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ORIGINAL ARTICLE

Confusing boundaries of the Labrador tea species: dispersal history explains the lack of clear species structure

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• **Background and Aims** The Labrador teas (genus *Rhododendron*, subsection *Ledum*) are a complex of species widely distributed in the Northern Hemisphere. They occupy cold-resistant plant communities from highlands to forest understorey and wetland habitats almost circumboreally and they are especially abundant in Northeast Asia and northern North America, yet there are no clear species boundaries in this group. The genetic structure of species of subsect. *Ledum* from Eurasia and North America as well as the dispersal history of the group require clarification.
• **Methods** The phylogeny and biogeography of subsect. *Ledum* of the genus *Rhododendron* were assessed using phylogenetic trees constructed based on the analysis of variation in chloroplast *pet*B-*pet*D, *trn*V-*ndh*C, *trn*H-*psb*A, K2R-K707, *atp*B oligo2 – *rbc*L oligo5 and nuclear (ITS1) markers of four Eurasian and one American species (65 populations, 408 individuals). The data were evaluated with maximum parsimony and Bayesian analysis. Molecular dating and reconstruction of ancestral areas were performed.
• **Key Results** Dense sampling revealed widespread presence of shared haplotypes and ribotypes among *Ledum* populations and species. Two American, three mixed and one Eurasian lineage diversified during the Neogene climate cooling and then rapidly dispersed during the Pleistocene. The ability to accumulate high genetic diversity and to preserve it across distribution ranges and generations prevented *Ledum* from lineage sorting. As a result, a species complex with a reserve of genetic variability appeared.
• **Conclusions** Although no clear phylogenetic inference can be obtained at present, the plastid genealogy is consistent with the nuclear genealogy and demonstrates the processes involved in speciation in the *Ledum* species complex.

Key words: Circumboreal, ITS, *Ledum*, North America, Northeast Asia, plastid DNA, phylogeography, *Rhododendron*.

INTRODUCTION

The Labrador tea species belong to the genus *Rhododendron* L., subgenus *Rhododendron*, section *Rhododendron*, subsection *Ledum* (L.) Kron&Judd of the family Ericaceae Juss. (Chamberlain *et al*., 1996). While contemporary species diversity of rhododendrons is highest in mountainous regions of the Himalayas up to China and the islands between Asia and Australia, Northeast Asia, northern Europe and North America have much lower species diversity (Irving and Hebda, 1993; Shrestha *et al*., 2018). The Labrador teas are among the most widespread *Rhododendron* species in cool temperate and subarctic regions with a nearly circumboreal distribution (Hultén, 1937; Tolmachev, 1953; Kron and Judd, 1990). The Labrador teas exhibit broad ecological plasticity, allowing species of this group to occupy various habitats from the subalpine mountain belt and tundra to peat bogs and undergrowth of boreal forests (Tolmachev, 1953; Hart *et al*., 2017; Amada *et al*., 2024).

Ledum was previously considered as a genus of the family Ericaceae, but a revision of phylogenetic relationships within the genus *Rhododendron* using both morphological characters (Kron and Judd, 1990) and DNA markers (Kurashige *et al*., 2001; Gao *et al*., 2002; Goetsch *et al*., 2005; Khan *et al*., 2021) required *Ledum* to be included in the genus *Rhododendron*. Harmaja (1990, 1991, 1998, 2002) recognized six species in Eurasia and three species in North America. However, other sources distinguish four to five species in Eurasia and from two to four species in North America (Hultén, 1937; Savile, 1969; Viereck and Little, 1972; Kron and Judd, 1990; Khokhrjakov, 1991; Usenko, 2009; Hébert and Thiffault, 2011). The

controversial taxonomic interpretations are associated with a wide range of variability of morphological traits that often overlap among different species of this group.

The Labrador teas include species with different patterns of distribution. Some exhibit wide distributions, for example *Rhododendron tomentosum* Harmaja and *R. subarcticum* Harmaja in Eurasia and *R. groenlandicum* (Oeder) Kron & Judd in North America. Other species have narrow ranges such as *R. tolmachevii* Harmaja, *R. hypoleucum* (Kom.) Harmaja, *R. diversipilosum* (Nakai) Harmaja and *R. subulatum* (Nakai) Harmaja in Northeast Asia, and *R. columbianum* (Piper) Harmaja and *R. neoglandulosum* Harmaja in western North America. Therefore, this relatively small group of taxa with a peculiar pattern of distribution in the Northern Hemisphere and wide ecological amplitude is an interesting model system for uncovering evolutionary processes in closely related species.

Although Hart *et al.* (2017) first performed a molecular phylogenetic analysis of subsect. *Ledum* based on variation in nuclear DNA (nrDNA) and plastid DNA (ptDNA) markers, the sampling level has not been exhaustive. The nuclear data indicated a monophyletic origin of subsect. *Ledum*, but chloroplast data indicated a paraphyletic origin, meaning that the North American taxa have an evolutionary history separate from the Asian taxa. Such incongruence suggested a hybrid nature of the Asian and American lineages and could imply that some of the existing species were composite artificial taxa. These initial data showed the importance of intensive sampling to capture the complex genetic and biogeographical structure of *Ledum* taxa.

In the present study, to unravel the confusing evolutionary background of five species of susect. *Ledum* and to understand intrasectional relationships, we set several goals. Since most of the species are described from Eurasia, we first attempted to conduct a dense sampling of subsect. *Ledum* taxa in the Far East of Russia, where four currently recognized species (*R. tomentosum*, *R. subarcticum*, *R. diversipilosum* and *R. hypoleucum*) coexist. We further undertook detailed sampling in Siberia and western Russia where only *R. tomentosum* and *R. subarcticum* are described intending to reveal a detailed geographical structure for these species. We also included some sample sets of *R. groenlandicum* from the north of North America to trace the history of the distribution of subsect. *Ledum* between the continents. Finally, we chose two types of molecular markers: maternally inherited chloroplast markers, which reflect the geographical distribution patterns of species and allow us to avoid unpredictability with polyploids (Petit and Vendramin, 2007) and a biparentally inherited nrDNA marker whose structure is more reflective of relatedness.

Our questions were: (1) What is the genetic structure of populations in the Labrador teas group? (2) Does the discovered genetic structure correspond to the division into species? (3) How are the American and Eurasian branches of the Labrador teas related genetically?

MATERIALS AND METHODS

Taxon sampling

High polymorphism of vegetative organs and the ability to hybridize between species of subsect. *Ledum* (Khokhrjakov and Mazurenko, 1986; Shiotani and Kudo, 2023) and with members of other subsections (Dalgaard and Fredskild, 1993; Kihlman, 2004) complicate clear species identification and the delimitation of species distribution boundaries. In this study we use the nomenclature of H. Harmaja accepted within the monograph on rhododendrons (Chamberlain *et al.*, 1996). We distinguish four Eurasian and two North American taxa of subsect. *Ledum* based on the distributional patterns, ecological preferences and morphological traits described in regional floras (Savile, 1969; Tolmachev, 1974; Judd and Kron, 2009b; Usenko, 2009).

Our taxon sampling included 65 populations of five species (Table 1; see Fig. 2I). Four species were from Russia, namely *R. tomentosum* (26 populations), *R. subarcticum* Harmaja (11 populations), *R. hypoleucum* (13 populations) and *R. diversipilosum* (10 populations), and two species from North America (NAm), *R. subarcticum* (one population) and *R. groenlandicum* (four populations). Although H. Harmaja distinguished two more species in Asia, *R. tolmachevii* and *R. subulatum*, we reliably identified only four species from the entire complex of Northeast Asian (NEA) species. Populations were numbered sequentially in strict order from North America to Eurasia (from east to west), regardless of apparent species identity, because the patterns of genetic structure that we identified in the course of the study are of a geographical nature to a greater extent than of a species one. This numbering (1G to 65T) provides an easier way to match populations in the table and figures. The number of individuals sampled per population was 1–5 for nrDNA markers and 2–21 for ptDNA markers. Specimens of the American species *R. columbianum* were not available. Hence, we included sequences from GenBank (KX676772 for K2R-K707, MG223104 for *atp*B-*rbc*L, KY356299 for *trn*V-*ndh*C) available from Manton (2016), Kuzmina *et al.* (2017) and Hart *et al.* (2017), respectively. For outgroups, *Therorhodion camtschaticum* (Maxim.) Small, *R. simsii* Planch. (subgenus *Tsutsusi*, section *Tsutsusi*), *R. aureum* Georgi (subgenus *Hymenanthes*, section *Ponticum*, subsect. *Pontica*), *R. parvifolium* Adams (subgenus *Rhododendron*, section *Rhododendron*, subsect. *Lapponica*) and *R. dauricum* L. (subgenus *Rhododendron*, section *Rhododendron*, subsect. *Rhodorastra*) were employed. Sequences from all plastid regions were obtained for *T. camtschaticum* (PP789575, OQ689304, OQ689321, OQ689338, OQ689355) and ITS1 sequence for *R. dauricum* (PP694615) and *R. parvifolium* (HM854160). For the remaining outgroups ptDNA data were taken from GenBank (AY494177, KT696942, ON508850, AB038931, JF956097, HQ415427, KJ161973, KT696941, KY486321). Some regions were unavailable, and they were treated as missing data in DNA contig for the analysis.

DNA extraction, sequencing and phylogenetic analyses

Leaf material was collected from the wild and dried in silica gel prior to laboratory work. Total genomic DNA was isolated following a protocol with cetyltrimethylammonium bromide (CTAB) (Devey *et al.*, 1996) with some modifications due to the presence of secondary metabolites (phenols, essential oils, etc.) that the Labrador teas contain. As a modification of the standard protocol, we used double purification with a mixture of chloroform and isoamyl alcohol, ammonium acetate and PVP.

Table 1. *Location of the examined populations of five species of the Labrador teas and variability of ptDNA and nrDNA markers*

Code	Population name, distribution	Species	Lat.	Long.	ptDNA				ITS1	
					N_1	Hap	Unique hap	H_e	N_2	Rib
1G	Twin Lakes Bog state Natural area, Taylor county, Wisconsin, USA	groen	45.278	−90.441	8	h27:4 h31:4	h31	0.57	4	R1a
2G	Deer Fly Trail road, Chippewa county, Wisconsin, USA	groen	45.189	−91.32	8	h27:8		0	2	R1a
3G	Douglas Island, near Juneau, USA	groen	58.299	−134.674	8	h32:8	h32	0	4	R1
4G	Chena Hot Springs, Fairbanks, Alaska, USA	groen	65.05	−146.05	12	h1:7 h20:2 h27:3	h20	0.62	2	R1
5S	Chena Hot Springs, Fairbanks, Alaska, USA	sub	65.05	−146.05	3	h18:2 h19:1	h19	0.67	–	–
6S	Sireniki, Providensky rayon, Chukotka	sub	64.416	−173.933	8	h1:5 h4:2 h18:1		0.61	1	R1b
7S	Novo-Chaplino, Providensky rayon, Chukotka	sub	64.497	−172.894	7	h1:4 h4:2 h18:1		0.67	2	R1
8S	Pinakul, Chukotsky rayon, Chukotka	sub	65.718	−171.144	7	h1:1 h9:5 h16:1		0.52	1	R1
9S	Avacha, Nalychevsky Park, Kamchatka	sub	53.272	158.839	6	h1:5 h4:1		0.33	2	R1
10S	Esso, Kamchatka	sub	55.769	158.713	8	h1:3 h4:3 h9:1 h16:1		0.78	3	R1
11S	Talaya, Hasansky rayon, Magadan oblast	sub	61.112	152.265	9	h1:3 h2:2 h3:1 h4:1 h14:1 h35:1		0.89	3	R1:2 R2:1
12T	Magadan, Magadan oblast	tom	59.556	150.808	8	h1:2 h2:3 h14:2 h35:1		0.82	3	R1:2 R2:1
13S	Arman, Magadan oblast	sub	59.666	150.133		–		–	2	R1
14D	Tserkovnaya Bay, Shikotan, Kuril Islands	div	43.833	146.695	8	h1:5 h12:1 h13:1 h21:1		0.69	1	R1
15D	Delfin Bay, Shikotan, Kuril Islands	div	43.845	146.694	8	h1:5 h4:1 h12:1 h23:1		0.64	1	R1
16D	Mendeleev Volcano, Kunashir, Kuril Islands	div	43.979	145.732	7	h1:4 h12:1 h21:1 h23:1		0.71	1	R1
17D	Golovina Volcano, Kunashir, Kuril Islands	div	43.843	145.504	4	h1:3 h4:1		0.5	1	R1
18H	Pervomaisk, Smirnykhovsky rayon, Sakhalin	hyp	49.966	143.366	6	h1:3 h8:3	h8	0.6	-	-
19H	Nogliki, Sakhalin	hyp	51.796	143.120	5	h3:1 h4:1 h5:1 h15:1 h16:1	h5	1	1	R2
20D	Nabilsky ridge, Tymovsky rayon, Sakhalin	div	50.846	142.98	3	h2:3		0	–	–
21H	Gastello, Sakhalin	hyp	49.107	142.954	3	h1:1 h7:1 h22:1	h7 h22	1	–	–
22T	Sabo, Okhinsky rayon, Sakhalin	tom	53.133	142.933	10	h1:3 h2:6 h3:1		0.6	2	R2
23H	Mayskoye, (Leonidovo), Poronaisky rayon, Sakhalin	hyp	49.291	142.877	7	h2:6 h4:1		0.28	2	R2
24T	Gornoye, Makarovsky rayon, Sakhalin	tom	48.807	142.874	3	h1:1 h2:2		0.67	–	–
25T	Chekhov Peak, Yuzhno-Sakhalinsk, Sakhalin	tom	47.005	142.840	2	h4:2		0	2	R1
26D	Starodubskoye, (Naiba), Dolinsky district, Sakhalin	div	47.405	142.789	2	h1:1 h2:1		1	2	R1:1 R2:1
27T	Sokol, Dolinsky rayon, Sakhalin	tom	47.248	142.726	7	h1:5 h4:1 h12:1		0.52	–	–
28T	Onor-2, Smirnikhovsky rayon, Sakhalin	tom	50.197	142.726	2	h2:1 h3:1		1	–	–
29D	Uskovo, Sakhalin	div	50.957	142.649	7	h2:5 h4:2		0.48	–	–
30D	Zhdanko, Makarovsky rayon, Sakhalin	div	48.240	142.573	7	h3:7		0	2	R1
31D	Tsapko, Makrovsky rayon, Sakhalin	div	48.099	142.510	2	h1:2		0	1	R2
32S	Mount Krasnova, Uglegorsky rayon, Sakhalin	sub	48.735	142.113	6	h1:4 h4:2		0.53	–	–
33T	Mount Krasnova, Uglegorsky rayon, Sakhalin	tom	48.735	142.113	3	h1:1 h2:2		0.67	–	–
34T	Krasnogorsk, Tomarinsky district, Sakhalin	tom	48.422	142.103	3	h4:2 h9:1		0.67	1	R1

Table 1. *Continued*

Code	Population name, distribution	Species	Lat.	Long.	ptDNA				ITS1	
					N_1	Hap	Unique hap	H_e	N_2	Rib
35H	Cape Davydova, De-Kastri, Khabarovsk Krai	hyp	51.424	140.888	4	h1:2 h2:2		0.67	–	–
36H	Cape Orlova, De-Kastri, Khabarovsk Krai	hyp	51.425	140.885	6	h2:6		0	2	R2
37H	Nikolaevsk-on-Amur, Khabarovsk Krai	hyp	53.148	140.741	10	h2:8 h25:2	h25	0.36	2	R2
38S	De-Kastri 10 km, Khabarovsk Krai	sub	51.468	140.598	6	h2:1 h4:4 h15:1		0.6	–	–
39H	Mount Kekurnaya, Sovetskaya Gavan, Khabarovsk Krai	hyp	49.01	140.15	21	h1:5 h2:11 h13:1 h26:2 h29:2	h26 h29	0.68	2	R2
40H	De-Kastri 40 km, Khabarovsk Krai	hyp	51.375	140.117	6	h1:2 h2:4		0.53	–	–
41H	Bikin, Bikinsky rayon, Khabarovsk Krai	hyp	47.066	137.133	8	h1:2 h2:2 h4:2 h33:2	h33	0.86	2	R2
42S	Brusnichnaya mount, Sikhote-Alinsky reserve, Terneysky rayon, Primorsky Krai	sub	45.809	136.604	2	h4:1 h10:1	h10	1	–	–
43H	Brusnichnaya mount, Sikhote-Alinsky reserve, Terneysky rayon, Primorsky Krai	hyp	45.809	136.604	6	h1:1 h4:1 h11:1 h13:3	h11	0.8	2	R1:1 R2:1
44H	Snezhnyy stream, Krasnoarmeysky rayon, Primorsky Krai	hyp	46.370	136.485	7	h1:1 h2:2 h28:2 h34:2	h28 h34	0.86	–	–
45H	Malinovyy stream, Krasnoarmeysky rayon, Primorsky Krai	hyp	46.390	136.446	6	h1:5 h2:1		0.33	–	–
46D	Anisimovka, Livadia ridge, Primorsky Krai	div	43.098	132.765	14	h6:14	h6	0	2	R2
47S	The Lena river, Yakutia	sub	72.365	126.456	11	h1:6 h2:1 h4:3 h24:1		0.67	3	R1b:2 R1:1
48T	Novaya Chara, Zabaikal oblast	tom	56.783	118.266	7	h1:2 h2:5		0.48	2	R1
49T	Bukukun, Sokhondiysky Reserve, Zabaikal oblast	tom	49.638	111.358		–		–	2	R1:1 R2:1
50T	Putorana, lake Lama, Krasnoyarsk Krai, Siberia	tom	69.550	89.859	4	h1:3 h13:1		0.5	5	R1:3 R2:2
51T	Sotyy pereval, Khakassia, Zapadnyy Sayan, Siberia	tom	51.638	89.799	4	h2:4		0	2	R1:1 R2:1
52T	Turukhansk, Krasnoyarsk Krai, Siberia	tom	65.801	87.91	4	h2:4		0	–	–
53T	Aktash, Altai, Siberia	tom	50.389	87.667	4	h2:4		0	–	–
54T	Surgut, Khanty-Mansi Autonomous Okrug, Siberia	tom	61.777	72.386	4	h1:2 h2:2		0.67	2	R1
55T	Sangymgort, Yamalo-Nenets Autonomous Okrug, Siberia	tom	64.789	67.336	4	h24:4		0	1	R2
56T	Shuryshkari, Yamal-Nenets Autonomous Okrug, Siberia	tom	65.902	65.365		–		–	2	R2
57T	Kosmakova, Sverdlovsk oblast, Ural	tom	56.333	60.85	8	h1:4 h2:2 h24:1 h30:1	h30	0.75	1	R1
58T	Lake Chusovskoye, Sverdlovsk oblast, Ural	tom	56.742	60.288	4	h1:4		0	2	R1:1 R2:1
59T	Vorkuta, Komi Republic	tom	63.658	53.705	4	h13:4		0	2	R1
60T	Sandarmokh, Medvezhyegorsk, Karelia	tom	62.886	34.633	14	h2:8 h24:6		0.53	2	R2
61T	Apatity, Murmansk oblast, Kola Peninsula	tom	67.563	33.450	18	h1:4 h2:4 h13:6 h24:4		0.78	2	R2
62T	Murmansk, Murmansk oblastõ Kola Peninsula	tom	68.963	33.082	4	h1:4		0	2	R1:1 R2:1
63T	Sestroretsk swamps, Leningrad oblast	tom	60.106	30.040	8	h13:3 h24:5		0.54	–	–
64T	Lomonosov rayon, Leningrad oblast	tom	59.952	29.326	5	h24:5		0	–	–
65T	Schwendlund Swamp, Zelenogradsk, Kaliningrad oblast	tom	54.966	20.5	8	h1:3 h13:3 h24:2		0.75	2	R1:1 R2:1
	Total				408				90	

Code: abbreviated population name, consisting of the number and the first letter of the species name. Species' abbreviations: groen – *R. groenlandicum*, hyp – *R. hypoleucum*, div – *R. diversipilosum*, tom – *R. tomentosum*, sub – *R. subarcticum*. N_1 – number of specimens examined in plastid data analysis; N_2 – number of specimens examined in nuclear data analysis; Hap – obtained haplotypes and their frequencies; Unique hap – haplotypes obtained from this population only; H_e – gene diversity; Rib – obtained ribotypes and their frequencies.

We carried out amplification with universal primers for five non-coding ptDNA regions, i.e. *pet*B-*pet*D (Löhne and Borsch, 2005), 3′*trn*V (UAC)-*ndh*C (Shaw *et al*., 2007), *trn*H-*psb*A and K2R-K707 (Jiang *et al*., 2016), and *atp*B oligo2 – *rbc*L oligo5 (Manen *et al*., 1994). To improve amplification, we designed new primers based on the plastid genome of *Rhododendron pulchrum* Sweet (NCBI number MN182619.1) for *trn*H-*psb*A and K2R-K707. Two more primers were designed based on the plastid genome of *Rhododendron tomentosum* (NCBI number OK586901.1) for *atp*B-*rbc*L and *pet*B-*pet*D. Primers details are given in Supplementary Data Table S3. The single temperature touchdown profile of PCR was adopted for all ptDNA fragments: 10 min denaturation at 95 °C; 10 cycles of touchdown phase amplification of 30 s for denaturation at 95 °C, 30 s of initial annealing at 61 °C and 2 min elongation at 72 °C; 25 amplification cycles for 30 s for denaturation at 95 °C, 30 s annealing at 56 °C and 2 min elongation at 72 °C; and a final elongation for 10 min at 72 °C.

The ITS 1 region was amplified using the primers 'ITS1' and 'ITS2' (White *et al*., 1990) with the following conditions: 10 min denaturation at 95 °C; 35 amplification cycles for 30 s for denaturation at 95 °C, 45 s annealing at 56 °C and 1 min elongation at 72 °C; and a final elongation of 10 min at 72 °C.

Before sequencing PCR products were checked on 1.0 % agarose gels and were purified using an ExS-Pure PCR Enzymatic Purification Kit (Nimagen, Netherlands), then used as templates for sequencing using a Brilliant Dye v.3.1 kit (Nimagen, Netherlands). We obtained sequences of these fragments using a NANOFOR 05 genetic analyser (Syntol, Russia).

Data from all five plastid fragments (2040 sequences) were combined into a single contig for each individual and the final data matrix contained 408 contigs; *T. camtschaticum* was treated as an outgroup in phylogenetic analysis. An ITS1 phylogenetic tree was constructed from 90 sequences with good sequencing quality. Accessions from *R. dauricum* and *R. parvifolium* were used as outgroups. Nucleotide sequence data for each plastid haplotype and for each ribotype are available in the GenBank database (Supplementary Data Tables S1 and S2).

Sequence alignments of ptDNA and nrDNA regions were performed using BioEdit v.7.0.9 software (Hall, 1999). Phylogenetic relationships among ptDNA haplotypes and ITS1 ribotypes were evaluated separately by maximum parsimony (MP) with the heuristic search algorithm, implemented in PAUP*4.0b10 (Swofford and Sullivan, 2003), and a Bayesian inference (BI) with MrBayes v.3.2.3 (Ronquist and Huelsenbeck, 2003). The BI estimates were performed using the GTR+G+I model of nucleotide substitutions. Insertions, inversions and deletions were considered as single events and were encoded as a binary data matrix consisting of zeros and ones. The binary data from ptDNA haplotypes were processed to reconstruct an MP network with the MJ algorithm and MP option in NETWORK v.4.6.1.2 (Bandelt *et al*., 1999). A hierarchical analysis of molecular variation (AMOVA) was performed using Arlequin v.3.5.1.2 (Excoffier and Lischer, 2010) to examine the partitioning of genetic variation within and between populations and between species (F_{ST}, F_{CT}, F_{CT} (AMOVA, SP)). We also calculated the partitioning of genetic variation between geographical regions, determined by several approaches: first, by the proportion of 'key' haplotypes (haplotype proportion, F_{CT} (AMOVA, HP)), and second, using both genetic data and geographical coordinates of the sampled populations in the programs SAMOVA and BAPS. SAMOVA 2.0 (Dupanloup *et al*., 2002) is based on a simulated annealing procedure. A process was run until we obtained the configuration of K groups that maximizes F_{CT} (the proportion of total genetic variance due to differences among groups of populations) and minimizes single-population groups, with 1000 simulated annealing steps. BAPS 5.4 (Corander *et al*., 2004) performs a population mixture analysis based on the clustering of individuals with non-identical allele frequencies, taking into account the geographical information of the samples. When testing for population clusters, we ran ten replicates for each K value from 2 to 10.

To detect historical population expansion events, mismatch distributions were calculated using Arlequin v.3.5.1.2. A total of 1000 parametric bootstrap replicates were used to generate an expected distribution under a model of sudden demographic expansion (Rogers and Harpending, 1992). Neutrality tests (Fu's Fs and Tajima's D indices) also implemented in Arlequin v.3.5.1.2 were assessed to test for deviation from neutrality (Tajima, 1989; Fu, 1997).

Estimates of divergence time

The combined ptDNA dataset was used to estimate divergence times using the Bayesian Yule model method implemented in BEAST v.1.7.5 (Drummond and Rambaut, 2007). Using MrModeltest v.2.2 software the K81 (123321) model with invariant sites and gamma distribution was evaluated as the best fitting model of nucleotide substitutions from the data. A relaxed molecular clock with uncorrelated lognormally distributed substitution rates for each branch of the phylogenetic tree was used to estimate age divergence within *Ledum* taxa. Priors for the most recent common ancestor (MRCA) were set to lognormal distributions with logmean = 1, lognormal s.d. = 0.5 and offset = 56 Mya for *T. camtschaticum* (Xia *et al*., 2022). The first 10 % simulations of Markov chain Monte Carlo (MCMC) chains were discarded (burn-in) in a total of 10^7 generations. The effective sample size (ESS) assessed in Tracer v.1.7.2 (Rambaut *et al*., 2018) was >200 as a quality-measure of the resulting sample sequence. TreeAnnotator 1.6.1 (Drummond and Rambaut, 2007) was used to summarize the trees with a criterion of the maximum clade reliability using the mean heights option. The final consensus tree was drawn by FigTree 1.4.3 (Rambaut, 2009).

Reconstruction of ancestral areas

We performed a statistical dispersal-vicariance analysis (S-DIVA) to reconstruct the geographical diversification of *Ledum*, as implemented in RASP v.4.4 (Yu *et al*., 2015). Four geographical regions were identified for examined species of subsect. *Ledum*: (A) North America; (B) northeast of the Verkhoyansk Range in Eurasia; (C) northwest of the Verkhoyansk Range in Eurasia; and (D) southeastern regions of Russia, Eurasia (see Fig. 4). These groupings were defined according to geographical and biogeographical criteria. We used 1000 trees from the Bayesian phylogenetic analyses as input for S-DIVA to compute the condensed tree, and subsequently

the ancestral areas and potential vicariance and dispersal events were inferred with RASP.

RESULTS

Nuclear data

The aligned ITS1 sequence had a length of 280 nucleotide positions and resulted in five parsimony-informative positions through 90 accessions. Bayesian analysis of the resulting four ribotypes divided all samples into two clusters according to the predominance of ribotype R1 or R2 (Fig. 1A–D; Table 1), differing from each other by three mutations. Several accessions differing by one mutation from R1 have ribotype R1a (*R. groenlandicum* from NAm: 1G, 2G) and R1b (*R. subarcticum* from NEA: 8S, 47S). The identified ribotypes are not species-specific and have a geographically structured distribution. Ribotype R1 was obtained in all five species – *R. groenlandicum*, *R. subarcticum*, *R. tomentosum*, *R. hypoleucum* and *R. diversipilosum*. R1 was the only ribotype found in populations from Alaska (3G, 4G, 5S), Chukotka (6S, 7S), Kamchatka (9S, 10S), Kuril Islands (14D–17D) and southern Sakhalin (25T, 30D, 34T). Ribotype R2 was predominant in three species, *R. tomentosum*, *R. hypoleucum* and *R. diversipilosum*, and was found once in *R. subarcticum.* R2 was spread in northern and middle Sakhalin (19H, 22T, 23H, 31D), Khabarovsk Krai (36H, 37H, 39H, 41H), Primorsky Krai (46D), Siberia (55T, 56T) and European part of Russia (60T, 61T). Both ribotypes coexisted in populations of four species except *R. groenlandicum.* These populations are from Magadan oblast (11S, 12T), southern Sakhalin (26D), Primorsky Krai (43H), Siberia (49T–51T), Ural (58T) and further to the European part of Russia (62T, 65T). Specimens with intragenomic polymorphism were observed in a number of populations of *R. tomentosum* and *R. subarcticum*. Their sequencing chromatograms showed double peaks at variable sites, indicating the presence of both ribotypes in one specimen. Such individuals were identified in Magadan oblast (11S, 12T), Siberia (51T) and in the north of European part of Russia (59T, 62T). They were excluded from the data employed for the phylogenetic tree. The presence of recombinant ribotypes is marked with a star in Fig. 1A. The outgroup *R. dauricum* and *R. parvifolium* differed from ribotypes of the subsect. *Ledum* accessions by ten mutations. On the phylogenetic tree (Fig. 1D), several clades were identified, none of which corresponded to any member

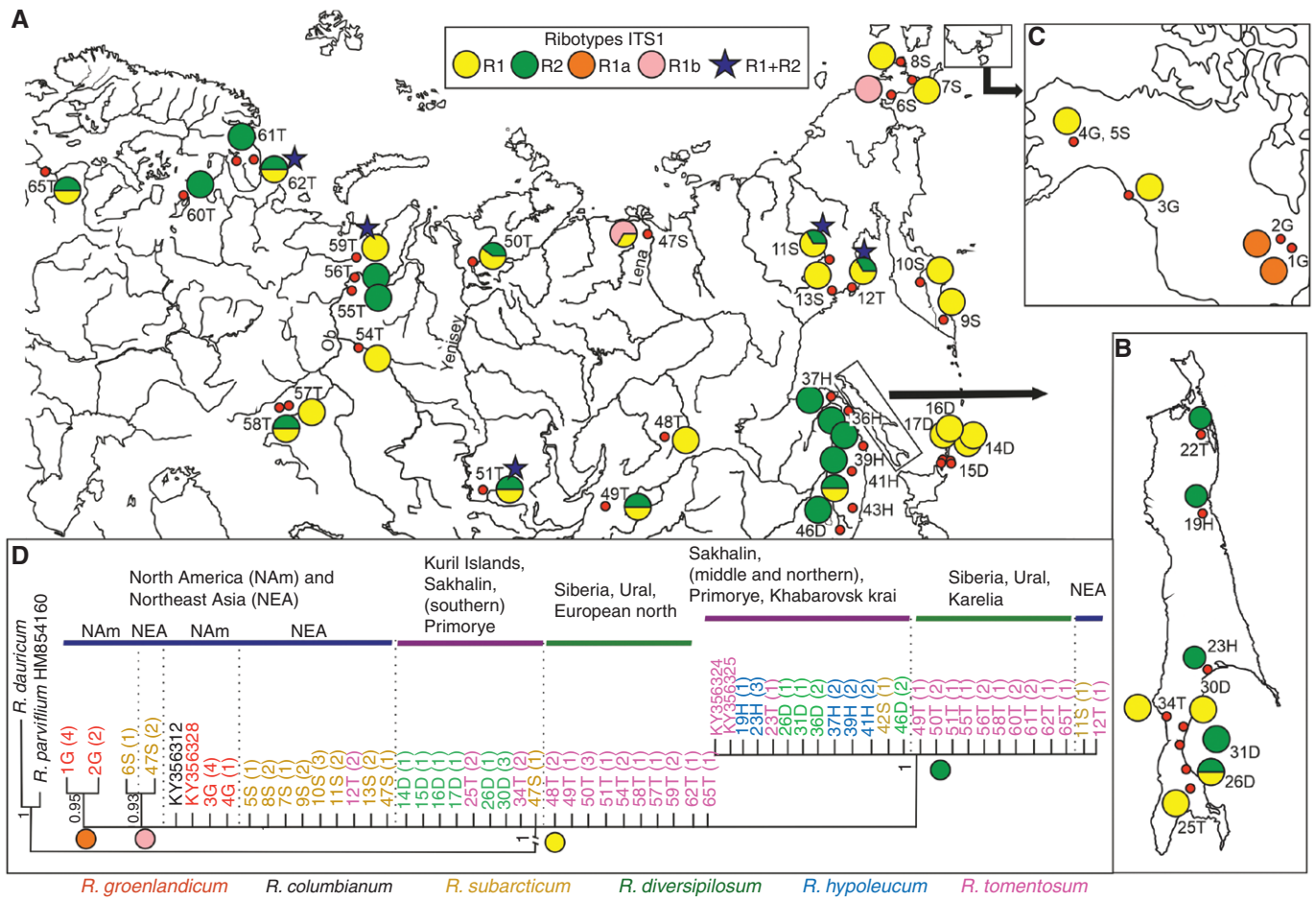

FIG. 1. (A–C) Distribution of the ITS1 region ribotypes in the assessed populations of subsect. *Ledum* taxa. Population numbers on the map are depicted according to Table 1. (D) Bayesian phylogenetic tree of 90 examined specimens. The number of sequenced individuals in the population is given in parentheses. Support values of Bayesian posterior probabilities are indicated below branches. Recombinant ribotypes are marked with an asterisk.

of five species. All the accessions with ribotype R2 formed a well-supported clade [posterior probability (PP) = 1.00] as well as a few accessions with ribotypes R1a and R1b (PP = 0.95 and 0.93, respectively) while all the accessions with ribotype R1 did not form a clade with support.

Plastid data

ptDNA diversity Single nucleotide polymorphisms (SNPs) and insertions/deletions (indels) were identified in the sequences of five intergenic spacers of ptDNA. The fragment *pet*B-*pet*D (452 bp) contains two indels and one SNP; 3′*trn*V (UAC)-*ndh*C (400 bp) contains two indels and nine SNPs; *atp*B-*rbc*L (428 bp) contains one indel and five SNPs; *trn*H-*psb*A (342 bp) contains one indel and eight SNPs; and K2R-K707 (650 bp) contains nine SNPs. The total length of the alignment was 2272 bp, and 34 haplotypes (H1–H16, H18–H35) were derived from all polymorphic alignment sites. Haplotype H36 corresponds to *R. columbianum* compiled from GenBank accessions. The outgroup *T. camtschaticum* (H17) differed from haplotypes of thesubsect. *Ledum* accessions by 78 mutations. The number of haplotypes and their frequencies (N_h), unique haplotypes and also the haplotype diversity index (H_e) for each population are given in Table 1 and the geographical distribution of haplotypes is shown in Fig. 2A–C.

Eighteen (H5–H8, H10, H11, H19, H20, H22, H25, H26, H28, H29, H30–H34) out of the 34 haplotypes were unique and were identified only once or twice. The remaining haplotypes were common to several species (Table 1; Fig. 2). The most widespread haplotype H1 was shared by all five species *R. groenlandicum*, *R. subarcticum*, *R. tomentosum*, *R. hypoleucum* and *R. diversipilosum*. Haplotypes H2 and H4 were common to four species, *R. subarcticum*, *R. tomentosum*, *R. hypoleucum* and *R. diversipilosum.* Haplotypes H3 and H13 were found in three species, *R. tomentosum*, *R. hypoleucum* and *R. diversipilosum.* Haplotype H9 was shared by *R. subarcticum* and *R. diversipilosum.* Although some haplotypes were shared by several species, other haplotypes were species-specific. For example, *R. groenlandicum* (1G–3G) had several specific haplotypes (H27, H31 and H32) and *R. subarcticum* (5S–7S) had specific haplotype H18, distributed in Alaska and Chukotka.

Haplotypes H1 and H2 (yellow and blue in Fig. 2A) are spread evenly almost throughout the whole examined populations of the four species in Eurasia. H1 was also found in Alaska (4G) in the North American species *R. groenlandicum*. Haplotype H4 (green) is spread in the Far East of Russia and its western distribution border runs along the Lena River including population 47S from Yakutia. Haplotype H24 (pink) first occurs in the same population of *R. subarcticum* (47S) and then becomes highly frequent in the western part of Russia in *R. tomentosum* (55T, 57T, 60T, 61T, 63T, 64T, 65T). Haplotype H13 (light beige) is distributed in the northwest of Russia in *R. tomentosum* (50T, 59T, 61T, 63T and 65T), as well as in the south of the Russian Far East in *R. diversipilosum* and *R. hypoleucum* (14D, 39H, 43H). Some haplotypes were highly localized and they occurred in closely associated localities. For example, H12, H21 and H23 occurred only on southern Sakhalin and the Kuril Islands (15D, 16D, 27T) and H14 was obtained only in Magadan oblast (11S, 12T).

The number of detected haplotypes varies, but the mean haplotype diversity (H_e) is approximately the same for all five species (Fig. 2H): ten *R. tomentosum* haplotypes (H_e = 0.775), 18 *R. hypoleucum* haplotypes (H_e = 0.740), ten *R. diversipilosum* haplotypes (H_e = 0.826), 11 *R. subarcticum* haplotypes (H_e = 0.826) and five *R. groenlandicum* haplotypes (H_e = 0.826).

The H_e index decreases from the Far East to the West in Eurasian populations (Table 1). The highest genetic diversity (H_e = 0.82–1.00) is observed in populations from Magadan oblast (11S, 12T), Khabarovsk Krai (41H), Primorsky Krai (42S, 43H, 44H) and Sakhalin (19H). Populations from Siberia (average H_e = 0.24) and northwestern part of Russia (average H_e = 0.37) have a smaller number of haplotypes.

Phylogeographical patterns in subsect. Ledum

The haplotype network reveals several haplogroups (Fig. 3A) with a star-like structure formed by one or several dominant haplotypes and a set of genetically close rare haplotypes. They are haplogroup I (yellow, H1, H6, H9, H12, H13, H18, H21, H23, H28, H34), haplogroup II (blue, H2, H3, H5, H7, H8, H14, H15, H22, H25, H26, H29, H33) and haplogroup III (green, H4, H10, H11, H16, H20, H35). The two main Eurasian haplotypes H1 and H2 are separated by a branch of seven mutational steps. Other haplogroups are smaller and do not have a star-like structure. Haplogroup IV includes haplotypes from North America and Eurasia (H19, H24, H30). Haplogroup V includes haplotypes exclusively from North America (H27, H31). North American haplotype H32 occupies a basal position in relation to all other haplotypes.

Since the distribution of ptDNA variability has a more pronounced geographical rather than species-specific pattern, we applied several approaches to reveal phylogeographical structure. First, we distinguished several geographical groups based on the distribution and proportion of ‘key’ haplotypes (Fig. 2A–C, F). *Rhododendron groenlandicum* and *R. subarcticum* (1G–5S) form the geographical group NAm (Fig. 2B, F), because they have haplotypes unique to North America (H19, H20, H27, H31, H32). Another geographical group is distributed in Northeast Asia and is called NEA1 (Fig. 2A, F); it includes populations of *R. subarcticum* from Chukotka (6S–8S) and Kamchatka (9S, 10S), populations of *R. diversipilosum* from the Kuril Islands (14D–17D), populations of *R. diversipilosum* and *R. tomentosum* from Southern Sakhalin (25T, 27T, 31D, 34T), and populations of *R. subarcticum*, *R. hypoleucum* and *R. diversipilosum* from Primorskii Krai (42S, 43H, 46D). A distinctive feature of NEA1 populations is the predominance of haplogroup I haplotypes and the absence of haplogroup II haplotypes. Conversely, NEA2 populations predominantly have haplogroup II haplotypes and haplogroup I haplotypes are less represented. NEA2 (Fig. 2A, C) includes almost all populations of *R. hypoleucum* from Khabarovskii Krai (35H–37H, 38S, 39H–41H) and the north of Primorskii Krai (44H, 45H), populations of *R. hypoleucum*, *R. subarcticum*, *R. diversipilosum* and *R. tomentosum* from northern and central Sakhalin (18H, 19H, 20D, 21H, 22T, 23H, 24T, 26D, 28T, 29D, 30D, 32S, 33T); populations of *R. subarcticum* and *R. tomentosum* from Magadan oblast (11S, 12T) and populations of *R. tomentosum*

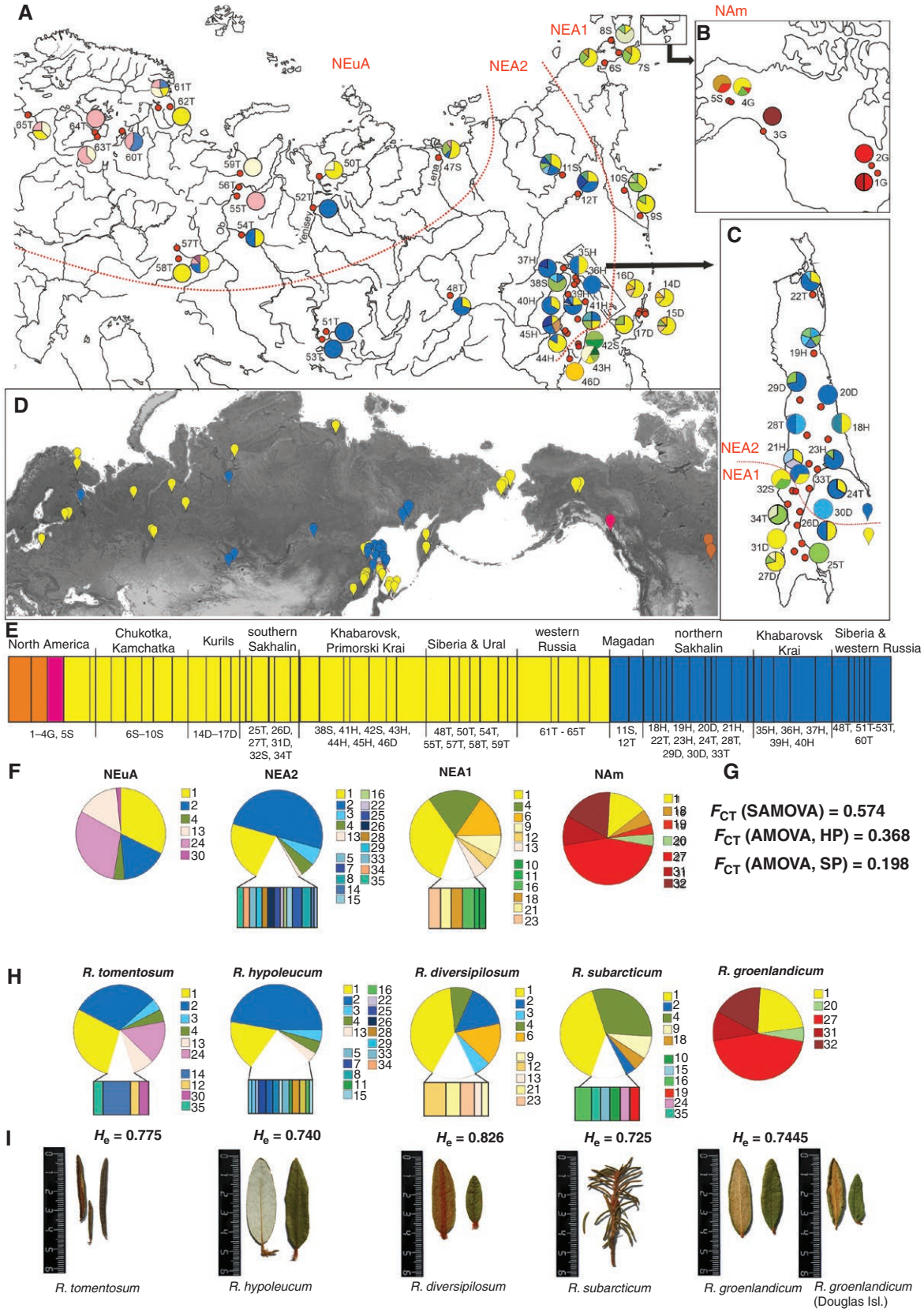

FIG. 2. (A–C) ptDNA haplotype distribution of 408 individuals from 65 populations of subsect. *Ledum* taxa. Population numbers on the map are depicted according to Table 1. (D) Distribution of genetic groups according to SAMOVA. (E) Assignment of populations (bar graphs) among the four regions using Bayesian analysis of population structure (BAPS). (F) Pie charts of the ratio of haplotypes pooled across regions, identified by the distribution and proportion of 'key' haplotypes. Geographical clusters are separated by dashed lines: North America – NAm, Northeast Asia with two clusters – NEA1 and NEA2, north Eurasia group – NEuA. (G) Distribution of ptDNA genetic diversity among four geographical regions by SAMOVA (F_{CT} (SAMOVA)); among four geographical regions based on proportion of 'key' haplotypes (F_{CT} (AMOVA, HP)); among five species (F_{CT} (AMOVA, SP)). (H) Pie charts of the ratio of haplotypes of five species, where H_e is mean haplotype diversity. (I) Photographs of leaf morphology of studied *Ledum* species.

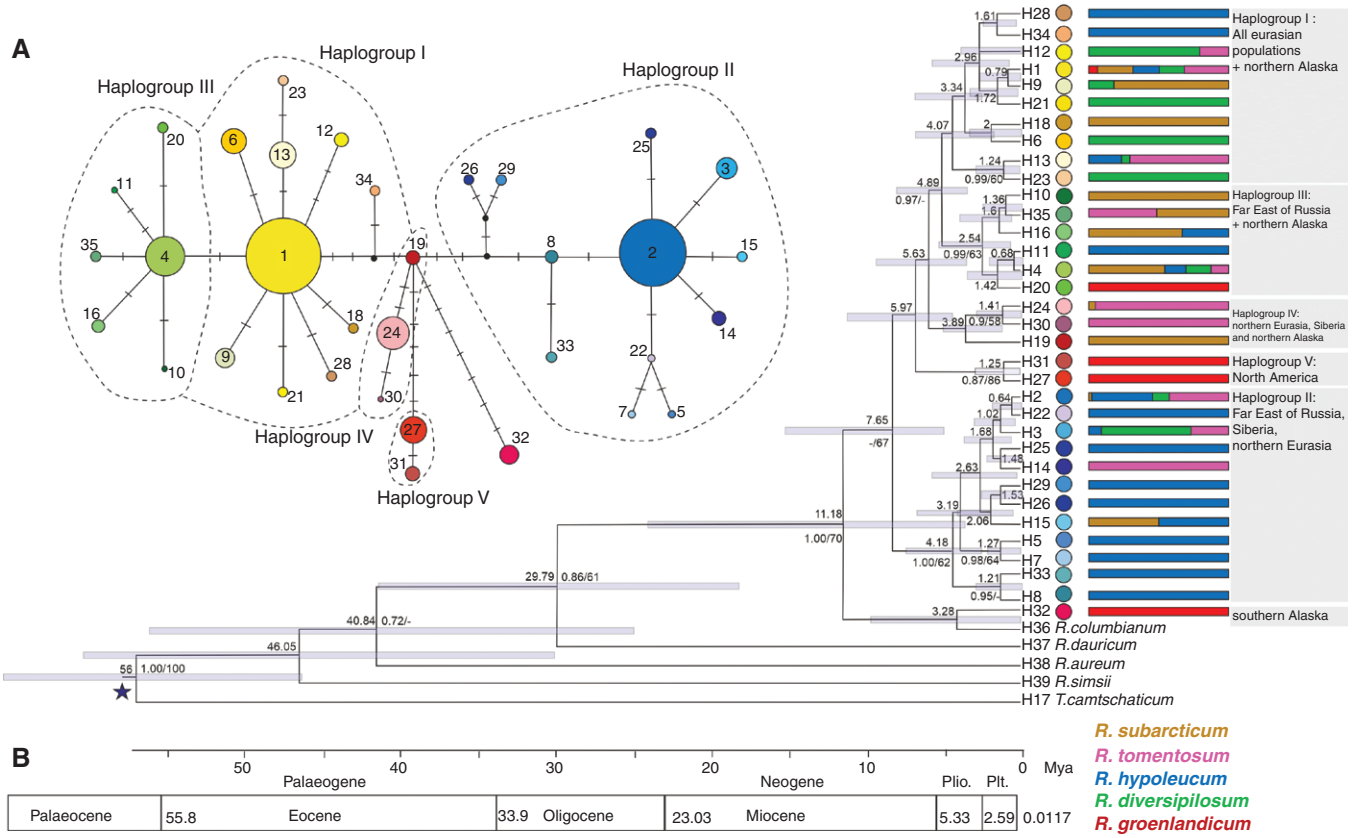

FIG. 3. Phylogenetic relationships of ptDNA haplotypes created with several approaches. (A) Maximum parsimony using NETWORK. The size of a circle is proportional to the frequencies of occurrence of the corresponding haplotype. Cross-strokes are mutational events. Black dots are hypothetical haplotypes. Dashed lines correspond to haplogroups. (B) The best-scoring Bayesian inference tree is shown with posterior probabilities (MrBayes) as well as bootstrap values (PAUP) shown below the branches (PP/BS). Estimates of the divergence times of subsect. *Ledum* obtained in BEAST. Shaded bars on tree nodes represent 95 % credible intervals for the time estimates relative to the scale at the bottom. Horizontal bars show the species membership by each haplotype. Star denotes the calibration point used. The geographical distribution of each haplogroup is shown by grey shading. Plio., Pliocene; Plt., Pleistocene.

from Siberia (51T–54T). The North Eurasian group NEuA includes the remaining populations, mainly *R. tomentosum* from the Lena River (47S) to Kaliningrad (65T) where only haplotypes H1, H2, H13 and the region-specific haplotype H24 exists. The population from Yakutia (47S) is located on the border between zones NEA2 and NEuA. A comparison of the proportions of haplotypes in each geographical group versus each species is presented in Fig. 2F and H.

AMOVA revealed that about 55 % ($F_{ST} = 0.548$, $P < 0.05$) of genetic differentiation was apportioned among all examined populations. Less than 20 % ($F_{CT} = 0.198$, $P < 0.05$) of genetic differentiation was detected among the five species *R. groenlandicum*, *R. hypoleucum*, *R. diversipilosum*, *R. tomentosum* and *R. subarcticum*. About 37 % ($F_{CT} = 0.368$, $P < 0.05$) was among the four geographical groups NAm, NEA1, NEA2 and NEuA.

SAMOVA showed that genetic differentiation among groups was highest ($F_{CT} = 0.574$) for $K = 4$ (Fig. 2D). The group configuration was the same for BAPS analysis (Fig. 2E). The first group included populations from Wisconsin, USA (1G, 2G); the second included the population from Douglas Island (3G); the third included populations from Alaska (4G, 5S) and those previously identified as the NEA1 group, with two additional populations from Khabarovsk Krai (38S, 41H) and most of populations from the NEuA group; and the fourth group included the majority of populations from the NEA2 group in addition to several populations from the NEuA group.

Analyses of mismatch distribution were conducted for each of the five species. The sum of squared deviations (SSD) from the observed mismatch distribution and the expectation under the expansion model suggest a stable population size in all species. Fu's Fs and Tajima's *D* indices indicate that deviation from neutrality was non-significant (Supplementary Data Table S4).

Phylogenetic relationships and divergence time

The topology of the evolutionary tree obtained using MrBayes, PAUP and BEAST was the same. The consensus tree (Fig. 3B) does not resolve each of the five species as monophyletic and reflects the evolution of haplotypes rather than species. Bayesian estimates of the divergence time for ptDNA haplotype clades with several outgroups, *R. columbianum* (H36), *R. dauricum* (H37), *R. aureum* (H38), *R. simsii* (H39) and *T. camtschaticum* (H17), suggest (Fig. 3B) that the *Ledum* group diverged from the closest outgroup *R. dauricum* of the subgenus *Rhododendron* during the Oligocene about 29.79 Mya. An American haplotype, H32, grouped with another American *Ledum* species, *R. columbianum*, and together they occupied

a basal position to the remaining genetic lineages with a divergence time estimated at ~11.18 Mya. The split between two major clades, one including haplotypes of haplogroup II and the other including haplotypes of haplogroups I, III, IV and V, was estimated at 7.65 Mya. Divergence within each clade began at the boundary of the late Miocene – Early Pliocene, 5.97–4.18 Mya, and the rapid radiation began near the Pleistocene after 2.63 Mya.

Ancestral area reconstructions

Results of S-DIVA suggest a complex phylogeographical history of the species of subsect. *Ledum* in Eurasia and North America. Pie charts at nodes indicate the relative frequencies of ancestral area optimization across the entire ancestral area reconstruction tree shown in Fig. 4. Both the Russian Far East and North America (AD, marginal probability 53.37, relative probability 0.27) are resolved to be the most probable ancestral area(s) for the MRCA of *Ledum*. Based on S-DIVA, at least two dispersal events and two vicariance events are hypothesized to have led to the current distribution of the major haplogroups, and these events are depicted in Fig. 4. During the Miocene, a vicariance event gave rise to two distinct lineages in North America and Eurasia. The most likely putative dispersal event is from Asia back to North America in the Late Miocene, followed by diversification in these two regions with further migration westward into Eurasia. Two major Eurasian lineages achieved their present distribution through different type of events. In haplogroup I, vicariance events most probably occurred, and in haplogroup II, dispersal events often occurred.

DISCUSSION

Past climatic changes in the Northern Hemisphere have altered the distribution of species: they have influenced the contraction and expansion of populations and species ranges, and the formation of contact zones between them (Hewitt, 2000). Biogeographical patterns identified for subsect. *Ledum* species coincide with those described previously for other species of cold-resistant flora of Northeast Asia and, in particular, Beringia (Polezhaeva *et al*., 2010; Hantemirova *et al*., 2023). Although previous studies have attempted to clarify species boundaries of subsect. *Ledum*, using a limited sample set (Hart *et al*., 2017) and a specific geographical area (Iunusova *et al*., 2023), in the present study we carried out a phylogeographical analysis of the subsection with a large sample set of several species across their geographical ranges in Eurasia and North America. Our analyses have shown that genetic diversity in subsect. *Ledum* did not correspond to the division into species, but have gradual geographical patterns probably explained by the history of dispersal of members within the current distribution.

Genetic structure of the Labrador tea populations: a melting pot of genetic variability

Our current study of 65 populations within subsect. *Ledum* showed high levels of genetic variation in five ptDNA sequences and moderate variation in one ITS1 nrDNA fragment. Only four nrDNA ribotypes have been identified, two of which were frequent, with a weak geographical and species-specific structure and 34 ptDNA haplotypes with geographical structure. There is much clearer differentiation of ptDNA genetic diversity between geographical regions [F_{CT} (SAMOVA) = 0.574; F_{CT} (AMOVA, HP) = 0.369] than between the five species [F_{CT} (AMOVA, SP) = 0.198], with genetic diversity tending to decrease from northeast Eurasia to the west. The limited amount of genetic variability in Western Eurasia is consistent with only two species, *R. tometosum* and *R. subarcticum*, described for this area. The highest haplotypic diversity was observed in the Russian Far East (Northeast Asia) including Sakhalin Island where four species, *R. tomentosum*, *R. subarcticum*, *R. hypoleucum* and *R. diversipilosum* (Harmaja, 1990, 1991), and greater (Barkalov and Taran, 2004) have been described. In North America, the only population assigned to *R. groenlandicum* from Alaska (4G) exhibited a high polymorphism while the others assigned to *R. groenlandicum* harboured only one or two haplotypes. Thus, the area of the Russian Far East, surrounding the Sea of Okhotsk and the Sea of Japan, appears to be a centre of both genetic and taxonomic diversity. A similar pattern of high species and genetic diversity in the Russian Far East was observed in other arboreal cold-tolerant species such as the *Larix* Mill. species complex (Polezhaeva *et al*., 2010) and the *Alnus alnobetula* (Ehrh.) K. Koch *s.l.* complex (Hantemirova *et al*., 2023). Since genetic diversity of species is a result of their evolutionary history and recent evolutionary processes, the high level of diversity and low genetic differentiation of species observed in our study suggest that these species have not undergone dramatic fluctuations in abundance over a long time. Although the results of mismatch distribution and neutrality tests indicate currently stable populations (Supplementary Data Table S4), star-like structures formed with high frequency and widespread haplotypes H1, H2 and H4 are consistent with rapid diversification of haplotypes and significant spatial and demographic expansion in the past. The large effective population size of the subsect. *Ledum* species complex in the Russian Far East may be the result of contiguous range expansion during climate cooling. Species of subsect. *Ledum* inhabiting similar ecosystems are widespread in mid- to high-elevation of forest and open communities in Northeast Asia due to high ecological plasticity and adaptation to harsh climates and poor soils (Mazurenko and Khokhrjakov, 1987; Rohrs-Richey and Mulder, 2007; Amada *et al*., 2024). In most populations we found an admixture of ribotypes and haplotypes. Highly frequent haplotypes are shared among all five or fewer species, and not many of them correspond to geographically close populations of the same species. This phenomenon could have stemmed from retention of ancestral polymorphisms caused by incomplete lineage sorting or introgression. Conservation and stochastic sorting of ancestral polymorphisms is particularly likely if the widths of lineages (i.e. the effective population size) are large compared to their length (i.e. the time between divergences). In this case, genetic drift is unlikely to have time to bring loci to fixation before subsequent divergences (Pamilo and Nei, 1988). Thus, it can be hypothesized that rapid expansion and large effective population size as a background for diversification due to temperature decline in the mid-Miocene (Graham, 2011) favoured the persistence of different genetic lineages (Pamilo and Nei, 1988; Maddison and Knowles, 2006; Jakob and Blattner, 2006; Chung *et al*., 2007;

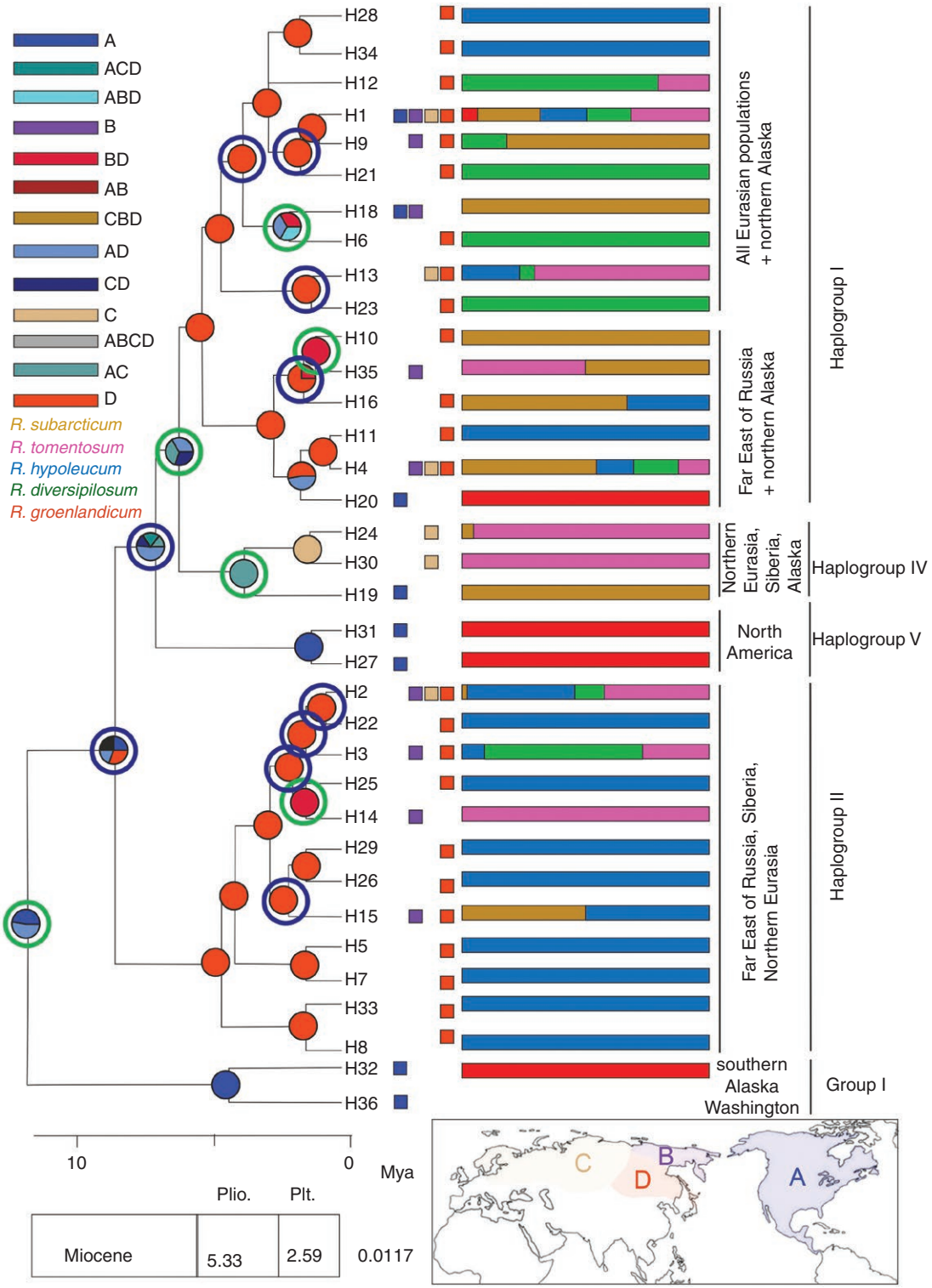

FIG. 4. Results of statistical dispersal–vicariance analysis (S-DIVA). Pie charts at each node correspond to probabilities of hypothetical ancestral areas: (A) North America; (B) northeast of the Verkhoyansk Range in Eurasia; (C) northwest of the Verkhoyansk Range in Eurasia; (D) southeastern regions of Russia, Eurasia (the borders of regions are depicted on the map). The coloured squares after each haplotype represent current geographical distributions. Horizontal bars show the species membership by each haplotype. The geographical distribution of each haplogroup is depicted. Plio., Pliocene; Plt., Pleistocene.

Yoichi *et al*., 2017; You *et al*., 2022) in closely related species. Sympatric coexistence could provide possibilities for introgression and genetic admixture. For *Rhododendron* in general and subsect. *Ledum* taxa in particular, hybridization events are well documented (Dalgaard and Fredskild, 1993; Milne *et al*., 1999; Zhang *et al*., 2007; Milne and Abbott, 2008; Zha *et al*., 2008; K. Theqvist, Arboretum Mustila, Finland, unpubl. res.). The lack of harsh restrictions on cross-breeding can lead to the formation of a species complex in which in extreme positions (geographically, ecologically) members are strikingly different from each other, but in sympatry it is almost impossible to draw a boundary between them. Hybridization events are also indirectly supported by an intragenomic polymorphism in the ITS1 marker found in several populations (11S, 12T, 51T, 59T, 61T). Such a deviation from concerted evolution in the ITS1 marker may occur in cases of hybridization or polyploidy (Álvarez and Wendel, 2003). Species of subsect. *Ledum* can be both diploids ($2n = 26$) and tetraploids ($2n = 52$). The American hypoarctic species *R. groenlandicum* is considered to be diploid (Löve, 1982; Sheik *et al*., 2019). The boreal Eurasian species *R. tomentosum* and *R. diversipilosum* are tetraploids (Gurzenkov, 1973; Mesícek and Javurková-Jarolímová, 1992; Lantai and Kihlman, 1995; Wakui, 2021). Diploid and tetraploid populations of the hypoarctic circumpolar species *R. subarcticum* are known (Zhukova and Petrovsky, 1976; Zhukova, 1980; Löve, 1982; Lantai and Kihlman, 1995; Wakui, 2021). In our chromatograms we observed intra-genomic variability for some individuals of *R. subarcticum* and *R. tomentosum* and we also observed the presence of two ribotypes in different individuals in the same population in these species. All this may suggest both polyploidy and hybridization and may finally indicate the recent divergence of these closely related species.

Divergence time and ancestral area reconstruction of the subsect. Ledum species complex

The genus *Rhododendron* is considered to have originated about 50–68 Mya with members existing in the understorey of evergreen mixed forests (Irving and Hebda, 1993; Brown *et al*., 2006; Shrestha *et al*., 2018). Diversification of the subsect. *Ledum* ancestral group probably started in the Miocene under the global climate cooling over the last 15 Myr (Donoghue and Smith, 2004; Pavlyutkin *et al*., 2016; Holbourn *et al*., 2018). During the Neogene cooling cold-tolerant forests spread widely across the Northern Hemisphere plains and the *Ledum* group developed within their understorey. BEAST analysis of ptDNA sequences estimated the crown age of core *Ledum* (divergence from *Rhododendron*) to be 29.79 Mya (Fig. 3), which matches with Xia *et al*.'s (2022) estimate of 27.78 Mya. This late Oligocene date coincides with events when high-latitude northern vegetation with deciduous/conifer forest underwent a major shift to vegetation more similar to extant boreal forest and taiga (Tiffney and Manchester, 2001). Further diversification of *Ledum* lineages began in the Middle Miocene (11.18–7.65 Mya).

The results of S-DIVA suggest that the ancestor of *Ledum* species inhabited either North America or Eurasia (Fig. 4). The oldest lineage is the North American lineage, consisting of haplotypes H32 and H36. Haplotype H32 corresponds to *R. groenlandicum* from Douglas Island, southern Alaska, and haplotype H36 consists of three *R. columbianum* sequences from GenBank. However, there are far fewer species of the genus *Rhododendron* in North America, and we therefore assume that the ancestral range was probably in Eurasia. The presumed common ancestor may have arisen in Beringia as part of future boreal communities. In the Miocene, about 11.18 Mya, the North American lineage may have migrated from Eurasia across the Bering Land Bridge to North America.

Divergence within the other two major clades occurred during the Pliocene and the Pleistocene (5.97–0.64 Mya) and was associated with the emergence and persistence of cold-adapted biomes to the present day. One of the clades includes only Eurasian haplotypes of haplogroup II and the other includes both Eurasian and North American haplotypes from haplogroups I, III, IV and V (Figs 3 and 4). Expansion into Eurasia through dispersal and vicariance apparently occurred from the Eurasian Far East (ancestral area D) during the Pliocene and the Pleistocene. The divergence of haplogroup IV haplotypes specific to *R. tomentosum* and *R. subarcticum* between Eurasia (H24, H30) and North America (H19) reflects an older event than the dispersal of haplotypes H1 and H18 (haplogroup I) from Eurasia to North America. Haplotypes (H27 and H31, haplogroup V) specific for *R. groenlandicum* appear to have originated and diverged after dispersal from Eurasia to North America. These trans-Beringian migration events support the 'out of Asia' hypothesis (Donoghue and Smith, 2004) for *R. subarcticum* and *R. groenlandicum*. Thus, haplogroup II and ribotype R2 are restricted to Eurasia, but repeated exchanges across the Beringian land bridge may have been possible for haplogroups I, III and IV and ribotype R1. Bidirectional migrations in Beringia at different periods of the past have been reconstructed for a number of plant groups, such as ferns, conifers and flowering plants (Wen *et al*., 2016).

Specific features of Ledum from southern Alaska (Douglas Island)

Of all the obtained ptDNA haplotypes of the five *Ledum* species studied, haplotype H32 of *R. groenlandicum* from Douglas Island (southern Alaska) unexpectedly revealed several mutations that unite it with haplotype H36 (the haplotype is composed of sequences available in GenBank for different *R. columbianum* specimens from Washington and California) and other rhododendrons from section *Rhododendron* and other sections. It was recognized as sister to all other *Ledum* haplotypes, with an estimated divergence split of 11.8 Mya. Previously, Hart *et al.* (2017) showed strong differentiation between Eurasian and North American clades within subsect. *Ledum* according to ptDNA data. In their study, *R. columbianum* and *R. groenlandicum* from Washington and *R. neoglandulosum* from California formed a single clade with other *Rhododednron* species from North America, but not with *Ledum* species from Eurasia. In contrast, all *Ledum* species were monophyletic based on nuclear markers. The authors hypothesized that American *Ledum* species may have arisen as a result of the chloroplast capture of rhododendrons of other subgenera existing in sympatry with *Ledum*. Although we were unable to analyse specimens from Washington and California, we do have *R. subarcticum* from northern Alaska and *R. groenlandicum* from Alaska and Wisconsin. All haplotypes

(H19, H20, H27, H31) specific to North American populations (except H32) were found to be related to Eurasian haplotypes of haplogroup I and diversified about 5.97 Mya. *Rhododendron subarcticum* and *R. groenlandicum* from northern Alaska (S5) even contain Eurasian haplotypes H1 and H18. However, nrDNA of *R. groenlandicum* from Wisconsin has a specific ribotype R1a, whereas Eurasian ribotype R1 occurs in both northern and southern Alaska (including H32). Thus, the only haplotype, H32 (from the west coast of North America), has an origin consistent with Hart's study, while the remaining American samples have different origins.

Specimens of *R. groenladicum* from Douglas Island are morphologically similar to specimens of *R. groenladicum* from northern Alaska, and from Wisconsin (Fig. 2I). *Rhododendron columbianum* can be distinguished by its greater plant height, slightly larger leaf blade, and white or pale green abaxial surface with less dense pubescence on the adaxial surface (Judd and Kron, 2009a). Unfortunately, the absence of a dense sampling of *R. groenlandicum*, as well as samples of *R. columbianum*, does not allow us to draw unambiguous conclusions regarding the isolated position of the population of *R. groenlandicum* from Douglas Island. However, we can speculate that such specific ptDNA differences can be explained by hybridization with *R. columbianum*, the distribution area of which, according to Flora of North America data (Judd and Kron, 2009a), may cover southern Alaska.

CONCLUSIONS

Genetic evaluation of *Ledum* taxa revealed that the species complex consists of one Eurasian lineage, two American lineages and three mixed lineages that separated after the mid-Miocene climatic optimum and then rapidly diversified. Multiple cycles of differentiation and hybridization/polyploidization, which presumably occurred during the Pleistocene, led to evolutionary reticulation in the *Ledum* complex. A key feature of this genetic structure is the parallel coexistence of several genetic lineages in most populations. Common genetic variants are widespread without narrow geographical or species localization in Eurasia. In North America there is genetic difference between *R. subarcticum* and *R. groenlandicum*. While *R. groenlandicum* occurred genetically isolated, there clearly was gene flow between *R. subarcticum* populations from North America and Eurasia. All this suggests Labrador teas as an actively evolving group colonizing various ecosystems including evolutionarily young ones (e.g. bogs and subarctic deserts). In general, due to the low level of genetic differentiation we refrain from recognizing genetic lineages at any taxonomic level and rather suggest the involvement of additional independently evolved molecular markers, ploidy-level analyses and niche modelling.

SUPPLEMENTARY DATA

Supplementary data are available at *Annals of Botany* online and consist of the following.

Table S1: GenBank accessions for plastid data. Table S2: GenBank accessions for nuclear data. Table S3: Primers for sequenced regions. Table S4: Results of Tajima's *D* and Fu's Fs tests and mismatch analyses of five species of subsect. *Ledum*

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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