we would expect substantial genetic differentiation between sampling sites. However, populations of *P. membranacea* are often large, implying that it would take an extended time period before differentiation could be observed between populations. The mycobiont could also disperse via microscopic ascospores and associate with local cyanobionts in new sites. Establishment of new individuals from ascospores would uncouple fungal and cyanobacterial genetic structures and could involve much larger distances, which could lead to low genetic differentiation. For example, ascospores reached on average two orders of magnitude further than symbiotic propagules of *Lobaria pulmonaria* (Ronnås *et al.* 2017). Here, we report 20 new microsatellites for the lichen-forming fungus *P. membranacea* to enable studies of population subdivision in this widespread terricolous species and 14 simple sequence repeat markers

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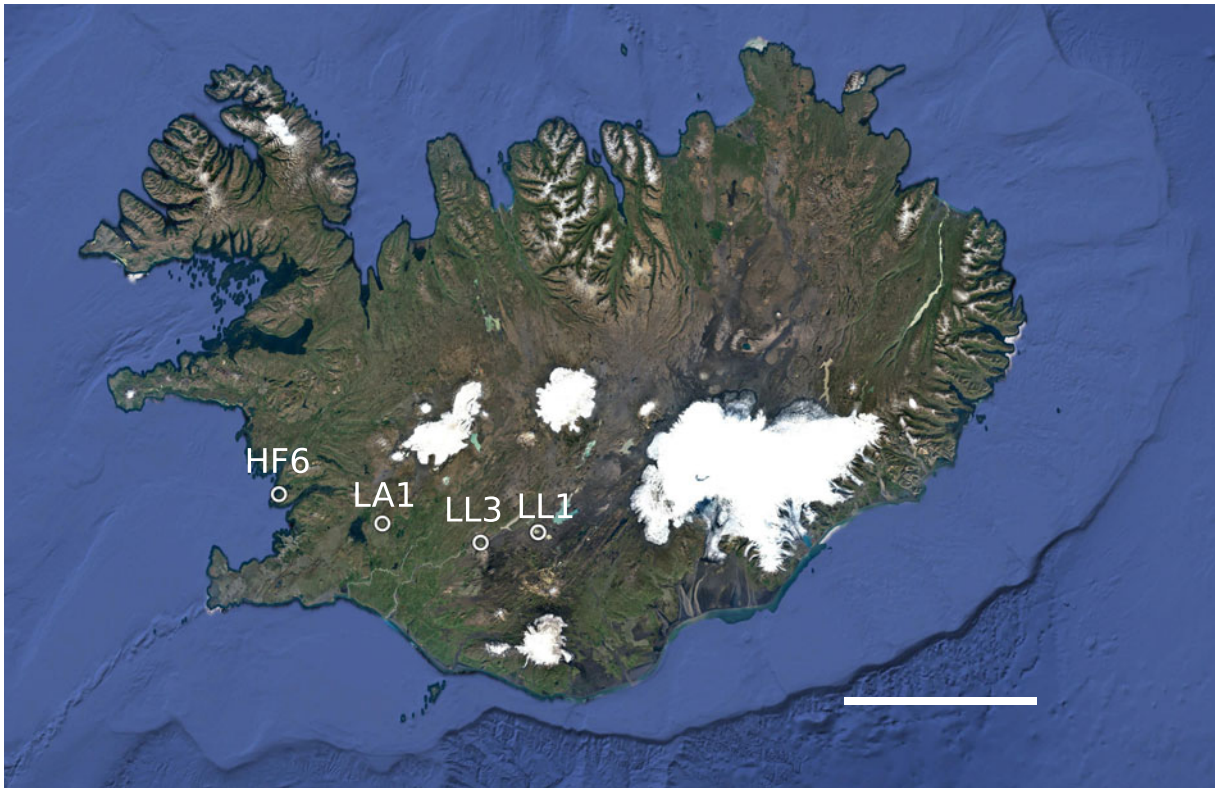

Fig. 1. Map of the collecting localities (labelled) in western Iceland. The largest distance between sites was 140 km. The map was made with Google Earth v.7.3.3.7786. Data attribution: SIO, NOAA, US Navy, NGA, GEBCO, Image Landsat/Copernicus, Image IBCAO. Scale = 100 km. In colour online.

for its *Nostoc* photobiont. We also report an assay based on the ITS region to check the species identity of *P. membranacea* against two of its morphologically similar congeners, *P. canina* and *P. praetextata*.

Materials and Methods

Study area and sampling

A total of 122 thalli of *P. membranacea* were collected from four sites in western Iceland (Fig. 1). We contrasted a coastal site (HF6: 64.34421°N, 22.01367°W, 3 m a.s.l.) with three sites located further inland (LA1: 64.21237°N, 20.85986°W, 184 m a.s.l.; LL1: 64.16659° N, 19.15357°W, 500 m a.s.l.; LL3: 64.12108°N, 19.78241°W, 285 m a.s.l.). We collected thalli while walking along a transect through the population, leaving at least 2 m between adjacent samples. The thalli were air-dried and stored at −20 °C until DNA extraction.

Microsatellite discovery and characterization

Metagenomic DNA of *P. membranacea* was used for 454 pyrosequencing, performed by Microsynth AG (Balgach, Switzerland) following standard protocols on a GS FLX instrument (Roche, Schlieren, Switzerland). Additionally, Illumina Solexa 2 × 35-bp reads were sequenced at a commercial facility, generating 1.4 GB of Illumina data, yielding *c.* 50-fold coverage of the *Nostoc* genome (Xavier *et al.* 2012; Kampa *et al.* 2013). The 454 data were assembled using the Newbler program, yielding 150 581 contigs with an average length of 900 nucleotides, 135 511 117 bases in total. Contigs were sorted by homology to known genomes and base composition and homology with poly-A cDNA (mycobiont) was also used. The contigs sorted as mycobiont were then assembled together with Illumina mate-pair data using MIRA v.3.2.1. Finally, SSPACE was used to scaffold the contigs, yielding 43 Mb in 2250 scaffolds for *Peltigera membranacea*. MSATCOMMANDER v.0.8.2 (Faircloth 2008) was used to find dinucleotide, trinucleotide, tetranucleotide, pentanucleotide, and hexanucleotide repeats in the *P. membranacea* metagenomic assembly; the search was restricted to retrieve only contigs containing ≥ 10 repeats in trinucleotides, and ≥ 9 in all others. In order to establish that the microsatellites found in the metagenomic assembly were specific to the mycobiont, we used only contigs that had a high coverage and blasted to *Ascomycota*, since high coverage could originate from either mycobiont or photobiont. We performed BLAST searches (Altschul *et al.* 1997) of the translated nucleotide sequences containing the microsatellite repeats (blastx) against protein databases (swissprot, refseq_protein, nr) to determine whether a given contig was of ascomycete origin.

The genome of *Nostoc* strain N6 originating from Keldur in Reykjavik, Iceland was sequenced on the Illumina platform and assembled in MIRA for a prior study (Gagunashvili & Andrésson 2018). This genome provided the *Nostoc* SSRs. TandemRepeatFinder v.4.08 (Benson 1999) was used to search the *Nostoc* N6 genome for SSRs. Primers were designed for an annealing temperature of 55 °C using the software PrimerBlast (Ye *et al.* 2012) which employed Primer3 for primer design (Rozen & Skaletsky 2000).

Species identity assay

During fieldwork it was noticed that *Peltigera membranacea* thalli in sparsely vegetated areas in Iceland tend to have narrower lobes and shorter, more branched rhizines than those in areas with substantial vegetation cover, thus becoming morphologically similar to *P. canina*. To verify the accuracy of our field species

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Table 1. Overview of SSR markers developed for the lichen fungus *Peltigera membranacea* (*Ascomycota*, *Peltigerales*) and its *Nostoc* (*Cyanobacteria*, *Nostocales*) photobiont.

Marker	N_A[1]	Fail[2]	Dye[3]	Forward primer[4]	Reverse primer[5]	Repeats[6]	Range [bp][7]	Multiplex[8,9]
Pme_ITS[10]	8	0.8	NED	TTTACTTGGGTKCGGTTT	TACGCTGGAATGCCTAACT	1	482–490	1b
Pme01153	5	16.5	VIC	TACGGAGCTGCTTTGGTTCG	GCGTGAACGACATCCGGTTA	3	400–412	2
Pme03072	21	0.8	VIC	TCACCAGTCACCACACTCCA	AGCCAAGCGCTGAAAGACAT	4	298–491	1b
Pme07630	5	2.5	NED	TGATAGAAAGTAACCGAATCGGA	ACAAGTAGGCGACTACATATGT	2	105–127	1a
Pme07704	3	1.7	PET	ACCAATGATTCCAGCGCCTC	CCGAACCCCAAATCGAGTCC	3	454–464	2
Pme112305	10	1.7	PET	CGAAGGCATTTGTTGCGCTT	TGTGCCGATCCATGCGATTT	2	245–272	2
Pme115956	10	2.5	FAM	TCCAGACCATACTCGGTGGG	TCCCGTAGTGTGTGCTGATTG	3	186–221	1a
Pme16703	5	0.8	VIC	CGAGGGCATTGTCTGGTCTC	CATTGACCGCGTGCATATCCT	2	185–206	1a
Pme17061	4	0.8	FAM	CTGCCGTCATTTGTTGCCAT	CCTGGTTTGGCGTCTACGAA	3	206–225	2
Pme17303	6	34.7	NED	AGTGAGAAAGGGAGAGCCGG	ATCCAGTCGCTAAGCTCACG	6	107–152	2
Pme20499	6	1.7	NED	CAACTGGGCAACGGGTACAA	GATTCCCGTCACGTAGCGAG	4	336–356	1a
Pme25414	8	1.7	PET	AGTTCGCGAAGTACTGCTGC	CTAATCGCTCTGCCCTGCTG	3	90–118	2
Pme32166	8	6.6	FAM	AGCTGTTGCGCAAAACATACAA	GCTGTCGCCCGAAATGGATT	2	455–474	2
Pme44221	13	2.5	FAM	GCAGTCCCGGCAAGATTGTT	TTTGGCGAAGGGAAGCAAGT	2	326–365	2
Pme44890	3	1.7	NED	AATGTGGGGGACACAGCAAC	TGTGTTCTTGACGGGGTCCT	5	408–418	2
Pme48804	8	0	PET	TGTAGCGGATATTCGATTTTGGT	TACGCTGGAATGCCTAACT	4	392–447	1b
Pme49183	17	0	FAM	CCGTTTACCGAGATGGTGACA	GCAAAACGCAAAGCAGGAGG	4	323–545	1b
Pme66801	9	1.7	NED	AGATAACGTCTGCGGGCGAA	TCTCCTGGTCATGTTGCATCC	3	82–116	2
Pme75971	4	0	FAM	TCTGAATGCTCTCTTGGCGC	CACGGGAGAGAGAGGTTTGC	2	85–102	2
Pme79082	6	1.7	VIC	GAGAAGAAAGGAACGGTTGGGG	ATGGTTACATATAAAATGGGGACCC	2	243–263	2
Pme85686	19	2.5	PET	TATGTCACGTGAGAACGCGG	ACATGAGGAGGTAACGAAGAAAGC	3	130–201	1a
Nos_01[11]	6	26.1	VIC	AGGGGATTTTAAACCTGTGG	TCGAGCAATTAGCACAGTAA	6	174–351	X3213
Nos_03	4	6.7	NED	CTAGGTGTCGGTGTAGGTT	AGAAGACTATCGACTCACCA	6	169–188	X3213
Nos_07	9	7.6	NED	GTGTTCCTCAGAGTTGTCAT	ATAGAACAACTCCCGCAAAT	6	287–323	X3213
Nos_08	6	21.0	FAM	ACCTTTTCTTTCTTTGTGACC	TTTACACGCAACCTGTGTTA	5	319–375	X3213
Nos_11	11	7.6	PET	GGTGGATGAAGACGGTAAAA	CGTCATTCGTAATGATGCTC	4	300–367	X3213
Nos_23	7	1.7	NED	GCGCCTCTACTAATACCATT	GAGTTTCGACTGAGCAACTA	7	306–329	X3212
Nos_41	8	22.7	PET	GAATGCCAACAAGTCGTTC	TGTCATCAGAAAACTTCTCAA	10	158–246	X3212
Nos_42	14	1.7	VIC	CGTCACTGTAACGTTTGATTT	CTAGTAACACTCCACTCTGC	13	193–364	X3212
Nos_43	4	0	FAM	CATCTATATGACGACGTTGC	GGAAGACATTAGGGACAAGG	9	99–121	X3212
Nos_44	14	9.2	NED	GCATGAAACGCAGTATACAA	AGCAACCCCTACTCTAAATG	8	126–288	X3212
Nos_46	6	10.9	PET	ACAAAATTGGATTCCCACCT	TGTTTGCAGAAGACGAGTAA	11	318–407	X3212

(*Continued*)

Table 1. (*Continued*)

Marker	N_A[1]	Fail[2]	Dye[3]	Forward primer[4]	Reverse primer[5]	Repeats[6]	Range [bp][7]	Multiplex[8,9]
Nos_48	6	30.3	NED	AAGTCTGGAGCATATCTGGA	GGCTAACATCACAGATCCAT	9	104–155	X3212
Nos_51	6	5.9	PET	GGGTAGAGGCATAAGTTACA	CCTAGCAAAACTTCAAGCTG	12	119–160	X3212
Nos_60	14	0	FAM	AGAAGGACTATCTACCACGG	TTTTAGCGTCTGTCTTCGAC	11	123–215	X3212

[1]Number of alleles.
[2]Percentage of PCR failures.
[3]Fluorescent dye.
[4]Sequence of M13(21)-labelled forward primer from 5′ to 3′.
[5]Sequence of reverse primer from 5′ to 3′.
[6]Number of repeated bp.
[7]Range of allele sizes in bp.
[8]Allocation to multiplex.
[9]Primers were designed for an annealing temperature of 55 °C. Sample sizes are $n = 121$ in *P. membranacea*, and $n = 119$ in *Nostoc*.
[10]'Pme' in the primer name stands for a primer of the mycobiont, *Peltigera membranacea*.
[11]'Nos' stands for a primer of the *Nostoc* cyanobiont of *P. membranacea*.

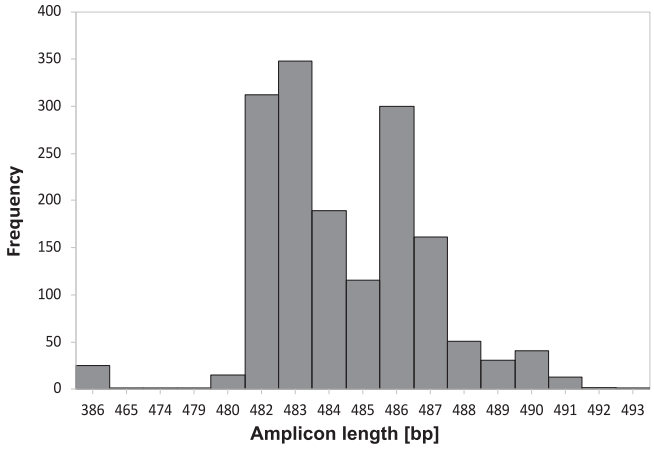

Fig. 2. Frequency of amplicon lengths (base pairs) of the internal transcribed spacer (ITS) region in 1606 thalli of *Peltigera membranacea* from Iceland, amplified with the Pme_ITS-primers (this study).

identifications, we developed a molecular assay based on the internal transcribed spacer (ITS) region, a part of the nuclear ribosomal gene cluster, based on sequences downloaded from GenBank. Part of the ITS region is hypervariable in members of the genus *Peltigera*, containing a microsatellite and many indels (Miadlikowska *et al.* 2003). We included all specimens of *P. membranacea*, *P. praetextata* and *P. canina* in GenBank (accessed 5 May 2021) which had a full coverage of the region spanned by our new primers, thus providing a representative sample. A number of the specimens included in the alignment were from Iceland (*P. membranacea*: 2 out of 17, *P. praetextata*: 3 out of 7, *P. canina*: 7 out of 9). Additional species of *Peltigera* occurring in Iceland that were analyzed included *P. islandica* (10 of 11 samples from Iceland), *P. monticola* (6 samples from Iceland) and *P. neckeri* (2 samples from Iceland). Each of these were aligned together with one sample of *P. membranacea* of known size. Sequences were aligned with Clustal Omega v.1.2.1. (Sievers *et al.* 2011) or with MUSCLE (Edgar 2004) implemented in MEGA X (Stecher *et al.* 2020) (see Supplementary Material Table S1, available online). Expected allele sizes were 386 or ≥ 469 bp in *P. membranacea*, 379–380 bp in *P. canina*, 380 bp in *P. praetextata*, 428–432 bp in *P. degenii*, 348 bp in *P. islandica*, 418 bp in Icelandic *P. monticola*, 408 bp in Icelandic *P. neckeri*, and 412–431 bp in *P.* '*scotteri*' (Miadlikowska *et al.* 2003), one of the species belonging to the *P. canina* complex (Supplementary Material Table S2, available online). *Peltigera* '*scotteri*' and *P. degenii* have not been reported from Iceland (Hansen 2009). The alignment of the relevant hypervariable part of the ITS region in the *P. canina* complex is shown in Supplementary Material Table S1. The ITS region includes several consecutive mononucleotide microsatellites in *P. membranacea* (Supplementary Material Table S1), which we included in the characterization of microsatellite polymorphism (locus name: Pme_ITS, Tables 1 & 2). We genotyped 1606 samples field-identified to *Peltigera membranacea* from different locations around Iceland and from two sites in Scotland with the ITS assay. Location information is included in Supplementary Material Table S3 (available online). Specimens are deposited in AMNH (Akureyri).

Laboratory procedures

DNA extraction was performed with the DNeasy Plant Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

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Table 2. Diversity statistics in 20 simple sequence repeat loci of *Peltigera membranacea* and 14 of its *Nostoc* cyanobiont, calculated for populations from western Iceland. HF6, LA1, LL1 and LL3 = collecting localities.

	Number of alleles				Nei's gene diversity H_E [1]			
Locus	HF6	LA1	LL1	LL3	HF6	LA1	LL1	LL3
Pme_ITS	6	6	5	5	0.787	0.711	0.750	0.742
Pme01153	5	4	4	4	0.717	0.576	0.749	0.495
Pme03072	12	10	8	12	0.878	0.836	0.816	0.837
Pme07630	4	4	3	3	0.641	0.633	0.438	0.628
Pme07704	2	2	3	1	0.399	0.064	0.207	0.000
Pme16703	3	2	4	2	0.231	0.064	0.328	0.114
Pme17061	3	2	4	3	0.529	0.498	0.645	0.594
Pme17303	3	3	4	4	0.723	0.927	0.687	0.641
Pme20499	6	5	5	6	0.722	0.658	0.771	0.803
Pme25414	6	4	6	5	0.734	0.676	0.594	0.522
Pme32166	5	4	7	6	0.748	0.584	0.883	0.691
Pme44221	6	4	4	9	0.729	0.704	0.630	0.803
Pme44890	2	2	3	3	0.457	0.480	0.322	0.522
Pme48804	4	4	3	6	0.379	0.596	0.203	0.599
Pme49183	11	8	11	10	0.855	0.793	0.796	0.817
Pme66801	4	6	7	4	0.643	0.758	0.757	0.599
Pme75971	3	3	3	2	0.545	0.527	0.359	0.367
Pme79082	2	5	4	4	0.430	0.720	0.586	0.527
Pme85686	12	10	11	12	0.810	0.871	0.888	0.880
Pme112305	7	7	5	5	0.839	0.760	0.595	0.584
Average	**5.3**	**4.75**	**5.2**	**5.3**	**0.640**	**0.622**	**0.600**	**0.588**
Nos_01	1	2	3	4	0.624	0.614	0.695	0.275
Nos_03	4	1	2	1	0.537	0.000	0.519	0.060
Nos_07	8	1	3	1	0.696	0.000	0.570	0.060
Nos_08	6	2	1	1	0.876	0.474	0.572	0.060
Nos_11	9	5	4	1	0.857	0.503	0.617	0.060
Nos_23	6	2	2	1	0.736	0.382	0.074	0.000
Nos_41	8	2	1	2	0.952	0.523	0.217	0.117
Nos_42	13	4	8	3	0.882	0.483	0.666	0.116
Nos_43	3	1	2	1	0.414	0.000	0.074	0.000
Nos_44	10	3	6	2	0.902	0.549	0.598	0.059
Nos_46	4	3	4	1	0.828	0.455	0.462	0.000
Nos_48	4	3	3	1	0.831	0.966	0.393	0.060
Nos_51	6	2	2	1	0.807	0.400	0.283	0.000
Nos_60	9	3	4	1	0.853	0.416	0.213	0.000
Average	**6.5**	**2.0**	**3.0**	**1.5**	**0.771**	**0.412**	**0.425**	**0.062**

[1]Nei's unbiased gene diversity H_E (Nei 1978).

Test-PCRs with M13(-21) dye labelling. Initial screening for amplification and polymorphism was performed in 5 µl reaction volumes using the M13 method (Schuelke 2000), adding 1 µl of primer mix containing all loci to be labelled with the same fluorescent dye (forward primer without M13-tail = 0.15 µM; M13(-21)-tailed forward primer = 0.01 µM; dye-labelled M13(-21) primer = $n \times 0.15$ uM, n = number of loci in multiplex; reverse primer = 0.15 µM), 2.5 µl Jump-Start Taq ReadyMix (Sigma-Aldrich, Buchs, Switzerland), 0.5 µl genomic DNA, and 1 µl ddH_2O. PCR products were labelled with four fluorescent dyes (6FAM, VIC, PET, NED; Life

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Technologies, Rotkreuz, Switzerland) via the M13(-21) primer and were pooled before fragment analysis, which proceeded with an internal size standard, GeneScan-500 LIZ (Life Technologies, Rotkreuz, Switzerland) on a 3130xl Genetic Analyzer (Life Technologies, Rotkreuz, Switzerland) at WSL. PCR amplifications used an initial denaturation of 94 °C for 5 min, followed by 30 cycles of 30 s at 94 °C, 45 s at the annealing temperature of 55 °C, 45 s at 72 °C; followed by 8 cycles of 30 s at 94 °C, 45 s at 53 °C, 45 s at 72 °C to incorporate the dye-labelled M13(-21) primer (5′- TGT AAA ACG ACG GCC AGT -3′); and a final extension at 72 °C for 30 min.

PCR with dye-labelled primers. In a second step, PCR and fragment analyses were performed with fluorescent dye-labelled primers by deCODE Genetics (Reykjavik, Iceland). Fluorescent dye-labelled primers were used in three multiplex PCRs. Microsatellite panels were created using a Packard MultiPROBE II pipetting robot. PCR set-up for microsatellite genotyping and post-PCR liquid handling was carried out using a Zymark Sciclone Alh500 pipetting robot. For primer sequences and multiplex setup, see Table 1. The PCR contained 0.496 × PCR Gold Buffer, 3.26 mM $MgCl_2$, 324.8 µM dNTPs, and 0.04 U/µl AmpliTaq Gold Polymerase. PCR cycling conditions were: 95 °C for 10 min; five cycles of 95 °C for 15 s, 63 °C for 30 s (−2.5 °C per cycle), 72 °C for 30 s; 12 cycles of 95 °C for 15 s, 55 °C for 30 s, 72 °C for 1 min; 23 cycles of 89 °C for 15 s, 55 °C for 30 s, 72 °C for 1 min; followed by a final extension at 72 °C for 3 min. Again, alleles were sized using GeneScan-500 LIZ as an internal size standard but this time a 3730 DNA Analyzer (Life Technologies, Rotkreuz, Switzerland) was utilized for capillary electrophoresis. Samples were genotyped using the Microsatellite Plugin v.1.4 in Geneious v. 7.1.9 (Biomatters Ltd, Auckland, New Zealand).

Protocol for the species identification assay in singleplex. PCR products were labelled with NED fluorescent dye (Life Technologies, Rotkreuz, Switzerland) via the M13(-21) primer (NED-5′- TGT AAA ACG ACG GCC AGT -3′). PCR was performed in 5 µl reaction volumes, adding 1 µl of primer mix (Pme_ITS-F = 0.15 µM; M13(-21)-Pme_ITS-F (5′- TGT AAA ACG ACG GCC AGT TTT ACT TGG GTK CGG TTT -3′) = 0.01 µM; NED-dye labelled M13(-21) primer = 0.15 µM; Pme_ITS-R = 0.15 µM), 2.5 µl Jump-Start Taq ReadyMix (Sigma-Aldrich, Buchs, Switzerland), 0.5 µl genomic DNA, and 1 µl ddH_2O. Fragment analysis proceeded as above with a GeneScan-500 LIZ internal size standard (Life Technologies, Rotkreuz, Switzerland) on a 3130xl Genetic Analyzer (Life Technologies, Rotkreuz, Switzerland) at WSL. PCR cycling conditions were: initial denaturation at 94 °C for 5 min; followed by 30 cycles of 30 s at 94 °C, 45 s at the annealing temperature of 55 °C, 45 s at 72 °C; followed by 8 cycles of 30 s at 94 °C, 45 s at 53 °C, 45 s at 72 °C to incorporate the NED-labelled M13(-21) primer; and a final extension at 72 °C for 30 min. Fragment lengths were determined with Geneious v.7.1.9 (Biomatters Ltd, Auckland, New Zealand) as above.

Data analysis

Twenty-one loci were checked for polymorphism based on 122 thalli of *Peltigera membranacea*, collected from four localities. Polymorphism was determined using our own code in R v. 3.0.0 (R Core Team 2018). We calculated Nei's unbiased gene diversity H_E (Nei 1978) and allelic richness A_R, the average number of alleles per locus. To test whether populations and coastal/inland groups of populations were subdivided, analysis of molecular variance and pairwise F_{ST} values were calculated with Arlequin v.3.5 (Excoffier & Lischer 2010).

For the molecular species identification assay, alignments were inspected using MEGA X (Stecher *et al.* 2020) to determine the length of the sequences spanned by the primers developed in this study. Furthermore, a bar plot was generated showing the frequency of ITS amplicon sizes.

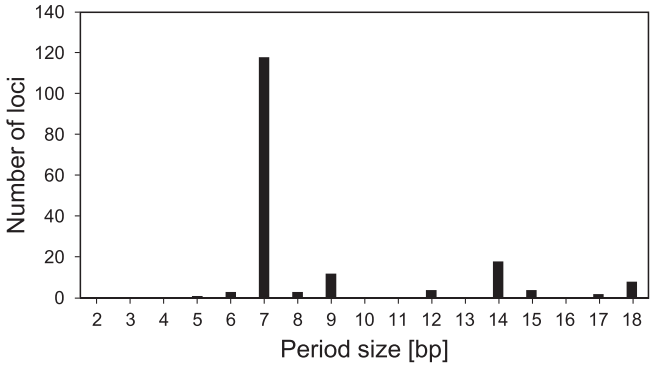

Fig. 3. Frequency of simple sequence repeat (SSR) loci with different period sizes [base pairs] for the genome of *Nostoc* N6, the strain used to design SSR markers. The figure is based on data from a search with TandemRepeatFinder v.4.08 (Benson 1999).

Results

The amplicon size of the ITS region allowed discrimination of 99.7% of Icelandic *Peltigera membranacea* specimens from two other common lichens in Iceland with similar morphology, *P. canina* and *P. praetextata*, as well as from *P. degenii*, *P.* '*scotteri*', *P. islandica* and *P. monticola* (Table 1; Supplementary Material Tables S1 & S2, available online). Screening of 1606 thalli revealed few (25, 1.5%) markedly shorter fragments (386 bp; Fig. 2), with a similar size to the expected amplicon size in *P. canina* or *P. praetextata*. When these samples were reinspected morphologically, 14 of these turned out to be *P. canina*, five were not *P. membranacea* but could not be identified further, and five (0.3%) had the typical morphology of *P. membranacea*.

Out of 355 contigs that contained microsatellites, 218 contigs blasted to ascomycetes (89 dinucleotides, 101 trinucleotides, 22 tetranucleotides, 2 pentanucleotides, 4 hexanucleotides). We tested polymorphism for *P. membranacea* in 20 SSR loci. All loci were polymorphic. Two loci had PCR failure rates > 10%. The number of alleles per locus ranged from 1–12, and gene diversity ranged from 0–0.927 (Table 2). The *Nostoc* N6 genome contained a total of 598 repeats, mostly with larger period sizes (average 61.9) and an average copy number of 6.3. There were 137 SSR with period sizes < 10 bp and 39 SSR with period sizes 10–20 bp. The distribution of period sizes in the *Nostoc* N6 genome was strongly asymmetrical (Fig. 3), with the most frequent period sizes for repeats under 20 bp being 7 (118 loci), 14 (18 loci), and 9 (12 loci).

The studied populations were significantly differentiated, both for the mycobiont and the photobiont (Tables 3 & 4), but the overall level of differentiation among populations was approximately four times higher in the photobiont (27.83% variance compared to 5.92% in the mycobiont; Table 3).

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Table 3. Analysis of molecular variance in *Peltigera membranacea* based on 20 SSR loci of the mycobiont and 14 of the *Nostoc* photobiont.

	df[1]	S[2]	V[3]	%[4]	F_{ST}[5]
Mycobiont					
Among populations	3	46.7	0.338	5.92	0.0591
Within populations	117	628.3	5.370	94.08	
Total	120	675.0	5.707		
Photobiont					
Among populations	3	24.2	0.250	27.83	0.2783
Within populations	115	74.6	0.649	72.17	
Total	118	98.9	0.899		

[1]Degrees of freedom.
[2]Sum of squares.
[3]Variance component.
[4]Percentage of variance.
[5]*F*-statistics. Both F_{ST} values were highly significant ($P < 0.01$).

Table 4. Pairwise F_{ST} values in four populations of the lichen *Peltigera membranacea* (below diagonal) and geographical distances (km) between them (above diagonal), based on 20 mycobiont and 14 cyanobiont SSRs. HF6, LA1, LL1 and LL3 = collecting localities.

	Mycobiont				Photobiont[1]			
	HF6	LA1	LL1	LL3	HF6	LA1	LL1	LL3
HF6	0	54.7	140.3	111.9	0	54.7	140.3	111.9
LA1	0.0477	0	84.5	54.1	0.2427	0	84.5	54.1
LL1	0.0716	0.0631	0	31.3	0.2717	0.0513	0	31.3
LL3	0.0400	0.0587	0.0801	0	0.4527	0.1839	0.1454	0

[1]Nonsignificant F_{ST} value underlined ($P = 0.05$).

Discussion

The size of the ITS region amplified by our new set of loci can act as a valuable proxy to sort out suspicious field-identified specimens. The ITS sizes of *P. membranacea* and *P. canina* overlap somewhat in Icelandic populations, but the short allele size is quite rare in *P. membranacea* (found in five or 0.3% of the specimens examined). Screening the ITS region of 1606 samples revealed that misidentifications did not represent a major concern in our data set; this differs from what has been reported for *Lobaria* section *Lobaria* from the Macaronesian Islands (Werth *et al.* 2010). While the ITS size assay works well in an area with comparatively little variability within *Peltigera membranacea* and allies such as Iceland, more work would be necessary before it can be applied to other places with higher variability in these species, or with more species belonging to the same group.

The variability of the microsatellite loci developed for the mycobiont *P. membranacea* was similar to that of microsatellites developed for other lichen-forming fungi, such as *Peltigera dolichorhiza* (Magain *et al.* 2010), which is the only species of *Peltigera* for which microsatellites are currently available. The four studied populations of *P. membranacea* exhibited moderate amounts of variability, a level that was comparable to that of populations of *Lobaria pulmonaria* in the Swiss Jura subjected to uneven-aged forestry (gene diversity $H_E \approx 0.5$) (Werth *et al.* 2006). The genetic diversity of the photobiont of *P. membranacea* varied markedly between populations, seemingly independent of the variation of the mycobiont populations.

We found significant differentiation between all studied populations of the mycobiont of *P. membranacea* and between all but one populations of the photobiont. This result was not unexpected as the localities studied were remote from each other (31–140 km; Fig. 1), at distances where we would not expect frequent gene flow via thallus fragments or ascospores. The new microsatellites provide a valuable tool to quantify population level variability in *Peltigera membranacea* and its *Nostoc* photobiont.

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Author Contribution. SW conceived the study, performed DNA isolations, genotyped the samples, analyzed the data and wrote the first draft of the manuscript. SÞP performed PCRs and fragment analyses. ÓSA contributed to manuscript writing.

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