Canadian Journal of Forest Research

Article

Biogeographic history of green alder (*Alnus alnobetula* (Ehrh.) K. Koch s.l.) in Eurasia and North America: evidence from genetic and morphological analyses

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Abstract

The green alder (*Alnus alnobetula* s.l.) is a cold-resistant boreal-arctic shrub species with a complex intraspecific taxonomy and a wide distribution range covering northern Eurasia and North America. In this study, we assess the level and distribution of diversity in five subspecies (*A. alnobetula* subsp. *fruticosa, kamtschatica*, *mandschurica*, *maximowiczii*, and *sinuata*) throughout 34 populations within the native species range by using 7 characters of leaf morphology and 11 nuclear microsatellites (nSSR). The differentiation in both sets of characters that we found has proven to be inconsistent with our previously obtained chloroplast DNA (cpDNA) data. Only three groups were identified using nSSR vs. five cpDNA haplogroups. Both morphological and nSSR analyses support the recognition of *A. alnobetula* subsp. *fruticosa* from the western part of the Eurasian distribution range (northwestern Russia, the Urals, and Siberia) and *A. alnobetula* subsp. *maximowiczii* from the eastern part (most of Sakhalin Island, the Kuril Islands, and most of Kamchatka). Among other East Asian subspecies such as *A. alnobetula* subsp. *kamtschatica* and subsp. *mandschurica*, as well as in *A.* subsp. *sinuata* from North America, considerable genetic and morphological admixture has been recorded. The discordance between the patterns inferred from cpDNA and nSSR data reflects limited gene dispersal via seeds and extensive gene flow via pollen between major glacial refugia.

Key words: *Alnus alnobetula*, biogeography, nuclear microsatellites, population structure, Far East, Siberia

Introduction

While adapting to various environmental conditions, plant populations accumulate genetic differences that manifest as variations in morphological characteristics. The major driving forces of population differentiation processes are geological events and climatic fluctuations. The existing taxonomic concepts have attempted to classify different levels of divergence of population groups, distinguishing either morphological races without explicit taxonomic status or subspecies and individual species. Thus, the uncertainty in the taxonomy of plant species is usually the result of their complex phylogeographic history.

The green alder, *Alnus alnobetula* (Ehrh.) K. Koch s.l., is one of the species with a complex intraspecific taxonomy. This boreal-arctic shrub or tree of the genus *Alnus*, subgenus *Alnobetula*, family Betulaceae, occupies an extensive range in Eurasia and North America. Having significant ecological and morphological plasticity, this species plays an important role in the composition of the vegetation cover of boreo-montane areas.

The subgenus *Alnobetula* (also described as the genus *Duschekia* Opiz) is clearly distinguished in the genus by its morphological and genetic characters. It appears to be basal in the genus *Alnus* due to the archaism of morphological features; it has a position close to the root of the phylogenetic tree due to low ITS divergence from the genus *Betula* (Navarro et al. 2003). But the number of species in the taxon is still subject of debate. Currently, two assumptions are considered: there exists either one circumpolar species, *A. alnobetula* (green alder), divided into geographic subspecies or several allopatric or parapatric species (Greuter and Raab-Straube 2011; Vanden Heuvel 2011). On the other hand, based on the results of molecular phylogeny (using cpDNA and ITS), all European, Asian, and North American species of this subgenus have been combined into a single complex, *Alnus viridis* s.l., which may indicate their recent divergence (Chen and Li 2004; Ren et al. 2010). The high morphological variability of green alder in Northeast Asia, a region considered the center of origin and diversification of the genus *Alnus*, has resulted in the descriptions of a number of geographic races or subspecies within the subgenus *Alnobetula* (Chen and Li 2004). The study of genetic relationships between them is complicated by introgressive hybridization (Banaev and Adelshin 2009).

Recently, we have studied the variability of chloroplast DNA (cpDNA) markers in *A. alnobetula* s.l. throughout its extensive modern range in Eurasia, with a reference population of *A. alnobetula* subsp. *sinuata* from North America (Alaska) also included (Hantemirova et al. 2018; Hantemirova and Marchuk 2021). A total of five haplogroups have been identified. The haplogroup corresponding to the subspecies *A. alnobetula* subsp. *sinuata* is significantly divergent from the others. The remaining four haplogroups are structured geographically and correspond to the subspecies *A. alnobetula* subsp. *fruticosa*, *A. alnobetula* subsp. *kamtschatica*, *A. alnobetula* subsp. *mandschurica*, and *A. alnobetula* subsp. *maximowiczii* (Greuter and Raab-Straube 2011; Chery 2015; Hantemirova et al. 2018).

The haplogroup of the subspecies *A. alnobetula* subsp. *fruticosa*, which is common in northern Eurasia from Arkhangelsk Oblast (Mezen River valley) to Yakutia (Verkhoyanskiy Range), contains one widely distributed cpDNA haplotype. Although the other three haplogroups corresponding to *A. alnobetula* subsp. *kamtschatica*, *A. alnobetula* subsp. *mandschurica*, and *A. alnobetula* subsp. *maximowiczii* have been recorded from the Russian Far East, the geographical boundaries of these subspecies with similar morphologies remain incompletely understood. The data on cpDNA variability indicated a mixing of gene flows and some contact zones between the subspecies. Obviously, the complex geological and glacial history of this part of the range associated with orogeny processes and sea level fluctuations is also reflected in the complex structure of the distribution of genetic variability within plant species (Milne and Abbott 2002).

For better understanding the most important genetic patterns in plant species, comparative studies using plant genomes with different types of inheritance are required. The haploid plastid genome in angiosperms is inherited down the maternal line and dispersed via seeds, while the nuclear genome has biparental inheritance and is dispersed by pollen over long distances, especially in wind-pollinated species (Reboud and Zeyl 1994; Mogensen 1996). The uniparental inheritance of cpDNA implies a twofold smaller effective population size compared with nuclear DNA, which means that the haploid plastid genome is more subject to random events such as genetic drift. Thus, the variability of cpDNA reflects geographic structure to a greater extent than that of nuclear DNA (Vendramin et al. 1999; Petit et al. 2005). However, there are cases where nuclear microsatellites reveal incongruent differentiation patterns compared with those inferred from chloroplast microsatellite markers, e.g., in *Fraxinus excelsior* L. in southeastern Europe, the genetic pattern identified at nuclear microsatellites was highly homogeneous compared with the cpDNA pattern (Heuertz et al. 2004). Cases of incongruence of genetic structures or phylogenetic trees obtained using different marker types may indicate hybridization and introgression in closely related species or subspecies (King and Ferris 2000; Du et al. 2011; Jia et al. 2012).

Nuclear microsatellite (nSSR) markers have been successfully applied in studies on some species of the genus *Alnus*, such as *A. incana* and *A. glutinosa* (Havrdová et al. 2015; Mandak et al. 2016; Mingeot et al. 2016). To the best of our knowledge, the genetic variation of *A. alnobetula* has not yet been considered using nSSR markers. For our study, we selected the primer pairs from these studies to analyze the variation of 11 nuclear microsatellite loci in populations of *A. alnobetula* throughout the range in northern Eurasia and North America. We conducted the study in the same populations where the variability of cpDNA markers had been analyzed before, with a few more populations involved. We also performed a morphological analysis of the leaf blade parameters on representatives of the four subspecies described from Eurasia.

Our study aimed to identify the genetic structure of the species throughout its range using data on nuclear DNA variability in combination with cpDNA variability and morphological data, interpreting the results from a phylogeographic viewpoint.

Materials and methods

Population sampling

The collected samples covered most of the range of *A. alnobetula* in Russia. In addition, one sample set was from northwestern China, and another one was from North America (Alaska). A total of 440 individuals were sampled from 34 natural populations (Table 1 and Fig. 1A). Each sample set contained 7–22 randomly selected plants. The plant sampling strategy was designed to avoid the collection of individuals closer than 15 m from one another, thus minimizing the collection of asexually (clonally) reproduced plants. The same sample sets were used as in the previous study (Hantemirova and Marchuk 2021). We also added sample sets from the Polar and Subpolar Urals, as well as from the Russian Far East (Sikhote-Alin Mountains) and from the westernmost part of the range in Archangelsk Oblast near the Mezen River. Collected leaves were stored in silica gel or dried in paper envelopes prior to DNA extraction. Since *A. alnobetula* does not form a continuous natural range and the sample sets taken were widely dispersed geographically, we assumed that each sample set represented a distinct population and used the term "population" later in the text when discussing genetic parameters.

DNA extraction and nSSR analysis

Genomic DNA was extracted from the silica-gel-dried or air-dried leaves using the CTAB method, according to Devey et al. (1996). The quality and quantity of DNA were measured using a spectrophotometer.

Since nSSR markers for *A. alnobetula* are not developed, we used the literature data for other species of *Betulaceae* (Wu et al. 2002; Kulju et al. 2004; Lance et al. 2009; Tsuda et al. 2009; Gürcan and Mehlenbacher 2010; Gürcan et al. 2010) tested by cross-amplification on *A. glutinosa* and *A. incana* (Drašnarová et al. 2014). To study *A. alnobetula* populations, a set of 19 microsatellite loci were chosen and tested for those previously reported (Drašnarová et al. 2014): A4, A5, A6, A7, A8, A9, A10, A11, A12, A13, A15, A22, A24, A25, A26, A32, A33, A35, and A38. Locus L13.1 was also used (Kulju et al. 2004).

Markers were initially tested in simplex reactions on four green alder DNA samples to check the green alder DNA amplification. PCRs were carried out in a final volume of

Table 1. Sampling locations and summary of genetic diversity statistics for 34 populations of *Alnus alnobetula* from nature.

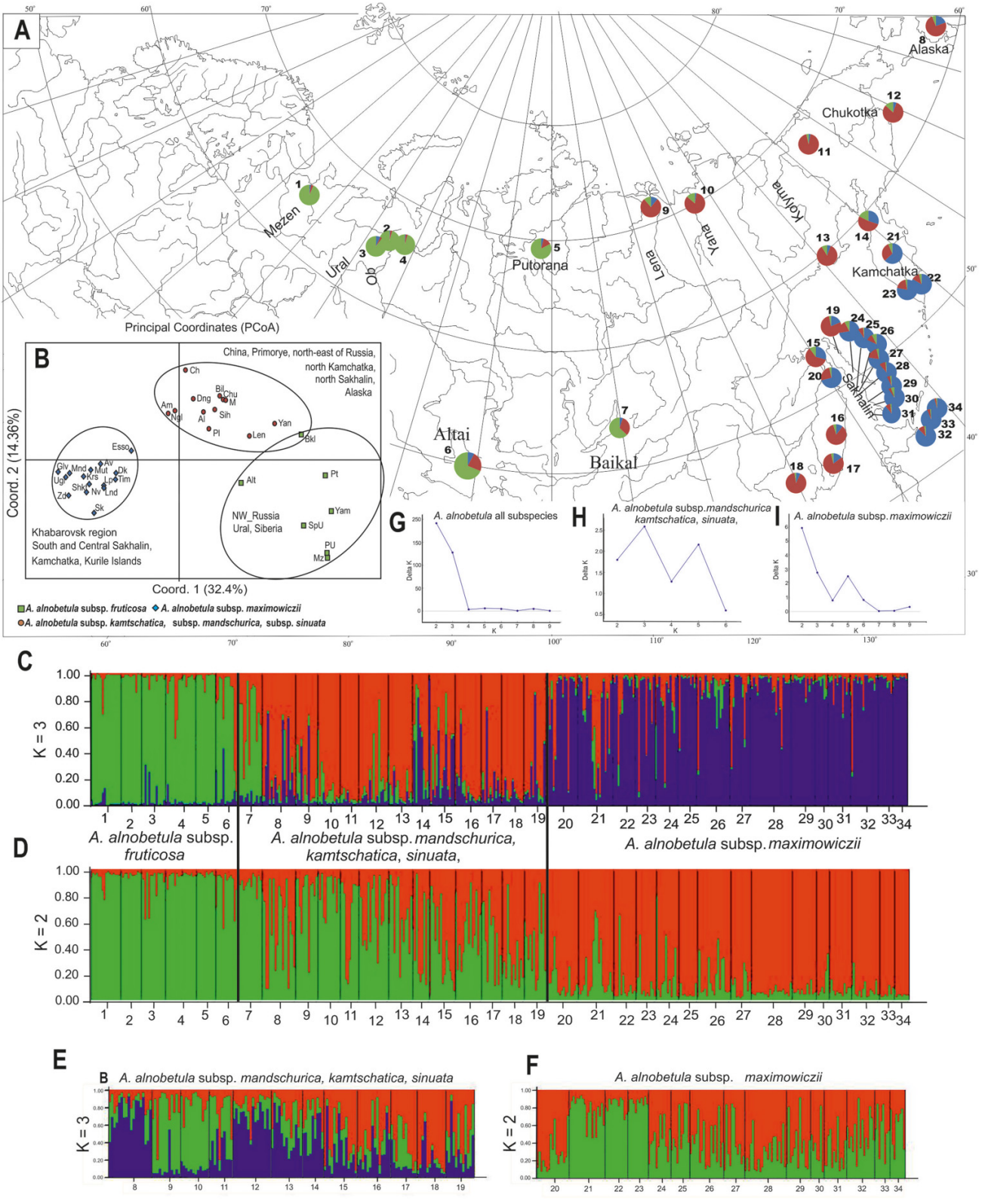
P	Locality	Name	Latitude/longitude	Alt., m	*N*	N_A	*Ho*	*He*	*F*
			A. alnobetula subsp. *fruticosa*						
1	Mezen river, north-west of Russia	Mz	N 65°40′/E 44°47′	16	16	4.73	0.617	0.586	−0.05
2	Polar Ural	PU	N 66°51′/E 65°20′	561	10	4.46	0.545	0.570	0.07
3	Subpolar Ural, Mt. Narodnaya	SPU	N 65°02′/E 60°07′	1853	13	4.27	0.483	0.559	0.09
4	Yamal peninsula, West Siberia	Yam	N 66°39′/E 66°24′	27	18	4.82	0.550	0.560	−0.003
5	Putorana Plateau, Middle Siberia	Pt	N 69°24′/E 90°46′	667	9	4.36	0.525	0.530	0.08
6	Altai Mountains	Alt	N 51°46′/E 87°17′	505	12	4.73	0.538	0.566	0.059
7	Lake Baikal	Bkl	N 52°59′/E 108°17′	486	13	5.73	0.580	0.660	0.143
			North America, *A. alnobetula* subsp. *sinuata*						
8	Alaska, Fairbanks	Al	N 64°56′/W147°59′	612	18	5.73	0.520	0.603	0.110
			A. alnobetula subsp. *kamtschatica, mandschurica*						
9	Lena River, north-east Siberia	Len	N 72°22′/E 126°28′	13	11	5.73	0.485	0.617	0.209
10	Yana River, north-east Siberia	Yan	N 71°27′/E 136°10′	15	12	5.09	0.553	0.572	0.012
11	Bilibino, Chukotka	Bil	N 68°04′/E 166°27′	342	10	4.73	0.503	0.577	0.097
12	Arinai Lagoon, Chukotka	Chu	N 62°32′/E 179°07′	12	16	5.91	0.556	0.585	0.025
13	Magadan region	M	N 59°35′/E 150°04′	118	13	6.09	0.580	0.588	0.082
14	Palana, north of the Kamchatka Peninsula	Pl	N 59°04′/E 159°50′	6	9	4.91	0.545	0.595	0.084
15	Nikolaevsk on Amur, Khabarovsk Region	Amu	N 53°05′/E 140°45′	48	14	6.91	0.640	0.664	0.055
16	North Sihote-Alin, Primorsky Krai	Sih	N 45°06′/E 136°13′	671	13	7.18	0.591	0.657	0.129
17	Dalnegorsk, Primorsky Krai	Dlg	N 44°31′/E 135°21′	1100	11	5.46	0.471	0.544	0.098
18	Mt. Changbai, China	Ch	N 41°44′/E 127°°57′	1690	12	5.91	0.621	0.639	0.054
19	Nogliki, north of Sakhalin	Ngl	N 51°49′/E 143°09′	29	16	4.82	0.538	0.573	0.055
			A. alnobetula subsp. *maximowiczii*						
20	De Kastri, Khabarovsk Region	Dk	N 51°28′/E 140°46′	44	17	6.73	0.640	0.672	0.118
21	Esso, Kamchatka pen.	Esso	N 55°55′/E 158°46′	1300	16	4.82	0.434	0.555	0.152
22	Mutnovsky volcano, Kamchatka pen.	Mut	N 52°20′/E 158°13′	131	11	5.27	0.541	0.554	0.033
23	Avachinsky volcano, Kamchatka pen.	Av	N 53°16′/E 158°44′	850	10	4.64	0.536	0.560	−0.002
24	Timovskoye, Sakhalin Is.	Tim	N 51°06′/E 143°00′	346	12	6.18	0.629	0.631	0.009
25	Uglegorsk, Sakhalin Is.	Ugl	N 49°03′/E 142°02′	34	10	4.10	0.476	0.516	0.125
26	Leonidovo, Sakhalin Is.	Lnd	N 48°38′/E 142°46′	36	18	7.36	0.626	0.675	0.071
27	Lopatino, Sakhalin Is.	Lp	N 48°23′/E 142°15′	213	11	5.82	0.579	0.596	0.013
28	Mt. Krasnova, Sakhalin Is.	Krs	N 42°44′/E 142°06′	519	22	7.46	0.553	0.616	0.096
29	Mt. Zhdanko, Sakhalin Is.	Zd	N 48°06′/E 142°30′	300	13	6.27	0.587	0.609	0.014
30	Sokol, Sakhalin Is.	Sk	N 47°4′/E 142°45′	17	7	4.82	0.468	0.585	0.191
31	Nevelsk, Sakhalin Is.	Nv	N 46°40′/E 141°51′	6	12	5.91	0.520	0.606	0.111
32	Shikotan, Kuril Is.	Shkt	N 43°52′/E 146°50′	37	15	5.91	0.580	0.579	−0.017
33	Kunashir, Kuril Is., Mendeleeva Volcano	Mnd	N 43°58′/E 145°43′	750	8	5.10	0.558	0.558	−0.028
34	Kunashir, Kuril Is., Golovnina Volcano	Glv	N 43°51′/E 145°30′	102	8	4.82	0.468	0.573	0.142

Note: *N*, total number of samples; N_a, mean number of allele per locus; H_o, observed heterozygosity; H_e, expected heterozygosity; *F*, Wright's fixation index.

10 μL using 1× Qiagen Multiplex PCR Master Mix (Qiagen, Germany), 0.3 μm forward and reverse primers, and 1 μL template DNA (20–25 ng/μL). The following conditions are used for PCR: 15 min of denaturation at 95 °C, followed by 35 cycles at 94 °C for 30 s, with two different annealing temperatures (57 and 60 °C) for 45 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. Amplification products were tested on a 6% denaturing polyacrylamide gel with subsequent silver staining. Thirteen nSSR loci (A4, A7, A8, A9, A10, A11, A15, A22, A26, A33, A35, A38, and L13.1) with high polymorphism and good reproducibility were selected. Markers that yielded clear bands were labeled with one of the fluorescent dyes FAM, HEX, or ROX (Syntol, Russia) and tested again by PCR on 16 samples from different geographical regions under the same conditions as before. The PCR product was diluted 10 times; 1 μL diluted PCR product was mixed with 9 μL HiDi formamide and 0.18 μL of 450 LIZ size standard (Gordiz, Russia) and electrophoresed using a NANOFOR 05 capillary sequencer (Syntol, Russia). Allele sizes were determined using GeneMapper v. 4.0. (Applied Biosystems, USA), first automatically and then manually.

We finally selected 11 nSSR that provided repeatable, unambiguous bands of expected sizes with sufficient polymorphisms from the set of 13 nSSR (data available). Loci A7 and

Fig. 1. Outlines of the study region (adapted from Bobrov 1978) with localities of the sampling sites of *Alnus alnobetula* s.l., distribution of genotypes, and graphs of estimates based on SSR polymorphisms. Population numbers and geographical coordinates are explained in Table 1. (A) Sampling sites and distribution of polymorphic frequencies in surveyed populations using STRUCTURE ($K = 3$). (B) Principal coordinates analysis (PCoA) of the 34 populations of *A. alnobetula*. The colors correspond to the STRUCTURE genetic groups ($K = 3$). Assignment of individuals to ancestral groups: (C, D) for 440 individuals of *A. alnobetula* s.l. ($K = 3$ and $K = 2$, respectively); (E) for group of subspecies of *A. alnobetula* subsp. *mandschurica*, *kamtschatica* and *sinuate* ($K = 3$); and (F) for *A. alnobetula* subsp. *maximowiczii* (K = 2). (G, H, I) Plots for detecting the number of K groups that best fit the data.



A8 were excluded. The selected loci were amplified in two multiplex PCRs, each in a total volume of 10 μL, with the following protocol: an initial denaturation cycle of 95 °C for 10 min, followed by 35 cycles of 90 °C for 30 s, 57 °C or 60 °C for 30 s, and 72 °C for 30 s, and then a final elongation cycle of 72 °C for 10 min.

Data analysis

The diversity statistics for each population included the mean number of alleles per locus (N_A), observed heterozygosity (H_o), and expected heterozygosity (H_e). Moreover, Wright's fixation index (F), which reflects inbreeding, was also determined. All calculations were performed in the GenAlEx 6.5 software (Peakall and Smouse 2012). Tests for Hardy–Weinberg equilibrium (HWE) were performed in the GENEPOP v.4.2 software (Raymond and Rousset1995). The frequency of null alleles was estimated using the Brookfield method (1996), as implemented in the Micro-Checker 2.2.3 software (van Oosterhout et al. 2004).

Several approaches were used to study the population structure. First, a principal coordinate analysis was performed using GenAlEx 6.5 (Peakall and Smouse 2012) based on the matrix of individual pairwise genetic distances (DA) (Nei et al. 1983). Furthermore, a Bayesian clustering approach was carried out in the STRUCTURE 2.3.4 software (Pritchard et al. 2000). The number of potential clusters (K) was assessed with 20 replications for each K value ranging from 2 to 10, with a burn-in length of 10,000 and 100,000 iterations of each chain using the admixture model along with the assumption of correlated allele frequencies between groups (Falush et al. 2003). The most likely number of clusters was estimated on the basis of the posterior probability of data for a given K [LN P(D)] or the ad hoc statistic Delta K described in Evanno et al. (2005) and was conducted using STRUCTURE Harvester (Earl and von Holdt 2012).

A hierarchical analysis of molecular variance was performed to measure the molecular divergence between populations and groups of populations using the Arlequin v3.5 software package (Excoffier and Lischer 2010). Genetic differentiation among populations was assessed by R_{ST} (Slatkin 1995) based on the stepwise mutation model. According to this model, alleles similar in size are less different in terms of mutational steps than alleles showing a greater difference in size (Jarne and Lagoda 1996). The R_{ST} value was obtained to measure the molecular divergence between populations and groups of populations. The correlation between genetic and geographic distances for pairs of samples was analyzed using the Mantel test (1967).

Morphological analysis

To study the variability of morphological characters of *A. alnobetula* s.l. and further comparative and morphological analysis of the subspecies, we used samples of green alder collected during the expeditions in 2018–2021. For measurements, we selected foliated shoots of adult generative individuals, collected from the middle part of the crown in the period of full leaf blade formation. We also analyzed specimens of green alder from the electronic herbarium (VBGI; e-Herbarium 2015–2022) of the BGI FEB RAS (http://botsad.ru/herbarium/). In total, we processed 169 shoots of four subspecies and made measurements of 645 leaves: for *A. alnobetula* subsp. *fruticosa*, 23 shoots and 122 leaves; for *A. alnobetula* subsp. *mandschurica*, 39 shoots and 132 leaves; and for *A. alnobetula* subsp. *kamtschatica*, 30 shoots and 135 leaves; for *A. alnobetula* subsp. *maximowiczii*, 77 shoots and 256 leaves. Subspecies affiliation was identified based on the descriptions of their geographical distributions available in literature: in Sverdlovsk Oblast, *A. alnobetula* subsp. *fruticosa*; in Primorsky Krai and Khabarovsk Krai, *A. alnobetula* subsp. *mandschurica*; in Kamchatka Krai, *A. alnobetula* subsp. *kamtschatica*; and in Sakhalin Oblast, *A. alnobetula* subsp. *maximowiczii*.

The samples were scanned on a Microtek ObjectScan 1600 flatbed scanner at the herbarium of the BGI FEB RAS (VBGI) with a resolution of 300 dpi at a scale of 1:1. Leaf characters were measured in the GIMP 2.10 (2022) graphics editor (https://www.gimp.org/).

For the comparative morphological analysis, we used five parameters of the leaf blade of green alder as characters that had previously shown differences between the species or subspecies of this complex taxon (Nedoluzhko and Skvortsov 1996; Banaev and Adelshin 2009). Two additional leaf indices were also calculated: leaf blade length to width ratio and petiole length ratio. The following parameters were used in the analysis: LL, leaf length (cm), measured from base to leaf apex; LW, leaf width (cm), measured at the widest section of leaf blade; PL, petiole length (cm), measured from base to apex of petiole; BA, leaf base angle (degree), measured from petiole base to two sides of leaf blade base; AA, leaf apex angle (degree), measured from top of leaf blade to the two sides of leaf blade; LL/LW, ratio of leaf blade length to its width; and LL/PL, ratio of leaf blade length to petiole length.

Statistical processing of morphological data and visualization of the results were performed in the R version 4.2.1 software (R Core Team 2022: https://www.R-project.org/) using the "tidyverse" (Wickham et al. 2019), "ggpubr", and "rstatix" packages. Differences between subspecies were tested by the Kruskal–Wallis test for non-parametric data. A post hoc test was performed using the Wilcoxon test with the Benjamini–Hochberg correction (Benjamini and Hochberg 1995). Principal component analyses (PCAs) were done on all five morphological characters of the leaf blade and two ratios, and also for a reduced dataset except for two characters (the base angle and the angle of the leaf blade top), which are the least significant for the subspecies differentiation.

Results

Molecular analysis

Genetic diversity and genetic structure

Alnus alnobetula s.l. shows remarkably high levels of diversity. An analysis of the variation of 11 microsatellite loci in 34 populations (440 samples; Table 1) revealed 152 allelic variants, of which 17 were unique. In the 374 HWE tests across all loci in all populations (11 loci × 34 populations), only five loci (A4, A11, A22, A26, and A33) showed a significant

deviation ($p < 0.01$), but these loci differed between populations. No one locus exhibits systematic deviations in the tests; therefore, all loci were retained in subsequent analyses. No null alleles were detected. The inbreeding coefficient values were small, mostly positive. All loci were highly polymorphic; the number of identified alleles per locus in all populations ranged from 6 (loci A10 and A15) to 25 (locus A33). The average number of alleles per locus ranged from 4.1 to 7.5 in different populations, and the heterozygosity (H_e) ranged from 0.530 to 0.675. The highest values of intrapopulation genetic diversity were observed in populations from Sikhote-Alin (Sih), Khabarovsk krai (Amu and Dk), and the central part of Sakhalin Island (Lnd, Krs, and Zd), located in the distribution area of the subspecies complex *A. alnobetula* subsp. *mandschurica*, *A. alnobetula* subsp. *kamtschatica*, and *A. alnobetula* subsp. *maximowiczii*. Lower values of intrapopulation genetic diversity were observed in all populations of the subspecies *A. alnobetula* subsp. *fruticosa*.

The genetic structure of *A. alnobetula* s.l. was determined using the Principal Coordinate Analysis based on genetic distances between populations (Fig. 1B). Differentiation was found between three groups of populations. The group of populations that belonged mostly to *A. alnobetula* subsp. *maximowiczii* was separated by the first principal coordinate (32.48% of total variability). These are populations from Sakhalin Island (central and southern parts, Ugl, Zd, Sk, Nv, Lnd, Lp, Krs, and Tim), the Kuril Islands (Gvl, Mnd, and Shkt), the Kamchatka Peninsula (Mut, Av, and Esso), except the northernmost part, and the mainland population from the coast of the Tatar Strait (Dk). According to the second main coordinate (14.29% of the total variability), a separate group was formed by the populations of the western part of the range from the Mezen River to the Lena River (Mz, PU, SPU, Yam, Pt, Alt, and Bkl), corresponding to the subspecies *A. alnobetula* subsp. *fruticosa*. The third group was mixed. It combined populations of the subspecies *A. alnobetula* subsp. *kamtschatica* and subsp. *mandschurica*. Geographically, these are populations of northeastern Russia, including the Kolyma region, Chukotka, and northern Kamchatka (Len, Yan, Bil, Chu, M, and Pl), as well as the populations from Khabarovsk Krai and Primorsky Krai (Amu, Sih, and Dlg), northern Sakhalin (Ngl), and China (Ch). The North American species *A. alnobetula* subsp. *sinuata* from Alaska (Al) also occurred in the same group.

The Bayesian clustering method in STRUCTURE software was applied to the SSR data to infer the genetic structure. The number of clusters that best explained the population genetic structuring based on the ΔK approach was two (Delta $K = 190$) and three (Delta $K = 128$) (Figs. 1C, 1D, and 1G). When two clusters are distinguished, populations of *A. alnobetula* subsp. *kamtschatica* from the North-East of Russia (Len, Yan, Chu, and M) and *A. alnobetula* subsp. *sinuata* from Alaska (Al) are combined with populations of *A. alnobetula* subsp. *fruticosa* into one cluster; populations of *A. alnobetula* subsp. *maximowiczii* (Ugl, Zd, Sk, Nv, Lnd, Lp, Krs, Tim, Gvl, Mnd, and Shkt) form another cluster; populations of *A. alnobetula* subsp. *mandschurica* (Amu, Sih, Dlg, Ngl, and Ch) are equally probable in the first and second clusters. Assignment to three clusters proved to be consistent with the results of principal coordinate analysis grouping (Figs. 1A, 1B, and 1C). Two clusters clearly separated two subspecies: *A. alnobetula* subsp. *maximowiczii* and subsp. *fruticosa*; the third cluster included the samples of *A. alnobetula* subsp. *kamtschatica*, subsp. *mandschurica* and subsp. *sinuata* with mixed genetic origins.

The differentiation among all populations based on R_{ST} amounted to 18%; approximately 82% of the diversity resided within populations. The differentiation among the two STRUCTURE groups was 8%, while the proportion of genetic diversity among the three clusters of populations (estimated by the PCoA and STRUCTURE algorithms) R_{CT} was 11.4%. The genetic and geographic distances were significantly correlated ($Rxy = 0.729$, $p = 0.01$).

Further analyses of differentiation using STRUCTURE within each of the three clusters revealed a finer genetic structure. The populations of *A. alnobetula* subsp. *fruticosa* showed a tendency to be structured with $K = 3$. However, the low mean of Delta $K = 0.9$ and unsignificant differentiation between these groups ($R_{CT} = 1.2\%$, $p = 0.282$) indicated homogeneity within the subspecies (figures are not shown). The populations of *A. alnobetula* subsp. *kamtschatica*, subsp. *mandschurica*, and subsp. *sinuta* are divided into three groups (Delta $K = 2.6$): the first included populations from the mainland Far East and northern Sakhalin (Sih, Dlg, Am, Ngl, and Ch); the second included populations from the northeastern part of Russia (Bil, Chu, M, Pl, and Al); and the third included populations from the Lena and Yana rivers (Len and Yan) (Figs. 1E and 1H). The differentiation between the groups was significant ($R_{CT} = 3.4\%$; $p = 0.014$). The populations of *A. alnobetula* subsp. *maximowiczii* formed two groups (Delta K = 5.9): southern and central Kamchatka (Mut, Av, and Esso) were separated from other populations from Sakhalin, the Kuril Islands, and Khabarovsk Krai (Ugl, Tim, Lnd, Lp, Krs, Zhd, Sk, Nv, Shkt, Mnd, Glv, and Dk) (Figs. 1F and 1I). The differentiation between the groups also has significant support ($R_{CT} = 4.3\%$, $p < 0.001$).

Morphological analysis

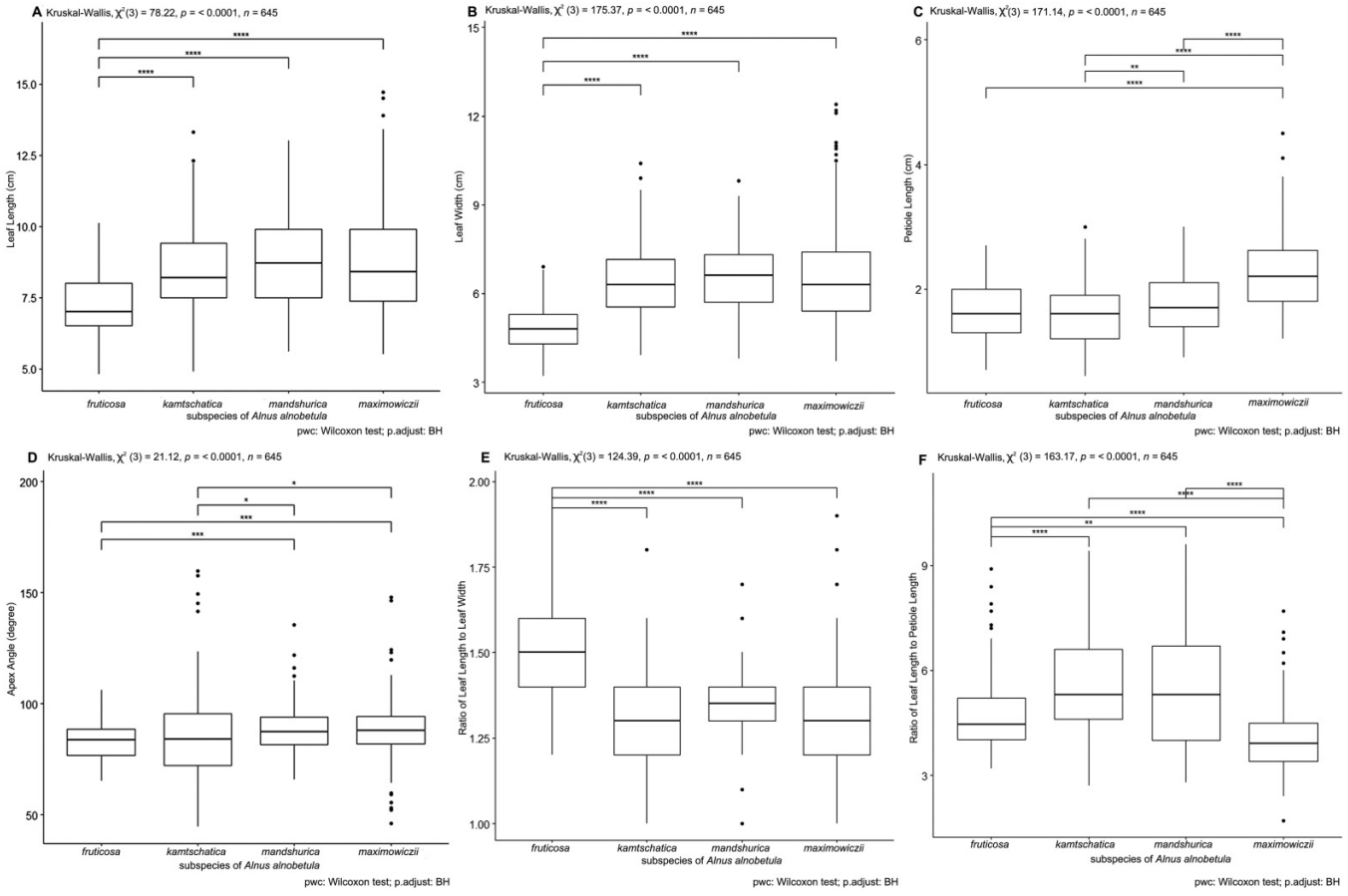
Figure 3 shows the boxplots of the values of the six leaf blade parameters characterized; the mean and standard deviation values are provided in Table 2. The boxplot for the parameter "leaf base angle" (BA) is not provided because this character proved to be less significant ($\chi^2 = 11.58$, $p < 0.01$) compared with other analyzed traits, in which the subspecies differ significantly at $p < 0.0001$. A morphological analysis of the green alder subspecies showed significant differences for *A. alnobetula* subsp. *fruticosa* in most of the characters analyzed, with the exception of "leaf apex angle" (AA) (Fig. 2). This subspecies is characterized by the smallest leaves: the mean length of the leaf blade was 7.28 cm and the mean width was 4.78 cm, while in the other subspecies the mean length was more than 8 cm and the mean width was more than 6 cm. The most useful character for distinguishing *A. alnobetula* subsp. *maximowiczii* was the petiole length (Fig. 2C). In this subspecies, it could reach 4.5 cm, with a mean of 2.28 cm, while in the other subspecies it did not exceed 3 cm, with a mean no greater than 1.78 cm. The most useful character for differentiation between the subspecies *A. alnobetula* subsp. *fruticosa* and *A. alnobetula* subsp. *maximowiczii* was such an

Table 2. Mean and standard deviation (s.d.) values for leaf characters.

Subspecies	*. fruticosa*			*. kamtschatica*			*. mandshurica*			*. maximowiczii*		
LL	7.28	1.11	a	8.49	1.53	b	8.78	1.52	b	8.75	1.84	b
LW	4.78	0.74	a	6.46	1.28	b	6.58	1.18	b	6.60	1.65	b
PL	1.62	0.45	a	1.58	0.45	a	1.73	0.42	a	2.28	0.62	b
AA	83.70	9.00	a	86.20	20.80	a	88.60	10.50	a	88.2	12.30	a
BA	135.0	33.2	a	33.60	148.00	a	92.00	123.00	a	80.10	129.00	a
LL/LW	1.53	0.15	a	1.32	0.13	b	1.35	0.12	b	1.35	0.16	b
LL/PL	4.74	1.12	a	5.70	1.49	b	5.41	1.62	b	3.98	0.86	c

Note: a, b, and c denote significantly different groups according to the results of paired comparisons by using Wilcoxon test with the Benjamini–Hochberg correction ($p < 0.0001$).

Fig. 2. Variation of six morphometric characters of four subspecies of *Alnus alnobetula* s.l. Significantly different groups according to the results of pairwise comparisons at $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, and $^{****}p < 0.0001$. Boxplots show medians, interquartile distances (IQR, 25%–75%), minimum, and maximum; the circles correspond to outliers (values exceeding 1.5 × IQR).

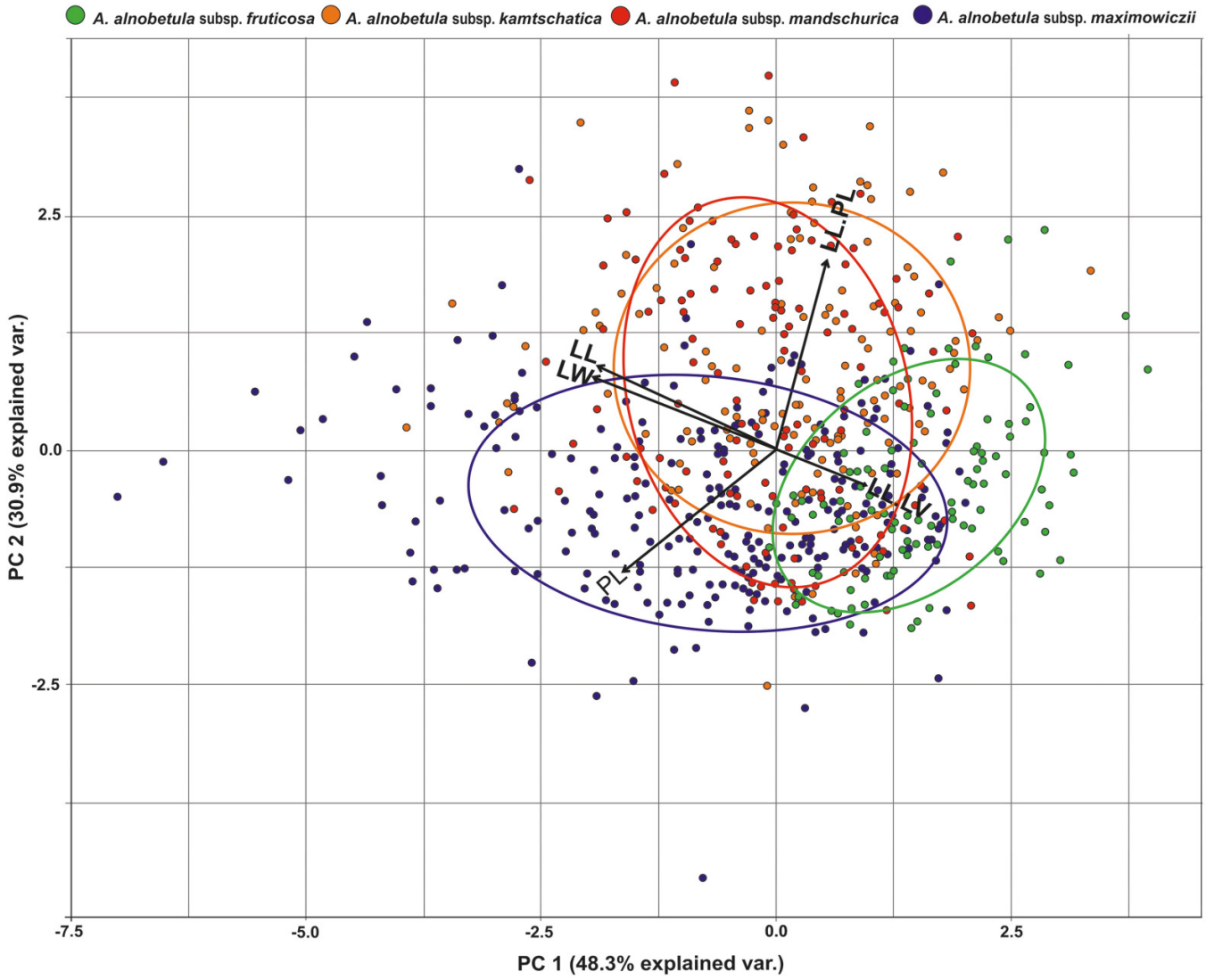


index as the leaf length to petiole length ratio (Fig. 2F). On the basis of this parameter, these two subspecies differed significantly both from each other and from the other subspecies. The subspecies *A. alnobetula* subsp. *mandschurica* and *A. alnobetula* subsp. *kamtschatica* showed no significant differences in all characters, and they had overlapping character values. The PCA did not reveal any clear differences between the subspecies in all characters. As shown in Fig. 3, when the least significant parameters "leaf base angle" (BA) and "leaf apex angle" (AA) are cut off, *A. alnobetula* subsp. *fruticosa* and *A. alnobetula* subsp. *maximowiczii* tend to separate from one another.

Discussion

In recent years, a number of studies have been conducted on the genetic diversity of cold-resistant species with wide ranges in northern Eurasia (Polezhaeva et al. 2010; Hantemirova et al. 2017; Polezhaeva et al. 2018; Knyazeva and Hantemirova 2020). A common feature of such studies is a large sample of populations covering almost the entire natural range of species. As a rule, those were species, including a complex of closely related species or subspecies with similar morphologies distinguished. For example, a study of the

Fig. 3. Principal component analysis of morphometric data for four subspecies of *Alnus alnobetula* based on the dataset including only significant characters for intraspecific differentiation.



genetic variability of *Larix* species from Siberia and the Far East of Russia has revealed certain patterns of variation in chloroplast and mitochondrial DNA markers that generally support the species described on the basis of morphology (Polezhaeva et al. 2010). Also, within *Rhododendron dauricum* s.l., a mountain shrub species distributed from the Altai Mountains to the Sikhote-Alin Mountains and China, several genetic groups were identified on the basis of cpDNA variation that geographically corresponded to the four closely related species described on the basis of morphology (Polezhaeva et al. 2018). However, additional analysis of nuclear DNA markers and morphological characters often results in cases of mismatch of genetic patterns obtained by different types of markers. Such a case was reported for *Juniperus communis* L., an evergreen coniferous species with a wide ecological niche. This species grows both in mountain tundra and in the undergrowth of boreal forests and is represented by various life forms, from creeping shrubs to erect trees. An analysis of the cpDNA of common juniper has revealed a genetic structure that well matches the geographic distribution of this species and, partially, the varieties described (Hantemirova et al. 2017). However, an isoenzyme analysis and further morpho-anatomical analysis have revealed a less distinct pattern: they have confirmed the isolation of only one variety in the Caucasus and no differences between the other varieties, including those with different life forms (Knyazeva and Hantemirova 2020). The ecological niche of *J. communis* is similar to that of *A. alnobetula*. The significant variations in the shape and size of alder leaves were first pointed out by Hultén (1927). He noted the continuity of variations in morphological characters within the range from west to east, from narrow-leaved forms with a wedge-shaped base (var. *fruticosa*) to broad-leaved ones with a heart-shaped base (var. *maximowiczii*) and emarginate edge (var. *sinuata*). According to Hultén, only var. *fruticosa* (Hultén 1927) grows in Eurasia (from Arkhangelsk to the Chukchi Peninsula and Korea Peninsula) and in North America (Alaska, east to Yukon and south to Washington State). Based on the results of a study of the morphological characters of leaves and flowering

earrings, Banaev and Adelshin (2009) also recommends considering the green alder growing in Asian Russia as a single highly polymorphic species, *A. alnobetula* subsp. *fruticosa* (this distinguishing it from subsp. *viridis* from Europe). According to our analysis of morphological data, there is no clearly pronounced clinal eastward and southeastward variation in the leaf shape of green alder in Asian Russia. However, the western subspecies of *A. alnobetula* subsp. *fruticosa* and one of the eastern subspecies, *A. alnobetula* subsp. *maximowiczii*, show a tendency toward differentiation. Although we cannot draw a clear conclusion as to whether these features reflect the modification variability and adaptive properties of the species or taxonomic differences, our data are consistent with the opinions of other specialists who recognize the presence of green alder, which differs from subsp. *fruticosa*, in the east. Thus, Maksimovich's alder is reported for Sakhalin Island, the Kuril Islands (eastern Russia), and Hokkaido Island (Japan) (Nedoluzhko and Skvortsov 1996; Barkalov and Taran 2004; Barkalov 2009; Takahashi 2015). In a humid monsoon climate, green alder forms the largest leaf blades, as was demonstrated in our analysis. This feature is a good diagnostic character for an independent taxon, which we refer to as *A. alnobetula* subsp. *maximowiczii*.

Genetic structure of nSSR marker variation compared with that of cpDNA markers

The total genetic diversity found among the studied *A. alnobetula* s.l. populations (Table 1) is in the range characteristic of long-lived woody perennial, wind-outcrossing species (Hamrick and Godt 1990). The range of heterozygosity is comparable with average values of nSSR marker variability ($H_e = 0.61$) for species with seeds dispersed by wind and/or water (Nybom 2004). The level of differentiation of green alder inferred from nuclear SSR markers ($R_{ST} = 18\%$) was lower than that inferred from cpDNA markers (88%) (Hantemirova and Marchuk 2021), but much higher than that, e.g., observed for *Alnus glutinosa* ($F_{ST} = 1.4\%$) (Mingeot et al. 2016). On the basis of both nuclear and cpDNA markers, genetic diversity was greater within populations than between them, and the highest values of intrapopulation genetic diversity were observed in the populations from the Sikhote-Alin Mountains (Sih), Khabarovsk Krai (Am and Dk), and the central part of Sakhalin Island (Lnd, Zhd, Krs, and Sk). The high population differentiation of *A. alnobetula* s.l. on the basis of cpDNA markers is most likely due to its maternal inheritance and limited distribution with seeds. The rate of gene flow via dispersal of *Alnus* pollen is considerably higher than that via seed dispersal. For example, the relative rate of pollen flow was 23-fold higher than that of seed flow for *A. glutinosa*, as estimated by King and Ferris (1998), and even more (194-fold, according to Mingeot et al. 2016). Indeed, seed dispersal occurs mainly via flowing water rather than wind. Although there is a high potential for seed dispersal over long distances by rivers or spring floods, seed dispersal in this case is expected to be low. Thus, different rates of gene flow via seed and pollen dispersal should be an important factor in the high level of population differentiation found in chloroplast DNA compared with that in the nuclear genome, and the pattern that we observe is mostly a reflection of past and current gene flows.

The results for highly variable nSSR markers agree with the structure inferred from cpDNA markers only in part, and the distribution of genotype groups is not fully consistent with the taxonomic varieties described by *A. alnobetula* s.l. Only one haplotype was identified on the basis of cpDNA markers in all the populations of *A. alnobetula* subsp. *fruticosa* (the western part of the range) from the Mezen River to the Lena River. The variability indices for nSSR markers within subsp. *fruticosa* were, in contrast, slightly lower but still quite high. Thus, the populations of subsp. *fruticosa* formed a separate genetic group, as confirmed by both morphological and genetic (nrDNA and cpDNA) analyses.

A more complex pattern is observed in the Pacific part of northern Eurasia. According to a previous study (Hantemirova and Marchuk 2021), in this part of the range, three highly divergent cpDNA lines corresponded to geographic regions and three subspecies, *A. alnobetula* subsp. *mandschurica*, subsp. *maximowiczii*, and subsp. *kamtschatica*, but the nuclear markers were less structured. Both nSSR markers and morphological analysis provided strong support only for one subspecies in this part of the range, *A. alnobetula* subsp. *maximowiczii*, with its distribution covering southern and central Sakhalin, the adjacent mainland of Khabarovsk Krai, the Kuril Islands, and most of Kamchatka. It is worth noting that, as evidenced by cpDNA marker analysis, the populations from southern and central Kamchatka had one haplotype from the group of haplotypes distributed in the Northeast of Eurasia and belonging to subsp. *kamtschatica.* This unexpected contradiction between the chloroplast and nuclear microsatellite data could be explained by the event of introgressive hybridization between populations that colonized the Kamchatka Peninsula by migration routes from the north (from northeastern Eurasia) and south (from the south of the Russian Far East via the Kuril Islands) in the past. Such cases of incongruent patterns of genetic structure for different types of markers were previously observed in Kamchatka for *Picea* (Aizawa et al. 2007) and *Larix* (Polezhaeva et al. 2010) and could be explained by the history of species dispersal.

The subspecies *A. alnobetula* subsp. *kamtschatica* and subsp. *mandschurica* form a single group inferred from the nSSR and morphological analyses and include samples from the south of the Far East of Russia and China (Amu, Sih, Dlg, Ngl, and Ch) and northeast Eurasia (Len, Yan, Bil, Chu, M, and Pl). Unexpectedly, the population of *A. alnobetula* subsp. *sinuata* from Alaska (Al) is assigned to the same group, though previously (Hantemirova and Marchuk 2021) it had a unique cpDNA marker haplotype, highly divergent compared with the haplotypes of the other subspecies. Among these populations, a large number of common nSSR alleles were found, indicating a common genetic pool. Hybridization is quite common in *Alnus* and has been reported previously. For instance, the phenomenon of hybridization has been detected between two North American green alder subspecies, *A. alnobetula* subsp. *crispa* and *A. alnobetula* subsp. *sinuata* (Bousquet et al. 1990), using allozyme markers. In the specific case of *A. alnobetula* studied here, the admixture of the nuclear genomes of several subspecies without replacement of the chloroplast genome may indicate a wide zone of introgression between them.

The increased diversity in the Sikhote-Alin region and central Sakhalin, both in genetic (cpDNA and nSSR markers) and morphological characters, is evidence for mixing and hybridization processes. Eastern Sikhote-Alin is known for its increased biodiversity with a large number of endemics (Pimenova 2016), which is characteristic of the continent–ocean transition zone in general (Bruchmann and Hobohm 2014) and supports the hypothesis of the existence of refugia in this part of the Far East during cooling periods (Harrison et al. 2001).

Therefore, we can hypothesize that the high differentiation in cpDNA markers reflects past fragmentation and an abundance decrease over a long period for populations of the ancestral species. Then the populations, when spreading under favorable conditions over a limited area, were subject to a founder effect, which manifested as a more pronounced regional structure. The nuclear SSR markers, spreading far away with pollen and vice versa, reflect a structure formed by the free mixing of genetic flows over wider territories. Thus, we see that the subsp. *maximowiczii* group mainly includes populations from islands and peninsulas around the Sea of Okhotsk, where genetic exchange with the mainland is difficult. The subsp. *fruticosa* is well differentiated beyond the Sikhote-Alin and Verkhoyansk mountain ranges, which are the biogeographic barrier separating the more southerly and easterly located territories. However, when we consider the two clusters from STRUCTURE (Fig. 1D), subsp. *fruticosa* combined with the mixed group of subspecies *kamtschatica*, *mandschurica*, and *sinuata,* in turn, reflects the easy gene exchange of the northeast part of Russia, with other mainland parts of the Russian Far East, Siberia, and Ural, the same as with Alaska.

The incongruence of genetic groups that we found in *A. alnobetula* s.l. when comparing the topologies reconstructed using different types of markers, nuclear and plastid, the so-called phylogenetic conflict, was also reported for many other species, e.g., of the genera *Zelkova* (Naciri et al. 2019), *Rhodiola* (Zhang et al. 2014), and *Stewartia* (Lin et al. 2019). The authors attribute this complex genetic pattern to a combination of ancient diversification and speciation events with more recent hybridization during recent glacial/interglacial retreats and recolonizations. The eastern Pacific part of northern Eurasia and North America are parts of the Beringia, known as the refugium, where biota survived unfavorable climatic periods (Tarasov 2013). This territory represents a complex center of montane and boreal plant and animal diversification, including hybridization events due to historical range shifts. Hybridization has caused much of the current morphological variability, generating a wide range of morphological intermediate forms between the two subspecies, subsp. *mandschurica* and subsp. *kamtschatica*.

Acknowledgements

We are grateful to Bessonova V.A. for providing *Alnus* shoots from Ural and to Tsarenko N.A. for samples from Khabarovsk Krai. We also thank Agafonov L., Chernyagina O., Gurskaya M., Kizhevatov Ya., Kirdyanov A., Krivenko D., Kukarskii V., Nikolaev A., Sokolov V., and Fedorov V. for their help with material collections.

Article information

History dates

Received: 27 February 2023
Accepted: 20 June 2023
Version of record online: 15 August 2023

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Data availability

doi: 10.6084/m9.figshare.22890146; available online: https://figshare.com/s/fee26ddff5a23519b817.

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Supervision: MAP
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Visualization: MAP
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Competing interests

The authors declare there are no competing interests.

Funding information

The study was performed under the State assignment to the Institute of Plant and Animal Ecology, Ural Branch, the Russian Academy of Sciences (No. 122021000090-5) and to the Botanical Garden-Institute, Far Eastern Branch, Russian Academy of Sciences (No. 122040800085-4), and also supported by the Russian Foundation for Basic Research (project No. 18-04-00616 A).

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