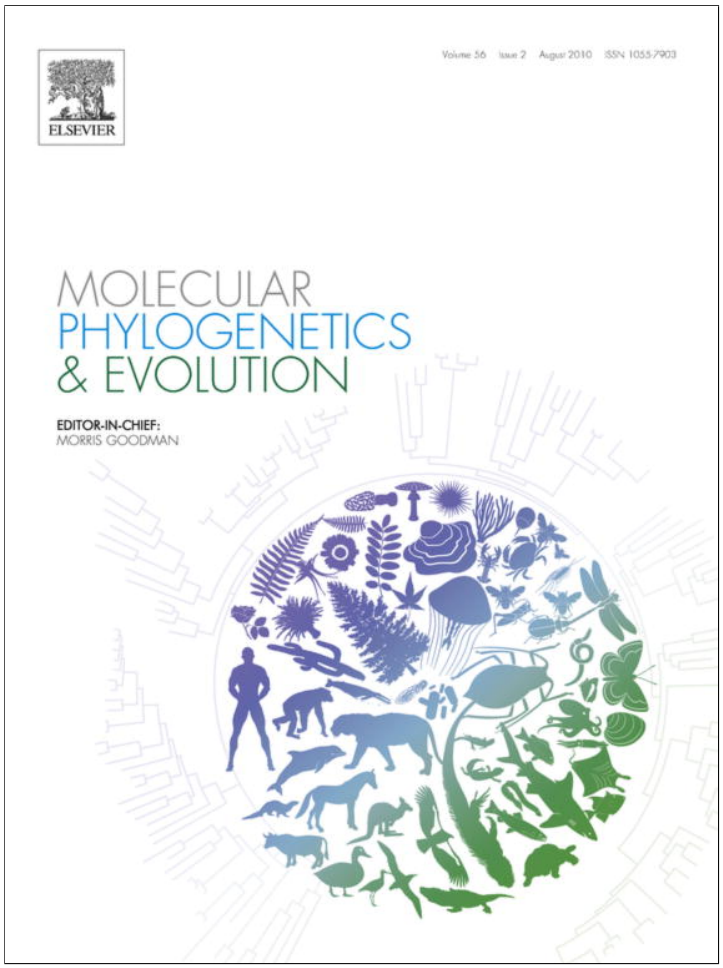

Molecular Phylogenetics and Evolution 56 (2010) 562–571

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

# Phylogeography and molecular adaptation of Siberian salamander *Salamandrella keyserlingii* based on mitochondrial DNA variation

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ARTICLE INFO

*Article history:*
Received 8 September 2009
Revised 6 April 2010
Accepted 6 April 2010
Available online 14 April 2010

*Keywords:*
Mitochondrial DNA
Cytochrome *b*
*Salamandrella keyserlingii*
Molecular phylogeography
Molecular adaptation

ABSTRACT

We assessed the phylogeographic pattern of Siberian salamander (*Salamandrella keyserlingii*, Dybowski, 1870), which appear to be the most northern ectothermic, terrestrial vertebrate in Northern Eurasia, by sequence analysis of a 611-bp fragment of the mitochondrial cytochrome *b* gene in 159 specimens from different localities (Khabarovsk region, Sakhalin, Yakutia, Magadan region, Chukotka, Kamchatka and others). The data revealed that cytochrome *b* lineages of *S. keyserlingii* are divided into haplogroups A, B and C. Haplogroup A and B sequences are widespread in the Far East region, whereas haplogroup C consisting of several phylogenetic clusters (C1, C2, C3) is present in the all range of *S. keyserlingii*. Among them, cluster C3 appears to be specific for Sakhalin; most likely, it has arisen *in situ* in this island, with the entry time of the founder mtDNA estimated at about 0.4 MY. Analysis of cytochrome *b* gene variation by using different neutrality tests (including those based on $K_A/K_S$-ratio) has shown that differences between haplogroups were statistically insignificant, thus suggesting selective neutrality. However, analysis of amino acid changes allowed us to detect a signature of molecular adaptation, which might have led to appearance of adaptive cytochrome *b* variants in haplogroup C, originating most likely at the end of Eopleistocene (about 0.64 MY based on the haplogroup C divergence level). It seems probable that this adaptive mechanism could promote subsequent populating of new regions.



## 1. Introduction

Siberian salamander (*Salamandrella keyserlingii* Dybowski, 1870) has the widest among amphibians geographic range in Eurasia, extending from Hokkaido in the east to the North-East of Europe in the west and from Chukotkan peninsula in the north to northern China in the south (Borkin et al., 1984). It has been recently found by means of analysis of mitochondrial DNA (mtDNA) cytochrome *b* gene variation that population of *S. keyserlingii* appears to be relatively homogeneous (Berman et al., 2005; Matsui et al., 2008; Poyarkov and Kuzmin, 2008). Substantial differentiation of *S. keyserlingii* was found only within Sakhalin population and between Sakhalin and Hokkaido (Matsui et al., 2008). Therefore, it has been suggested that the history of divergence of *S. keyserlingii* in the continent may be much younger than that within Sakhalin and that it is probable that some Sakhalin populations expanded their ranges in the continent in the late Pleistocene. Meanwhile, phylogeographic structure of *S. keyserlingii* in the neighboring regions of continental Asia is very poorly investigated. In addition, although nucleotide sequences of cytochrome *b* gene have been extensively used in phylogenetic studies, little attention has been devoted to the study of molecular adaptation on the protein level (Irwin et al., 1991; McClellan and McCracken, 2001; Fink et al., 2004; McClellan et al., 2005; da Fonseca et al., 2008; Willerslev et al., 2009). Cytochrome *b* in animals is composed of three functional domains: the intermembrane domain generated by four loops of the redox center $Q_o$, transmembrane domain composed by eight helices, and the matrix domain with the redox center $Q_i$ (Irwin et al., 1991; Degli Esposti et al., 1993; Zhang et al., 1998). The intermembrane domain has involved in the creation of a proton gradient and the transfer of electrons to the cytochrome *c* protein. It was shown that this domain evolves extremely slowly and appears to be very important for maintaining the mitochondrial bioenergetics (Howell, 1989; Irwin et al., 1991; Darrouzet et al., 2000; McClellan et al., 2005; Bell et al., 2007; da Fonseca et al., 2008).

To obtain more detailed information on phylogeographic structure of Siberian salamander, in context of molecular adaptation of this species, we present here new data on cytochrome *b* gene variation in populations of the Russian Far East, including the most extreme (considering climatic conditions) North-Eastern part of Asia.

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doi:10.1016/j.ympev.2010.04.005

## 2. Materials and methods

### 2.1. Samples collection, DNA extraction, PCR amplification and sequencing

We examined a total of 159 Siberian salamanders from 14 different regions of Eurasia (Table 1 and Table S1). Material was collected during the springs and summers of 2004–2008. Genomic DNA was isolated from various tissues of adults, larvae, and embryos, which were frozen or fixed with 70% ethanol. DNA was extracted by the standard method involving tissue lysis in a solution containing 100 mM Tris–HCl (pH 8.0), 10 mM EDTA, 100 mM NaCl, 1% sodium dodecylsulfate, and 0.2 mg/ml proteinase K (Sigma, USA) at 56 °C for 12–16 h, with subsequent phenol–chloroform deproteinization.

A segment (640 bp) of the cytochrome *b* gene was amplified by polymerase chain reaction (PCR) with the pair of primers MVZ15L and MVZ18H, proposed by Goebel et al. (1999). DNA was sequenced in the ABI 3130 genetic analyzer, using BigDye Terminator v. 3.1 cycle sequencing kit (Applied Biosystems) and primer MVZ15L. The nucleotide sequences gathered in the course of this study for the 611-bp region of *S. keyserlingii* cytochrome *b* gene were deposited in GenBank (Benson et al., 2010) under Accession Nos. GQ849138–GQ849190 (Table S1).

### 2.2. Data analysis

To reconstruct the tree topology, a neighbor-joining (NJ) method was used as implemented in MEGA 3.1 (Kumar et al., 2004). Maximum likelihood (ML) analysis was performed using PHYML v.3.0 (Guindon and Gascuel, 2003) with the General Time Reversible (GTR) model (Rodriguez et al., 1990). The confidence of branches in NJ and ML trees was assessed using non-parametric bootstrapping searches of 1000 replicates (Felsenstein and Kishino, 1993). Topologies of NJ and ML trees with bootstrap values 70% or greater were regarded as sufficiently resolved (Huelsenbeck and Hillis, 1993), and those between 50% and 70% as weakly supported.

Bayesian inference (BI) of phylogeny using the Markov Chain Monte Carlo technique (MCMC) was done with the BEAST v.1.4.7 (Drummond and Rambaut, 2007) with the GTR+I+G model of DNA substitution. We initiated three independent analyses with a random starting tree that ran for 20 million generations. We conservatively discarded the first two million generations from each run as "burn-in" and sampled one of every 1000 generations from the remaining 18 million generations to calculate posterior probabilities for each branch in the Bayesian tree. Posterior probabilities 95% or greater were considered significant support (Leache and Reeder, 2002). As an outgroup, we used the cytochrome *b* gene sequence of Schrenck Siberian salamander, *Salamandrella schrenckii* (Berman et al., 2005).

Maximum parsimony (MP) median networks were constructed by the median-joining (MJ) algorithm, using the Network 4.5 package (www.fluxus-engineering.com). Optimal trees were constructed using the MP Calculation option. Cladistic nomenclature for classification of mtDNA phylogenetic clusters of haplotypes (haplogroups) was used (Macaulay et al., 1999). The extent of mtDNA divergence was estimated using the $\rho$ distance, which corresponds to the averaged distance of the haplotypes within a phylogenetic cluster from the respective root haplotype (Bandelt et al., 1999). To estimate the time of divergence for a pairwise comparison of the clades, we calculated also the uncorrected percent sequence divergences (*p* distances) between samples in MEGA 3.1. We used the divergence rate of 0.64% per million years as suggested by Weisrock et al. (2001).

Haplotype and nucleotide diversity indices and their variances within populations were calculated using Arlequin 3.1 software package (Excoffier et al., 2005) and DnaSP version 5.0 (Librado and Rozas, 2009). We used the frequency distribution of the number of pairwise differences between haplotypes (the mismatch distribution) to estimate the timing of population expansion (Rogers, 1995).

The ratio of the number of nonsynonymous substitutions per nonsynonymous sites ($K_A$) to the number of synonymous substitutions per synonymous sites ($K_S$) indicates the level of selection against nonsynonymous substitutions relative to synonymous ones. $K_A/K_S$ distributions in pairwise comparisons between DNA sequences were calculated (Nei and Gojobori, 1986) using DnaSP 5.0. In the presence of positive selection, $K_A$ is higher than $K_S$ (Nielsen,

**Table 1**
Distribution of cytochrome *b* gene haplotypes of *S. keyserlingii* in Eurasian populations.

| Population | Haplogroups and haplotypes | | | | | |
|---|---|---|---|---|---|---|
| | A | B | C1 | C1a | C2 | C3 |
| Khabarovsk (17, 18, 19, 20) | k1 (1), k3 (4), k5 (2), k6 (1), k24 (1) | k19 (1), k20 (2), k21 (1), k68 (4), k79 (1) | k7 (9), k25 (1), k26 (2), k55 (1), k77 (2), k78 (1) | k23 (1), k39 (4), k40 (1), k41 (3), k42 (1), k43 (1), k52 (1), k53 (1) | k17 (1), k49 (18), k50 (1), k56 (1) | |
| Jewish autonomy (16) | k2 (1), k3 (1), k5 (1), k36 (1) | | k7 (2), k30 (1), k35 (1), k44 (1), k45 (1), k46 (2) | k34 (3) | k47 (1), k48 (1) | |
| Amur (21, 22, 23, 24) | | | k7 (2), k27 (1), k28 (1), k29 (1), k32 (1), k33 (1) | | | |
| Sakhalin (25) | k71 (1) | k18 (1), k68 (2), k75 (1) | k73 (1) | | k49 (1) | k11 (2), k12 (2), k13 (2), k13 (46), k14 (14), k15 (1), k16 (2), k57 (2), k58 (2), k59 (1), k60 (2), k61 (4), k62 (1), k63 (1), k64 (1), k65 (1), k66 (1), k67 (3), k70 (1), k72 (1), k76 (1) |
| Baikal (4, 5) | | | k7 (2) | | | |
| China | | | k37 (1), k38 (1) | | | |
| Tomsk (3) | | | k7 (5) | | | |
| Sverdlovsk (2) | | | k7 (8) | | | |
| Arkhangelsk (1) | | | k31 (2) | | | |
| Yakutia (6) | | | k7 (9), k10 (15) | | | |
| Magadan (7, 8, 11, 12) | | | k7 (24), k8 (2), k10 (12) | | | |
| Chukotka (9, 10, 13, 14) | | | k10 (3) | | | |
| Kamchatka (15) | | | k7 (2) | | | |

*Note:* Population location on the map (Fig. 5) is shown in parentheses. Number of specimens with certain haplotype is indicated in parentheses.

2001). The closer the $K_A/K_S$ is to 1, the weaker is the purifying selection.

Neutrality test by Elson et al. (2004) was performed manually to compare the ratio of the numbers of synonymous (S) and nonsynonymous (NS) substitutions in DNA sequences stratified into two classes on the basis of median-network analysis. The first class includes polymorphisms associated with haplogroups; each of these substitutions defines a subclade comprising of at least two mtDNA sequences. The second class concerns private polymorphisms; these substitutions occur at the tips of individual branches within a network.

By means of DnaSP application the McDonald–Kreitman test was performed for comparison of between-species divergence ($D$) and within-species polymorphism ($P$) at nonsynonymous ($D_A$, $P_A$) and synonymous ($D_S$, $P_S$) sites to infer adaptive protein evolution (McDonald and Kreitman, 1991). For between-species divergence estimates cytochrome *b* sequences of *S. schrenckii* (Malyarchuk et al., 2009) and *Onychodactylus fischeri* (Yoshikawa et al., 2008) were used.

Protein secondary-structure modeling for the cytochrome *b* sequences in different haplogroups of Siberian and Schrenck Siberian salamanders was performed using the TMpred software (www.ch.embnet.org). Amino acids with negative Kyte–Doolittle hydropathy index (Kyte and Doolittle, 1982) were defined as hydrophilic, and those with positive index as hydrophobic. To measure amino acid dissimilarity, we also used an average physicochemical distance taken from Grantham's matrix (Grantham, 1974). In addition, significant physicochemical amino acid changes among residues in mitochondrial protein coding genes were identified by the algorithm implemented in TreeSAAP 3.2 (Woolley et al., 2003).

The conservation index (CI) that is the percentage of species that have the consensus type of certain amino acid in cytochrome *b* sequence was calculated. The 27 different species of the family Hynobiidae used to calculate the conservation index are: *S. schrenckii*, *Batrachuperus tibetanus, B. yenyuanensis, B. taibaiensis, B. karlschmidti, B. pinchonii, B. londongensis, Pseudohynobius tsinpaensis, P. flavomaculatus, Hynobius chinensis, H. yiwuensis, H. amjiensis, H. leechii, H. formosanus, H. fuca, H. nebulosus, H. naevius, H. arisanensis, H. sonani, H. glacialis, H. quelpartensis, Liua shihi, Ranodon sibiricus, O. fischeri, Euproctus platycephalus, E. montanus, E. asper*. Nucleotide sequences of the cytochrome *b* gene of these species were extracted from GenBank (www.ncbi.nih.gov/entrez).

## 3. Results

### 3.1. Cytochrome b gene variability and phylogeography of S. keyserlingii

Cytochrome *b* gene sequences of a 611 bp region located between nucleotide positions 14,259 and 14,869 according to complete mtDNA sequence of *S. keyserlingii* (Genbank Accession No. DQ333814 (Zhang et al., 2006)) were analyzed. Analysis was performed in a total of 290 samples from different regions of Eurasia. Among them, 58 sequences were previously published by Berman et al. (2005) and 73 sequences were presented by Matsui et al. (2008). In total, 73 cytochrome *b* gene haplotypes defined by 83 nucleotide substitutions were revealed (Table 1).

Bayesian phylogeny (Fig. 1) appears poorly structured, with many unsupported or moderately supported grouping. Nevertheless, the tree comprises three highly supported clades, each of them comprising samples collected from a geographically distinct area—haplogroups A (100%), B (96%) and C (100%). Haplogroups A and B exclusively consist of haplotypes found among the Far Eastern Siberian Salamanders, whereas haplogroup C is present in the all range of *S. keyserlingii*. In the NJ analysis, statistical support for these haplogroups is less apparent being found at the level of 89% for A, 75% for B and 61% for C haplogroups (Fig. S1). However, in the ML tree (data not shown) bootstrap support was high only for haplogroup C (97%). In addition, both BI and NJ trees demonstrate that haplogroups A and B, as well as haplotype k69 detected by Matsui et al. (2008) in Hokkaido, are combined in the phylogenetic cluster labeled as AB in the present study. Although statistical support for such grouping is weak (61% in BI and 30% in NJ analyses), monophyly of haplogroups A and B and Hokkaido-specific haplotype k69 seems quite possible taking into account their geographic proximity.

Median-network analysis designed for the reconstruction of all possible MP trees from a given data set also allowed the identification of haplogroup AB, inclusive Hokkaido-specific haplotype k69, and large haplogroup C characterized by substantial substructure in the form of subhaplogroups (shg) C1, C2 and C3 (Figs. 2 and 3). The range of haplogroup AB (44 specimens, 16 haplotypes including Hokkaido-specific k69) appears to be restricted to Khabarovsk and neighboring Amur and Jewish autonomous regions as well as Sakhalin and Hokkaido (Table 1 and Fig. 4). The nucleotide diversity ($\pi$) within haplogroup AB was significantly higher than within haplogroup C (1.19% versus 0.38%) (Table 2). In the median network, haplogroups AB and C are also considerably distant, with the $\rho$ distance equal to 4.75 ± 2.12 substitutions. Application of the evolutionary rate for Hynobiidae (0.64% per million years (MY) per lineage) suggests that the age of mitochondrial gene pool of *S. keyserlingii* is about 1.6 MY ($\rho$ = 6.30 ± 1.76). Coalescence time estimates for haplogroups AB and C are about 1.25 MY ($\rho$ = 4.89 ± 1.19) and 0.64 MY ($\rho$ = 2.52 ± 1.12), respectively. Based on the uncorrected percent sequence divergences (as it implemented in MEGA 3.1), the divergence time was 1.5 MY between haplogroups AB and C ($p$ distance = 1.87%).

Despite the low within-group diversity (Table 2), geographic distribution of haplogroup C (246 specimens, 57 haplotypes) is very impressive—C-Siberian salamanders were found in the area extending from Sakhalin on the east to Arkhangelsk region on the west (Fig. 5). Meanwhile, the mentioned trend is characteristic mainly for shg C1 (except for C1a which is Khabarovsk region-specific), while shg C2 and C3 are specific for the Far East being found in Khabarovsk region and Sakhalin (Table 1 and Figs. 4 and 5). Median-network analysis of mtDNA haplotypes allowed us to identify two main migration routes of *S. keyserlingii* in Eurasia: (1) to the North-East of Asia (Yakutia, Magadan region, Chukotka and Kamchatka); this route is marked by haplotypes k7 and k10 (Fig. 3); and (2) to Sakhalin and Hokkaido; this route is marked by several haplotypes belonging to haplogroups A, B, C1, C2 and C3 (Figs. 2 and 3). Among them, the range of shg C3 is restricted solely to Sakhalin. This feature seems to be very important allowing us to estimate the age of C3 Sakhalin population at approximately 0.23 ± 0.08 MY ($\rho$ = 0.9 ± 0.31 from founder haplotype k13). However, phylogeographic data indicate that shg C3 was derived from haplotype k49, which is widespread in Khabarovsk region, thus suggesting that Siberian salamanders may have reached Sakhalin approximately 0.4 MY ago ($\rho$ = 1.57 ± 0.86 from founder haplotype k49). Much earlier arrival is seen for Hokkaido-specific branch k69, with evolutionary distance $\rho$ = 5.67 ± 2.31 (from hypothetical AB-ancestor) equal to 1.5 ± 0.6 MY. This dating seems to be in agreement with one previously proposed by Matsui et al. (2008). The estimated divergence time for haplogroup B was much lower (0.75MY based on $\rho$ = 2.89 ± 1.45), thus pointing to more recent arrival of B-haplotypes k18, k68 and k75 to Sakhalin.

At the same time, diversity of mtDNA sequences present in populations of North-East Asia is so low that evolutionary age of those haplotypes is only 0.125 MY ($\rho$ = 0.48 ± 0.08). However, low genetic diversity of *S. keyserlingii* in North-East Asia may also suggest

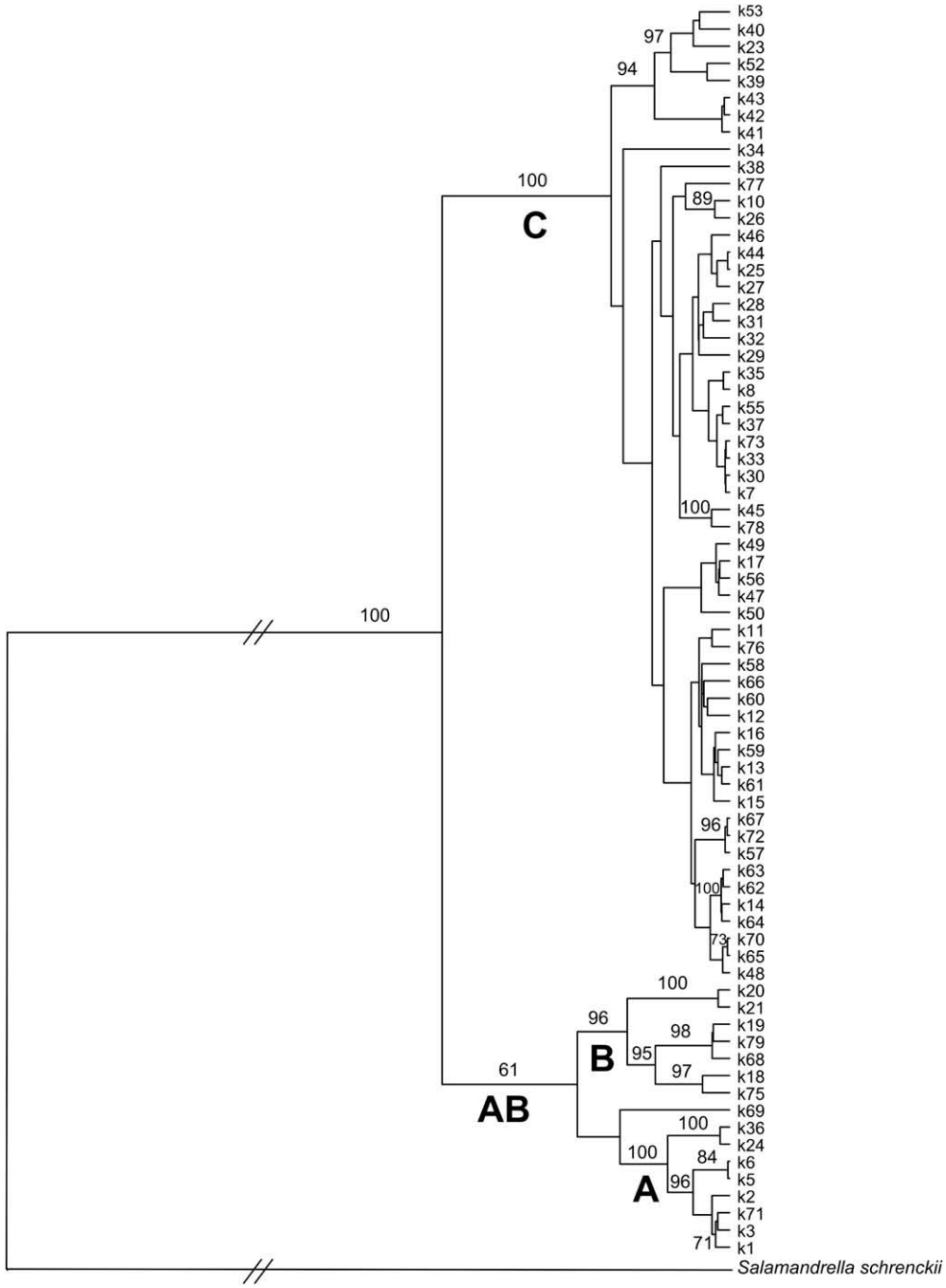


**Fig. 1.** Bayesian inference of the intraspecific phylogeny of *S. keyserlingii* under the GTR+I+G model of nucleotide substitutions. Schrenck Siberian salamander (*S. schrenckii*) was used as an outgroup. Support values are indicated for nodes that were supported in 60% or more of 18,000 sampled trees (burn-in = 2000).

that cytochrome *b* gene variation might subject to selective and adaptive processes.

### 3.2. Testing for the signatures of selection in cytochrome b gene

Haplogroup AB is much more diverse than haplogroup C taking into consideration such parameters as the average mean of pairwise nucleotide differences and nucleotide diversity (Table 2). However, genetic demographic data indicate that haplogroup C underwent a substantial increase in size, based on $\theta_S$ and $\theta_K$ indices, which have been estimated from the number of polymorphic sites and haplotypes in populations, respectively. Since $\theta_K$ reflects the effective size of a population, the data received indicate that haplogroups A, B and C substantially differed in effective size, which was several times greater in haplogroup C (Table 2). This haplogroup displayed an unimodal distribution of pairwise nucleotide differences as well as significantly negative values of sequence-based neutrality tests (Tajima's *D* and Fu and Li's *F**), suggesting recent demographic expansion or a series of expansions with a high migration rate between neighbor groups (Rogers and Harpending, 1992; Aris-Brosou and Excoffier, 1996; Ray et al., 2003). The timing of demographic expansion of haplogroup C was estimated at 0.3 MY ago (95% CI, 0.09–0.53 MY) by the mode of mismatch distribution ($\tau = 2.53$).

Meanwhile, negative values of Tajima's *D* reflect an excess of rare polymorphisms in a population, which may be also consistent

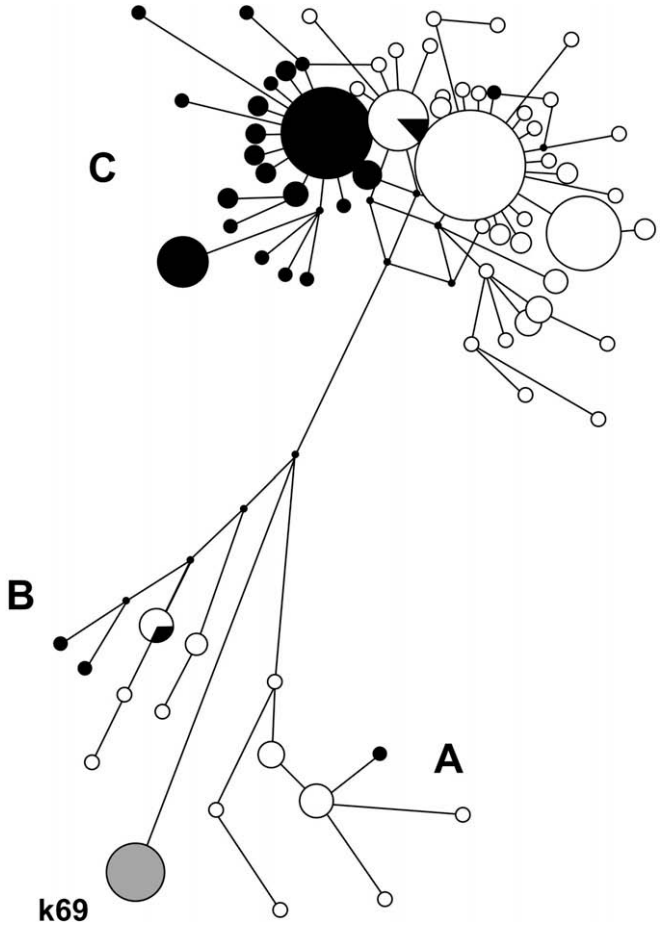


**Fig. 2.** Median-joining network of cytochrome *b* gene haplotypes observed in 290 specimens of *S. keyserlingii*. This network illustrates the relationships between the common haplogroups AB, which includes haplogroups A, B and Hokkaido-specific lineage k69, and C. The size of each circle is proportional to the haplotype frequency and geographical origins are indicated by different colors—those found in Sakhalin and Hokkaido are shown in black and grey, respectively. Haplotypes from remaining areas are shown in white.

with positive or weak negative selection, not only with an increase in population size (Tajima, 1989; Nielsen, 2005). We estimated the ratio of the number of nonsynonymous substitutions per nonsynonymous sites ($K_A$) to the number of synonymous substitutions per synonymous sites ($K_S$) and found that there is no excess of nonsynonymous substitutions in haplogroup C as well as in AB. $K_A/K_S$ values for both haplogroups were much lower than 1, indicating the influence of negative selection. Meanwhile, it is well known that negative selection effects (in the form of very low $K_A/K_S$ values) appear to be characteristic for mitochondrial genomes of many species (Popadin et al., 2007), thus pointing to the possibility that the observed departure from neutrality in haplogroup C of *S. keyserlingii* may be explained by demographic events rather than natural selection.

It is noteworthy that Sakhalin shg C3 demonstrates increased level of nonsynonymous substitutions (NS/S = 7/38) in comparison with the remaining C-haplotypes designated as C* (NS/S = 7/17); however, these differences were insignificant ($P = 0.152$, Fisher's exact test). Similar results were achieved using neutrality test by Elson et al. (2004) applied to haplogroups AB, C3 and C*. We compared the ratios of NS/S in two classes of polymorphisms: in haplogroup-associated substitutions and private substitutions occurring at the tips of individual branches within a network (Table 3). All comparisons demonstrate that the neutrality indices (NI) for analyzed cytochrome *b* gene region are >1, a result that indicates negative selection. However, these differences are statistically insignificant, thus suggesting that selection has not had an impact on the cytochrome *b* gene evolution in *S. keyserlingii*.

Some difficulties have arisen with explaining the low level of genetic diversity present in *S. keyserlingii* from North-East Asian populations of Yakutia, Magadan region, Chukotka and Kamchatka. Despite the relatively high sample size of specimens analyzed ($N = 67$), only three haplotypes defined by two nucleotide substitutions (both synonymous) were revealed in populations of North-East Asia. Positive values of Tajima's $D$ recorded in this geographic region ($D = 0.57$, $P > 0.1$) can result from balancing selection or population bottlenecks. To investigate the pattern of cytochrome *b* gene variation in *S. keyserlingii* we also performed the MacDonald–Kreitman test by the use of *S. schrenckii* as an outgroup for interspecies analyses. The data obtained show that despite the neutrality indices (NI) exceeded the value 1, these differences were statistically insignificant, thus suggesting selective neutrality (Table 4).

### 3.3. Amino acid substitutions and molecular adaptation

Using $K_A/K_S$ as the sole method for detecting natural selection is too conservative to detect single adaptive amino acid changes (McClellan et al., 2005). This is essentially true for analysis of evolutionary conservative genes, such as cytochrome *b*. Analysis of amino acid substitutions fixed between two sister taxons, *S. keyserlingii* and *S. schrenckii*, shows only one substitution from Met to Leu in amino acid position 97. In *S. keyserlingii*, virtually all substitutions occurred between neutral hydrophobic amino acids (Val ↔ Ile, Val ↔ Ala, Leu ↔ Met, Phe ↔ Leu) (Table 5). The only substitution from Ser to Leu in position 160 appears to be radical. Grantham distance (GD) for this amino acid change was 32, whereas all remaining changes were conservative, with GD > 57.9.

Analysis of amino acid changes with respect to the structural model for cytochrome *b* of *S. keyserlingii* indicates that most of the variable positions (11 from 16) are located within transmembrane helices. Only 3 and 2 amino acid changes were respectively revealed on the outer and inner surfaces of the protein (Table 5). Majority of amino acid replacements were singular, excluding changes in positions 119, 160 and 219 only which were multiple and/or cluster-specific. Isoleucine residue at amino acid site 119 appears to be a consensus variant in Hynobiidae phylogeny (CI = 0.78), however valine residue was found to be haplogroup-specific in haplogroup B members, and, in addition, a parallel amino acid change Ile-Val was detected in haplotype k52 of haplogroup C. Replacement of valine by isoleucine at site 219 (as parallel event) was found in two haplotypes (k21 and k79) of haplogroup B.

The most interesting case is radical amino acid change from serine to leucine at site 160. This replacement seems to be very rare in Hynobiidae (CI = 0.96) and it appears only once as a variant defining the whole haplogroup C. However, this change was probably reversed twice (Leu160Ser) via convergence in shg C1a, which is characteristic for Khabarovsk region, and in haplotype k57 belonging to Sakhalin-specific cluster C3. Comparison of amino acid sequences corresponding to haplogroups C (k7) and B (k68) performed by TreeSAAP application indicates that site 160 was affected by significant changes ($P < 0.05$) on six amino acid properties (bulkiness, hydropathy, long-range non-bonded energy, power to be at the N-terminal, surrounding hydrophobicity, thermodynamic transfer hydrophobicity). We performed also protein secondary-structure analysis of cytochrome *b* harboring the haplogroup C-specific amino acid changes (for haplotype k7) in comparison to haplogroup B sequence (for k68) using the TMpred application. This allowed us to find that amino acid replacement

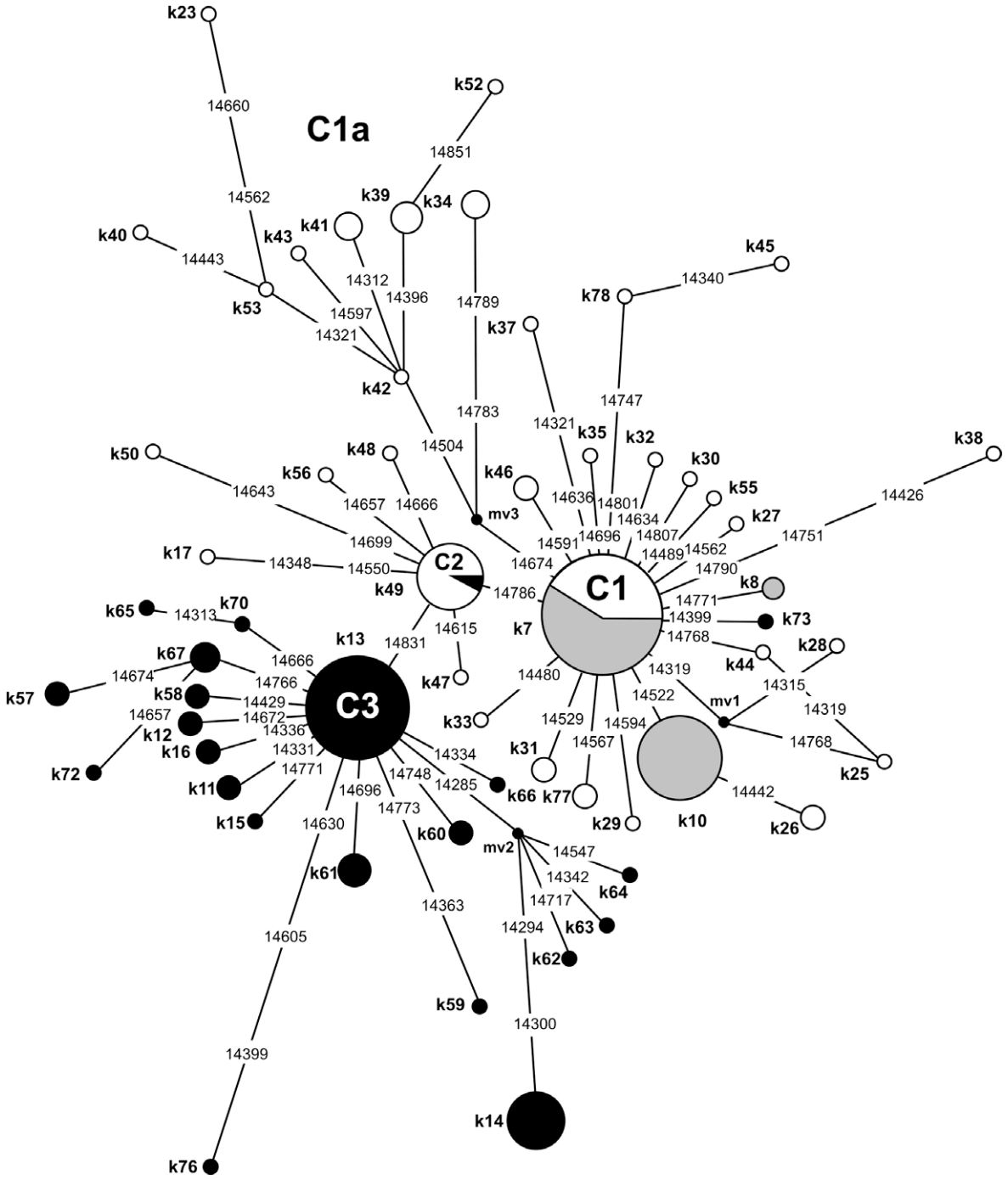


**Fig. 3.** Median-joining network of *S. keyserlingii* cytochrome *b* gene haplotypes belonging to haplogroup C. This haplogroup consists of subhaplogroups C1, C2 and C3. Nucleotide substitutions shown on the branches are numbered according to *S. keyserlingii* complete mitochondrial genome sequence (Zhang et al., 2006). Median vectors are designated as "mv". Haplotypes found in Sakhalin and North-East Asia (Yakutia, Magadan region, Chukotka and Kamchatka) are shown in black and grey, respectively. Haplotypes from remaining areas are shown in white.

from Ser to Leu increased the hydrophobicity in the cd-loop of the redox center $Q_o$, changing it from 374 to 581 units of Kyte–Doolittle index (Fig. S2). It seems also important that nucleotide sequences corresponding to the cd-loop of cytochrome *b* are characterized by considerable differences in Siberian salamanders of haplogroups C and AB (Table 6). Apart from nonsynonymous substitution leading to Ser160Leu change, there are three synonymous substitutions distinguishing these haplogroups. In general, the cd-loop region demonstrates the highest level of between-haplogroup divergence (up to 4%), thus suggesting that this region may be involved in the molecular adaptation process in Siberian salamanders belonging to haplogroup C (Table 7).

## 4. Discussion

The occurrence of three strongly supported phylogenetic haplogroups of Siberian salamanders (A, B and C) in the Far East region is consistent with observations of growing number of species of different taxa from this region demonstrating distinct genetic

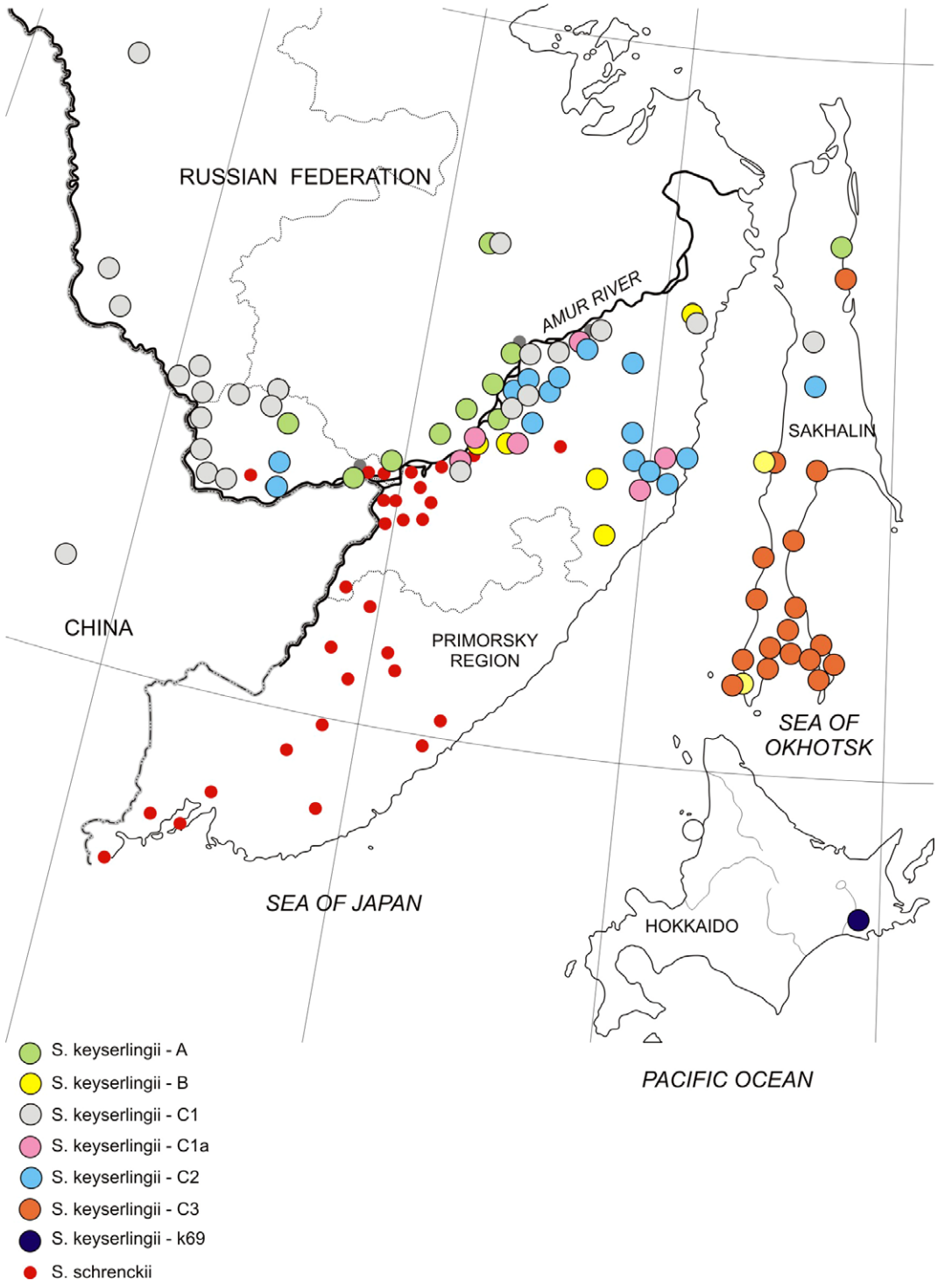


**Fig. 4.** Map showing sampled localities of *S. keyserlingii* and geographical distribution of the main haplogroups A, B and C in the Far East region.

subdivisions, which are explained with ecological factors accompanying the changing climatic conditions during the Pleistocene (Haring et al., 2007; Fedorov et al., 2008). Divergence between haplogroups AB and C of Siberian salamander has occurred approximately 1.5 MY ago. Therefore, a scenario involving glacial refugia, with survival of relic haplogroups A and B in the Far East region at least during the first glaciation of the Pleistocene, seems probable. At present, Siberian salamanders of all mtDNA haplogroups inhabit only the territory of the Far East (including Sakhalin), whereas the remaining area of northern Eurasia was colonized, probably during the last 0.125 MY, by the only haplogroup C1. Therefore, we suggest that the middle Amur River Basin and the northern Sikhote-Alin Mountains region can be considered as the center of genetic diversification and, possibly, origin of the ancestral *S. keyserlingii* (Fig. 4).

It is also probable that the glacial cycles might have led to appearance of cytochrome *b* variants that were advantageous in those climatic conditions and have allowed Siberian salamanders of haplogroup C to settle in new regions with extreme environments. Thus, it seems that radical amino acid change at site 160

**Table 2**
Diversity indices of cytochrome *b* gene in haplogroups A, B, AB and C of Siberian salamander.

| Genetic characteristics | Haplogroups | | | |
|---|---|---|---|---|
| | A | B | AB | C |
| Sample size, *n* | 14 | 13 | 44 | 246 |
| Number of haplotypes, *k* | 8 | 7 | 16 | 57 |
| Number of polymorphic sites, *s* | 12 | 9 | 30 | 59 |
| Haplotype diversity, *h* | 0.86 ± 0.08 | 0.80 ± 0.11 | 0.83 ± 0.05 | 0.88 ± 0.01 |
| Average number of pairwise differences, *i* | 2.55 | 2.82 | 7.27 | 2.31 |
| Nucleotide diversity, $\pi$ | 0.0042 | 0.0046 | 0.0119 | 0.00378 |
| Theta estimator, $\theta_S$ | 3.77 | 2.90 | 6.90 | 9.69 |
| Theta estimator, $\theta_K$ | 6.92 | 5.43 | 8.60 | 24.85 |
| *D* (Tajima's neutrality test) | −1.29 ($P > 0.1$) | −0.11 ($P > 0.1$) | 0.18 ($P > 0.1$) | −2.28 ($P < 0.01$) |
| $F^*$ (Fu, Li's neutrality test) | −1.28 ($P > 0.1$) | 0.39 ($P > 0.1$) | −0.11 ($P > 0.1$) | −4.95 ($P < 0.02$) |
| Number of synonymous changes | 10 | 8 | 26 | 48 |
| Number of nonsynonymous changes | 2 | 1 | 4 | 13 |
| $K_A/K_S$ | 0.040 | 0.036 | 0.029 | 0.039 |

*Note:* Haplogroup AB consists of haplogroups A and B and Hokkaido-specific haplotype k69.

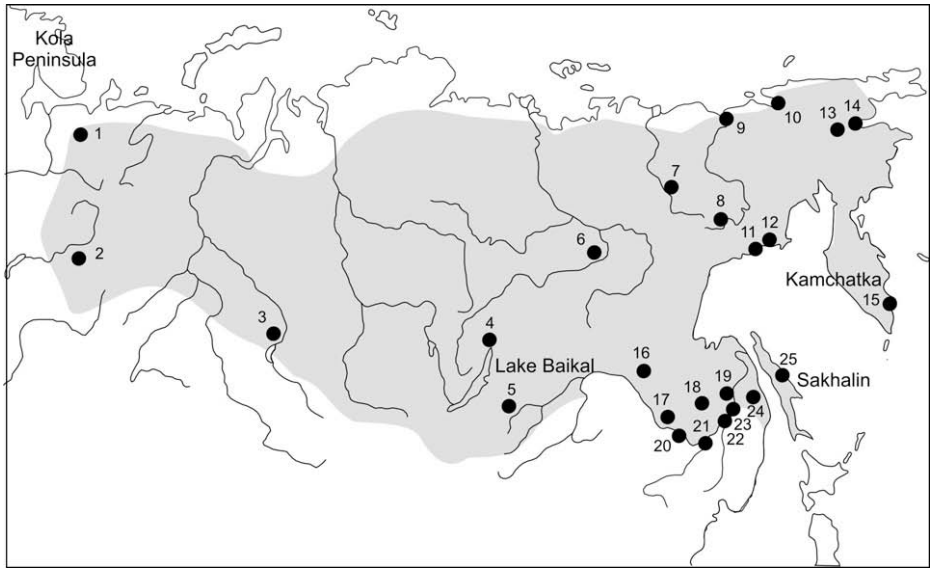


**Fig. 5.** Map showing sampled localities and geographical distribution of haplogroup C1 in the overall range of *S. keyserlingii* in northern Eurasia. Numbers on the map correspond to the population numbering shown in the Table 1.

**Table 3**
Analysis of nonsynonymous (NS) and synonymous (S) haplogroup-associated and private polymorphisms in haplogroups AB, C3 and C* of Siberian salamander.

| Haplogroup | *N* | $K_A/K_S$ | Number of haplogroup-associated substitutions | | | Number of private substitutions | | | *P* | NI |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | NS | S | NS/S | NS | S | NS/S | | |
| AB | 44 | 0.029 | 1 | 19 | 0.05 | 4 | 8 | 0.5 | 0.053 | 9.62 |
| C3 | 90 | 0.071 | 2 | 10 | 0.20 | 5 | 7 | 0.71 | 0.19 | 3.55 |
| C* | 156 | 0.062 | 2 | 14 | 0.14 | 5 | 24 | 0.21 | 0.52 | 1.50 |

*Note: N* is sample size. *P*-values were determined with Fisher's exact test. NI is neutrality index calculated as (NS-private/NS-haplo)/(S-private/S-haplo) (Elson et al., 2004). The NI value should be 1.0 under strict neutrality. In the presence of negative selection NI should be >1, whereas in the presence of positive selection NI should be <1. C* includes all haplotypes, with the exception of those belonging to Sakhalin-specific cluster C3.

of cytochrome *b* in Siberian salamanders may have some influence on the proton-output function of the complex that creates the proton gradient necessary for the production of ATP and, thus, cellular respiration (Degli Esposti et al., 1993; McClellan et al., 2005). It is also worth to note that amino acid changes in the cd-loop (at sites 144, 158, 159, 162, 166) have been previously detected as signatures of adaptive evolution in many mammalian species (McClellan and McCracken, 2001; McClellan et al., 2005; da Fonseca et al., 2008). It has been suggested that in mammalian species mutations occurring in cytochrome *b* may have functional implications which resulted in extreme amino acid properties variation in species with peculiar metabolic requirements (such as adaptation to low energy diet vs. large body size; and adaptation to extreme $O_2$ requirements, i.e. diving in cetaceans and high altitudes resistance in alpacas) (McClellan and McCracken, 2001; Fink et al., 2004; McClellan et al., 2005; da Fonseca et al., 2008). Balance tending toward less effective energy production and more heat production appears to be more beneficial for cold environmental conditions (Gershoni et al., 2009). Thus, the observed pattern of genetic and biochemical variation in *S. keyserlingii* may be interpreted as the result of adap-

**Table 4**
Analysis of selection in cytochrome *b* gene of Siberian salamander *via* the McDonald–Kreitman test (in comparison with Schrenck Siberian salamander).

| Species/haplogroup | $D_A$ | $D_S$ | $P_A$ | $P_S$ | $P$ | NI |
|---|---|---|---|---|---|---|
| Siberian salamander | 1 | 26 | 21 | 109 | 0.127 | 5.01 |
| Haplogroup AB | 3 | 35 | 12 | 79 | 0.550 | 1.77 |
| Haplogroup C3 | 2 | 40 | 14 | 71 | 0.09 | 3.94 |
| Haplogroup C* | 2 | 32 | 14 | 89 | 0.357 | 2.52 |
| Haplogroup C(NEA) | 4 | 41 | 9 | 62 | 0.567 | 1.49 |

*Note:* *P*-values were determined with Fisher's exact test. NI is neutrality index calculated as a ratio of $P_A/P_S$ to $D_A/D_S$, and its value should be 1.0 under selective neutrality. In the presence of negative selection NI should be >1, while in the presence of positive selection NI should be <1. C* includes all haplotypes, with the exception of those belonging to Sakhalin-specific cluster C3. C(NEA) is a group of haplotypes found in North-East Asian populations.

**Table 5**
Amino acid replacements in cytochrome *b* of Siberian salamander.

| Site | Replacement | Replacement type | Domain | CI |
|---|---|---|---|---|
| 40 | Val-Ile | Singular (C) | TM-A | 0.3 |
| 46 | Ile-Val | Singular (C) | TM-A | 0.93 |
| 47 | **Ala-Thr** | Singular (C) | TM-A | 1.0 |
| 83 | Val-Ile | Singular (C) | TM-B | 0.3 |
| 83 | Val-Ala | Singular (C) | TM-B | 1.0 |
| 97 | Met-Thr | Singular (A) | TM-B | 1.0 |
| 112 | **Glu-Lys** | Singular (C) | $Q_i$ | 1.0 |
| 118 | Val-Ile | Singular (C) | TM-C | 0.67 |
| 119 | Ile-Val | Multiple (B, C) | TM-C | 0.78 |
| 154 | **Ile-Met** | Singular (C) | $Q_o$-cd | 1.0 |
| 160 | **Ser-Leu*** | Cluster-specific (C) | $Q_o$-cd | 0.96 |
| 181 | **Ala-Thr** | Singular (A) | TM-D | 0.96 |
| 186 | Phe-Leu | Singular (C) | TM-D | 0.89 |
| 193 | Ala-Val | Singular (C) | TM-D | 0.85 |
| 219 | Val-Ala | Singular (C) | $Q_i$ | 1.0 |
| 219 | Val-Ile | Multiple (B) | $Q_i$ | 0.85 |

*Note:* CI is the conservation index calculated for 27 different species of the family Hynobiidae. Domains: TM is the transmembrane domain composed by eight helices; $Q_i$ is the matrix domain with the redox center $Q_i$; and $Q_o$ is the intermembrane domain composed by four loops of the redox center $Q_o$. Haplogroups are indicated in parentheses. Amino acid replacements leading to the polarity changes are shown in bold. Radical amino acid replacement is marked by an asterisk (*).

**Table 6**
Nucleotide sequence of cytochrome *b* gene fragment corresponding to cd-loop of redox center $Q_o$.

| Nucleotide position | *S. schrenckii* | *S. keyserlingii* | | |
|---|---|---|---|---|
| | | Haplogroup A | Haplogroup B | Haplogroup C |
| 14,648 | A | A | A | T |
| 14,657 | T | C | C | C |
| 14,660 | T | T | T | C |
| 14,663 | C | T | T | T |
| 14,669 | A | G | A | A |
| 14,672 | C | T | T | T |
| 14,674* | C | C | C | T |
| 14,690 | T | C | C | C |
| 14,693 | G | A | A | A |
| 14,696 | T | C | C | T |
| 14,699 | G | A | A | A |
| 14,702 | T | C | C | C |
| 14,711 | T | C | C | C |
| 14,717 | T | C | C | C |
| 14,723 | T | C | C | C |

*Note:* Nucleotide differences between haplogroups C and AB of *S. keyserlingii* are filled in grey. Fixed nucleotide differences between *S. schrenckii and S. keyserlingii* are shown as well. Nucleotide positions leading to the radical amino acid change (Ser160Leu) are marked by an asterisk (*).

**Table 7**
Nucleotide diversity and divergence of nucleotide sequences of cytochrome *b* gene in haplogroups of Siberian salamander, taking into account the protein secondary structure.

| Domain | Nucleotide positions | Haplogroups and nucleotide diversity $\pi$ | | Between-haplogroup divergence, $d_{XY}$ |
|---|---|---|---|---|
| | | AB | C | |
| TM-A | 14,286–14,363 | 0.0248 | 0.0020 | 0.0177 |
| $Q_o$-ab | 14,364–14,438 | 0.0065 | 0.0011 | 0.0057 |
| TM-B | 14,439–14,507 | 0.0007 | 0.0020 | 0.0014 |
| TM-C | 14,541–14,606 | 0.0204 | 0.0014 | 0.0147 |
| $Q_o$-cd | 14,607–14,729 | 0.0134 | 0.0024 | 0.0412 |
| TM-D | 14,730–14,798 | 0.0039 | 0.0100 | 0.0253 |
| $Q_i$ | 14,799–14,869 | 0.0075 | 0.0071 | 0.0105 |

*Note:* TM is the transmembrane domain composed by eight helices; $Q_i$ is the matrix domain with the redox center $Q_i$; and $Q_o$ is the intermembrane domain composed by four loops of the redox center $Q_o$.

tive selection that occured after the split of *S. keyserlingii* protopopulation in the glacial periods, most likely at the end of Eopleistocene taking into account that the age of Siberian salamanders belonging to haplogroup C is estimated at 0.64 MY. It seems also probable that this adaptive mechanism, which was advantageous in the periglacial areas, has allowed *S. keyserlingii* to populate later great part of Northern Eurasia.

## Acknowledgment

This research was supported by a grant from the Far-Eastern Branch of the Russian Academy of Sciences (10-III-B-06-136).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.04.005.

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