


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 & Andr sson 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; J riado *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-W rg tter 2013; Werth *et al.* 2013; Alors *et al.* 2014; Belinch n *et al.* 2014; Devkota *et al.* 2014; Nadyeina *et al.* 2014; T rra *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* (Ronn s *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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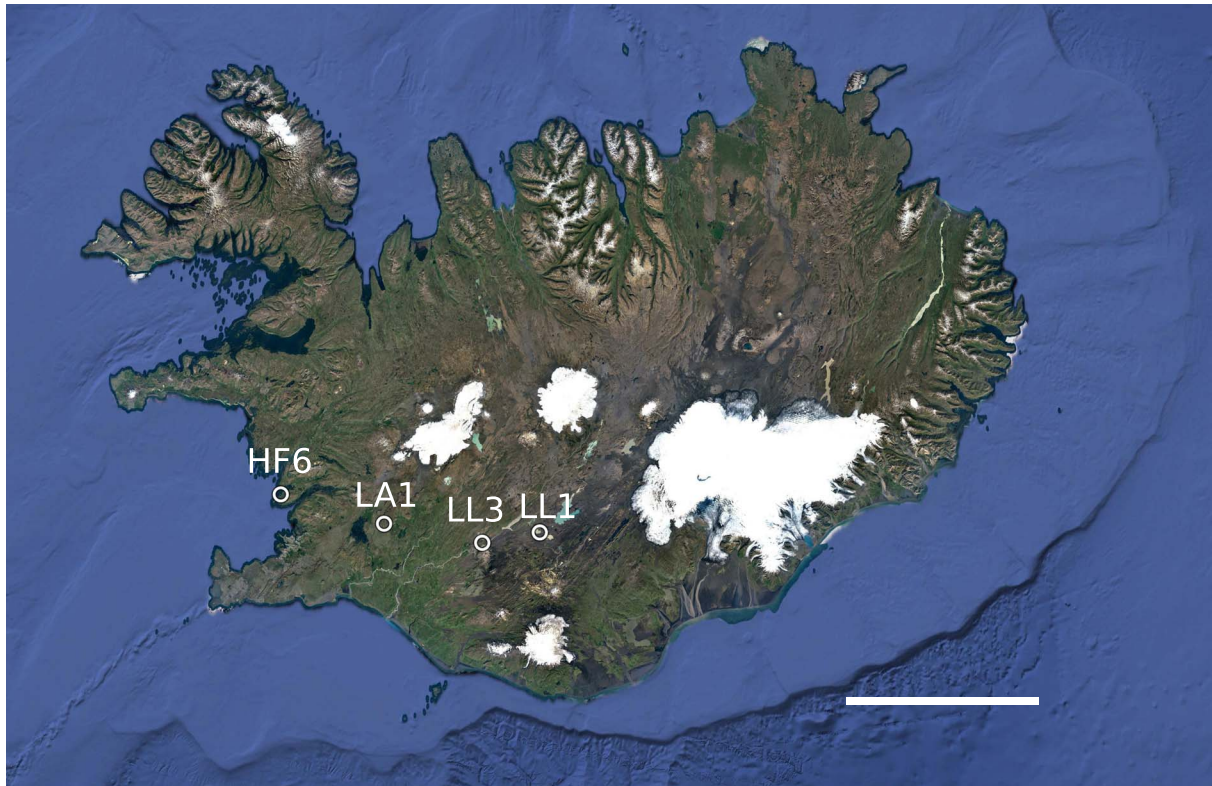


Fig. 1. Map of the collecting localities (labelled) in western Iceland. The largest distance between sites was 140 km. The map was made with Google Earth v.7.3.3.7786. Data attribution: SIO, NOAA, US Navy, NGA, GEBCO, Image Landsat/Copernicus, Image IBCAO. Scale = 100 km. In colour online.

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 & Andr sson 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

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 | TTTACTTGGGKCGGTTT | 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 | TGATAGAAAAGTAACCGAATCGGA | ACAAGTAGGCGACTACATATGT | 2 | 105–127 | 1a |
| Pme07704 | 3 | 1.7 | PET | ACCAATGATTCCAGCGCCTC | CCGAACCCAAATCGAGTCC | 3 | 454–464 | 2 |
| Pme112305 | 10 | 1.7 | PET | CGAAGGCATTTGTTGCGCTT | TGTGCCGATCCATGCGATTT | 2 | 245–272 | 2 |
| Pme115956 | 10 | 2.5 | FAM | TCCAGACCACTACTCGGTGGG | TCCCGTAGTGTGTGCTGATTG | 3 | 186–221 | 1a |
| Pme16703 | 5 | 0.8 | VIC | CGAGGGCATTGTCTGGTCTC | CATTGACCGCTGCATATCCT | 2 | 185–206 | 1a |
| Pme17061 | 4 | 0.8 | FAM | CTGCCGTCAATTTGTTGCCAT | CCTGTTTTGGCGTCTACGAA | 3 | 206–225 | 2 |
| Pme17303 | 6 | 34.7 | NED | AGTGAGAAAAGGGAGAGCCGG | ATCCAGTCGCTAAGCTCAG | 6 | 107–152 | 2 |
| Pme20499 | 6 | 1.7 | NED | CAACTGGGCAACGGGTACAA | GATTCCTCAGCTAGCGAG | 4 | 336–356 | 1a |
| Pme25414 | 8 | 1.7 | PET | AGTTCGCGAAGTACTGCTGC | CTAATCGCTCTGCCCTGCTG | 3 | 90–118 | 2 |
| Pme32166 | 8 | 6.6 | FAM | AGCTGTTGCGCAAAACATACAA | GCTGTGCGCCGAAATGGATT | 2 | 455–474 | 2 |
| Pme44221 | 13 | 2.5 | FAM | GCAGTCCCAGCAAGATTGTT | TTTGCGAAGGGAAGCAAGT | 2 | 326–365 | 2 |
| Pme44890 | 3 | 1.7 | NED | AATGTGGGGGACACAGCAAC | TGTGTTCTTGACGGGGTCT | 5 | 408–418 | 2 |
| Pme48804 | 8 | 0 | PET | TGTAGCGGATATCGATTTTGGT | TACGCTGGAATGCCTAACT | 4 | 392–447 | 1b |
| Pme49183 | 17 | 0 | FAM | CCGTTTACCAGATGGTGACA | GCAAAACGCAAGCAGGAGG | 4 | 323–545 | 1b |
| Pme66801 | 9 | 1.7 | NED | AGATAACGTCTGCGGGCGAA | TCTCCTGGTCATGTTGCATCC | 3 | 82–116 | 2 |
| Pme75971 | 4 | 0 | FAM | TCTGAATGCTCTTTGGCGC | CACGGGAGAGAGGTTTGC | 2 | 85–102 | 2 |
| Pme79082 | 6 | 1.7 | VIC | GAGAAGAAAAGAAACGGTTGGGG | ATGGTTACATATAAAATGGGGACCC | 2 | 243–263 | 2 |
| Pme85686 | 19 | 2.5 | PET | TATGTCACGTGAGAACGCGG | ACATGAGGAGGTAACGAAGAAAGC | 3 | 130–201 | 1a |
| Nos_01 ¹¹ | 6 | 26.1 | VIC | AGGGGATTTAAACCTGTGG | TCGAGCAATTAGCACAGTAA | 6 | 174–351 | X3213 |
| Nos_03 | 4 | 6.7 | NED | CTAGGTGTCGGTGTAGGTT | AGAAGACTATCGACTCACCA | 6 | 169–188 | X3213 |
| Nos_07 | 9 | 7.6 | NED | GTGTTCTCAGAGTTGTCAT | ATAGAACAACCTCCGCAAAT | 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 | GAATGCCAACAAGTCGTTT | TGTCATCAGAAAACCTCTCAA | 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 | AAGCTGGAGCATATCTGGA | GGCTAACATCACAGATCCAT | 9 | 104–155 | X3212 |
| Nos_51 | 6 | 5.9 | PET | GGGTAGAGGCATAAAGTTACA | CCTAGCAAAACTTCAAGCTG | 12 | 119–160 | X3212 |
| Nos_60 | 14 | 0 | FAM | AGAAGGACTATCTACCACGG | TTTTAGCGTCTGTCTTCGAC | 11 | 123–215 | X3212 |

¹Number of alleles.

²Percentage of PCR failures.

³Fluorescent dye.

⁴Sequence of M13(21)-labelled forward primer from 5' to 3'.

⁵Sequence of reverse primer from 5' to 3'.

⁶Number of repeated bp.

⁷Range of allele sizes in bp.

⁸Allocation to multiplex.

⁹Primers were designed for an annealing temperature of 55 °C. Sample sizes are n = 121 in *P. membranacea*, and n = 119 in *Mostoc*.

¹⁰'Pme' in the primer name stands for a primer of the mycobiont, *Peltigera membranacea*.

¹¹'Nos' stands for a primer of the *Mostoc* cyanobiont of *P. membranacea*.

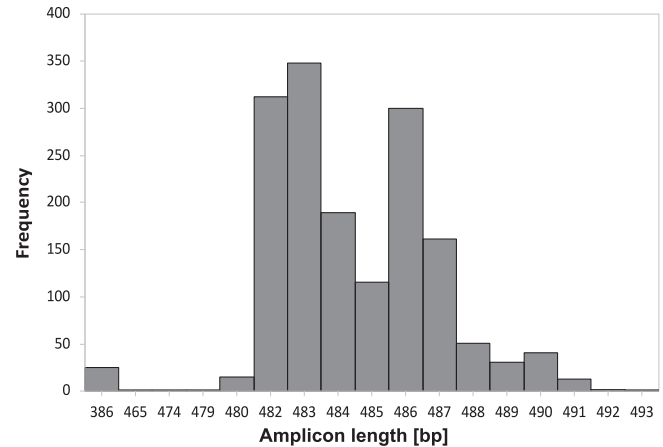


Fig. 2. Frequency of amplicon lengths (base pairs) of the internal transcribed spacer (ITS) region in 1606 thalli of *Peltigera membranacea* from Iceland, amplified with the Pme_ITS-primers (this study).

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.

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.

| Locus | Number of alleles | | | | Nei's gene diversity H_E ¹ | | | |
|----------------|-------------------|-------------|------------|------------|---|--------------|--------------|--------------|
| | 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 |

¹Nei'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 μ l reaction volumes using the M13 method (Schuelke 2000), adding 1 μ l of primer mix containing all loci to be labelled with the same fluorescent dye (forward primer without M13-tail = 0.15 μ M; M13(-21)-tailed forward

primer = 0.01 μ M; dye-labelled M13(-21) primer = $n \times 0.15$ μ M, n = number of loci in multiplex; reverse primer = 0.15 μ M), 2.5 μ l Jump-Start Taq ReadyMix (Sigma-Aldrich, Buchs, Switzerland), 0.5 μ l genomic DNA, and 1 μ l ddH₂O. PCR products were labelled with four fluorescent dyes (6FAM, VIC, PET, NED; Life

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 MgCl₂, 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 ddH₂O. 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

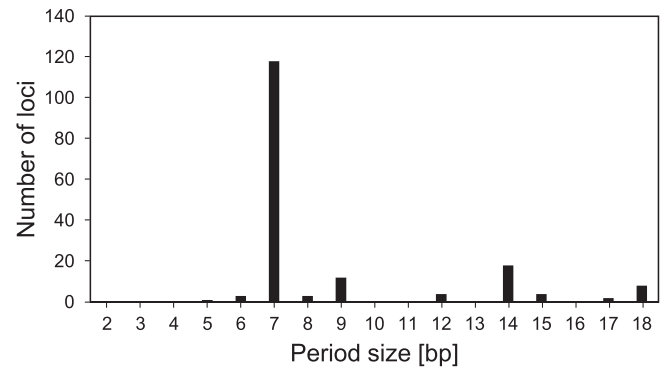


Fig. 3. Frequency of simple sequence repeat (SSR) loci with different period sizes [base pairs] for the genome of *Nostoc* N6, the strain used to design SSR markers. The figure is based on data from a search with TandemRepeatFinder v.4.08 (Benson 1999).

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.

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).

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 | | |

¹Degrees of freedom.²Sum of squares.³Variance component.⁴Percentage of variance.⁵F-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 | <u>0.0513</u> | 0 | 31.3 |
| LL3 | 0.0400 | 0.0587 | 0.0801 | 0 | 0.4527 | 0.1839 | 0.1454 | 0 |

¹Nonsignificant 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. SPP performed PCRs and fragment analyses. ÓSA contributed to manuscript writing.

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References

- Alors D, Dal Grande F, Schmitt I, Kraichak E, Lumbsch HT, Crespo A and Divakar PK (2014) Characterization of fungus-specific microsatellite markers in the lichen-forming fungus *Parmelina carporrhizans* (Parmeliaceae). *Applications in Plant Sciences* 2, 1400081.
- Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25, 3389–3402.
- Belinchón R, Ellis CJ and Yahr R (2014) Microsatellite loci in two epiphytic lichens with contrasting dispersal modes: *Nephroma laevigatum* and *N. parile* (Nephromataceae). *Applications in Plant Sciences* 2, 1400080.
- Benson G (1999) Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acid Research* 27, 573–580.
- Dettki H (1998) Dispersal of fragments of two pendulous lichen species. *Sauteria* 9, 123–132.
- Devkota S, Cornejo C, Werth S, Chaudhary RP and Scheidegger C (2014) Characterization of microsatellite loci in the Himalayan lichen fungus *Lobaria pindarensis* (Lobariaceae). *Applications in Plant Sciences* 2, 1300101.
- Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acid Research* 32, 1792–1797.
- Excoffier L and Lischer H (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10, 564–567.
- Faircloth BC (2008) MSATCOMMANDER: detection of microsatellite repeat arrays and automated, locus-specific primer design. *Molecular Ecology Resources* 8, 92–94.
- Gagunashvili AN and Andrésson ÓS (2018) Distinctive characters of *Nostoc* genomes in cyanolichens. *BMC Genomics* 19, 434.
- Guzow-Krzeminska B and Stocker-Wörgötter E (2013) Development of microsatellite markers in *Protopermaliopsis muralis* (lichenized Ascomycete) – a common lichen species. *Lichenologist* 45, 791–798.
- Hansen ES (2009) A contribution to the lichen flora of Iceland. *Folia Cryptogamica Estonica* 46, 45–54.
- Heinken T (1999) Dispersal patterns of terricolous lichens by thallus fragments. *Lichenologist* 31, 603–612.
- Jones TC, Green TGA, Hogg ID and Wilkins RJ (2012) Isolation and characterization of microsatellites in the lichen *Buellia frigida* (Physciaceae), an Antarctic endemic. *American Journal of Botany* 99, e131–e133.
- Jüriado I, Kaasalainen U and Rikkinen J (2017) Specialist taxa restricted to threatened habitats contribute significantly to the regional diversity of *Peltigera* (Lecanoromycetes, Ascomycota) in Estonia. *Fungal Ecology* 30, 76–87.
- Jüriado I, Kaasalainen U, Jylhä M and Rikkinen J (2019) Relationships between mycobiont identity, photobiont specificity and ecological preferences in the lichen genus *Peltigera* (Ascomycota) in Estonia (northeastern Europe). *Fungal Ecology* 39, 45–54.
- Kampa A, Gagunashvili AN, Gulder TAM, Morinaka BI, Daolio C, Godejohann M, Miao VPW, Piel J and Andrésson ÓS (2013) Metagenomic natural product discovery in lichen provides evidence for a family of biosynthetic pathways in diverse symbioses. *Proceedings of the National Academy of Sciences of the United States of America* 110, E3129–E3137.
- Lagostina E, Dal Grande F, Ott S and Printzen C (2017) Fungus-specific SSR markers in the Antarctic lichens *Usnea antarctica* and *U. aurantiacoatra* (Parmeliaceae, Ascomycota). *Applications in Plant Sciences* 5, 1700054.
- Lutsak T, Fernández-Mendoza F, Greshake B, Dal Grande F, Ebersberger I, Ott S and Printzen C (2016) Characterization of microsatellite loci in the lichen-forming fungus *Cetraria aculeata* (Parmeliaceae, Ascomycota). *Applications in Plant Sciences* 4, 1600047.
- Magain N, Forrest LL, Serusiaux E and Goffinet B (2010) Microsatellite primers in the *Peltigera dolichorhiza* complex (lichenized ascomycete, Peltigerales). *American Journal of Botany* 97, e102–e104.
- Magain N, Tniong C, Goward T, Niu D, Goffinet B, Serusiaux E, Vitikainen O, Lutzoni F and Miadlikowska J (2018) Species delimitation at a global scale reveals high species richness with complex biogeography and patterns of symbiont association in *Peltigera* section *Peltigera* (lichenized Ascomycota: Lecanoromycetes). *Taxon* 67, 836–870.
- Mansournia MR, Wu B, Matsushita N and Hogetsu T (2011) Genotypic analysis of the foliose lichen *Parmotrema tinctorum* using microsatellite markers: association of mycobiont and photobiont, and their reproductive modes. *Lichenologist* 44, 1–22.
- Martinez I and Burgaz AR (1996) Anatomical study of *Peltigera canina*, *P. membranacea* and *P. praetextata* (lichenized Ascomycotina). *Annales Botanici Fennici* 33, 223–229.
- Miadlikowska J and Lutzoni F (2000) Phylogenetic revision of the genus *Peltigera* (lichen-forming Ascomycota) based on morphological, chemical, and large subunit nuclear ribosomal DNA data. *International Journal of Plant Sciences* 161, 925–958.
- Miadlikowska J and Lutzoni F (2004) Phylogenetic classification of peltigeralean fungi (Peltigerales, Ascomycota) based on ribosomal RNA small and large subunits. *American Journal of Botany* 91, 449–464.
- Miadlikowska J, Lutzoni F, Goward T, Zoller S and Posada D (2003) New approach to an old problem: incorporating signal from gap-rich regions of ITS and rDNA large subunit into phylogenetic analyses to resolve the *Peltigera canina* species complex. *Mycologia* 95, 1181–1203.
- Miao VPW, Rabenau A and Lee A (1997) Cultural and molecular characterization of photobionts of *Peltigera membranacea*. *Lichenologist* 29, 571–586.
- Nadyeina O, Cornejo C, Boluda CG, Myllys L, Rico VJ, Crespo A and Scheidegger C (2014) Characterization of microsatellite loci in lichen-forming fungi of *Bryoria* section *Implexae* (Parmeliaceae). *Applications in Plant Sciences* 2, 1400037.
- Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89, 583–590.
- O'Brien HE, Miadlikowska J and Lutzoni F (2009) Assessing reproductive isolation in highly diverse communities of the lichen-forming fungal genus *Peltigera*. *Evolution* 63, 2076–2086.
- Prieto M, Romera L, Merinero S, Aragón G and Martínez I (2015) Development and characterization of fungal specific microsatellite markers in the lichen *Lobaria scrobiculata* (Lobariaceae, Ascomycota). *Lichenologist* 47, 183–186.
- R Core Team (2018) *R: a Language and Environment for Statistical Computing* (version 3.6.3). R Foundation for Statistical Computing, Vienna, Austria [WWW resource] URL <https://www.R-project.org>.
- Ronnäs C, Werth S, Ovaskainen O, Várkonyi G, Scheidegger C and Snäll T (2017) Discovery of long-distance gamete dispersal in a lichen-forming ascomycete. *New Phytologist* 216, 216–226.
- Rozen S and Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In Krawetz S and Misener S (eds), *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Totowa, New Jersey: Humana Press, pp. 365–386.
- Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology* 18, 233–234.
- Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, et al. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology* 7, 539.
- Stecher G, Tamura K and Kumar S (2020) Molecular Evolutionary Genetics Analysis (MEGA) for MacOS. *Molecular Biology and Evolution* 37, 1237–1239.
- Törta T, Cornejo C, Cheenacharoen S, Grande FD, Marmor L and Scheidegger C (2014) Characterization of fungus-specific microsatellite markers in the lichen fungus *Usnea subfloridana* (Parmeliaceae). *Applications in Plant Sciences* 2, 1400034.
- Vitikainen O (1994) Taxonomic revision of *Peltigera* (lichenized Ascomycotina) in Europe. *Acta Botanica Fennica* 152, 1–96.
- Walser JC, Sperisen C, Soliva M and Scheidegger C (2003) Fungus-specific microsatellite primers of lichens: application for the assessment of genetic variation on different spatial scales in *Lobaria pulmonaria*. *Fungal Genetics and Biology* 40, 72–82.
- Wedin M, Wiklund E, Jørgensen PM and Ekman S (2009) Slippery when wet: phylogeny and character evolution in the gelatinous cyanobacterial lichens (Peltigerales, Ascomycetes). *Molecular Phylogenetics and Evolution* 53, 862–871.
- Werth S, Wagner HH, Holderegger R, Kalwij JM and Scheidegger C (2006) Effect of disturbances on the genetic diversity of an old-forest associated lichen. *Molecular Ecology* 15, 911–921.

- Werth S, Cornejo C and Scheidegger C** (2010) A species-specific real-time PCR assay for identification of three lichen-forming fungi, *Lobaria pulmonaria*, *Lobaria immixta*, and *Lobaria macaronesica*. *Molecular Ecology Resources* **10**, 401–403.
- Werth S, Cornejo C and Scheidegger C** (2013) Characterization of microsatellite loci in the lichen fungus *Lobaria pulmonaria* (Lobariaceae). *Applications in Plant Sciences* **1**, 1200290.
- Widmer I, Dal Grande F, Cornejo C and Scheidegger C** (2010) Highly variable microsatellite markers for the fungal and algal symbionts of the lichen *Lobaria pulmonaria* and challenges in developing biont-specific molecular markers for fungal associations. *Fungal Biology* **114**, 538–544.
- Xavier BB, Miao VPW, Jónsson ZO and Andrésón ÓS** (2012) Mitochondrial genomes from the lichenized fungi *Peltigera membranacea* and *Peltigera malacea*: features and phylogeny. *Fungal Biology* **116**, 802–814.
- Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S and Madden TL** (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**, 134.