Introgressive Hybridization between Southern Asian Dolly Varden, Salvelinus curilus, and Northern Dolly Varden, S. malma malma, on Sakhalin Island

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ANIMAL GENETICS

Introgressive Hybridization between Southern Asian Dolly Varden, *Salvelinus curilus*, and Northern Dolly Varden, *S. malma malma*, on Sakhalin Island

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Abstract—Genetic characteristics of the southern Asian Dolly Varden, *Salvelinus curilus*, and northern Dolly Varden, *S. malma malma*, in northeastern Asia and the Aleutian Islands were examined using mitochondrial (mt) and microsatellite (ms) DNA markers. The maximum-likelihood tree based on mtDNA control region haplotypes showed two well-supported monophyletic lineages for these species, but the haplotypes found in the Sakhalin Island populations were an admixture of the two mtDNA lineages. Bayesian clustering using msDNA indicated that all individuals from Sakhalin Island grouped with the *S. curilus* populations, regardless of their mtDNA haplotype. Incongruence between mtDNA and msDNA markers provided strong evidence of historical mtDNA introgression from *S. m. malma* to *S. curilus*. Secondary contact by postglacial colonization from different refugia is a plausible explanation for the introgressive hybridization detected in Sakhalin Island.

Keywords: genetic admixture, microsatellite DNA, mitochondrial DNA, secondary contact

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INTRODUCTION

Climate oscillations related to the Pleistocene glacial-interglacial cycles had enormous impacts on the population structure and phylogeographic history of many freshwater fishes, especially species distributed in high-latitude regions where the impacts of glaciation on hydrology were particularly severe [1]. Salmonid fishes are typical cold water-adapted fish, and have complex evolutionary and demographic histories that result from the effects of climate oscillations, such as secondary contact, gene flow, and hybridization between diverging populations and/or different species [2]. Introgressive hybridization between different lineages or species as result of historical secondary contact has been well documented for several salmonid fishes [e.g., 3–7]. Introgressive hybridization has received considerable attention, because the geographical extent of hybrid zones has provided a valuable framework for examining distribution limits and evolutionary barriers to genetic exchange [8, 9]. Genetic analysis of sympatric populations from different lineages also provides important insight into the identification of reproductive isolation and species status based on the biological species concept [10].

Northern Dolly Varden, *Salvelinus malma malma*, occurs widely along the North Pacific Rim and into the Pacific northwestern of North America and northeastern Asia, and neighboring islands. Southern Asian Dolly Varden, *S. curilus* (syns. *S. malma kraschenininkovi*, *S. malma curilus*), has a parapatric distribution with *S. malma malma* in northeastern Asia. The range of *S. curilus* covers the continental coast from the Amur River mouth to the North Korean rivers, which includes the Shantar Islands, Sakhalin Island, Kuril Islands, and south to Hokkaido Island [11–13]. Previous genetic study on the *S. malma* subspecies-complex throughout their distribution range, which includes that of *S. curilus*, revealed the existence of two discrete mitochondrial (mt) DNA lineages, called the Western

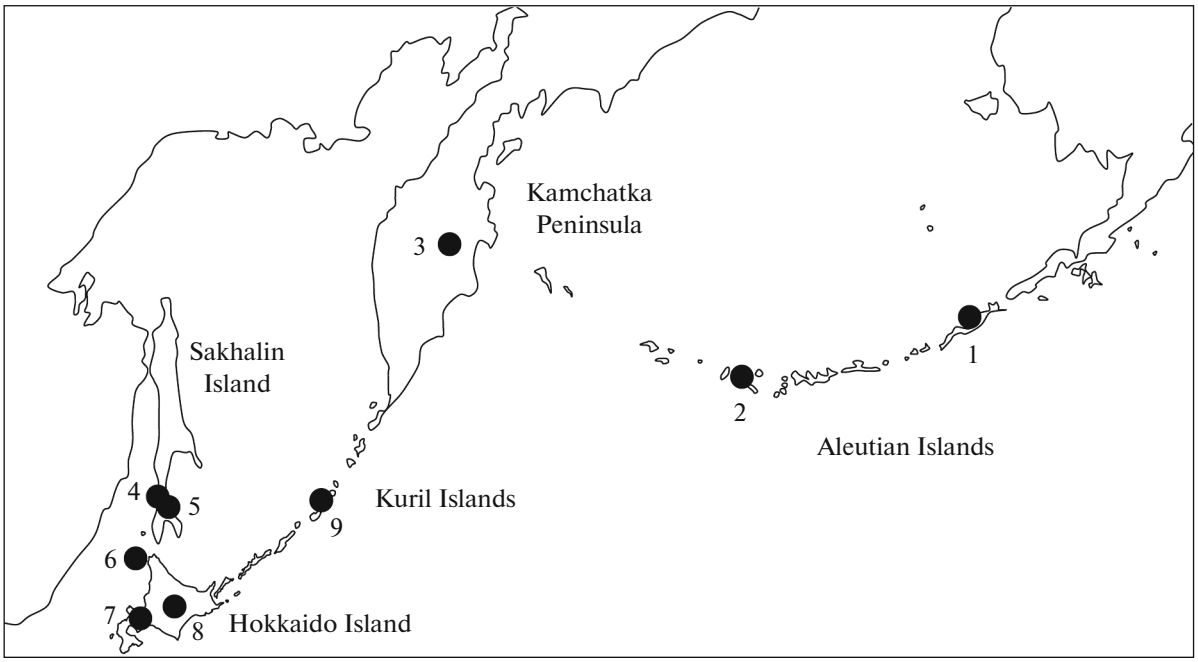

Fig. 1. Locations of the nine populations sampled for genetic analyses. Details for each sample are listed in Table 1.

and Central Lineages, in northeastern Asia; these lineages had a degree of geographical affiliation with *S. curilus* and *S. malma malma*, respectively [14]. These two mtDNA lineages were found sympatrically in Sakhalin Island, the Kuril Islands, and some populations along the coast of the Okhotsk Sea [5, 14–17]. It was suggested that contact zone of the two mtDNA lineages originated from postglacial colonization from different refugia [14].

Although the two species exhibit substantial differences in karyotypes [18, 19], morphological traits [11, 12], ribosomal DNA [19], and mtDNA [14, 16, 20, 21], their species and taxonomical status is still debated and controversial, because of some inconsistent results [22, 23]. Because of the lack of simultaneous analyses of mtDNA and nuclear DNA, it also remains unknown if coexistence of the two mtDNA lineages were "footprints" of historical isolation and re-colonization, or if this results from contemporary interactions between the two species. In this study, we examined mtDNA and microsatellite (ms) DNA to assess genetic characteristics among the two populations from Sakhalin Island, and allopatric populations of both *S. curilus* and *S. malma malma*. Specifically, we investigated if there was evidence of reproductive isolation between the two species in their contact zone.

MATERIALS AND METHODS

Sample Collection and DNA Analyses

Fish were collected in the Aniva and Naiba rivers in the southern Sakhalin Island (Fig. 1). Additional samples from the Higashi-Nottodomari River on Rishiri Island, near northern Hokkaido Island, were also collected for DNA analyses. After clipping the adipose fin for DNA analyses, the fish were released at the capture site.

Total genomic DNA was extracted using a QIAGEN Blood & Tissue DNeasy Kit (Qiagen, Inc.). Following previous genetic study on the *S. malma* subspecies-complex [14], the mtDNA control region was amplified using the primers LRBT-25 (5'-AGA GCG CCG GTG TTG TAA TC-3') and LRBT-1195 (5'-GCT AGC GGG ACT TTC TAG GGT C-3'), which are located in the tRNA gene regions that flank the control region [24]. Amplified DNA was purified using ExoSap-IT (Thermo Fisher, Inc.) and directly sequenced using BigDye Terminator Cycle Sequencing Ready Reaction Kit ver. 3.1 (Applied Biosystems, Inc.). Mitochondrial DNA sequences were aligned with the multiple sequence editor Clustal W [25], and trimmed to a 552-bp fragment of the mtDNA control region to be consistent with the sequences analyzed by Yamamoto et al. [14] and Brunner et al. [26].

MsDNA analysis was carried out for the nine populations that spanned the distributions of both *S. curilus* and *S. malma malma* (Fig. 1, see also Yamamoto et al. [14]). Samples were taken and re-analyzed from Yamamoto et al. [14] except for those from the Aniva, Naiba, and Higashi-Nodottomari rivers. We used eight msDNA loci for population genetic analyses: *Saleu*-2, *Saleu*-8, *Saleu*-22, *Saleu*-25 [27], *Sco*-211, *Sco*-214 [28], *Sfo*-12 [29], and *Ssa*-197 [30]. MsDNA amplifications were performed using a Type-it Micro-

satellite PCR Kit (Qiagen, Inc.). The 10-μL reaction mixture contained 1× Type-it Microsatellite PCR master mix, 0.2 μM of each primer, 2 μL of distilled water, and 1 μL of DNA solution. Amplified products were analyzed using an ABI PRISM 3730xl sequencer (Applied Biosystems, Inc.). Allele size was determined using GeneMapper ver. 4.1 (Applied Biosystems, Inc.).

Data Analysis

Phylogenetic relationships among the mtDNA haplotypes from the nine populations were inferred from a maximum-likelihood (ML) analysis using MEGA ver. 7 [31]. The Tamura 3-parameter plus invariant sites plus gamma model was chosen as the most appropriate model using the Bayesian information criterion. Sixty-eight haplotypes reported by Yamamoto et al. [14] detected from the species' range of the *S. malma* subspecies-complex and *S. curilus* were retrieved from DNA Data Bank of Japan (DDBJ: accession numbers AB206973, AB684779–AB684845) to add to the analysis for resolving the phylogenetic position of each haplotype. The ML tree was rooted with four haplotypes of white-spotted charr, *S. leucomaenis*, from Hokkaido Island, Japan [6] as an outgroup. ML tree support was evaluated by 1,000 bootstrap replicates.

The msDNA genetic diversity of each population was quantified based on allelic richness using FSTAT ver. 2.9.3 [32]. The observed and expected heterozygosities were calculated in Arlequin ver. 3.5 [33]. Within-population deviations from Hardy–Weinberg equilibrium (HWE) were tested using Fisher's exact test with the Markov chain method (Markov chain steps, 10^6; dememorization, 10^5) using Arlequin. Critical significance levels for multiple tests were corrected following the sequence Bonferroni correction [34].

A Bayesian clustering approach, implemented in STRUCTURE ver. 2.3.4 [35], was used to estimate the population structure for the nine populations of *S. curilus* and *S. malma malma*. The program was run with the admixture/allele frequency model and without prior information of source populations. After a burn-in period of 100,000 replicates, an additional 500,000 Markov chain Monte Carlo replicates were used to simulate the posterior probabilities to identify the number of distinct populations (K) from the data. We performed 10 independent runs for each K ($K = 1$–9) to verify our posterior probability estimates. The K values showing the optimal subdivision of the data were estimated by the ΔK method [36]. STRUCTURE results were imported into StructureSelector [37] to estimate ΔK and for graphical representation. We also used the NEIGHBOR program in PHYLIP ver. 3.695 [38] to generate a neighbor-joining (NJ) tree based on Cavalli-Sforza and Edwards chord distances to represent genetic relationships among the nine populations of *S. curilus* and *S. malma malma*. Bayesian clustering analysis using the STRUCTURE was also conducted for the Aniva and Naiba populations with the admixture/allele frequency model. The genetic relationships among individuals from the Aniva and Naiba populations were also assessed by principal coordinates analysis based on the covariance matrix of genetic distance using GenAlEx ver. 6.5 [39].

RESULTS AND DISCUSSION

Five new haplotypes, SM74–76, 77, and 78, were found in the Naiba, Aniva, and Higashi-Nodottomari populations, respectively. The associated sequences were deposited in DNA Data Bank of Japan (DDBJ: accession numbers LC538384–LC538388). The ML tree showed that a total of 73 haplotypes from the *S. malma* subspecies-complex and *S. curilus* formed three main monophyletic groups with high bootstrap support (Fig. 2), which are referred to as the Western, Central, and Eastern Lineages (see Yamamoto et al. [14]). The Western Lineage was sister to the group that contained the Central and Eastern Lineages. Based on the degree of geographic affiliation, the Western, Central, and Eastern Lineages were assigned to *S. curilus, S. malma malma,* and *S. malma lordi* ([14], see also [19, 40–42]), respectively. Of the nine populations studied, the SummerBay, Dock, and Azabache populations had haplotypes assigned to the Central Lineage (*S. malma malma*); the Higashi-Nodottomari, Chihase, Ishikari, and Unnamed populations had haplotypes assigned to the Western Lineage (*S. curilus*); and the Aniva and Naiba populations were an admixture of the Central and Western Lineage haplotypes (Table 1, Fig. 2). The numbers of haplotypes of the Central and Western Lineages within the Aniva and Naiba populations were 2/46 and 10/38, respectively.

All eight msDNA loci were polymorphic, with the total number of alleles detected at each locus ranging from 2 (*Saleu*-2, *Sco*-214) to 69 (*Saleu*-25), and the expected heterozygosity ranging from 0.03 (*Sco*-214) to 0.96 (*Sco*-211). Among 54 HWE tests, only one (the *Sco*-211 locus, Azabache population) was significant following Bonferroni correction. There were variations in intrapopulation diversity, with average allelic richness ranging from 1.74 (Higashi-Nodottomari population) to 6.64 (Naiba population), and expected heterozygosity ranging from 0.44 (Higashi-Nodottomari population) to 0.81 (Dock population). In the Bayesian clustering analysis (STRUCTURE; Fig. 3), $K = 5$ was the most likely division for the nine populations following the approach of Evanno et al. [36]. A genetically distinguishable cluster belonged to the Central Lineage (i.e., *S. malma malma* in the SummerBay, Dock, and Azabache populations). The Aniva and Naiba populations were grouped with the Ishikari population (*S. curilus*, Western Lineage). The other three populations, Higashi-Nodottomari, Chihase, and Unnamed (*S. curilus*, Western Lineage), were each assigned as independent clusters. The topology of the NJ tree based on Cavalli-Sforza and

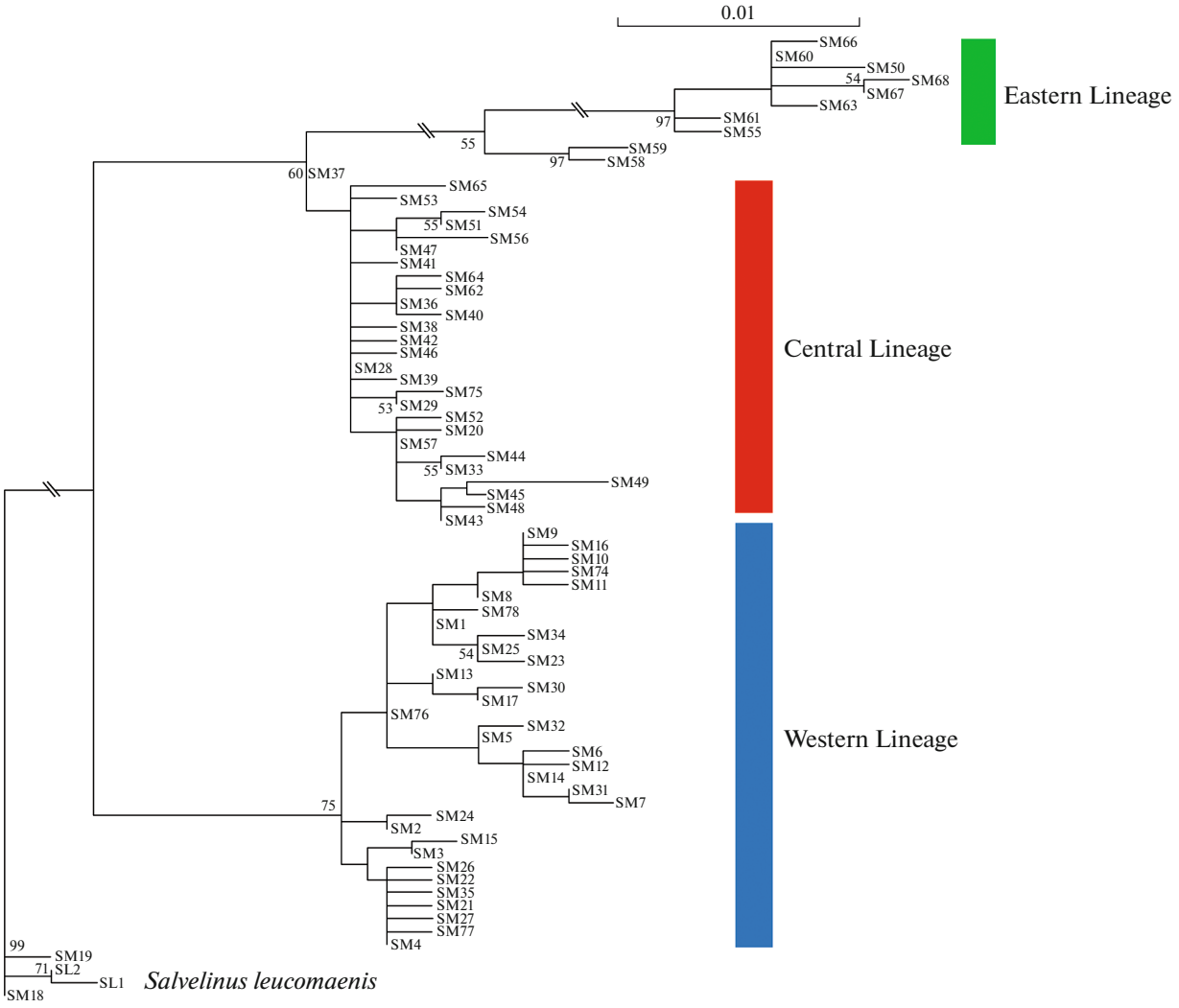

Fig. 2. Maximum-likelihood tree of the phylogenetic relationships of 73 mtDNA haplotypes of *Salvelinus curilus*, *S. malma malma*, and *S. malma lordi* based on Tamura 3-parameter plus invariant sites plus gamma model of nucleotide substitution. Haplotype abbreviations correspond to those listed in Table 1. Numbers on major branches indicate bootstrap values from 1,000 replicates. The tree was rooted with four *S. leucomaenis* haplotypes.

Edwards chord distances among the populations was nearly congruent with the Bayesian clustering results, although differences in population clustering between the Western and Central Lineage populations were not definitive. As in the Bayesian clustering, the Aniva and Naiba populations were grouped with the Ishikari population, and then grouped with the other Western Lineage populations (i.e., Higashi-Nodottomari, Chihase, and Unnamed).

In the Aniva and Naiba populations of Sakhalin Island, STRUCTURE analysis revealed that ΔK was the highest when $K = 4$ (Fig. 4). However, population clustering was somewhat unclear. Individuals were not specifically clustered by population nor by mtDNA lineage. The principal coordinates analysis also showed that individuals were not grouped by mtDNA lineage.

Our mtDNA analyses using a wide range of *S. curilus* and *S. malma malma* samples revealed that the two species are characterized by haplotypes that are differentiated by large nucleotide substitutions. The two mtDNA lineages were shaped by historical isolation in glacial refugia during the Pleistocene, which were in or around the Sea of Japan and/or Sea of Okhotsk for the Western Lineage [14, 15, 43] and the Beringian Refugium for the Central Lineage [44]. Historical secondary contact by postglacial colonization from different refugia, therefore, seems to be a plausible explanation for the coexistence of diverging mtDNA haplotypes in the Sakhalin Island populations.

The STRUCTURE clustering and NJ tree using msDNA markers were also congruent with the mtDNA genealogy, although differences in divergence between *S. curilus* and *S. malma malma* were not as

Table 1. Sampling locations, sample size, mitochondrial (mt) DNA haplotypes, and genetic diveristy statistics of microsatellite (ms) DNA analyses for the nine populations of *Salvelinus curiles* and *S. malma malma*. Numbers assigned to the populations correspond to the locations shown in Fig. 1. mtDNA haplotypes with and without an asterisk represent those assigned to the Westren Lineage and Central Lineage, respectively (see also Fig. 1)

Population no.	Location	Population	Sample size for mtDNA analyses	mtDNA haplotype(*n*)	Sample size for msDNA analyses	H_E	H_O	*A*	*AR*
1	Aleutian islands (Unalaska Island)	SummerBay L. (an inlet stream)	38	SM28(18), SM36(15), SM43(2), SM52(2), SM53(1)	24	0.79	0.75	13.00	6.27
2	Aleutian islands (Atka Island)	Dock R.	51	SM20(11), SM28(4), SM36(5), SM50(1), SM51(24), SM52(2), SM53(1), SM54(3)	30	0.81	0.79	11.88	5.83
3	Kamchatka Peninsula	Azabache L.	32	SM28(24), SM36(5), SM37(1), SM38(1), SM39(1)	30	0.78	0.71	13.00	6.04
4	Sakhalin Island	Aniva R.	48	SM1(12)*, SM4(12)*, SM5(4)*, SM9(17)*, SM29(2), SM77(1)*	48	0.68	0.68	13.50	6.04
5	Sakhalin Island	Naiba R.	48	SM1(4)*, SM4(4)*, SM5(2)*, SM9(10)*, SM14(12)*, SM27(1)*, SM28(3), SM29(5), SM30(1)*, SM74(2)*, SM75(2), SM76(2)*	48	0.68	0.68	17.00	6.64
6	Rishiri Island	Higashi-Nodottomari R.	38	SM78(38)*	40	0.44	0.39	2.29	1.74
7	Hokkaido Island	Chihase R.	22	SM2(22)*	33	0.50	0.47	3.00	2.42
8	Hokkaido Island	Ishikari R.	33	SM1(29)*, SM2(4)*	30	0.66	0.66	6.38	4.32
9	Kuril islands (Simushir Island)	Unnamed R.	30	SM9(1)*, SM25(8)*, SM34(13)*, SM35(8)*	30	0.72	0.69	6.63	4.80

H_E, average expected heterozygosity; H_O, average observed heterozygosity; *A*, average number of alleles; *AR*, average allelic richness.

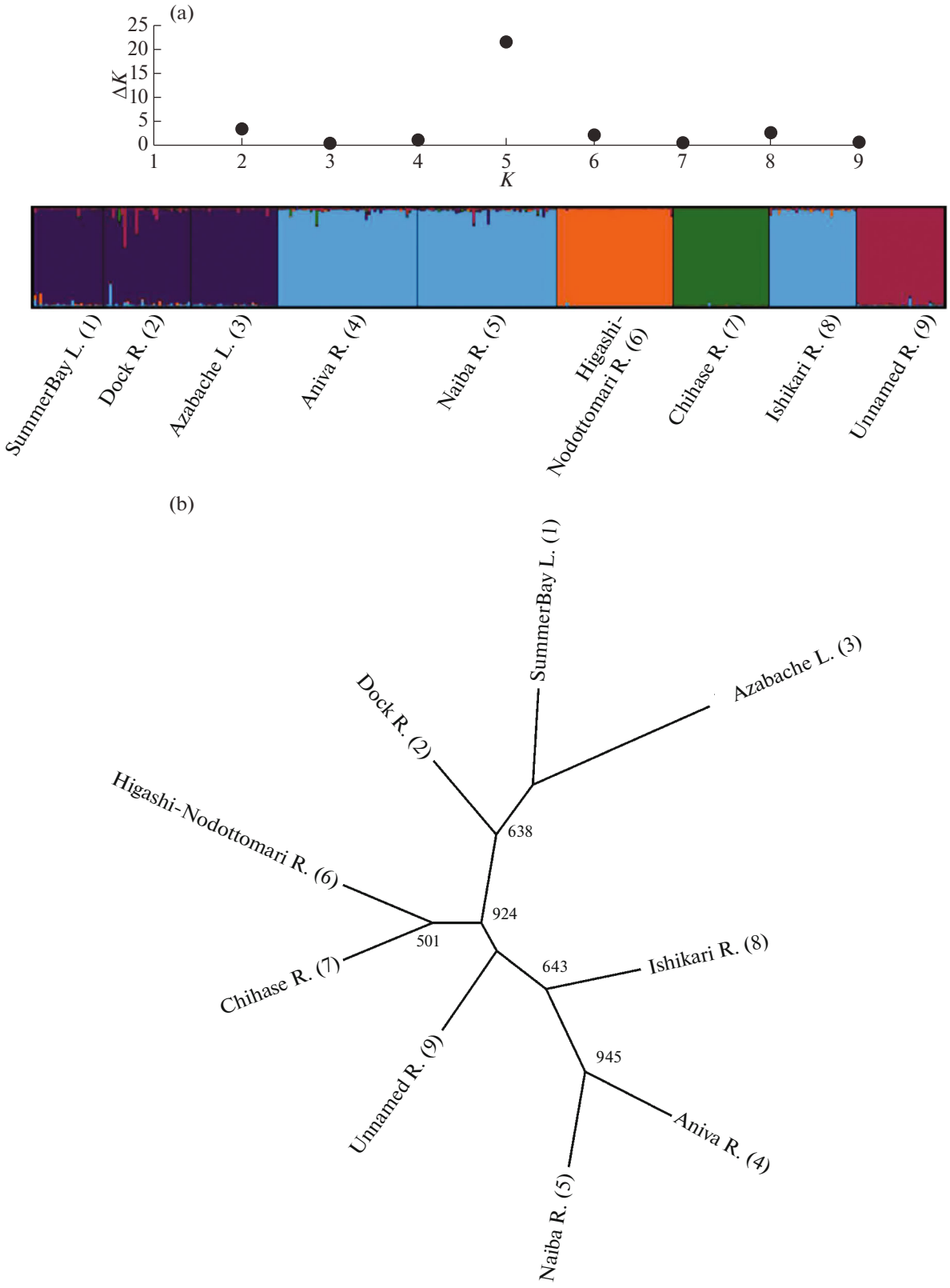

Fig. 3. (a) Bayesian population assignment using admixture/dependent allele frequency model of 313 *Salvelinus curilus* and *S. malma malma* individuals using microsatellite DNA markers. The ΔK estimates [36] with different K are also shown. (b) Unrooted neighbor-joining tree based on Cavalli-Sforza and Edwards chord distances of the nine populations using microsatellite DNA markers. Numbers represent bootstrap values of ≥50% from 1,000 replicates. The numbers in parentheses assigned to populations correspond to those listed in Fig. 1 and Table 1.

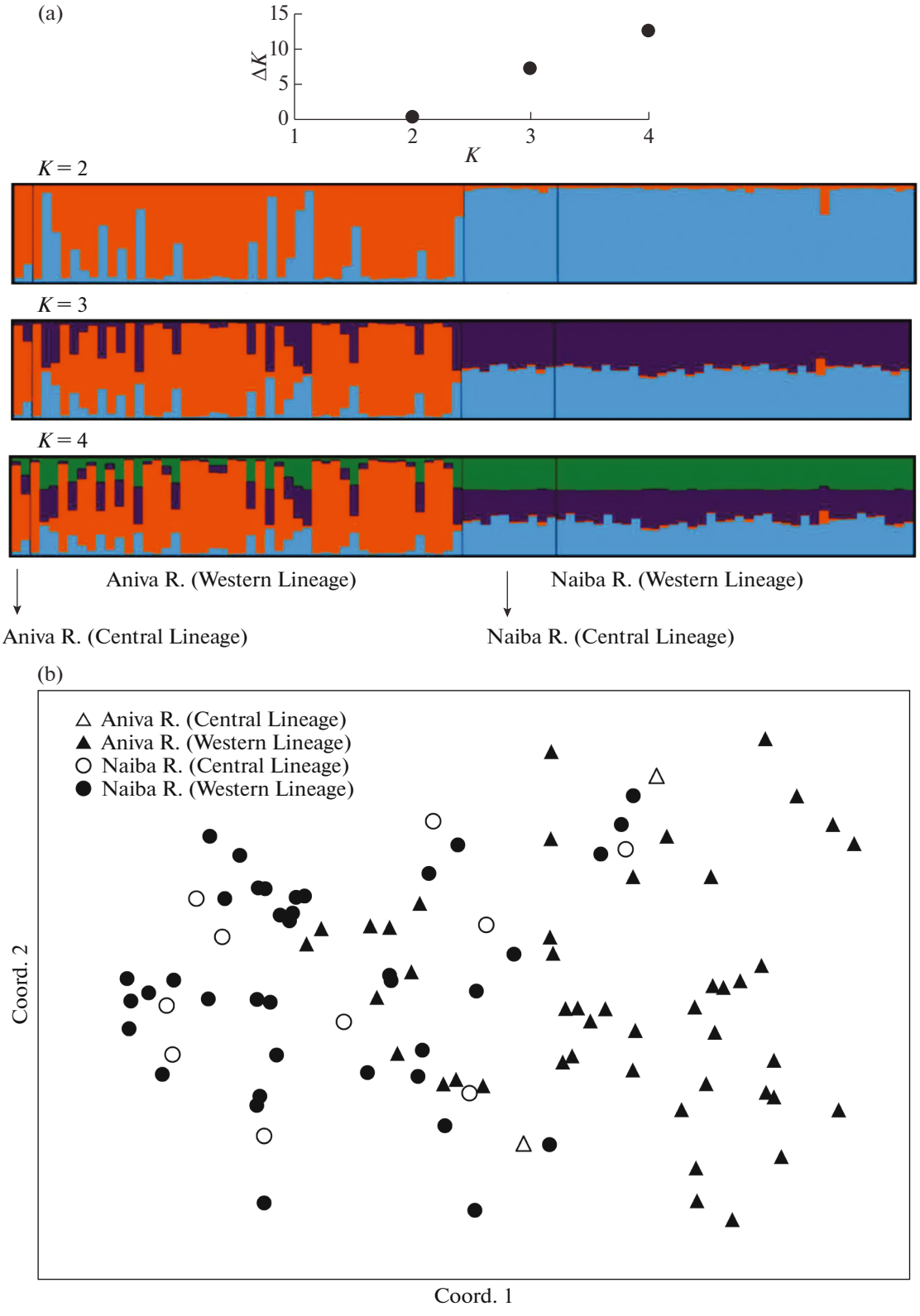

Fig. 4. (a) Bayesian population assignments of 96 individuals from the Aniva and Naiba rivers based on the three possible genetic clusters ($K = 2, 3, 4$). The ΔK estimates [36] with different K are also shown. (b) Two-dimensional plot of the principal coordinates analysis for 96 individuals from the Aniva and Naiba rivers. Approximately 18.6% of the total variation was explained by the first two principal components. Coord. 1 (11.3%) and Coord. 2 (7.3%) refer to the first and second principal components, respectively.

prominent (see also Gordeeva et al. [45]). It should be noted that all individuals from Sakhalin Island grouped with the *S. curilus* populations, regardless of their mtDNA lineage. Incongruence between mtDNA and nuclear DNA markers of allopatric species provided strong evidence of historical mtDNA introgression, which was promoted by divergent patterns of gene flow between the two genomes [46]. MtDNA introgression between *S. curilus* and *S. malma malma* from Sakhalin Island is thought to have occurred as a result of southward dispersal of *S. malma malma,* which then introgressed into the *S. curilus* genome. Unidirectional hybridization from *S. malma malma* mtDNA to *S. curilus* could be attributed to sex-biased mating or offspring production, which represents a constraining factor of hybridization [47]. Most *S. curilus* individuals typically have a resident life history in rivers and mature at a smaller size [48, 49], whereas *S. malma malma* have a migratory life history in which they grow in the sea and mature at a larger size, and then return to their natal river to spawn [50–52]. Migratory individuals are usually predominantly females [51]. Those large *S. malma malma* females might have reproductive success with *S. curilus* males, because females of a rare species often become less discriminating and may eventually mate with males of a more common species [47]. Then, hybrid nuclear DNA would be replaced and diluted by long-term backcrossing with individuals of the father species, *S. curilus*. Historical hybridization and introgression have also occurred between *S. curilus* and *S. leucomaenis* on Hokkaido Island, which is the southernmost distribution limit of *S. curilus* [6]. Indeed, substantial evidence of unidirectional hybridization has been reported for other salmonid fishes, such as bull trout, *S. confluentus*, and Dolly Varden, *S. malma* [53]; bull trout and brook trout, *S. fontinalis* [54]; steelhead trout, *Oncorhynchus mykiss*, and cutthroat trout, *O. clarki* [55]; and *S. leucomaenis* and *S. fontinalis* [56, 57].

Bayesian clustering, principal coordinates analysis, and HWE test using msDNA all indicated that there was genetic admixture between the two mtDNA lineages on Sakhalin Island. It is, therefore, still not evident whether reproductive isolation between the two species currently occurs in their contemporary contact zones. It also remains unknown if there are selection mechanisms against hybridization, such as reproductive isolation, between the two species (e.g., the degree of sterility and developmental incompatibilities of hybrid individuals). Further detailed examinations, such as by artificial cross-breeding experiments and field verifications in contemporary contact zones, are needed to elucidate the pre- and post-mating selection mechanisms of these species to confirm their species status.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals have been observed.

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