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PRIMER NOTE

Fourteen polymorphic microsatellite markers for the widespread Labrador tea (*Rhododendron groenlandicum*)

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PREMISE: Microsatellite markers were developed for Labrador tea (*Rhododendron groenlandicum*, Ericaceae) to facilitate downstream genetic investigation of this species and the extremely closely related, circumboreal *Rhododendron* subsect. *Ledum*.

METHODS AND RESULTS: Forty-eight primer pairs were designed using Illumina data and screened for excellent amplification. Sixteen successful pairs were developed as microsatellite markers using fluorescently labeled amplification to generate chromatogram data. These data were evaluated for intrapopulation and interpopulation variability in three populations from Alaska and Maine, USA, and the Northwest Territories, Canada. Fourteen polymorphic markers genotyped reliably, each with one to eight alleles. Cluster analysis indicates that across the range, populations can be easily discriminated. Cross-amplification in other *Rhododendron* subsect. *Ledum* species shows broad application of the developed markers within this small, well-supported clade.

CONCLUSIONS: These microsatellite markers exhibit significant variability and will be useful in population genetics within *R. groenlandicum* and for investigation of species boundaries across *Rhododendron* subsect. *Ledum*.

KEY WORDS Ericaceae; Labrador tea; *Rhododendron groenlandicum*; *Rhododendron* subsect. *Ledum*; species boundaries.

Rhododendron groenlandicum (Oeder) Kron & Judd (Labrador tea) is one of eight named species within *Rhododendron* subsect. *Ledum* (L.) Kron & Judd (Ericaceae). *Rhododendron groenlandicum* is widespread across northern North America in damp habitats such as bogs and rocky alpine slopes. Although the related species commonly known as Labrador tea were long considered closely related to *Rhododendron*, Kron and Judd (1990) first demonstrated, using morphological cladistic analyses, that these species should not be maintained as the separate genus *Ledum*, but included within *Rhododendron*. Hart et al. (2017) confirmed the monophyly of subsect. *Ledum* in a molecular phylogenetic study. However, this study also demonstrated clear conflict between the nuclear and chloroplast genomes, suggesting likely recent hybridization involving multiple species within this lineage. Indeed, the named species in subsect. *Ledum* have a complex nomenclatural history that mirrors this reticulate evolutionary history, with little consensus about what taxa should be recognized. Therefore, the evolutionary history of this lineage remains unclear, particularly at the population scale. Löve and Löve (1982) reported a sporophytic chromosome count of $2n = 26$ for *R. groenlandicum*; however, recent flow cytometry data (K. T. Theqvist, unpublished) suggests that at least some populations may be tetraploid. A close relative, *R. tomentosum* Harmaja, was reported by Lantai and Kihlman (1995) to have populations of mixed ploidy ($2n = 26, 52$). Therefore, the possibility of tetraploid *R. groenlandicum* populations is reasonable.

Currently, there are no microsatellite markers available for use in any member of *Rhododendron* subsect. *Ledum*. The absence of rapidly evolving markers for this lineage limits our ability to investigate boundaries among these recently diverged and likely reticulate species. Because of the young age of this lineage and the high likelihood of hybridization, it is appropriate to investigate relationships among species at the population level by documenting population-level ploidy, zones of hybridization, and genetic diversity alongside phylogenetic investigation. Development of microsatellite markers for *R. groenlandicum*, the most widespread species within subsect. *Ledum*, will likely provide novel tools for use across this entire closely related lineage.

METHODS AND RESULTS

All bioinformatics aspects of this project followed Gillespie et al. (2017). DNA from one *R. groenlandicum* individual (Appendix 1)

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was extracted following a modified cetyltrimethylammonium bromide (CTAB) approach (Doyle and Doyle, 1987) followed by $CsCl_2$ purification (Palmer, 1986). A microsatellite sequencing library using the MiSeq v2 protocol and 2 × 250-bp paired-end sequencing was performed on an Illumina MiSeq at Cornell Life Sciences Sequencing and Genotyping Facility (Ithaca, New York, USA). Out of 3,882,418 raw sequence reads (GenBank Sequence Read Archive no. PRJNA577479) that were trimmed of vector and low-quality sequence using the BBduk 1.0 plugin within Geneious 11.1.5 (Kearse et al., 2012; Biomatters Ltd., Auckland, New Zealand), 605,089 reads included microsatellite regions. Of this subset of reads, 16,420 permitted design of unique primers using MSATCOMMANDER (Faircloth, 2008) with mostly default settings, but mononucleotide motifs were excluded, primer length was 20–22 bp, and primer GC maximum content was 50%. A PIG-tail sequence (Brownstein et al., 1996) was added to reverse primers for stability.

Details of both amplification and polymorphism screens followed Kasireddy et al. (2018). DNA from seven silica-preserved *R. groenlandicum* individuals (Appendix 1) was extracted using a QIAGEN Plant Mini Kit (QIAGEN, Hilden, Germany) modified for use with herbarium material (Drábková et al., 2002). These seven DNAs were used to screen 48 markers representing diverse motifs and repeat numbers via PCR amplification (1× GoTaq Flexi Buffer, 2.5 mM $MgCl_2$, 800 μM dNTPs, 0.5 μM of each primer, 0.5 units GoTaq Flexi DNA Polymerase [Promega Corporation, Madison, Wisconsin, USA], and ~20 ng DNA, in a 10-μL reaction). Touchdown PCR (94°C for 5 min; followed by 13 cycles of 45 s at 94°C, 2 min at touchdown temperature [68–55°C], and 1 min at 72°C; followed by 24 cycles of 45 s at 94°C, 1 min at 55°C, and 1 min at 72°C; and followed by 5 min at 72°C) was employed.

After the amplification screen, 16 primer pairs (Table 1) that amplified exactly one distinct amplicon were genotyped at the Georgia Genomics and Bioinformatics Core (University of Georgia, Athens, Georgia, USA) and scored for polymorphisms using DNA of 68 well-spaced individuals from three populations representing the broad range of *R. groenlandicum* (Sitka, Alaska, USA; Northwest Territories, Canada; and Washington County, Maine, USA). For PCR reactions used to genotype individuals, 50% of forward primer was replaced with fluorescently tagged (6-FAM, VIC, NED, or PET; Life Technologies, Grand Island, New York, USA) M13 universal primers.

Resulting chromatograms were manually scored using Geneious 11.1.5. We employed strict criteria for calling peaks. First, a peak was called only if the relative fluorescence unit (RFU) was ≥3000 and exhibited little background noise relative to signal. Additionally, a second peak (i.e., a heterozygote) was called only if the secondary peak's RFU was ≥90% of the first peak. Consequently, our measurements of genetic diversity are conservative. Descriptive statistics, including

TABLE 1. Characteristics of 16 microsatellite primer pairs developed for *Rhododendron groenlandicum*.

Locus	Primer sequences (5′–3′)[a]	Repeat motif	Allele size range (bp)	T_a (°C)	Fluorescent label	GenBank accession no.
RGROE001	F: TTCACCCTCTTCAGATCTTCGG R: GTTTACAACTCTAGACATCGGATCAC	$(AAAAAC)_6$	149–167	59.2	NED	MN428531
RGROE002	F: AGGCTTGTGGGAGTAGTAAGTG R: GTTTCTGCATAGTGTGTCCATGC	$(AAAAC)_6$	340–350	59.8	PET	MN428532
RGROE003	F: AGGCTTGTGGGAGTAGTAAGTG R: GTTTCTGCGTAATGTGTCCATGC	$(AAAAC)_6$	340–350	60.1	PET	MN428533
RGROE004	F: AATTTGGCTTTGTTCGGTAGC R: GTTTGGTTGTGTTTGGTTGGC	$(AAAACT)_6$	190–202	58.6	NED	MN428534
RGROE012	F: AGGAAGTGTTTGAATGGGTTGG R: GTTTCCTCGCCTTGATTTGTGC	$(AAC)_8$	347–365	59.8	VIC	MN428535
RGROE015	F: AAATTCGAAGCCACCATAGTTG R: GTTTGTTGGCTATCCTCTTCCG	$(AAG)_8$	139–160	58.1	6-FAM	MN428536
RGROE019	F: TGAATGTTGAATCGGGTGCG R: GTTTAGTGGATGGGACTTGTTCTTC	$(AAGGAC)_8$	NA	59.1	VIC	MN428537
RGROE020	F: TGCGCAATATGTGGACGTAC R: GTTTGTTCAATGGCGGAGTGG	$(AAGGAG)_6$	233–275	59.6	PET	MN428538
RGROE021	F: TGCAGTAGACTCATTGCAGC R: GTTTCCTCGGTGCCAAGAATTG	$(AAT)_9$	115–130	59.1	6-FAM	MN428539
RGROE027	F: GCGACACGTATAGGCAAATTG R: GTTTGGTGATTTCTTGGCCGATC	$(ACC)_8$	245–260	58.9	PET	MN428540
RGROE036	F: CAAGGCGTTGTAAAGGATTTCC R: GTTTCCCTCTGGTTTGGTGTG	$(AG)_{36}$	305–377	58.8	PET	MN428541
RGROE041	F: AGCAACTATAATGGCGGAGG R: GTTTAACTAGAGCCAAGACTGCG	$(AGG)_8$	119–125	58.4	6-FAM	MN428542
RGROE042	F: ACAATTGTCAGTGGCCAGAAC R: GTTTCAACACCCATGGCAAGTG	$(AGG)_{11}$	NA	60.1	6-FAM	MN428543
RGROE045	F: TGTCGCCGTTATAACCATCG R: GTTTACACGCAACTCCACTGATC	$(AT)_{21}$	343–357	60.0	VIC	MN428544
RGROE046	F: TGGTTGGAGGCCTATGGTTATC R: GTTTGTCGGAGTGGTTGCTATG	$(ATC)_9$	212–236	60.0	NED	MN428545
RGROE047	F: AACCATTGACAAGCAGCATTAC R: GTTTACCATTCTTGACCCTGCTAG	$(ATCC)_6$	160–176	58.4	NED	MN428546

Note: NA = markers did not genotype well and are not included in analyses; T_a = annealing temperature.
[a]Pigtail sequence is underlined on reverse primers.

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Hardy–Weinberg equilibrium (HWE) deviations, multilocus matches analysis (MMA) and principal coordinate analysis (PCoA) (Orloci, 1978), were calculated using GenAlEx version 6.503 (Peakall and Smouse, 2006, 2012). Two markers, RGROE019 and RGROE042, did not genotype consistently and were not developed further.

Although some past studies have allowed the possibility that *R. groenlandicum* is polyploid, 14 loci revealed chromatograms with one to two peaks per individual. Our scoring of peaks is conservative in terms of genetic diversity, and therefore may underscore alleles associated with dosage differences. Although there was very little background noise/stutter in our data set, failure to detect polyploidy using this methodology is acknowledged. Overall, however, we conclude that individuals sampled here are diploid.

Fourteen polymorphic loci exhibited one to eight alleles per population (mean 2.81) (Table 2). No more than two peaks per individual were observed. Observed heterozygosity ranged from 0.000–0.636 (mean 0.125). HWE expectations were not met for 11 loci (78.6%) in at least one population including RGROE045, which violated HWE assumptions in all three sampled populations. The 14 polymorphic loci easily differentiated the populations, demonstrated by genetic distance followed by PCoA (not shown). The first three axes of the PCoA explained 52.61% of the variation and showed a clear division between the Sitka, Alaska, USA, population and the other two populations, which were moderately differentiated. The MMA of the 14 polymorphic loci revealed two sets of identical individuals within the Sitka population, suggesting limited clonality. The MMA and PCoA results together suggest considerable population structure within *R. groenlandicum*. The 14 developed markers were cross-amplified within a phylogenetic context following Hart et al. (2017). This included 12 individuals from *Rhododendron* subsect. *Ledum* (five *R. columbianum* (Piper) Harmaja, three *R. tomentosum*, and one each of *R. diversipilosum* (Nakai) Harmaja, *R. hypoleucum* (Kom.) Harmaja, *R. palustre* (L.) Kron & Judd, and *R. tolmachevii* (Tolm.) Harmaja). Amplification of all developed markers (Table 3) was successful in all species

TABLE 2. Descriptive statistics for 14 microsatellite loci developed for *Rhododendron groenlandicum*.[a]

	NW Territory, Canada (N = 24)				Sitka Co., AK (N = 22)				Washington Co., ME (N = 22)			
Locus	A	H_o	H_e	HWE[b]	A	H_o	H_e	HWE[b]	A	H_o	H_e	HWE[b]
RGROE001	2	0.174	0.287	NS	1	0.000	0.000	M	2	0.091	0.087	NS
RGROE002	3	0.188	0.498	**	2	0.091	0.087	NS	3	0.095	0.316	**
RGROE003	3	0.100	0.515	***	2	0.053	0.049	NS	2	0.000	0.245	***
RGROE004	2	0.050	0.049	NS	1	0.000	0.000	M	1	0.000	0.000	M
RGROE012	2	0.125	0.117	NS	2	0.636	0.496	NS	3	0.143	0.125	NS
RGROE015	2	0.083	0.153	*	4	0.273	0.674	***	4	0.381	0.690	*
RGROE020	2	0.150	0.219	NS	4	0.000	0.449	***	2	0.000	0.397	***
RGROE021	2	0.042	0.041	NS	2	0.000	0.091	***	1	0.000	0.000	M
RGROE027	3	0.042	0.322	***	3	0.048	0.291	***	3	0.182	0.169	NS
RGROE036	8	0.238	0.773	***	7	0.333	0.373	NS	7	0.429	0.532	***
RGROE041	2	0.000	0.080	***	1	0.000	0.000	M	1	0.000	0.000	M
RGROE045	5	0.000	0.753	***	2	0.000	0.408	***	6	0.238	0.706	***
RGROE046	3	0.000	0.277	***	4	0.545	0.518	NS	3	0.455	0.577	NS
RGROE047	2	0.042	0.043	NS	3	0.045	0.208	***	1	0.000	0.000	M
Mean	2.929	0.088	0.295		2.714	0.144	0.260		2.786	0.144	0.275	

Note: A = number of alleles detected across all individuals; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium.
[a]Voucher and locality information are provided in Appendix 1.
[b]Asterisks (*) indicate statistically significant deviation from HWE (*P < 0.05; **P < 0.01; ***P < 0.001). M = monomorphic marker; NS = not statistically significant.

TABLE 3. Cross-amplification of 14 primer pairs developed for *Rhododendron groenlandicum* in representatives from *Rhododendron* subsect. *Ledum*.[a]

Locus	Rcol1	Rcol2	Rcol3	Rcol4	Rcol5	Rtom1	Rtom2	Rdiv	Rhyp	Rpal	Rtol
RGROE001	149	155	143	149	149	149	149	155	155	149	161
RGROE002	340	—	—	345	345	—	—	345	335	335	340
RGROE003	345	—	—	340	335	—	350	335	345	355	345
RGROE004	202	—	—	202	196	202	196	184	196	184	184
RGROE012	353	350	—	344	350	356	353	350	356	356	347
RGROE015	133	—	—	136	133	133	133	136	136	142	133
RGROE020	236	248	272	245	257	253	257	257	263	248	263
RGROE021	—	112	115	124	115	112	127	133	127	130	124
RGROE027	260	248	257	242	248	245	242	242	242	248	254
RGROE036	311	323	309	367	365	311	325	305	307	313	311
RGROE041	116	131	116	116	116	125	113	110	116	116	131
RGROE045	343	349	343	345	345	—	349	341	349	341	341
RGROE046	215	209	215	212	212	218	212	212	215	215	221
RGROE047	160	160	160	164	168	168	160	172	172	160	164

Note: — = no observable amplification; Rcol = *Rhododendron columbianum*; Rtom = *Rhododendron tomentosum*; Rdiv = *Rhododendron diversipilosum*; Rhyp = *Rhododendron hypoleucum*; Rpal = *Rhododendron palustre*; Rtol = *Rhododendron tolmachevii*.
[a]Ranges of allele sizes are given when at least two individuals per species were sampled.

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except marker RGROE002, which failed to amplify in any *R. tomentosum* individual.

CONCLUSIONS

These newly developed microsatellite markers represent the first such tool for use in Labrador tea and its close relatives. The markers will allow population-level investigation within *R. groenlandicum* but are likely to also aid in clarifying the evolutionary history of *Rhododendron* subsect. *Ledum*, including investigation of species boundaries and putative hybridization events. The markers presented here are collectively able to demonstrate considerable genetic structure in just three populations of *R. groenlandicum* and genotype well in all sampled species within *Rhododendron* subsect. *Ledum*, likely because of inter-species similarity resulting from recent and ongoing divergence of these species.

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AUTHOR CONTRIBUTIONS

K.L.L. and E.M. conducted all fieldwork (but see Acknowledgments). E.L.G. carried out all bioinformatics and project design aspects and analyzed the data. M.L.S. conducted the majority of the lab work with assistance from E.L.G. M.L.S. drafted the manuscript for submission, and all co-authors commented on and edited the manuscript.

DATA AVAILABILITY

The raw sequence reads are deposited in the National Center for Biotechnology Information (NCBI; GenBank Sequence Read Archive accession no. PRJNA577479). Sequence information for the developed primers has been deposited to NCBI; accession numbers are provided in Table 1.

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APPENDIX 1. Voucher information for *Rhododendron* individuals included in this study.

Species	Voucher (Herbarium)	Geographic coordinates		Elevation (m)	State (Country)	County/unit	*N*
		Latitude	Longitude				
Rhododendron groenlandicum (Oeder) Kron & Judd	Antieau 01-29 (WTU)[a]	47.40	−121.92	228	Washington (USA)	King	1
Rhododendron groenlandicum	Beaulieu s.n. (BUT)[b]	61.17	−113.68	158	NW Territories (CAN)	Fort Smith	24
Rhododendron groenlandicum	LaBounty s.n. (BUT)[b]	57.06	−135.19	151	Alaska (USA)	Sitka	22
Rhododendron groenlandicum	Mitchell 473 (BUT)[b]	44.56	−67.61	8	Maine (USA)	Washington	22
Rhododendron columbianum (Piper) Harmaja	Arnot 73 (WTU)[c]	48.52	−120.67	1654	Washington (USA)	Chelan	1
Rhododendron columbianum	Denton 4271 (WTU)[c]	41.01	−123.08	1584	California (USA)	Trinity	1

(Continues)

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APPENDIX 1. (Continued)

Species	Voucher (Herbarium)	Geographic coordinates		Elevation (m)	State (Country)	County/unit	*N*
		Latitude	Longitude				
Rhododendron columbianum	Denton 3144 (WTU)[c]	42.04	−123.02	938	Oregon (USA)	Curry	1
Rhododendron columbianum	Smith 3172 (WTU)[c]	45.63	−115.68	1615	Idaho (USA)	Valley	1
Rhododendron columbianum	Kruckeberg 6547 (WTU)[c]	48.96	−119.80	2134	Washington (USA)	Okanogen	1
Rhododendron tomentosum Harmaja	Putnam 24 (WTU)[c]	70.48	−155.06	1	Alaska (USA)	North Slope	1
Rhododendron tomentosum	LaBounty s.n. (WTU)[c]	59.26	−135.84	244	Alaska (USA)	Haines	1
Rhododendron tomentosum	Gustafsen s.n. (WTU)[c]	69.361	−145.08	866	Alaska (USA)	North Slope	1
Rhododendron diversipilosum (Nakai) Harmaja	Kihlman 20040770 (ARS)[c]	43.35	−142.91	837	Hokkaido (Japan)	NA	1
Rhododendron hypoleucum (Kom.) Harmaja	Larsen 87/04 (ARS)[c]	—	—	—	—	NA	1
Rhododendron palustre (L.) Kron & Judd	Chase MWC869 (K)[c]	50.14	−86.30	1052	Siberia (Russia)	Kurai	1
Rhododendron tolmachevii (Tolm.) Harmaja	Theqvist 20040806 (ARS)[c]	53.56	−127.41	365	Amur (Russia)	NA	1

Note: — = horticulture specimen of uncertain provenance; *N* = number of individuals; NA = not available; ARS = American Rhododendron Society Rhododendron Species Botanical Garden, Federal Way, WA; BUT = Friesner Herbarium (Butler University); K = Royal Botanic Garden Kew Herbarium; WTU = Burke Museum (University of Washington).
[a]Voucher for Illumina sequencing.
[b]Voucher for marker development (separate collection effort).
[c]Voucher for cross-amplification.

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