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

Matthew L. Sheik
Kitty L. LaBounty
Erika Mitchell
Emily L. Gillespie

Follow this and additional works at: https://digitalcommons.butler.edu/facsch_papers

Part of the Biology Commons
Fourteen polymorphic microsatellite markers for the widespread Labrador tea (*Rhododendron groenlandicum*)

Matthew L. Sheik1, Kitty L. LaBounty2, Erika Mitchell3, and Emily L. Gillespie4,5

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*. 

**KEYWORDS:** 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)
was extracted following a modified cetyltrimethylammonium bromide (CTAB) approach (Doyle and Doyle, 1987) followed by CsCl₂ 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., 2018). DNA from seven silica-preserved individuals (Appendix 1) was extracted using a QIAGEN Plant Mini Kit (QIAGEN, Hilden, Germany) modified for use with herbarium material (Drábková et al., 2002).

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 a wide 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 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; 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′) | Repeat motif | Allele size range (bp) | Tₘ (°C) | Fluorescent label | GenBank accession no. |
|---------|--------------------------|--------------|------------------------|---------|------------------|-----------------------|
| RGROE001| F: TTGACCCTCTTTGATGCTTGC | (AAAAAC)₆     | 149–167                | 59.2    | NED              | MN428531              |
|         | R: GTTTACACCTGCTGAGTCG   |              |                        |         |                  |                       |
| RGROE002| F: AGGCTTGGAGGATGTTAAGTG | (AAAAAC)₆     | 340–350                | 59.8    | PET              | MN428532              |
|         | R: GTTTCTGCATAGTGGTCCATGC|              |                        |         |                  |                       |
| RGROE003| F: AGGCTTGGAGGATGTTAAGTG | (AAAAAC)₆     | 340–350                | 60.1    | PET              | MN428533              |
|         | R: GTTTCTGCATAGTGGTCCATGC|              |                        |         |                  |                       |
| RGROE004| F: AAATTGCTTTTGTCTGGTAGC | (AAAAACT)₆    | 190–202                | 60.6    | VIC              | MN428534              |
|         | R: GTTTGTGTTTGTTTGTTGTCG |              |                        |         |                  |                       |
| RGROE012| F: AGGAAGTTTGGATAGTGGTGG | (AAC)₆        | 347–365                | 59.6    | VIC              | MN428535              |
|         | R: GTTTCTGCGCTGTTAGTTGTC |              |                        |         |                  |                       |
| RGROE015| F: AAATTTGGAACCCCATAGTTG | (AAG)₆        | 139–160                | 59.1    | F-AM             | MN428536              |
|         | R: GTTTGTGGCATCTCCTTCCG |              |                        |         |                  |                       |
| RGROE019| F: TGGTATGGAATAGGGTTGCG | (AAGGAC)₆     | NA                     | 59.1    | VIC              | MN428537              |
|         | R: GTTTAGTGTGATGATGGTTGC |              |                        |         |                  |                       |
| RGROE020| F: TGGGCAATATGTTGACGCTAC| (AAGGAG)₆     | 233–275                | 59.6    | PET              | MN428538              |
|         | R: GTTTGTGTTGCTGTTGGGATGC|              |                        |         |                  |                       |
| RGROE021| F: TGGCTAGTACCTATGCTCAC | (AAT)₉        | 115–130                | 59.1    | F-AM             | MN428539              |
|         | R: GTTTCTGCCTGTGTCCTGGTG |              |                        |         |                  |                       |
| RGROE026| F: GCCACAGCTATAGCCAAATTC | (ACC)₆        | 245–260                | 59.8    | PET              | MN428540              |
|         | R: GTTTGTGTTGCTGTTGGGATGC|              |                        |         |                  |                       |
| RGROE041| F: ACAAGGTGTAAGAGATTTC | (AG)₆         | 305–377                | 58.9    | PET              | MN428541              |
|         | R: GTTTCTGCTCTGTTGGGATGC|              |                        |         |                  |                       |
| RGROE042| F: AGCAAAATGGTCTCCAGGCAG | (AGG)₆        | 119–125                | 58.4    | VIC              | MN428542              |
|         | R: GTTCTAACAGGACCAAGTCC |              |                        |         |                  |                       |
| RGROE045| F: TGTCGCGTATATACCATCGC | (AT)₁₁        | 343–357                | 60.0    | VIC              | MN428544              |
|         | R: GTTACACCGCAATCCCACGTGC|              |                        |         |                  |                       |
| RGROE046| F: TGTCGCGTATATACCATCGC | (ATC)₉        | 212–236                | 60.1    | NED              | MN428545              |
|         | R: GTTTGTGGGATGTTGCTATGC|              |                        |         |                  |                       |
| RGROE047| F: ACCATTGCAGAGGCAGATTAC | (ATCC)₆      | 160–176                | 58.4    | NED              | MN428546              |
|         | R: GTTTACCATCCTGACCCCTTAG |              |                        |         |                  |                       |

*Note: NA = markers did not genotype well and are not included in analyses; Tₘ = annealing temperature.

*Pigtail sequence is underlined on reverse primers.*
Hardy–Weinberg equilibrium (HWE) deviations, multilocus matches analysis (MMA) and principal coordinate analysis (PCoA) (Otloci, 1978), were calculated using GenAIEx 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.
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 *Rhododendron 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.

ACKNOWLEDGMENTS

The authors acknowledge Ms. Gail Beaulieu and Ms. Suzanne Carriere (Government of Northwest Territories) for collections from Northwest Territories, Canada. Startup funding to E.L.G. was provided by Butler University and Marshall University. We acknowledge iNaturalist (www.inaturalist.org) for publicly available observation data that were critical in identifying localities and collaborators to accomplish field collections for this widespread species.

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.

LITERATURE CITED

Brownstein, M. J., J. D. Carpten, and J. R. Smith. 1996. Modulation of non-templated nucleotide addition by Taq DNA polymerase: Primer modifications that facilitate genotyping. *Biotechniques* 20: 1004–1006, 1008–1010.

Doyle, J. J., and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

Drábková, L., J. Kirschner, and C. Vlček. 2002. Comparison of seven DNA extraction and amplification protocols in historical herbarium specimens in Junceae. *Plant Molecular Biology Reporter* 20: 161–175.

Faircloth, B. C. 2008. MSATCOMMANDER: Detection of microsatellite repeat arrays and automated, locus-specific primer design. *Molecular Ecology Resources* 8: 92–94.

Gillespie, E. L., A. G. Pauley, M. L. Haffner, N. M. Hay, M. C. Estep, and Z. E. Murrell. 2017. Fourteen polymorphic microsatellite markers for a widespread limestone endemic, *Carex eburnea* (Cyperaceae: Carex sect. Albae). *Applications in Plant Sciences* 5(8): 1700031.

Hart, A., K. Kron, and E. L. Gillespie. 2017. Molecular phylogenetic analysis of the north-temperate Labrador Teas (Ericaceae: *Ledum* subsect. *Ledum*) suggests a complex genetic history. *Journal of the Botanical Institute of Texas* 11(1): 53–65.

Kasireddy, V., E. Mitchell, Z. E. Murrell, and E. L. Gillespie. 2018. Fifteen microsatellite markers for the Appalachian rockcap fern, *Polypodium appalachianum* (Polypodiaceae), and its relatives. *Applications in Plant Sciences* 6(11): e1195.

Kearse, M., R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, S. Buxton, et al. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28(12): 1647–1649.

Kron, K. A., and W. S. Judd. 1990. Phylogenetic relationships within the Rhodoreae (Ericaceae) with specific comments on the place of *Ledum*. *Systematic Botany* 15(1): 57–68.

Lantai, K., and B. Kihlman. 1995. The chromosome number of *Ledum palustre* ssp. *decumbens* and of some related taxa. *Hereditas* 122: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.

Löve, A., and D. Löve. 1982. IOPB chromosome number reports LXXV . *Hereditas* 98: 181–184.
APPENDIX 1. (Continued)

| Species | Voucher (Herbarium) | Geographic coordinates | Latitude | Longitude | Elevation (m) | State (Country) | County/unit | N |
|---------|---------------------|------------------------|----------|-----------|--------------|----------------|-------------|---|
| *Rhododendron columbia* | Denton 3144 (WTU)\(^a\) | 42.04 | -123.02 | 938 | Oregon (USA) | Curry | 1 |
| *Rhododendron columbia* | Smith 3172 (WTU)\(^a\) | 45.63 | -115.68 | 1615 | Idaho (USA) | Valley | 1 |
| *Rhododendron columbia* | Kruckeberg 6547 (WTU)\(^a\) | 48.96 | -119.80 | 2134 | Washington (USA) | Okanogen | 1 |
| *Rhododendron tomentosum* | Putnam 24 (WTU)\(^a\) | 70.48 | -155.06 | 1 | Alaska (USA) | North Slope | 1 |
| *Rhododendron tomentosum* | LaBounty s.n. (WTU)\(^b\) | 59.26 | -135.84 | 244 | Alaska (USA) | Haines | 1 |
| *Rhododendron tomentosum* | Gustafson s.n. (WTU)\(^b\) | 69.361 | -145.08 | 866 | Alaska (USA) | North Slope | 1 |
| *Rhododendron diversipilum* (Nakai) Harmaja | Kihlman 20040770 (ARS)\(^c\) | 43.35 | -142.91 | 837 | Hokkaido (Japan) | NA | 1 |
| *Rhododendron hypoleucum* (Kom.) Harmaja | Larse 87/04 (ARS)\(^c\) | — | — | — | — | NA | 1 |
| *Rhododendron palustre* (L.) Kron & Judd | Chase MWCB69 (K)\(^c\) | 50.14 | -86.30 | 1052 | Siberia (Russia) | Kurai | 1 |
| *Rhododendron tolmachevii* (Tolm.) Harmaja | Theqvist 20040806 (ARS)\(^c\) | 75.36 | -127.41 | 365 | Amur (Russia) | NA | 1 |

\(^a\)Voucher for Illumina sequencing.
\(^b\)Voucher for marker development (separate collection effort).
\(^c\)Voucher for cross-amplification.

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).