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CHARACTERIZATION OF FUNGUS-SPECIFIC MICROSATELLITE MARKERS IN THE LICHEN FUNGUS *USNEA SUBFLORIDANA* (PARMELIACEAE)¹

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- **Premise of the study:** Microsatellite loci were developed for the haploid lichenized fungal species *Usnea subfloridana* to study its population subdivision and the species' response to forest disturbance, fragmentation, and environmental pollution.
- **Methods and Results:** We developed 14 polymorphic microsatellite markers using 454 pyrosequencing data of *U. subfloridana*. The number of alleles per locus ranged from three to 15, and Nei's unbiased gene diversity averaged over nine markers without null alleles ranged from 0.64 to 0.67. Evaluation of the cross-species amplification in *U. glabrescens* and *U. wasmuthii* indicates that these markers are also informative in other *Usnea* species.
- **Conclusions:** These markers will allow us to investigate the effects of forest management and environmental pollution on genetic population structure of *U. subfloridana* and closely related species. Moreover, they will help facilitate phylogeographic studies of *U. subfloridana* across the species' distribution area in Europe.

Key words: Ascomycetes; lichen-forming fungi; microsatellites; population subdivision; *Usnea florida*; *Usnea subfloridana*.

Conspicuous epiphytic lichens like beard lichens (*Usnea* Dill. ex Adans.) are valuable indicators of forest ecosystems, hence contributing to monitoring the conservation value of forest landscapes (Will-Wolf et al., 2002). *Usnea subfloridana* Stirton is a widely distributed species occurring in Europe (Törta and Randlane, 2007; Randlane et al., 2009), appearing from the northern boreal to temperate regions (Halonen et al., 1998). The sexually reproducing *U. florida* (L.) Weber ex F. H. Wigg. and *U. subfloridana*, which has a predominantly asexual reproduction with symbiotic propagules, represent a typical species pair, as they share many morphological characters but differ by the characters associated with their dissimilar dispersal strategies (Articus et al., 2002; Randlane et al., 2009). Here, we develop 14 microsatellite markers to study the impact of land use and habitat fragmentation on the lichen's dispersal and population subdivision.

METHODS AND RESULTS

We selected three *U. subfloridana* specimens sampled from Norway, Finland, and Lithuania and two *U. florida* specimens sampled from the United

Kingdom (Appendix 1). The central axis, which is of pure fungal origin (haploid), was manually separated and used for DNA extraction with the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's protocol. Library preparation and whole genome 454 pyrosequencing of pooled DNA samples was performed by Microsynth (Balgach, Switzerland) using a Roche GS FLX sequencer to generate enough random sequences and to isolate a sufficient number of microsatellite loci. Shotgun libraries were prepared using the GS FLX Titanium Rapid Library Preparation Kit (Roche Diagnostics, Basel, Switzerland), and Microsynth provided barcode adapters. We obtained 85,718 reads with an average length of 391 bases and a total of 27,344,042 bases out of a 2/16th run. We screened for all sequence motifs of di-, tri-, tetra-, and pentanucleotide microsatellites in the unassembled reads using MSATCOMMANDER 1.0.2 alpha (Faircloth, 2008). Microsatellites with motifs repeated at least eight times (for di- and trinucleotides) or six times (for all others) were selected. Primer pairs were developed with Primer3 (Rozen and Skaletsky, 2000), implemented in the software MSATCOMMANDER 1.0.2 alpha using the default parameters except for the following: optimal primer size 20 bp, product size 150–450 bp, melting temperature (T_m) 58–65°C. We found 132 primer pairs that fulfilled the specified primer parameters, but 68 pairs were later discarded either because they were duplicates, which were detected after alignment using CLC DNA Workbench 5 (CLC bio, Aarhus, Denmark), or because they contained mononucleotide repeats in the flanking region.

Additionally, we set up axenic algal cell cultures of the photobiont of *U. subfloridana* to assess the symbiont specificity of the newly designed markers. The culture was established under sterile conditions on 1/4 of strength of original *Trebouxia* Organic Nutrient Medium–I according to Ahmadjian (1967). Algal cells were taken from the algal layer of *U. subfloridana* thalli and inoculated on the medium. The cultivation took place under diurnal light (12 h) and darkness (12 h) for four months before the algal culture was harvested and deposited at the Swiss Federal Research Institute WSL (cultures TTA1 and TTA2) at –80°C. Algal cells were disrupted and DNA was extracted with MO BIO PowerPlant DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California, USA) according to the manufacturer's protocol. The three loci that produced positive PCR reactions were excluded from further analyses because

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they were considered alga-specific rather than fungus-specific. For PCR amplification, forward primers were labeled with an M13 tag (5'-TGTAACGACGGCCAGT-3') (Schuelke, 2000). PCR reactions were performed in a total volume of 10 µL containing 1 µL of ~1–5 ng genomic DNA, 0.5 µL of 5 µM forward and reverse primers, and 2× Type-it Multiplex PCR Master Mix (QIAGEN). All PCRs were performed on Veriti Thermal Cyclers (Life Technologies, Carlsbad, California, USA). The PCR reactions were assessed using a temperature gradient with one-degree increments from 56–61°C, and under the following conditions: denaturation for 5 min at 95°C, followed by 33 cycles of 30 s at 95°C, 90 s at 56–61°C, and 30 s at 72°C; then for the M13-tag binding an additional eight cycles of 30 s at 95°C, 90 s at 53°C, and 30 s at 72°C, with a final extension of 30 min at 60°C were run.

Only primers failing to amplify with DNA extracted from the axenic algal culture were considered of fungal origin. These 61 primers were tested for variability under the same conditions as above and using the total DNA of eight specimens of *U. subfloridana* collected from EVO population from southern Estonia (Appendix 1), resulting in 14 loci with satisfactory amplification. Cross-species amplification of two closely related species (Saag et al., 2011) was tested with 12 specimens of *U. glabrescens* (Vainio) Vainio and 14 specimens of *U. wasmuthii* Räsänen, which were collected from the same site. Approximately 50 mg dry weight of each lichen thallus was lyophilized overnight and ground in a Retsch MM2000 mixer mill (Düsseldorf, Germany) for 3 min at 30 Hz, and total DNA was extracted with the same procedure as the algal cells.

To characterize the 14 polymorphic *U. subfloridana* loci (Table 1), we analyzed PCR products of 174 individuals from three populations (Table 2, Appendix 1). Fluorescent forward primers were used for the PCR protocol and the reaction was adjusted to: 5 min at 95°C, followed by 28 cycles of 30 s at 95°C, 90 s at 57°C, and 30 s at 72°C, with a final extension of 80 min at 60°C. PCR products were multiplexed (Table 1) and run on a 3130xl DNA Analyzer with GeneScan 500 LIZ Size Standard (G5 dye set) for fragment analysis (both by Life Technologies). Alleles were determined

using GeneMapper version 3.7 (Life Technologies). To characterize the variability of each microsatellite locus, we counted the number of alleles and calculated Nei's unbiased gene diversity using Arlequin version 3.11 (Excoffier et al., 2005).

Sequences of the 14 polymorphic loci were deposited in GenBank as they appear in the original pyrosequencing sample (Table 1). Five loci (Us10–Us14) had more than 10% of null alleles, possibly because of mutations in the primer regions, and were therefore omitted from the population analyses. The nine microsatellite loci that had no null alleles (Us1–Us9) produced three to 15 alleles per locus with a mean of 8.78. Nei's unbiased gene diversity, averaged over nine markers, ranged from 0.64 to 0.67 (Table 2). After PCR optimization for the annealing temperature (Table 1), all nine primers successfully amplified and were polymorphic in *U. subfloridana*, *U. wasmuthii*, and *U. glabrescens*, except marker Us07, which showed no polymorphism in *U. glabrescens*. As is often the case in populations of highly clonal organisms such as lichens (Walser et al., 2004; Dal Grande et al., 2012), significant linkage disequilibrium was found using Arlequin version 3.11 in 41 *U. subfloridana* distinct multilocus genotypes for two pairs of markers (i.e., Us02–Us06 and Us05–Us08).

CONCLUSIONS

The manual separation of the purely fungal central axis of the genus *Usnea* did not provide pure fungal DNA as expected. This preparation led to mixed DNA of the two fungal and algal symbionts and thus symbiont-specificity of genetic markers has to be tested in lichens (Devkota et al., 2014) even if lichens contain purely fungal plectenchyma. The newly developed, highly variable fungus-specific markers are currently being

TABLE 1. Characteristics of 14 microsatellite loci developed for the lichen fungus *Usnea subfloridana*.^a

Locus	Primer sequences (5'–3')	Repeat motif	Fluorescent dye	T _a (°C)	Allele size range (bp)	Primer conc. (µM)	Multiplex ^b	GenBank accession no.
Us01	F: CCCTCCAATCCCATATATAC R: GATCCATCGTCTCCATGATA	(CA) ₁₀	PET	59	181–303	0.02	1	KC912190
Us02	F: GTTTGCGAGGGATACTTTAC R: GTAGAAAGACAAGGACGCAC	(TA) ₁₀	NED	57	83–89	0.06	2	KC912191
Us03	F: CCATCTCAAACCCGATAGTC R: CCTCTTATCCTTTGGCAAAT	(CTT) ₁₁	PET	57	192–218	0.12	2	KC912192
Us04	F: ATTCCTCCGAGTCAAGTAG R: CTTTACTTTGCGAGCTTCAG	(CAT) ₉	VIC	57	181–196	0.06	2	KC912193
Us05	F: TCTGGGATGGATGGCATT R: GCGGGAAAGCCACAGATA	(CA) ₉	FAM	57	161–175	0.20	2	KC912194
Us06	F: CGATGGCGTAATGATCAG R: CAAACATTTCGCGTCAGTTAC	(GAT) ₉	FAM	57	106–127	0.12	2	KC912195
Us07	F: TCCTGAGTTTGAGCCTCTTG R: GTCCAACGCAGCTACTCTCT	(GTC) ₈	VIC	57	123–135	0.02	3	KC912196
Us08	F: CCTTCACCTTCCCTATACACC R: GAATCCAGTCAATGCACTC	(AAC) ₁₀	NED	57	192–217	0.03	3	KC912197
Us09	F: GGAAGGTATGAGGTGAGGTG R: AGAATTTGCAGCACGGGTA	(GGAT) ₆	FAM	57	341–372	0.30	3	KC912198
Us10	F: ACGAGGGAGGGTAGTTAGG R: GTATGATTGGTTGTGATGCG	(GA) ₉	PET	59	220–318	1.0	1	KJ446958
Us11	F: AGCATCCACAACACACTTC R: CACTCTTTATCCTGCTCGC	(GCA) ₉	NED	59	242–245	1.0	1	KJ446959
Us12	F: CCCTTCTTCTACTCGGTATC R: CCTACTTCTACCATGCGTCC	(TTC) ₈	PET	57	281–287	0.2	3	KJ446960
Us13	F: CCATTTACGGCAGAAGACTC R: CGTAGATGGGAGTCCGATAT	(CTT) ₁₁	FAM	57	122–176	0.1	3	KJ446961
Us14	F: GACCATTAGCCAGGTTAGAG R: CACAGGGTCTCATGATTCTG	(GCTT) ₆	FAM	59	189–205	0.1	1	KJ446962

Note: T_a = annealing temperature.
^aSee Appendix 1 for specimens used.
^bMultiplex indicates loci that were mixed in the same capillary electrophoresis run.

TABLE 2. Characteristics of nine polymorphic microsatellite loci developed for *Usnea subfloridana*^a and screened in 174 individuals.

Locus	Total (n = 174)		EPV (n = 56)		EVO (n = 60)		EVY (n = 58)	
	A	H _e	A	H _e	A	H _e	A	H _e
Us01	15	0.81	9	0.81	14	0.84	9	0.74
Us02	6	0.78	6	0.75	5	0.79	5	0.76
Us03	14	0.78	9	0.73	10	0.77	12	0.83
Us04	3	0.43	3	0.48	3	0.41	3	0.39
Us05	9	0.63	7	0.69	7	0.63	6	0.55
Us06	7	0.71	6	0.72	6	0.72	6	0.71
Us07	6	0.66	4	0.65	6	0.68	4	0.67
Us08	7	0.72	7	0.77	6	0.70	6	0.68
Us09	12	0.42	6	0.43	9	0.43	5	0.41
Average	8.78	0.66	6.33	0.67	7.33	0.66	6.22	0.64

Note: A = number of alleles; H_e = Nei's unbiased gene diversity; n = total number of samples analyzed.
^aSee Appendix 1 for populations used in the study.

used to study the genetic differentiation and diversity in *U. subfloridana*, *U. florida*, and related species and will allow us to investigate effects of forest management and environmental pollution on genetic population structure in epiphytic lichens.

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APPENDIX 1. Location information, number of individuals sampled, and voucher specimens of *Usnea* species used in this study.

Species	Locality	Population	Latitude	Longitude	n	Herbarium ^a	Date
<i>U. florida</i> *	United Kingdom: Devon, Dartmoor, Lydford	UK	50°33'20"N	04°02'58"W	1	TU-FLO 02	07.10.2006
<i>U. florida</i> *	United Kingdom: Devon, Morwellham Quay, river Tamar	UK	50°33'20"N	04°02'58"W	1	TU-FLO 03	06.10.2006
<i>U. glabrescens</i>	Estonia: Community of Põlva Vald	EPV_16a	58°06'13.6"N	27°04'29.1"E	1	WSL-21566	29.06.2007
<i>U. subfloridana</i> *	Norway: Hordaland, Voss, Tvinde	NOR	60°43'43"N	06°29'13"E	1	TU-SUB 01	12.07.2006
<i>U. subfloridana</i> *	Lithuania: Birzai district, Birzai forest, Buginiai forestry	LIT	56°14'N	24°58'E	1	TU-SUB 05	13.05.2006
<i>U. subfloridana</i> *	Finland: Northern Savonia, Suonenjoki, Mustolanmäki	FIN	62°44'39"N	27°01'28"E	1	TU-SUB 10	10.07.2007
<i>U. subfloridana</i>	Estonia: Community of Põlva Vald	EPV	58°06'13.6"N	27°04'29.1"E	56	WSL-21521-21583	29.06.2007
<i>U. subfloridana</i>	Estonia: Community of Vastse-Kuuste Vald	EVO	58°07'23.7"N	26°59'22.0"E	60	WSL-21773-21834	03.07.2007
<i>U. subfloridana</i>	Estonia: Community of Vastse-Kuuste Vald	EVY	58°08'29.3"N	27°02'57.9"E	58	WSL-21899-21961	04.07.2007
<i>U. wasmuthii</i>	Estonia: Community of Põlva Vald	EPV_7a	58°06'13.6"N	27°04'29.1"E	1	WSL-21539	29.06.2007

Note: n = number of samples analyzed; Herbarium = Herbarium accession number, Date = collection date.
^aTU = University of Tartu, WSL = Swiss Federal Research Institute WSL. Specimens stored at WSL are kept frozen at –20°C. All specimens were collected by T.T. and L.M.
*Specimens used for shotgun sequencing.