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ANIMAL GENETICS

Phylogeography of the Siberian Newt *Salamandrella keyserlingii* by Mitochondrial DNA Sequence Analysis

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Abstract—Differentiation of geographical populations of the Siberian newt *Salamandrella keyserlingii* throughout the species range was analyzed using a fragment of the cytochrome b gene. The population of the Primorye region (Russian Far East) is separated to the greatest extent; the Japanese and South Kuril populations are the next most separate. These populations are possibly subspecies. Geographical differentiation of populations in the Siberian part of the species range is lower, lacks a clinal variation, and is irregular. The molecular variation of *S. keyserlingii* supports the hypothesis that several primary vicarious refugia of pre-Pleistocene differentiation of a common ancestor of *Salamandrella* occurred in the southeastern part of its current distribution range and that northern and western regions were gradually colonized via repeated steps of expansion and retreat in the Siberian part of the modern species range.

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INTRODUCTION

The Siberian newt *Salamandrella keyserlingii* belongs to the primitive family Hynobiidae. Among all Palearctic amphibians, *S. keyserlingii tridactyla* has the greatest species range and is found in the northeastern part of European Russia, Siberia, the northern part of Kazakhstan, Mongolia, China, Korea, and Japan. At the same time, the species is characterized by an extreme morphological uniformity without a distinct geographical variation, which has given grounds for considering the taxa described for this species to be invalid [1, 2]. Differences in ecology have been reported for individual populations from different geographical localities, as well as morphological and ontogenetic modifications [1, 3–6] and a genetic variation [7, 8]. A special status has been proposed for the populations of Primorye [4, 5, 8, 9]. Berman et al. [10, 11] have assumed that the Primorye populations represent a separate species. Analysis of a mitochondrial cytochrome b gene fragment has led to the conclusion that molecular differentiation between the populations of Primorye and Siberia is high enough to isolate two species: former *S. keyserlingii tridactyla* [8, 9] has been termed *schrenckii* (Primorye and southern Khabarovsk krai) and the other populations (almost total range, mostly Siberia) have been termed *keyserlingii.*

Owing to the vast distribution and the irregular geographical variation of molecular markers, Siberian newt provides an appealing model for phylogeographical studies. Moreover, Siberian newt is one of the few amphibian species that efficiently survive in adverse polar environments. The molecular variation of the species throughout the entire species range has not been studied as of yet, although such studies are necessary for understanding the mechanisms of differentiation of geographical populations and reconstructing the history of the species range formation. We focused on this problem in our work. Since the taxonomy and the species status of the Siberian newt population from Primorye are beyond the scope of this paper, the above two taxa are hereafter designated as Primorye and Siberian forms.

MATERIALS AND METHODS

Material. Tissue specimens were obtained from newts of mainland and island (Sakhalin, Paramushir, Kunashir, and Hokkaido) populations. In total, we examined 173 newts from 30 mainland and four island populations (table, Fig. 1). The mean sample size was five newts per population, which is usually considered to be sufficient for phylogeographical studies [12, 13]. The maximum sample (ten newts) was from the Kedrovaya Pad' nature reserve, and the minimum sample (one newt) was from Paramushir (this was caused by the poor quality of the material). In addition to the original data, our analysis included the published sequences of a cytochrome b gene fragment of 86 newts [10, 11] (NCBI GenBank accession nos. AY701904–AY701989, http://www.ncbi.nlm.nih.gov/entrez). The cytochrome b sequence of the closely related Korean salamander *Hynobius leechii* (family Hynobiidae) was established using an individual from Korea; the

Sites of material collection

No.	Collection site	Region	Coordinates		*N*
			N.	E.	
1	Lekma, Slobodskoi raion	Kirov oblast	59°02′	49°56′	8
2	Yekaterinburg region	Sverdlovskaya oblast	56°51′	60°36′	8
3	Yekaterinburg	″	56°51′	60°36′	8
4	Tomsk	Tomsk oblast	56°29′	84°57′	4
5	Tomsk region	″	56°29′	84°57′	9
6	Village of Marusino, Tolmachevskii raion	Novosibirsk oblast	55°02′	82°45′	4
7	Taz River, near the village of Kikkiaki	Yamalo-Nenetskii Autonomous District	63°48′	82°46′	8
8	Novosibirsk region	Novosibirsk oblast	55°02′	82°56′	6
9	Krasnoyarsk region	Krasnoyarsk krai	56°00′	92°49′	3
10	Village of Kultuk, Slyudyanskii raion	Irkutsk oblast	51°43′	103°39′	4
11	Kamniokan Pier	Buryatia	56°34′	113°34′	4
12	Village of Khasurta	″	52°17′	108°52′	4
13	Onon River, Khapcherangskii raion	Chita oblast	49°38′	112°32′	2
14	Yakutsk region	Sakha (Yakutia)	62°02′	129°44′	8
15	Main River valley (1), Markovskii raion	Chukotskaya oblast	64°53′	171°46′	1
16	Main River (2, 3), Markovskii raion	″	64°28′	171°45′	4
17	Yuzhno-Kuril'skoe Lake, Ozernovskii raion	Kamchatskaya oblast	51°24′	157°5′	8
18	Paramushir	″	50°38′	156°07′	2
19	Magadan region	Magadan oblast	59°35′	150°48′	9
20	Obluch'e	Evreiskaya Autonomous District	49°01′	131°03′	3
21	Village of Preobrazhenovka	″	48°04′	131°54′	3
22	Khailar region	Internal Mongolia	48°50′	120°31′	4
23	Norskii nature reserve, Selemdzhinskii raion	Amurskaya oblast	52°38′	130°19′	8
24	23 km south to Krasnaya Rechka, Khabarovskii raion	Khabarovsk krai	48°12′	135°6′	4
25	Kholmsk raion	Sakhalinskaya oblast	47°03′	142°04′	4
26	Serebryanka River, Kunashir	″	44°03′	145°49′	2
27	Kusiro Marsh, Kusiro region	Hokkaido, Japan	43°01′	144°22′	2
28	Kedrovaya Pad' nature reserve	Primorskii krai	43°04′	131°33′	10
29	Shkotovskoe Plateau	″	43°21′	132°24′	8
30	Artem	″	43°22′	132°10′	2
31	Village of Kondratenovka	″	43°37′	132°10′	2
32	Vladivostok	″	43°07′	131°55′	5
33	Village of Pavlo-Fedorovsk, Sungacha River, Kirovskii raion	″	45°08′	133°16′	8
34	Baishan, region of the village Huadian	People's Republic of Korea			4

Note: Site numbers correspond to the population numbers (Fig. 1). *N* is the sample size.

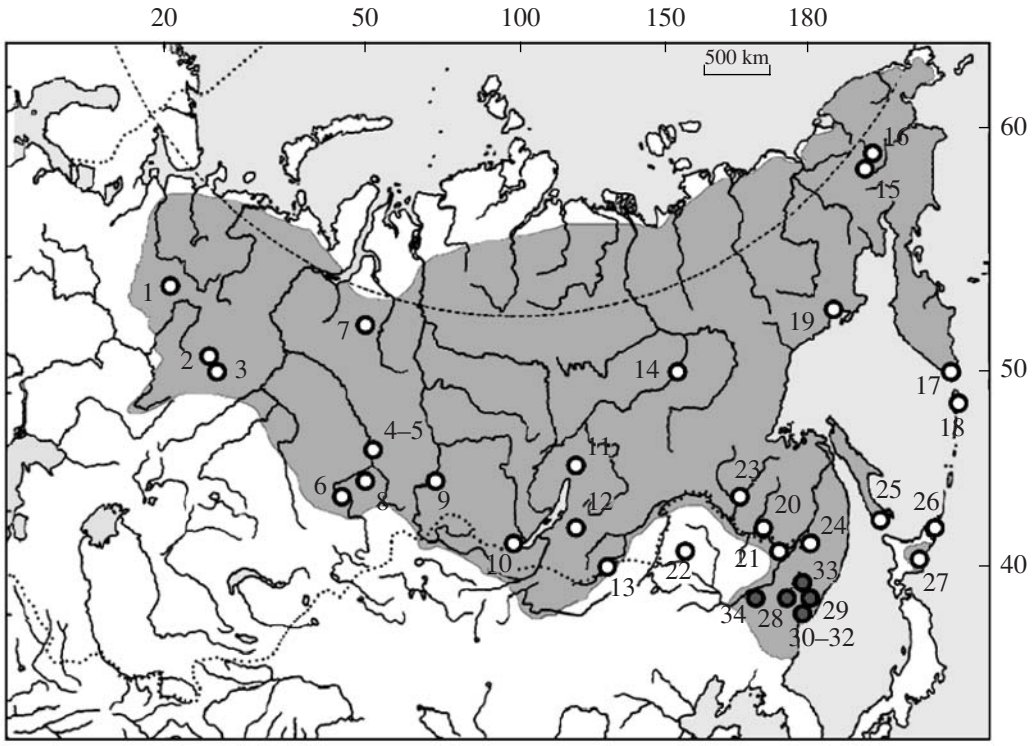

Fig. 1. Distribution of Siberian newt *Salamandrella keyserlingii* and the positions of the populations under study in the species range. Population numbers corresponds to the site numbers given in the table. Populations of the Siberian and Primorye forms are shown with open and filled circles, respectively.

sequence of another individual of the same species was extracted from GenBank (NC 008079).

DNA isolation. We used muscle tissue (tail, limbs, and tongue) or the liver for DNA isolation. Total genomic DNA was isolated from frozen, fresh, or fixed (96% ethanol) tissue with SDS, proteinase K, and phenol–chloroform [13, 14]. In some cases, DNA was additionally isolated using a Diatom DNA-Prep kit (IsoGen, Russia). A 100-μl DNA preparation was obtained from each specimen. Total DNA was resolved by agarose gel electrophoresis in the presence of ethidium bromide and visualized in UV light along with positive and negative controls. The DNA concentration was measured spectrophotometrically by a standard method.

Polymerase chain reaction (PCR) and sequencing. A fragment of the cytochrome b gene was amplified with two modified primers, forward MVZ15 (5'-GAA CTA ATG GCC CAC ACA/T A/TTA CGT/A AAA/T-3') and reverse MVZ16 (5'-AAA TAG GAA A/GT/AA TTA T/CTC/T TGG TTT A/GA/GT-3'), which were based on the primers designed for lungless salamanders (family Plethodontidae) [15] and successfully employed in studies of salamanders of the genus *Batrachuperus* [16]. Amplification was performed with a Gene Pack kit (IsoGen) and *Taq* DNA polymerase. PCR was performed in a 20-μl reaction mixture and included initial denaturation at 92°C for 3 min; 35 cycles of denaturation at 92°C for 30 s, primer annealing at 45–49°C for 45 s, and elongation at 72°C for 60 s. The fragments of the cytochrome b gene were resolved by electrophoresis and visualized in UV light in the presence of positive and negative controls. The amplicon concentration was measured spectrophotometrically by a standard method. The PCR product was purified using a PCR purification kit (Millipore, United States). The cytochrome b gene fragment was sequenced in an ABI 377 automated sequencer (United States), using a Big-Dye Ready-Reaction kit (United States). Sequencing was always performed with the forward primer (MVZ15); when the resulting sequence was short, the reaction was repeated with the reverse primer (MVZ16).

DNA sequence analysis. The resulting sequences of the cytochrome b gene fragment were viewed using the Chromas v. 1.45 program and manually aligned using the BioEdit v. 5.0.9 program. Dendrograms were constructed by the neighbor-joining algorithm on the basis of Kimura's genetic distances [35], using the TreeCon v. 1.3b and TreeView v. 1.6.6 programs [17]. As an outgroup, we used the cytochrome b gene sequences of other Hynobiidae (*Hynobius leechii*, Chinese sala-

mander *Hynobius chinensis*, and Semirechensk salamander *Ranodon sibiricus*). The reliability of branches was estimated by bootstrap analysis with 1000 replicates. Phylogenetic analysis, including construction of cladograms by the maximum parsimony method [18], computation of pairwise genetic differences (*p*-distances), and estimation of the genetic diversity of the sample under study, was performed using the Mega 3 software package [19]. Median haplotype networks and a pairwise mismatch distribution were constructed using the Arlequin v. 2.000 (2000) and Network v. 4.1.1.2 (2004) programs. Analysis of the distribution of pairwise differences has been successfully used by other authors [20, 21]. This analysis is believed to report the past fluctuations of the population size [22]: an unimodal distribution suggests an increase in population size, while a polymodal distribution is interpreted as evidence for a long-term demographic stability of the population [12]. Although errors may arise because of the uneven character of samples, it is still possible to compare the pairwise mismatch distribution for individual phyletic lineages even when the samples involved differ in size. This analysis is advantageous for studying individual genealogical lineages or populations and has already been used with success to examine the specific demographic history of caudate amphibians [23].

The sequences observed in 173 *S. keyserlingii* individuals and corresponding to 63 haloptypes of the cytochrome b gene fragment were deposited in GenBank (http://www.ncbi.nlm.nih.gov/entrez) under accession nos. EU156965–EU157028.

RESULTS

The aligned fragment of the cytochrome b gene was 780 bp. In total, analysis of 173 newts from 34 samples revealed 63 haplotypes of the cytochrome b gene fragment. Of these, 44 were observed in the populations of Siberia and the island part of the species range (27 samples) and 19 were specific to the populations of the Russian Far East and the adjacent region of the People's Republic of Korea (seven samples). We did not detect stop codons, deletions, and insertions in the cytochrome b gene fragment. The overall mean distance for the total sample was $d = 0.0645$ (Kimura 2-parameter). The divergence of the gene fragment in the Primorye populations was substantially higher than in Siberian populations ($d = 0.0249$ vs. $d = 0.0138$, Kimura 2-parameter). The maximum intrapopulation haplotype divergence was observed in the populations of the

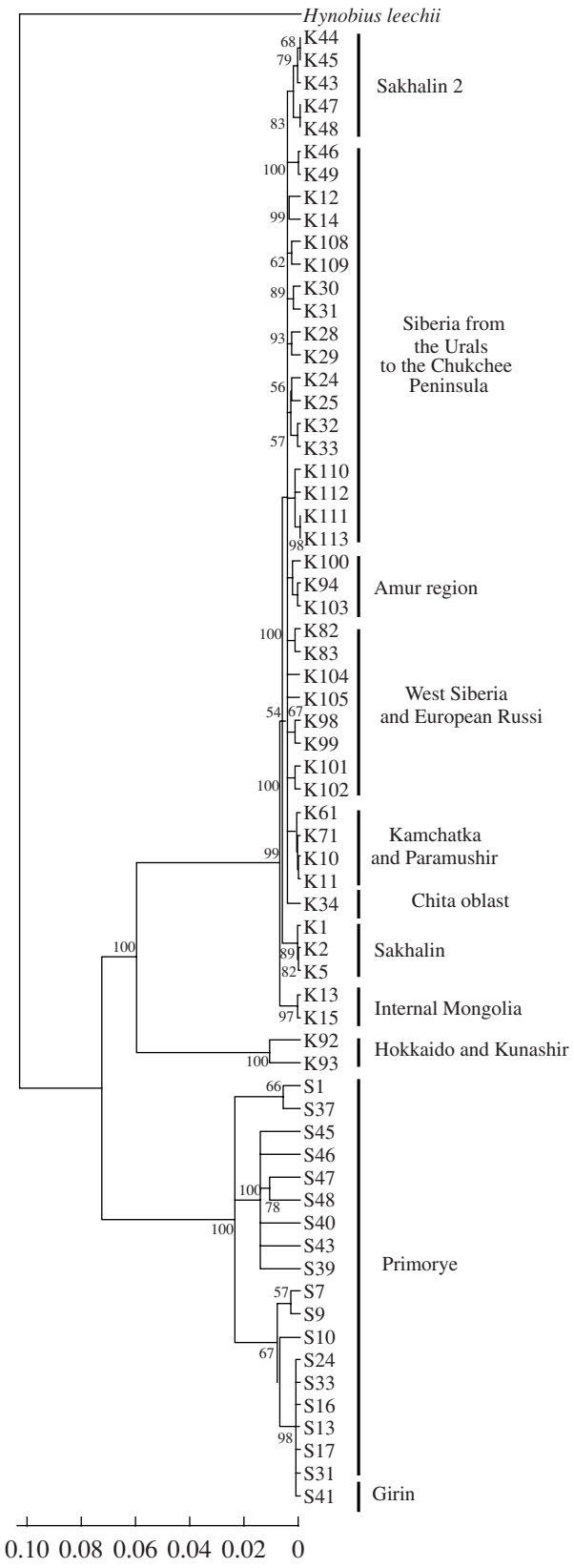

Fig. 2. Cladogram of the phylogenetic relationships among the 63 cytochrome b gene haplotypes observed in the *Salamandrella* samples. The cladogram was constructed by the neighbor-joining algorithm. Genetic distances were estimated according to Kimura [35]. Bootstrap support values exceeding 50% are shown.

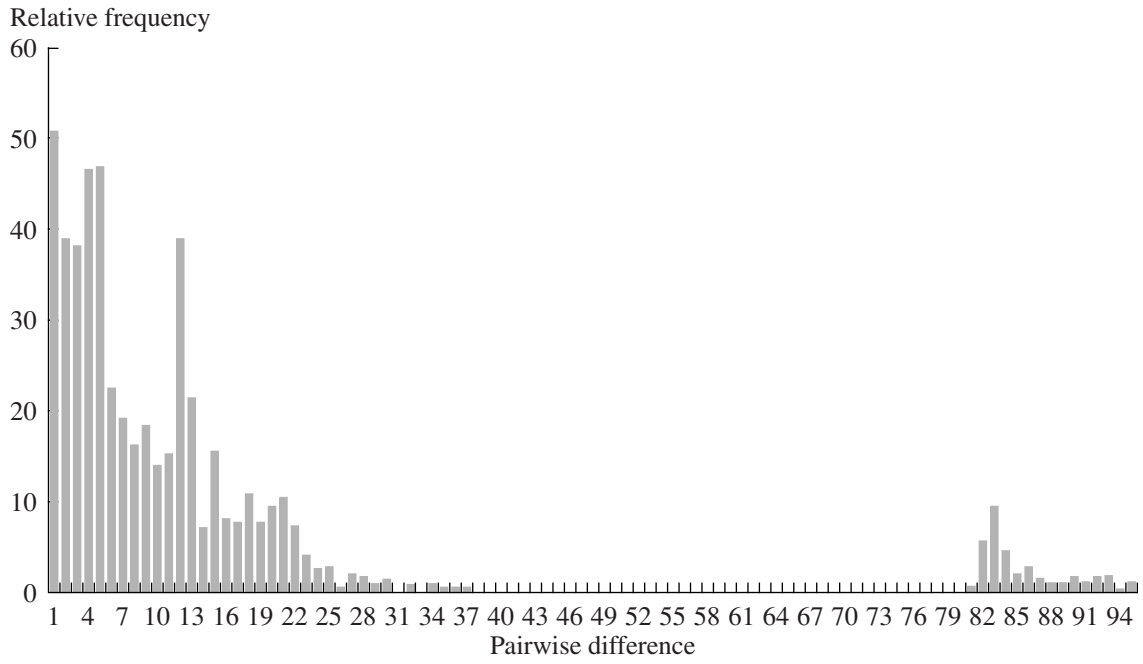

Fig. 3. Distribution of pairwise differences in the sequence of the cytochrome b gene fragment for the Siberian form of *S. keyserlingii.* The distribution is far from bell-shaped. The unweighted mean pairwise difference was 11.188.

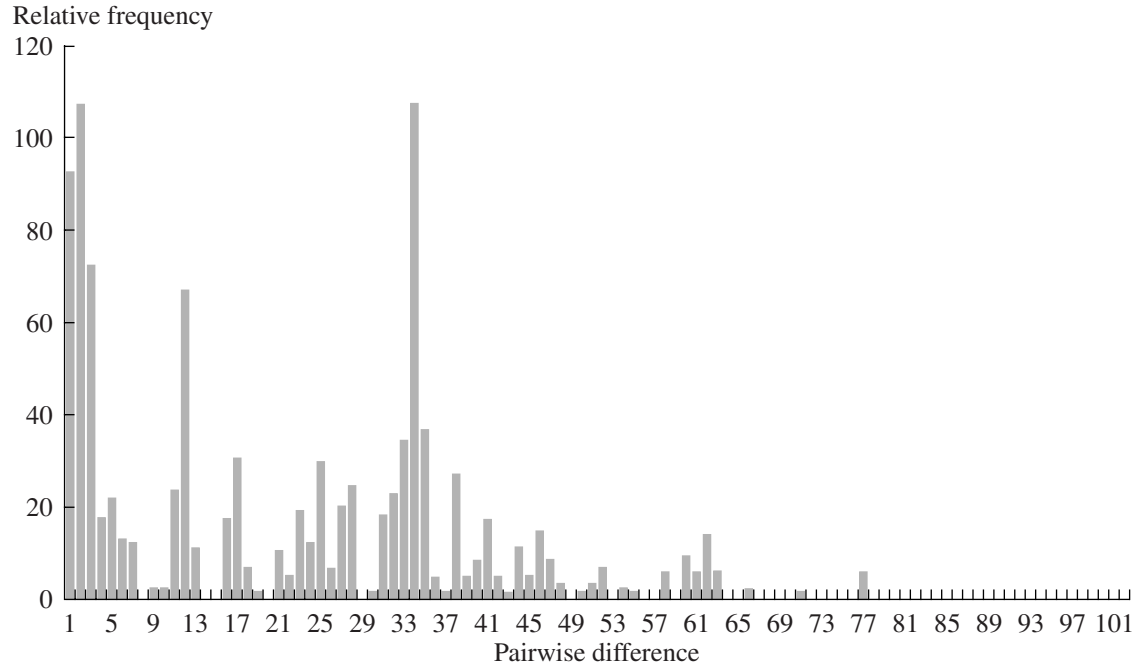

Fig. 4. Distribution of pairwise differences in the sequence of the cytochrome b gene fragment for the Primorye form of *S. keyserlingii.* The distribution has a distinct peak in region 31–36 (modal values of pairwise differences). The unweighted mean pairwise difference was 20.472.

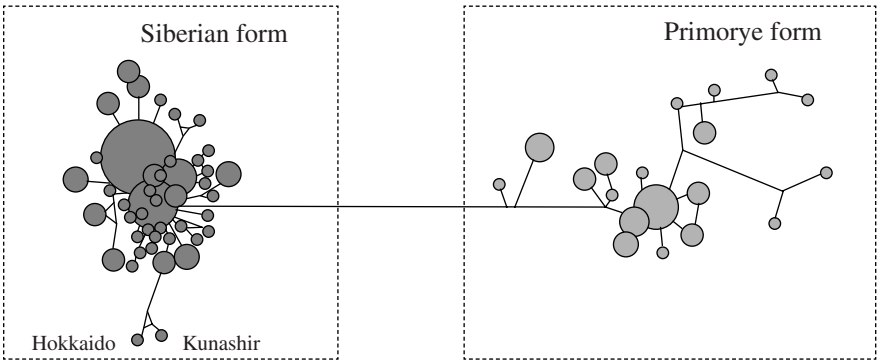

Fig. 5. Median-joining network of the haplotypes observed in *S. keyserlingii.* The haplotypes are shown with circles; the diameter reflects the number of newts having the given haplotype. The haplotype network reflects two, Primorye and Siberian, phyletic lineages of Siberian newt. The Primorye lineage includes several divergent haplotypes, while the Siberian lineage has a few central haplotypes, occurring at maximum frequencies, and several peripheral rare haplotypes. The populations of Hokkaido and the Kurils are distinctly separate.

Primorye form (d = 0.058), which agrees with the data that intrapopulation polymorphism of the cytochrome b gene haplotypes is extremely high in the Primorye form [10, 11].

The mean genetic distance between the Primorye and Siberian forms was rather high, d = 0.1338; the mean intrapopulation diversity was d = 0.0193 (Kimura 2-parameter).

The cladogram constructed (Fig. 2) was stable; its main branches had a high bootstrap support (BS > 75%). The *Salamandrella* haplotypes formed a monophyletic group including two clades, which were substantially separated from each other, were certainly monophyletic (BS = 100%), and corresponded to the Primorye and Siberian forms. The clade of the Primorye populations displayed a more distinct subdivision, including three haplotype clusters with a high bootstrap support.

In the clade of the Siberian form, most of the haplotypes found in the Siberian part of the species range grouped to form a poorly subdivided clade. The most basal position in this clade is occupied by a long branch combining the Hokkaido and Kunashir samples (BS = 100%). The next basal branch combined the samples from the southern and eastern populations (Sakhalin, internal Mongolia, and Chita oblast). The other haplotypes of the Siberian form were highly monomorphic and formed a poorly subdivided branch. Some geographically close populations formed clades with a low bootstrap support. For instance, this was observed for the populations of Paramushir and Kamchatka or those of the Amur region (Evreiskaya Autonomous District, Khabarovsk krai, and Amurskaya oblast).

Geographical differentiation of the populations of the Siberian form displayed no clinal variation and was irregular; the distance between the most geographically remote marginal populations was surprisingly low. For instance, one group included the samples from Kirovskaya oblast, Khanty-Mansiiskii Autonomous District, the Sakha Republic (Yakutia), the Chukchee Peninsula, and Kamchatka. The intrapopulation haplotype variation in the populations close to the northern boundary of the species range was appreciably lower: d= 0.0049 (Kimura 2-parameter).

The distribution of pairwise differences in the sequence of the cytochrome b gene fragment clearly differed between the Siberian and Primorye forms. The distribution obtained for the Siberian form was distinctly unimodal and far from bell-shaped; the maximum frequencies were observed for comparisons with low differences between haplotypes (Fig. 3). The unweighted mean pairwise difference between sequences wa estimated at 11.188. In the case of the Primorye form, the distribution of pairwise differences was not unimodal but had several distinct peaks in the regions of pairwise differences of 1, 11, 16, 26, 31–36, and 61, corresponding to different modal values of pairwise differences (Fig. 4). The unweighted mean pairwise difference was 20.472; i.e., a shift toward a greater difference between haplotypes was observed as compared with the Siberian form.

Median-joining network analysis illustrates how some haplotypes originate from others. A median network of the haplotypes found in Siberian newt is shown in Fig. 5. The haplotypes of the Siberian and Primorye forms had two distinct centers and principally different branching patterns.

In the case of the Primorye form, several haplotypes were clearly separate from each other, formed a series, and lacked a distinct center. Most of the haplotypes occurred at comparable frequencies. In the case of the Siberian form, a few haplotypes had extremely high frequencies and formed a median network center, which gave origin to all other haplotypes. A distinct

separation was observed only for the Hokkaido and Kunashir populations. Most haplotypes occurred at substantially lower frequencies as compared with the central haplotypes.

DISCUSSION

Our findings support the conclusion that the Primorye geographical population of Siberian newt is rather separate from the other populations [9–11]. Note, however, that the restoration of the name *schrenckii* for the Primorye population [10] generates a taxonomic collision, because the original description of *Isodactylium schrenckii* Strauch, 1870 states that this species had earlier been described as *Salamandrella keyserlingii* Dybowski, 1870. Hence, *I. schrenckii* has been published as a junior synonym of *S. keyselringii*. In this case, it is illegitimate to use the name *schrenckii* for the Primorye population according to the International Code of Zoological Nomenclature (2004).

The phylogenetic relationships in the family Hynobiidae have recently been studied using the complete mitochondrial genome sequences of animals representing the majority of the genera of the family [24]. However, the data on molecular differentiation of Hynobiidae at the specific and intraspecific levels have not been summarized as of yet. Our preliminary findings indicate that intraspecific differentiation of several forms of the family Hynobiidae is rather high (interpopulation and intrapopulation genetic distances d exceed 0.05). Berman et al. [10] have also observed extremely high genetic distances between haplotypes found in one population of the Primorye form of Siberian newt. These distances correspond to or even exceed the interspecific distances reported for caudate amphibians of the family Salamandridae. This agrees with the hypothesis that genetic differentiation proceeds at different rates in different phyletic groups of the family Hynobiidae. A level of differences that would correspond to the specific differentiation level and would be universal for the total family Hynobiidae has not been established. A reliable molecular clock calibration is also unavailable for the family. The molecular evolution rate estimates that are based on the divergence of three *Euproctus* species and are broadly used in studies of Salamandridae [11, 25] are inapplicable to Hynobiidae, because the family Salamandridae belongs to another branch of Caudata. Recent data suggest that the genus *Euproctus* is polyphyletic [26], which complicates the interpretation of molecular differentiation among various groups of Caudata on the basis of published estimates [25].

Molecular differentiation between the Primorye and Siberian forms of *Salamandrella keyserlingii* is extremely high and exceeds that observed in many species of the genus *Hynobius*. Intermediate haplotypes have not been found as of yet in our and other works, suggesting early differentiation. These data support the species status of the Primorye form of Siberian newt according to the current views of the molecular phylogeny and taxonomy of caudate amphibians [27].

However, we think it necessary to use the biological concept of species to verify this status. For instance, the species status has been proposed for the northern and southern forms of the marbled newt *Triturus marmoratus* (Salamandridae) on the basis of their substantial molecular differentiation [28]. However, the samples have been collected in Spain, where the two forms are geographically isolated from each other. The region of contact between the two forms has been studied more recently in Portugal, and the gene flow proved to be constricted to sympatric populations in both forms, providing evidence for their species status [29].

Barriers between the Primorye and Siberian forms of Siberian newt are unknown. Since the two forms may have a parapatric distribution, it is necessary to study a possible gene flow between their marginal populations.

It is of interest that the Primorye haplotypes have been observed in populations of Khabarovsk krai [10, 11], in the vicinity of populations carrying Siberian haplotypes (our data). This finding suggests a contact region between the two forms for the middle and lower stretches of the Ussuri River. It cannot be excluded that hybridization of the two forms have led to a partial introgression of mitochondrial haplotypes. After this paper had been submitted, a Siberian newt population with egg batches morphologically similar to typical batches of both forms was found (Berman, personal communication). This finding suggests sympatry for the Siberian and Primorye forms and a contact region between them.

Thus, the species status of the Primorye form remains an open question. Its detail analysis is beyond the scope of this paper. To clarify the status of the Primorye form of Siberian newt, it is necessary to further study the contact region between the two forms and to estimate the gene flow between the phyletic branches.

Our results indicate that differentiation between the two Siberian newt clades is high and suggests a pre-Pleistocene separation of the Siberian and Primorye forms. The Primorye form is genetically more variable than the Siberian form (2.49 and 1.38%, respectively). This result agrees with the published data [10, 11]. The high levels of intrapopulation and interpopulation polymorphism in the samples from Primorye indicate that the local populations of southern Primorye existed for a long time in demographically stable conditions, which is supported by the polymodal character of the pairwise difference distribution (Fig. 4). The same is also evident from the median network (Fig. 5); i.e., the presence of several haplotypes substantially separated from each other in the Primorye clade suggests a long-term differentiation of individual populations in local microrefugia. The unique history, high biodiversity, and biogeographical specifics of Primorye determine the relic character of this refugium. Of all continental terri-

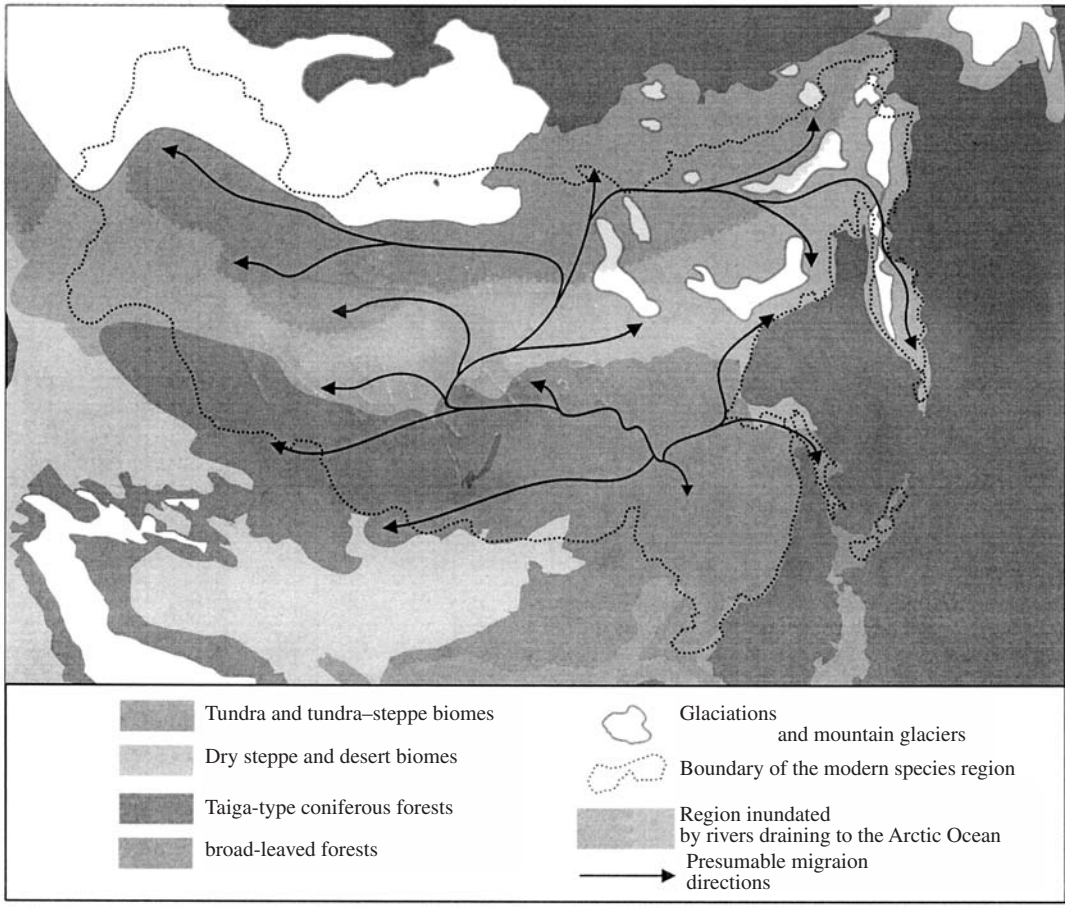

Fig. 6. Modern range of *Salamandrella* and possible pathways of colonization of the territory by the Siberian form of *S. keyserlingii* at the end of the past glacial period (25000 years ago). The margins of habitats unsuitable for Siberian newt (glaciations and tundra–steppe biomes), the region of inundation due to hindered drainage of northern rives to the Arctic Ocean, and the presumable migration pathways are shown.

tories of eastern Russia, Primorye was least affected by Pleistocene glaciations and preserved the autochthonous batrachofauna. Highlands facilitated the isolation of individual populations and the formation of high interpopulation diversity.

The modern distribution of the species suggests at least two autochthonous centers for the formation of the phyletic branches of Siberian newt. One center probably corresponds to southern Primorye and the adjacent northern Korean and southern Manchu territories, giving origin to the Primorye form. It is unclear where and when the ancestral Siberian newt population was divided. This event might be associated with gradually increasing aridity and climatic changes in continental Manchuria during the Pleistocene.

Presumably, the territory between the Khingan Ridge and the interfluve of the rivers Amur, Sungari, and Ussuri was the place where ancestral populations were formed to give origin to the branch that colonized the main part of the modern species range, that is, to the Siberian form. The low haplotype diversity of Siberian form populations from the main part of the species range suggests that the population size dramatically decreased and then rapidly increased again. In contrast to the Primorye form, the Siberian form displays a strongly unimodal distribution of pairwise differences with a distribution mode substantially shifted toward lower differences (Fig. 3). Such a distribution suggests a recent expansion and a consequent rapid increase in population size [12, 22]. This assumption is supported by analysis of the median haplotype network (Fig. 5). The network structure is indistinct: the network includes three central haplotypes, which occur at maximum frequencies, and many single or extremely rare haplotypes, which differ from the central haplotypes by several substitutions. The most distant haplotypes were found in the populations of internal Mongolia, Sakhalin, and Chita oblast.

Note that the island populations of Siberian newt from Hokkaido and the southern Kurils are separated

from the others. These populations form a cluster of closely related haplotypes. The cluster certainly belongs to the Siberian form but is rather distant from others (a long branch). This pattern indicates that the islands were colonized long ago from the mainland (probably, via Sakhalin) and that their populations were then separated. The isolation and small sizes of the island populations might facilitate an accumulation of mutations. We observed that these populations are genetically separated to a substantial extent from the other populations of the Siberian clade, which is associated with their biochorological specifics [30].

Thus, our data on molecular differentiation of the two Siberian newt forms suggest fundamentally different demographic histories for the corresponding phyletic lineages. The Primorye form is characterized by long-term demographic stability, a small range, and high divergence between individual genotypes. In contrast, the Siberian form inhabits a vast area; its gene pool is obviously homogenous, suggesting a bottleneck; and only the island populations display a certain extent of separation. The distribution of pairwise differences suggests a recent rapid increase in population size, and the vast area suggests rapid colonization. The pathways and mechanisms of this process are the most interesting issues in this context.

The relationships of the centers of the formation of the two *S. keyserlingii* branches are unclear. Possibly, these centers correspond to the centers of the formation of the north Palearctic taiga belt [31]. Since the species inhabits a vast territory, its low morphological polymorphism and the observed pattern of molecular differentiation indicates that most of the species range was colonized within a relatively short time. Moreover, a relatively low haplotype and nucleotide diversity of the Siberian form suggests a bottleneck; i.e., the population size was dramatically reduced, which possibly led to a sorting of genealogical lineages so that only a few closely related haplotypes were preserved. This event might be associated with the climatic fluctuations of the Pleistocene, when the northern hemisphere was periodically covered with glaciers. During the last glacial maximum, a main part of the modern species range included cold and dry habitats, such as tundra and tundra-steppe regions, and Siberia was covered by many local, especially highland, glaciations (e.g., see [32]), which prevented amphibians from crossing watersheds. On the other hand, fluctuations of the global sea level produced dry pathways to islands, allowing their colonization. During the past 250 000 years, Siberia experienced at least three critical cold spell periods with a decrease in the global sea level and about 19 local drops of temperature [32]. The observed differentiation of Siberian populations probably results from repetitive stages of expansion through new territories in the interglacial periods and a subsequent retreat to moderate climatic regions during the next glaciation.

If so, it is possible to assume that populations of the south Siberian part of the species range (Mongolia and, apparently, north Manchuria) were least affected by glaciations. In view of the unique temperature adaptations of the Siberian form, it is possible to assume that the southern margin of the tundra and tundra–steppe belt was gradually colonized during a main part of the Pleistocene. This assumption is supported by the cladogram topology; i.e., the most basal haplotype branches of the Siberian clade seem to correspond to the two primary pathways of Pleistocene colonization. One (western) pathway led to colonization of Siberia, while the other (eastern) one led to colonization of Sakhalin.

It is noteworthy that two different haplotype lineages were found in the Sakhalin population. The haplotypes of newts from northern Sakhalin reliably grouped with Siberian haplotypes, while the haplotypes observed in southern Sakhalin occupied a more basal position in the Siberian clade. Theoretically, Sakhalin, Hokkaido, and the southern Kuril islands could be colonized at different periods, since the islands were more than once bridged to the mainland during the past two million years. Apparently, newts migrated to Hokkaido and, then, to the southern Kuril islands from Sakhalin. It is possible that their populations were isolated from the Sakhalin geographical populations earlier than Sakhalin was separated from the mainland. This scenario would explain why the Sakhalin population genetically differs from the Hokkaido and south Kuril populations to a greater extent than from the mainland populations.

The most part of the species range, including northern and central Siberia and, probably, western regions, was colonized after the last glacial period (within the past 25 000 years). The haplotype diversity in north Siberia is extremely low, suggesting rapid colonization of this region in the last interglacial period. Following Hewitt [21], colonization of new territories by a species involves a series of bottlenecks, since the populations inhabiting the edge of the species range continuously experience the effect of isolation because gene exchange with the main part of the range is hindered. This phenomenon, known as the leading edge effect, provides an appealing hypothesis to explain the extremely low genetic diversity of a species expanding during an interglacial period, in our case, Siberian newt expanding northwards and westwards.

It is possible that, after the peak of the past glaciation, Siberian newt first colonized eastern Siberia and that colonization further proceeded in two directions, westwards (to the Urals) and eastwards (to Beringia and Kamchatka, including Paramushir in the north Kurils) (Fig. 6). Since Siberian newt has a low mobility, it is important to understand the mechanisms of its expansion through a vast area in a short time. An important role in expansion could be played by passive distribution; i.e., adults, larvae, or egg sacks possibly moved

northwards with floods and rivers. Siberian newt is found predominantly in river valleys at the northern margin of its species range [2]. Note, moreover, that the drainage of Siberian rivers to the Arctic Ocean was hindered after the peak of the past glaciation [32], which generated what is known as the Giant Siberian Lake, a system of marshes and lakes at the southern edge of the glacier in Europe, West Siberia, and the northern region of East Siberia. We believe that this circumstance substantially promoted a rapid expansion of Siberian newt from the southern regions of East Siberia northwards and through Arctic regions.

The differentiation patterns of various vertebrates in Siberia agree with our assumption. For instance, tundra shrew *Sorex tundrensis* occupies a vast Siberian area and has three main phyletic branches, only one of which probably colonized the Arctic region of Siberia (A.A. Bannikova, personal communication). Stepwise northward expansion has been demonstrated for the Baikal chromosome race of common shrew *Sorex araneus*, which most likely migrated from the Baikal region northwards and colonized East Siberia [34].

To summarize, the molecular variation of *S. keyserlingii* supports the hypothesis that two primary vicarious refugia of pre-Pleistocene differentiation of a common ancestor of *Salamandrella* occurred in the southeastern part of its current distribution range and that a main part of the modern *S. keyserlingii* range was colonized by the Siberian form. Pacific islands were probably colonized in several steps, which was associated with repeated steps of expansion and retreat in the Siberian part of the modern species range. The northern and western regions were colonized at an extremely high rate, which could be explained by a contribution of passive expansion factors. Colonization was accompanied by a dramatic increase in population size and a drop of genetic diversity, which was possibly due to the leading edge effect.

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