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Phylogeography and genetic structure of disjunct *Salix arbutifolia* populations in Japan

Teruyoshi Nagamitsu · Takeshi Hoshikawa · Takayuki Kawahara · Vyacheslav Yu. Barkalov · Renat N. Sabirov

Abstract Disjunct geographic distributions of boreal plant species at the southern edges of their ranges are expected to result in low genetic diversity and high genetic differentiation in the disjunct populations. This prediction was tested in a riparian willow, *Salix arbutifolia,* distributed in the northeastern Eurasian continent and the Sakhalin, Hokkaido, and Honshu Islands, using chloroplast DNA haplotypes and nuclear microsatellite genotypes. Hokkaido and Honshu populations shared a chloroplast haplotype identical to a closely related species, *S. cardiophylla*. This haplotype was divergent from haplotypes in the Eurasian continent (Primorsky) and the Sakhalin Island. In the nuclear microsatellites, most Hokkaido populations were genetically closer to Primorsky populations than to Sakhalin populations in spite of the geographical vicinity between Sakhalin and Hokkaido. The unexpected genetic divergence between Sakhalin and Hokkaido implies a complicated history of migration and colonization. The most peripheral populations in Honshu had the lowest genetic diversity and were most differentiated from the others. Thus, low genetic diversity and high genetic differentiation at the range periphery suggest substantial effects of genetic drift on genetic structure in the disjunct populations of *Salix arbutifolia* at the southern edge of its range.

Keywords Chloroplast DNA · Disjunct distribution · Genetic drift · Nuclear microsatellites · Salicaceae

Introduction

Boreal plant species, which grow in subarctic (taiga) biomes in the northern hemisphere and in subalpine zones in temperate areas (Olson et al. 2001), often have disjunct geographic distributions at the southern edges of their ranges. These distributions are attributed to isolated habitats where these plants can survive at the low latitude in the present postglacial period (Birks 2008). Boreal plants have experienced environmental changes during the Quaternary

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climatic oscillations, which have influenced the formation of their disjunct distributions through colonization and extinction (Schönswetter 2003; Gaudeul 2006; Kropf et al. 2008). Boreal plants migrated southward and colonized available habitats during glacial periods, which they survived in or otherwise disappeared from during interglacial periods. In many cases, populations surviving at the southern edges have experienced bottleneck and isolation events (Hampe and Petit 2005), and thus they are expected to have both decreased genetic diversity and increased genetic differentiation due to genetic drift at selectively neutral loci in the edge populations (Lesica and Allendorf 1995; Vucetich and Waite 2003; Hamilton and Eckert 2007; Eckert et al. 2008; Meeus et al. 2012). The effects of disjunction on genetic structure occur at various spatial scales, and higher magnitudes of disjunction may have larger effects. These processes were likely to occur throughout the northern hemisphere, including Eastern Asia, where mountains on islands around the Eurasian continent harbor subalpine plants that are thought to have originated from such boreal plants (Hewitt 2004).

A suitable example of such boreal plant species with disjunct populations is a riparian willow, *Salix arbutifolia*. This species grows on braided gravel beds along rivers on alluvial fans at the foot of high, steep mountains (Shin and Nakamura 2005) and is distributed across the northeastern Eurasian continent, including Siberia east of the Lake Baikal, Primorsky, northeastern China, and northern Korea, and on the Sakhalin, Hokkaido, and Honshu Islands (Kuzeneva 1985) (Fig. 1). Access to the Hokkaido and Honshu Islands in the Japanese archipelago from the Eurasian continent was through three land bridges (the Kuril Islands, the Sakhalin Island, and the Korean peninsula; Fig. 1) available during the glacial periods (Fujii and Senni 2006). Boreal plants are found in alpine and subalpine habitats along the mountain ranges in the Japanese archipelago. In several alpine and subalpine plant species, populations on the mountains in central Honshu genetically differed from those in both Hokkaido and northern Honshu, which often belong to lineages on the Sakhalin and Kuril Islands (Fujii and Senni 2006; Ohsawa and Ide 2011). These phylogeographic patterns suggest colonization through the Sakhalin and Kuril Islands and subsequent isolation in central Honshu (Ikeda et al. 2009a, 2012). In *S. arbutifolia*, main populations are in Hokkaido (the Tokachi, Monbetsu, and Hidaka districts) (Kawabe and Saito 1991; Nagamitsu and Kawahara 2002; Nagamitsu et al. 2003), and disjunct populations are in central Honshu (Nagano Prefecture), isolated over 700 km from Hokkaido (Shin 2009). With regard to the phylogenetic history of the boreal plants, disjunct *S. arbutifolia* populations in central Honshu are expected to show lower genetic diversity and higher genetic differentiation than those in Hokkaido, which are
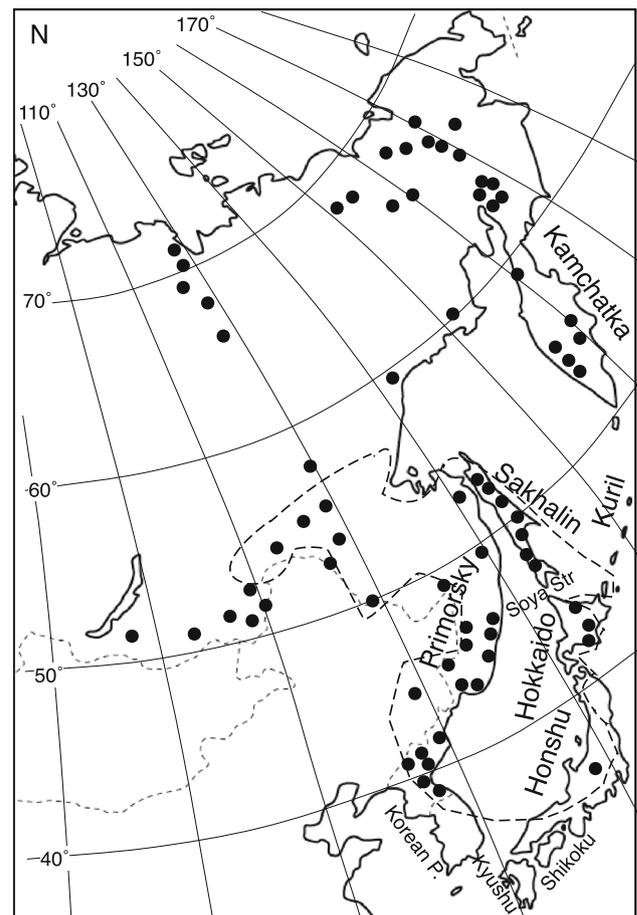

Fig. 1 Geographic distribution of *Salix arbutifolia* specimens (*circles*) drawn after Kuzeneva (1985). Distribution range of a closely related species, *S. cardiophylla*, (*thick broken lines*) is also shown after Skvortsov (1999)

closer to the Sakhalin and Kuril Islands (Fig. 1). However, these predictions have not been verified yet.

Genetic variation in the disjunct populations can be examined in nuclear and organellar genomes. Haplotypes of chloroplast (cp) DNA are useful for reconstructing colonization history because of their maternal inheritance and dispersal by seeds in most angiosperms. The relatively slow evolutionary rate in cpDNA is suitable for estimating the phylogenetic relationship among related species (Palmé et al. 2003). On the other hand, nuclear DNA inherited from both parents is available to assess the extent of gene flow between the disjunct populations. The relatively high variation in nuclear microsatellites is suitable for estimating recent events affecting genetic structure (Hoshikawa et al. 2012). We aimed to verify the low genetic diversity and high genetic differentiation expected in disjunct populations of *S. arbutifolia* in Japan and to elucidate the phylogeographic origin of these populations through the analyses of cpDNA haplotypes and nuclear microsatellite genotypes.

Table 1 Locations of sampling sites, information on voucher specimens, and the number of samples (Cp: chloroplast DNA sequences and nSSR: nuclear microsatellite genotypes) for *Salix arbutifolia*

Site	Region	District or prefecture	River	Latitude (N)	Longitude (E)	Altitude (m)	Voucher specimen	Number of samples	
								Cp	nSSR
K	Kamchatka		Bysmraja	55°56′	158°43′	460		2	2
S1	Sakhalin		Tym	50°39′	143°04′	300	T. Kawahara 200211310	6	32
S2	Sakhalin		Smirnykh	49°46′	142°50′	80	T. Kawahara 200211316	5	32
S3	Sakhalin		Nituy	48°53′	142°55′	20	T. Kawahara 200211199	7	32
P1	Primorsky		Shkotovka	43°21′	132°47′	200	T. Kawahara 200611161	10	32
P2	Primorsky		Frolovka	43°13′	133°18′	100	T. Kawahara 200611581	9	32
HM	Hokkaido	Monbetsu	Shokotsu	44°17′	143°17′	30	T. Nagamitsu 10062	5	32
HT1	Hokkaido	Tokachi	Otofuke	43°05′	143°12′	150	T. Nagamitsu 10041	8	32
HT2	Hokkaido	Tokachi	Tokachi	42°58′	142°56′	140	T. Nagamitsu 10042	8	32
HT3	Hokkaido	Tokachi	Satsunai	42°42′	143°11′	130	T. Nagamitsu 10043	8	32
HT4	Hokkaido	Tokachi	Rekifune	42°28′	143°20′	50	T. Nagamitsu 10058	7	32
HT5	Hokkaido	Tokachi	Saruru	42°07′	143°17′	60	T. Nagamitsu 10060	7	32
HH	Hokkaido	Hidaka	Hidakahorobetsu	42°12′	142°52′	70	T. Nagamitsu 10061	7	32
N1	Honshu	Nagano	Azusa (Kamikochi)	36°15′	137°40′	1,550	T. Nagamitsu 10040	9	32
N2	Honshu	Nagano	Azusa (Hata)	36°13′	137°52′	650	T. Nagamitsu 10044	8	32
							Total	106	450

Materials and methods

Sampling and DNA extraction

Materials were sampled from disjunct habitats in the Japanese archipelago and from habitats near past land bridges to the Eurasian continent (Table 1, Fig. 2a). Nagano Prefecture in the Honshu region and the Monbetsu, Tokachi, and Hidaka districts in the Hokkaido region were selected from Japan. The Kamchatka, Sakhalin, and Primorsky regions were also chosen as locations near the past land bridges through the Kuril Islands, the Sakhalin Island, and the Korean peninsula, respectively. Shoots with leaves and buds of 32 trees were collected from three sites (S1, S2, and S3) in Sakhalin, two sites (P1 and P2) in Primorsky, seven sites (HM in Monbetsu, HT1, HT2, HT3, HT4, and HT5 in Tokachi, and HH in Hidaka) in Hokkaido, and two sites (N1 and N2 in Nagano) in Honshu (Table 1, Fig. 2a). In each site, one to three places where *S. arbutifolia* trees were abundant were selected within a 10-km section along a river. At each place, more than 10 trees were sampled haphazardly within a 200-m section. Representative specimens from the the above 14 sites (Table 1) were deposited in the Herbarium of Forestry and Forest Products Research Institute (FFPRI; TF). Because *S. arbutifolia* is absent in the Kuril Islands, the past land bridge from the Kamchatka region does not seem important. Thus, samples of only two trees were obtained from a site (K) in Kamchatka (Table 1).

As outgroups of *S. arbutifolia* Pall. (synonym: *Chosenia arbutifolia* (Pall.) A. K. Skvortsov, Salicaceae) (Ohashi 2000), a closely related species, *S. cardiophylla* Trautv. and Mey (Azuma et al. 2000), and two other species, *S. nummularia* Andersson and *S. caprea* L., were examined. *Salix nummularia* and *S. caprea* belong to the subgenera *Chamaetia* and *Vetrix* (Skvortsov 1968), respectively, which are sister groups of both *S. arbutifolia* and *S. cardiophylla* (Azuma et al. 2000). Shoots of two *S. cardiophylla* trees (Voucher specimen: T. Nagamitsu 10049 and 10050) and a *S. caprea* tree (T. Nagamitsu 10051) were sampled from the Arboretum of Hokkaido Research Center of FFPRI and deposited in the Herbarium of FFPRI (TF). Shoots of a *S. nummularia* shrub were collected from Keundai on the Taisetsu Mountains (43°33′N, 142°52′E, altitude: 1,900 m) and deposited in the herbarium of Tohoku University Botanical Garden (TUSG 090716001).

The collected shoots were stored at −20 °C until DNA extraction. Total DNA was extracted from leaves or buds of the stored shoots using the DNeasy Plant Mini Kit (Qiagen, Hilden). Nuclear microsatellite genotypes were determined for 32 trees in each of 14 sites (S1, S2, S3, P1, P2, HM, HT1, HT2, HT3, HT4, HT5, HH, N1, and N2) and two trees in Kamchatka (K; Table 1). Sequences in cpDNA regions were determined for 2–10 trees in each of these sites (Table 1) and for samples of the three outgroup species.

Sequencing chloroplast DNA

Two cpDNA regions, one around tRNA-Lys (UUU) 5′ exon (region a) and the other from *mat*K exon to tRNA-Lys
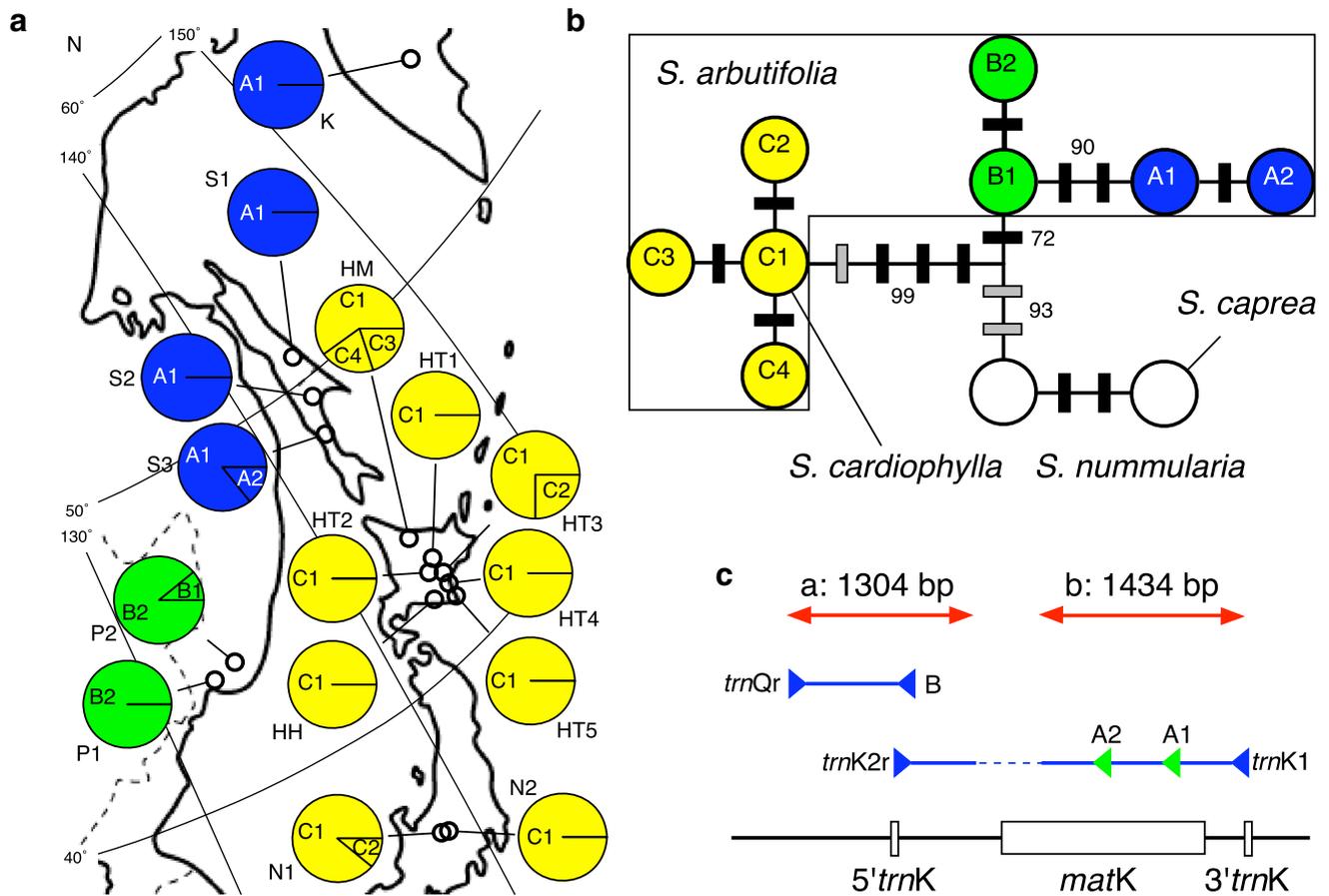
Fig. 2 Chloroplast DNA haplotypes in *Salix arbutifolia*: **a** geographic distribution and frequency of haplotypes (*blue*, *green*, and *yellow pie charts*); **b** a parsimony network of haplotypes (*blue*, *green*, *yellow*, and *white circles*) in *S. arbutifolia* and three *Salix* species (*black bars* indicate nucleotide substitutions, *gray bars* indicate insertions or deletions, and *numbers* at internal nodes indicate bootstrap support exceeding 25 % with 1,000 iterations); and **c** two target cpDNA regions (*red lines*) that span coding (*black boxes*) and intergenic (*black lines*) regions were amplified in two fragments (*solid* and *broken blue lines*) by primers *trn*K1, *trn*K2r, *trn*Qr, and B (*blue triangles*) and sequenced using either the amplification primers or internal sequencing primers A1 and A2 (*green triangles*)

(UUU) 3′ exon (region b), were selected because variable sites were frequently found in these regions (Fig. 2c). Six primers were used for amplification and sequencing of the two regions. They included three universal primers, *trn*K[tRNA-Lys (UUU) exon 1] and *trn*K[tRNA-Lys (UUU) exon 2] (*trn*K1 and *trn*K2r, respectively) (Demesure et al. 1995), and *trn*Qr (Dumolin-Lapegue et al. 1997). In addition to them, two primers, A1: 5′-CAGTACTTTT GTGTTTACGA-3′ and A2: 5′-GGCGTATCCTTTGAGA CAA-3′, were newly designed to read sequences of a long amplified fragment, and a new primer, B: 5′-ATTCTCCA TTGATACGACAT-3′ was designed to amplify and read a sequence overlapped with that from *trn*K2r. A fragment (part of region a) was amplified and sequenced using primers *trn*Qr and B (Fig. 2c). The other fragment (part of region a and region b) was amplified and sequenced using primers *trn*K2r and *trn*K1, and A1 and A2 were used for internal sequencing primers (Fig. 2c).

A polymerase chain reaction (PCR) was performed in 25 µL of a mixture with 1 mM MgSO₄, 1 unit of KOD-Plus DNA Polymerase in PCR buffer for KOD-Plus (Toyobo, Tokyo), 0.3 µM of each primer, 0.2 mM of each dNTP, and 1 µL of the template DNA solution. The PCR was performed using a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City) programmed for 2 min at 94 °C, followed by 30 cycles consisting of 15 s at 94 °C, 30 s at 56 °C, and 1.5 min at 68 °C. The PCR products were purified using the Wizard SV PCR Clean-Up System (Promega, Madison) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing reaction products were purified using the Agencourt CleanSEQ (Beckman Coulter, Brea) and separated on an ABI PRISM 3100-Avant Genetic Analyzer with POP4 in 36-cm capillaries (Applied Biosystems). The electropherograms were examined using Sequencing Analysis software (Applied Biosystems).

Genotyping nuclear microsatellites

Genotypes at 10 microsatellite loci characterized in *S. arbutifolia* (Hoshikawa et al. 2009) were determined. Multiplex PCR was performed for each set of three or four loci (set 1: Cha392, 579, and 591; set 2: Cha415, 472, and 580; and set 3: Cha437, 500, 506, and 603) in 12 μL of a mixture containing 6 μL of Qiagen Multiplex PCR Master Mix (Qiagen), 0.2 μM of each primer, and 1 μL of the template DNA solution using a GeneAmp 9700 thermal cycler (Applied Biosystems) programmed for 15 min at 95 °C, followed by 21–27 cycles, which were optimized to each primer set (Hoshikawa et al. 2009), consisting of 30 s at 94 °C, 90 s at 60 °C, and 60 s at 72 °C, and finished by 30 min at 60 °C. The size of the PCR products were measured using an ABI PRISM 3100-Avant Genetic Analyzer with POP4 in 36-cm capillaries and GeneScan Analysis software (Applied Biosystems).

Data analysis

All cpDNA sequences were aligned using Clustal X 2.0.10 (Thompson et al. 1997). Insertions or deletions (indels) at consecutive sites were treated as single mutations and evenly weighted with substitutions. Haplotypes of the cpDNA sequences consisting of both regions a and b were determined on the basis of both indels and substitutions. Parsimony networks of the haplotypes in *S. arbutifolia* and the three outgroup species were drawn using TCS 1.06 (Clement et al. 2000) with 1,000 bootstrap replications over polymorphic sites of indels and substitutions. The frequency of the cpDNA haplotypes was obtained for each sampling site (Table 1).

For the 10 microsatellite loci across the 14 sites, from each of which 32 samples were collected (Table 1), departure from Hardy–Weinberg equilibrium (HWE) at each locus and linkage disequilibrium between loci were tested by exact tests at significance levels corrected by the sequential Bonferroni method using FSTAT 2.9.3 (Goudet 1995). Loci that significantly departed from HWE and loci indicating significant linkage disequilibrium were excluded from the following analyses.

At the selected loci, allelic richness (*AR*) in 32 diploids, expected heterozygosity (H_S), and fixation index (F_{IS}) at polymorphic loci were obtained from each population in the 14 sites using FSTAT 2.9.3. The difference in *AR* and H_S between populations was examined by the Friedman tests (Hollander and Wolfe 1973) with multiple comparisons based on the values at all selected loci using R 2.15.1 (R Development Core Team 2012). The difference in F_{IS} from zero in each population was examined by a randomization test using FSTAT 2.9.3. To detect a reduction of the effective population size in the recent past, the Wilcoxon sign rank test was performed in each population with the infinite allele model (IAM), the two-phased mutation model (TPM), and the stepwise mutation model (SMM) using Bottleneck 1.2.02 (Piry et al. 1999). Excessive heterozygosity over allelic richness indicates a recent bottleneck because allelic richness decreases more rapidly than heterozygosity when population size is reduced (Maruyama and Fuerst 1985).

To cluster the 14 populations, a neighbor-joining dendrogram was drawn on the basis of genetic distance D_A (Nei and Chesser 1983) between sites using Populations 1.2.30 (Langella 1999) with 1,000 bootstrap replications over loci. Bayesian clustering for all samples from the 14 sites and Kamchatka (Table 1) was also performed using Structure 2.3.1 (Falush et al. 2003) with 20 independent runs for each number of clusters (*K*) ranging from 1 to 13. The runs included burn-in lengths of 50,000 iterations and sampling lengths of 100,000 iterations. Convergence of the runs were confirmed based on time-series plots of the log-likelihood L(*K*). The correlated allele frequency was assumed, and asymmetric admixture was allowed. Appropriate *K* values were inferred using L(*K*) and $\Delta K = \text{mean}[2L(K) - L(K - 1) - L(K + 1)]/\text{standard deviation}[L(K)]$ that indicates the second-order rate of change in L(*K*) at *K* (Evanno et al. 2005).

Genetic variation and differentiation among the inferred clusters of populations were examined. Division of genetic variation with regard to the clusters was assessed with an analysis of molecular variance (AMOVA) using GenAlEx 6.1 (Peakall and Smouse 2006). Pairwise genetic differentiation in two measures, G_{ST} dependent on heterozygosity (Nei and Chesser 1983) and D_{est} independent from heterozygosity (Jost 2008), was obtained for every combination of populations using DEMEtics 0.8.1 in R 2.15.1. The difference in G_{ST} and D_{est} between clusters was examined by the Kruskal–Wallis tests with multiple comparisons based on the values for all population pairs between the clusters using R 2.15.1.

Results

Chloroplast DNA haplotypes

In 106 trees sampled from the 15 sites (Table 1), 2,731- or 2,738-bp sequences consisting of both regions a and b in cpDNA were determined (Fig. 2c). Among these sequences, eight haplotypes (A1 and A2, B1 and B2, and C1, C2, C3, and C4) were distinguished (accession numbers: AB530660-AB530670). Haplotypes C1 and C2 were common in Hokkaido and Honshu, but haplotypes C3 and C4 were found in only Hokkaido (Fig. 2a). Haplotypes C2, C3, and C4 diverged by a substitution from haplotype C1,

Table 2 Genetic diversity (*AR*: allelic richness and H_S: expected heterozygosity), fixation index (F_{IS}), and *P* values of bottleneck tests under three mutation models (IAM: infinite allele model, TPM: two-phased mutation model, and SMM: stepwise mutation model) to detect heterozygosity deficit (def) or excess (ex) as compared with allelic richness at eight selected loci of nuclear microsatellites in 14 populations of *Salix arbutifolia*

Site	Region	District or prefecture	Diversity			Bottleneck test		
			AR	H_S	F_{IS}	IAM	TPM	SMM
S1	Sakhalin		6.71 ab	0.668 ab	0.012	0.098 ex	0.422 def	0.014 def
S2	Sakhalin		8.14 ab	0.648 ab	−0.027	0.156 ex	0.191 def	0.020 def
S3	Sakhalin		6.57 ab	0.686 ab	−0.007	0.004 ex	0.098 ex	0.422 def
P1	Primorsky		7.71 ab	0.721 ab	0.025	0.004 ex	0.027 ex	0.098 def
P2	Primorsky		9.57 a	0.753 a	−0.001	0.014 ex	0.473 ex	0.125 def
HM	Hokkaido	Monbetsu	4.86 ab	0.607 ab	0.003	0.014 ex	0.191 ex	0.527 ex
HT1	Hokkaido	Tokachi	5.86 ab	0.661 ab	0.085	0.014 ex	0.422 ex	0.010 def
HT2	Hokkaido	Tokachi	6.29 ab	0.655 ab	0.046	0.125 ex	0.473 def	0.037 def
HT3	Hokkaido	Tokachi	6.00 ab	0.643 ab	−0.058	0.037 ex	0.098 ex	0.004 def
HT4	Hokkaido	Tokachi	6.43 ab	0.709 ab	0.031	0.002 ex	0.020 ex	0.014 def
HT5	Hokkaido	Tokachi	4.71 ab	0.587 ab	0.115	0.020 ex	0.191 ex	0.191 def
HH	Hokkaido	Hidaka	2.86 ab	0.523 ab	0.021	0.002 ex	0.004 ex	0.020 ex
N1	Honshu	Nagano	3.29 ab	0.362 ab	−0.026	0.406 ex	0.344 def	0.039 def
N2	Honshu	Nagano	1.71 b	0.217 b	0.278*	0.031 ex	0.031 ex	0.063 ex

Significant (*P* < 0.05) heterozygosity excess indicates recent bottlenecks. Different letters for genetic diversity (a, b) indicate significant differences in multiple comparisons. An asterisk by the fixation index indicates significant deviation from the Hardy–Weinberg equilibrium

which differed from haplotype B1 in Primorsky by four substitutions and an indel (Fig. 2a, b). In Primorsky, abundant haplotype B2 diverged from haplotype B1 by a substitution (Fig. 2a, b). Haplotype A1, which was common in Kamchatka and Sakhalin, differed from haplotype B1 in Primorsky by two substitutions (Fig. 2a, b). Haplotype A2 in Sakhalin diverged from haplotype A1 by a substitution (Fig. 2a, b). Haplotype C1 was identical to a haplotype found in *S. cardiophylla*, and haplotypes found in *S. nummularia* and *S. caprea* diverged from an unobserved haplotype on the branch between haplotypes B1 and C1 (AB543610-AB543617; Fig. 2b).

Nuclear microsatellite genotypes

In 450 trees sampled from the 15 sites (Table 1), microsatellite genotypes at the 10 loci were determined (see Electronic Supplementary Material). Among the 10 microsatellite loci in the 14 sites, from each of which 32 genotypes were obtained, the fixation index was low ($F_{IS} = 0.031$ on average) except for Cha392 ($F_{IS} = 0.085$), and significant deviation from HWE was detected at Cha392 ($P = 0.021$), suggesting null alleles at this locus. Significant linkage disequilibrium was detected in two of 45 pairs of the loci (Cha437 and 591, Cha506 and 591; $P < 0.031$), indicating genotypes at Cha591 associated with those at other loci. Therefore, Cha392 and Cha591 were removed from the following analyses.

Among populations in the 14 sites, the highest genetic diversity at eight selected loci was observed in Primorsky ($7.71 \leq AR \leq 9.57$, $0.721 \leq H_S \leq 0.753$; Table 2). The second highest diversity was found in Sakhalin ($6.57 \leq AR \leq 8.14$, $0.648 \leq H_S \leq 0.686$) and in Tokachi in Hokkaido ($4.71 \leq AR \leq 6.43$, $0.587 \leq H_S \leq 0.709$; Table 2). In Hokkaido, genetic diversity in Monbetsu ($AR = 4.86$, $H_S = 0.607$) and Hidaka ($AR = 2.86$, $H_S = 0.523$) was lower than that in Tokachi (Table 2). The lowest diversity was observed in Honshu ($1.71 \leq AR \leq 3.29$, $0.217 \leq H_S \leq 0.362$), particularly site N2 (Table 2). Among the population pairs, significant differences in both *AR* and H_S were found between sites P2 and N2 ($P < 0.034$; Table 2). In Honshu, locus Cha506 in site N1 and four loci Cha415, 500, 506, and 579 in site N2 were not polymorphic. Among the 14 sites, the fixation index was highest and significantly positive in site N2 ($F_{IS} = 0.278$, $P < 0.041$ at the remaining four polymorphic loci; Table 2). In all the three mutation models, recent bottlenecks were detected in site HH significantly ($P < 0.020$) and in site N2 at a marginal significance ($P < 0.063$), owing to an excess of heterozygosity over allelic richness (Table 2).

In a neighbor-joining dendrogram based on genetic distances D_A, populations in Honshu were grouped at a high bootstrap value (100 %), and grouping of populations in Sakhalin, Primorsky, and Tokachi and Hidaka in Hokkaido, respectively, were weakly supported (<61 % bootstrap
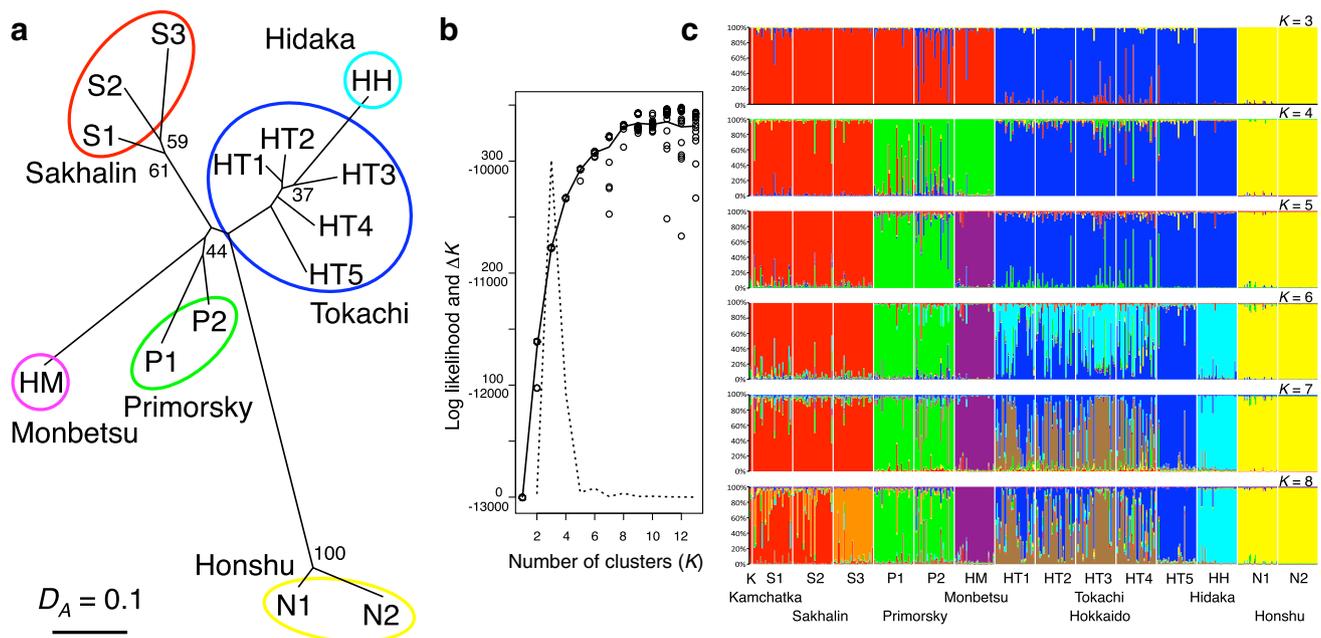
Fig. 3 Genetic structure in *Salix arbutifolia* based on nuclear microsatellite variation at eight selected loci: **a** a neighbor-joining dendrogram of 14 populations in six regions/districts on the basis of standard genetic distance D_A (*numbers* at internal nodes indicate values; Fig. 3a). Monbetsu and Hidaka populations in bootstrap support exceeding 25 % with 1,000 iterations), **b** changes in the log-likelihood (*solid line* and *circles*) and ΔK (*broken line*) as the number of clusters K ranges from 1 to 13, and **c** bar plots of 450 genotypes as K changes from 3 to 8 using STRUCTURE analysis

values; Fig. 3a). Monbetsu and Hidaka populations in Hokkaido were located at the end of long branches (Fig. 3a). In Bayesian clustering, ΔK was highest ($\Delta K = 301.0$) at three clusters ($K = 3$): (1) Honshu; (2) Kamchatka, Sakhalin, Primorsky, and Monbetsu; and (3) Tokachi and Hidaka (Fig. 3b, c). At $K = 4$ ($\Delta K = 93.0$), Monbetsu samples merged with Primorsky samples, and Kamchatka and Sakhalin samples diverged from Monbetsu and Primorsky samples. ΔK indicated a small peak ($\Delta K = 7.9$) at six clusters ($K = 6$) that corresponded to the six geographical regions/districts (Fig. 3b, c). At $K = 8$ ($\Delta K = 3.5$), the log-likelihood reached the highest values, and subclusters were found in Sakhalin and Tokachi samples (Fig. 3b, c). Throughout the clustering, admixture occurred between Primorsky (especially in site P2) and Tokachi (Fig. 3c). Admixture between Sakhalin and Primorsky and that between Tokachi and Hidaka were also found (Fig. 3c).

The analysis of molecular variance (AMOVA) for populations in the 14 sites showed that genetic variation was divided into 32 % among the six regions/districts, 9 % among populations within the regions/districts, and 59 % within the populations. Pairwise genetic differentiation was highest between populations in Honshu and the other populations ($0.20 \leq G_{ST} \leq 0.41$, $0.58 \leq D_{est} \leq 0.83$; Table 3). Monbetsu and Hidaka populations also showed high genetic differentiation to the other populations excluding Honshu populations ($0.06 \leq G_{ST} \leq 0.18$, $0.20 \leq D_{est} \leq 0.60$; Table 3). Among Tokachi, Sakhalin,

and Primorsky, population-pairwise genetic differentiation between Tokachi and Sakhalin ($0.07 \leq G_{ST} \leq 0.17$, $0.32 \leq D_{est} \leq 0.64$) was significantly higher than that between Tokachi and Primorsky ($0.03 \leq G_{ST} \leq 0.09$, $P < 0.001$; $0.18 \leq D_{est} \leq 0.42$, $P < 0.001$), but that between Sakhalin and Primorsky ($0.05 \leq G_{ST} \leq 0.08$, $0.32 \leq D_{est} \leq 0.41$) did not significantly differ from that between Tokachi and Sakhalin and that between Tokachi and Primorsky ($P > 0.076$ in G_{ST}, $P > 0.221$ in D_{est}; Table 3).

Discussion

Salix arbutifolia is broadly distributed in the northeastern Eurasian continent, and the southern edge of its range is located in Japan (Kuzeneva 1985) (Fig. 1). This geographic distribution suggests that *S. arbutifolia* migrated from the Eurasian continent through land bridges and colonized the Japanese archipelago during glacial periods (Fujii and Senni 2006; Ohsawa and Ide 2011). Thus, we expect that the Hokkaido and Honshu populations in the Japanese archipelago share cpDNA haplotypes with at least one of the Kamchatcka, Sakhalin, and Primorsky populations near the past land bridges. Such haplotype sharing suggests potential migration routes. However, haplotypes observed in the Hokkaido and Honshu populations differed from those found in the Kamchatcka, Sakhalin, and Primorsky populations (Fig. 2). The most abundant haplotype in both

Table 3 Pairwise genetic differentiation (upper triangle: G_{ST}, lower triangle: D_{est}) at eight selected loci of nuclear microsatellites in 14 populations of *Salix arbutifolia* in the Sakhalin, Primorsky, and Honshu regions and in the Monbetsu, Tokachi, and Hidaka districts in the Hokkaido region

| Population | Sakhalin | | | Primorsky | | Monbetsu | Tokachi | | | | | Hidaka | Honshu | |
	S1	S2	S3	P1	P2	HM	HT1	HT2	HT3	HT4	HT5	HH	N1	N2
S1		0.02	0.03	0.07	0.05	0.13	0.07	0.08	0.07	0.07	0.14	0.10	0.26	0.35
S2	0.13		0.04	0.08	0.07	0.15	0.11	0.11	0.09	0.08	0.17	0.12	0.31	0.39
S3	0.15	0.17		0.07	0.06	0.12	0.09	0.09	0.09	0.08	0.15	0.12	0.27	0.35
P1	0.39	0.34	0.41		0.02	0.09	0.05	0.06	0.05	0.05	0.09	0.11	0.25	0.31
P2	0.32	0.35	0.36	0.16		0.10	0.03	0.03	0.04	0.03	0.07	0.09	0.21	0.25
HM	0.52	0.57	0.54	0.46	0.48		0.13	0.14	0.13	0.12	0.18	0.18	0.30	0.34
HT1	0.35	0.47	0.41	0.29	0.20	0.55		0.01	0.03	0.03	0.06	0.06	0.26	0.32
HT2	0.35	0.46	0.42	0.31	0.18	0.57	0.05		0.02	0.02	0.03	0.07	0.26	0.31
HT3	0.32	0.41	0.43	0.31	0.25	0.47	0.12	0.09		0.03	0.06	0.06	0.27	0.34
HT4	0.36	0.43	0.45	0.33	0.19	0.54	0.14	0.09	0.13		0.04	0.06	0.20	0.25
HT5	0.54	0.64	0.61	0.42	0.34	0.60	0.18	0.13	0.18	0.15		0.11	0.21	0.26
HH	0.38	0.47	0.47	0.50	0.38	0.57	0.22	0.21	0.21	0.20	0.29		0.35	0.41
N1	0.64	0.70	0.73	0.66	0.65	0.77	0.70	0.70	0.70	0.58	0.58	0.79		0.09
N2	0.79	0.83	0.83	0.72	0.65	0.74	0.71	0.71	0.76	0.61	0.61	0.80	0.15	

Hokkaido and Honshu was identical to a haplotype of a closely related species, *S. cardiophylla* (Azuma et al. 2000), which hybridizes with *S. arbutifolia* and results in a natural hybrid, *S. × kamikotica* Kimura (Ohashi 2000). These findings imply replacement of a chloroplast genome introduced from hybridization or incomplete lineage sorting of an ancestral polymorphism after speciation of these species (Palmé et al. 2003). Because *S. cardiophylla* is distributed not only in Hokkaido and Honshu but also Sakhalin and Primorsky (Skvortsov 1999), it is not clear where the chloroplast genome was replaced (Fig. 1). In willows, many hybrid taxa are recognized in Japan (Ohashi 2000), and the phylogeny is inconsistent between cpDNA and nuclear ribosomal DNA (Hardig et al. 2010), suggesting frequent chloroplast genome capture and incomplete lineage sorting. Therefore, it is difficult to reconstruct the migration routes of Japanese *S. arbutifolia* from cpDNA haplotypes.

Among the three past land bridges, the closest connection is between the Sakhalin and Hokkiado Islands, which are separated by only 40 km across the Soya Strait that is only 60 m deep (Fig. 1) and are thought to have been connected by a land bridge during 10,000–75,000 years ago (Ono 1990). Because of the geographical vicinity, barriers to historical gene flow have been unlikely between the Sakhalin and Hokkaido populations. However, the cpDNA haplotypes showed the largest divergence between the Sakhalin and Hokkaido populations (Fig. 2). Nuclear microsatellite genotypes also indicated that the Tokachi populations in Hokkaido were more differentiated from the Sakhalin populations than from the Primorsky populations

(Table 3). In hierarchical clustering for the nuclear microsatellite genotypes, admixture was more frequent between Tokachi and Primorsky than between Tokachi and Sakhalin (Fig. 3c). When the number of clusters was four, the Monbetsu (HM) genotypes, the northernmost population in Hokkaido, merged with the Primorsky genotypes but diverged from the Sakhalin genotypes (Fig. 3c). Because there has been no direct land bridge between Primorsky and Hokkaido, gene flow between these regions must be through the Sakhalin Island or through the Honshu Island from the Korean peninsula (Fig. 1). Thus, a complicated history, such as a circumventing migration through Honshu to Hokkaido or a replacement of Sakhalin populations after colonization of Hokkaido populations through Sakhalin, is implied from the observed pattern. However, any findings supporting recent invasion in Sakhalin, such as bottleneck and founder events, were not found (Table 2). Because both cpDNA haplotypes and nuclear microsatellite genotypes were shared between Kamchatka and Sakhalin (Figs. 2, 3c), evidence for migration from Kamchatka cannot be discriminated from that from Sakhalin. Thus, it is difficult to evaluate the migration route through the Kuril Islands. The clear genetic divergence between Hokkaido and Sakhalin in *S. arbutifolia* is unique in comparison with other boreal plants in Hokkaido, most of which show close genetic relationship with those in the Sakhalin and Kuril Islands, indicating migration from these past land bridges (Fujii and Senni 2006; Aizawa et al. 2007, 2009; Ohsawa and Ide 2011).

On the other hand, the cpDNA haplotypes were common between the Hokkaido and Honshu populations (Fig. 2).

Unless the same haplotypes were captured or sorted independently in the two regions, the Hokkaido and Honshu populations belong to the same maternal lineage and had merged once through seed dispersal. Present-day seed dispersal is unlikely between the present habitats in the Hokkaido and Honshu regions, which are over 700 km away from each other, since the maximum distance of seed dispersal has been recorded to be 30 km (Kawabe and Saito 1994). Thus, it is reasonable that the disjunct distribution between Hokkaido and Honshu habitats resulted from extinction between these habitats. This explanation assumes that *S. arbutifolia* has been able to survive only within Nagano Prefecture in the Honshu region. The largest population in Nagano Prefecture is in Kamikochi (N1), a wide flood plain at high altitude surrounded with high, steep mountains, which seems a suitable habitat for *S. arbutifolia* (Shin et al. 1999; Shin and Nakamura 2005). The Kamikochi flood plain was formed 26,000 years ago as a result of the damming of the Azusa River by volcanic eruptions (Oikawa 2002). Because this habitat appeared before the end of the last glacial period, a part of *S. arbutifolia* population that had colonized the Honshu Island during the glacial period has been able to survive in that habitat.

In consequence of reduced population and enhanced isolation, disjunct populations at distributional peripheries are expected to show low genetic diversity and high genetic differentiation at selectively neutral loci due to genetic drift, according to Kimura's stepping-stone model (Kimura and Weiss 1964; Lesica and Allendorf 1995; Eckert et al. 2008). As expected, the lowest genetic diversity and the highest genetic differentiation were found in disjunct populations in the Honshu region (Tables 2, 3; Fig. 3). Additionally, in the Hokkaido region, disjunct populations in the Monbetsu and Hidaka districts tended to exhibit lower genetic diversity and higher genetic differentiation than the Tokachi populations (Tables 2, 3; Fig. 3). Thus, the effect of disjunction on genetic variation is likely to occur at various geographic scales in corresponding magnitudes. Extreme reduction in allelic richness and expected heterozygosity in the Honshu populations (Table 2) suggest substantial effects of genetic drift on genetic structure in disjunct populations. In *S. arbutifolia*, fast turnover of generations (Nakamura et al. 2007) and variable size of regenerating populations (Shin and Nakamura 2005) could accelerate genetic drift. Although each disjunct population of alpine and subalpine plants in Japan often has reduced genetic diversity, some species have larger genetic variation among populations in central Honshu than in northern Japan (Fujii and Senni 2006; Ohsawa and Ide 2011). This genetic variation may result from genetic divergence among spatially separated mountain areas in the central Honshu region since the last interglacial period (Ikeda et al.

2009a). In *S. arbutifolia*, however, disjunct populations have been survived in a single mountain area, which results in the extremely reduced genetic diversity in the Honshu populations.

Recent bottlenecks were estimated in two disjunct populations in Hokkaido (HH, the Hidakahorobetsu River) and Honshu (N2, Hata along the Azusa River; Table 2). A significantly positive fixation index was also found in the Hata population (N2; Table 2), suggesting population substructuring and/or biparental inbreeding. In contrast, Kamikochi (N1), the other population along the Azusa River, has neither a sign of recent bottlenecking nor a positive fixation index in spite of its low expected heterozygosity (Table 2). In addition to geographical disjunction, habitat quality also seems to affect genetic structure. The environmental conditions of the habitats are quite different between the two populations along the Azusa River. The altitude is 900 m higher (Table 1), and thus the climate conditions are more suitable for *S. arbutifolia* in Kamikochi than in Hata. The flood plains are wider, with higher gravel supply, and regeneration sites are more abundant in Kamikochi than in Hata. Furthermore, the invasion of exotic trees was intensive in Hata (Maekawa and Nakagoshi 1997). Thus, the Hata population is more reduced and fragmented than the Kamikochi population, which may result in the recent bottleneck and the positive fixation index observed in Hata.

Recently, *S. arbutifolia* was removed from the list of endangered species in Japan (Ministry of the Environment 2012). A demographic simulation indicates that the Hokkaido populations are unlikely to become extinct within 50 generations (Nagamitsu et al. 2003). On the other hand, frequent colonization and extinction of local populations around Kamikochi have been observed (Yokouchi 1998), suggesting that only the Kamikochi population is a single source in metapopulation dynamics. Thus, degradation of the habitat in Kamikochi is likely to threaten the persistence of disjunct populations in the Honshu region. The conservation priority of disjunct populations at range peripheries has been debated (Lesica and Allendorf 1995). The Honshu populations seem to have low potential of evolvability due to reduced genetic variation based on selectively neutral loci. However, peripheral populations of alpine plants in the central Honshu region are known to have evolved under natural selection in some functional genes (Ikeda et al. 2009b). Thus, if the Honshu populations of *S. arbutifolia* have locally adapted genes, the populations have particular conservation value as genetic sources of adaptive evolution and demographic persistence during future environmental change (Hardie and Hutchings 2010). It is necessary to understand such local adaptation in disjunct populations at range peripheries to consider the conservation priority of disjunct *S. arbutifolia* populations.

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