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GENOMICS. TRANSCRIPTOMICS. PROTEOMICS

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Genetic Structure of Schrenck Newt *Salamandrella schrenckii* Populations by Mitochondrial Cytochrome *b* Variation

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Abstract—The nucleotide sequence variation of the mitochondrial cytochrome *b* gene was studied in Schrenck newt *Salamandrella schrenckii* (Strauch, 1870) from populations of Primorye and the Khabarovsk region. Phylogenetic analysis revealed two haplotype clusters, southern cluster 1 and northern cluster 2, with a divergence of 3%. Analysis of the mtDNA and cytochrome *b* amino acid sequence variations made it possible to assume that the modern range of Schrenck newt was colonized from south Primorye northwards. In contrast to the southern cluster, the northern one demonstrated all the signs of demographic expansion (a unimodal distribution of pairwise nucleotide differences, specific results of tests for selective neutrality of mtDNA variation, and a good correspondence of genetic parameters to those expected from demographic expansion models).

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Key words: mitochondrial DNA, cytochrome *b*, molecular evolution, cryptic species

INTRODUCTION

Molecular genetic studies aimed at detecting cryptic species are an important field of evolutionary biology, since other methods have failed to reconstruct their developmental history. Earlier we had analyzed the nucleotide sequence variation of the mitochondrial cytochrome *b* gene in broad-range Siberian newt *Salamandrella keyserlingii* Dybowski, 1870, and identified Schrenck newt *Salamandrella schrenckii* Strauch, 1870, as a new cryptic species that inhabits Primorye and the Khabarovsk region [1]. The populations of southeastern Russia displayed a dramatic genetic difference from nearly homogeneous *S. keyserlingii* populations from various north Eurasian regions, suggesting high intraspecies variation for *S. schrenckii.* Scarce data on the *S. schrenckii* range were available at that time: the species was known to inhabit the southern parts of Primorye and the Khabarovsk region. Our molecular data indicated that *S. schrenckii* is far older than *S. keyserlingii* (2.5 and 0.49 Myr, respectively). Moreover, two *S. schrenckii* subclusters were detectable, with a divergence between them estimated at 3.2% on average [1].

To demarcate the *S. schrenckii* species range, we performed large-scale field studies and collected newts in Primorye, the Khabarovsk region, and adjacent territories. Analysis of variation in the nucleotide sequence of the mitochondrial cytochrome *b* gene allowed us to identify *S. schrenckii* in the collection material. Hence, the objective of this work was to study the intraspecific structure and molecular evolution of *S. schrenckii*, a new vertebrate of the Russian fauna.

EXPERIMENTAL

Material was collected from 2004 to 2007 in more than 65 localities of the Amur and Jewish autonomous oblasts, Primorye, and Khabarovsk region; *S. schrenckii* was found in 35 localities (Fig. 1). Genomic DNA was isolated from various tissues of adults, larvae, and embryos, which were frozen or fixed with 70% ethanol. Two cytochrome *b* gene regions were amplified with primers MVZ15L–MVZ18H and MVZ25L–ControlWH as described previously [1]. DNA was sequenced in an ABI 3130 genetic analyzer, using a BigDye Terminator v. 3.1 cycle sequencing kit (Applied Biosystems) and primers MVZ15L and MVZ25L. The resulting sequences were aligned and analyzed using the SeqScape v. 2.5 software package (Applied Biosystems). Genetic dis-

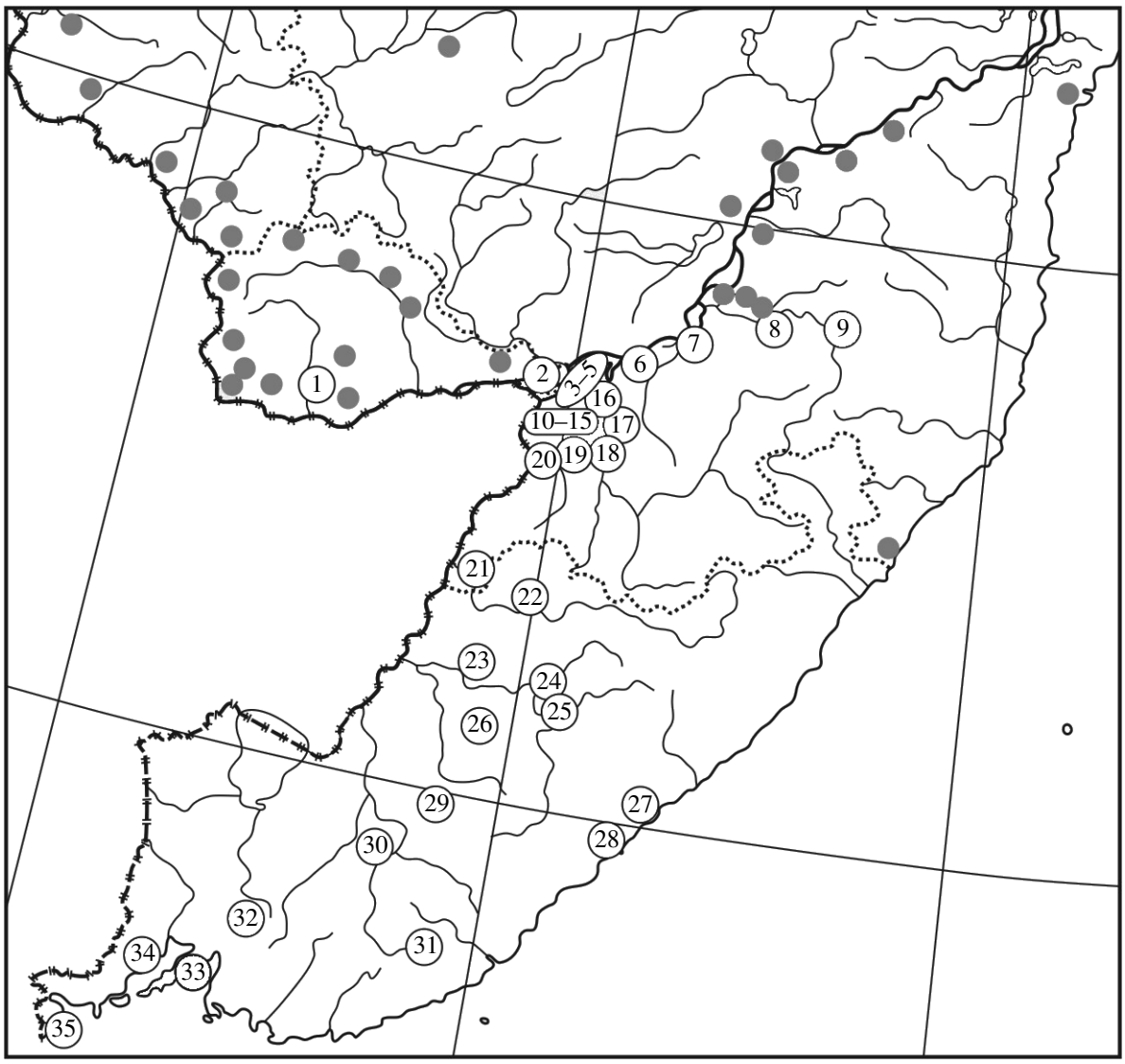

Fig. 1. Sites of *S. schrenckii* collection: 1, 2, Khabarovsk region and Jewish Autonomous District; 3–20, Khabarovsk region; 21–35, Primorye. Collection sites where *S. schrenckii* was not found are shown in gray.

tances (p-distances) between individual DNA sequences were calculated from the number of nucleotide substitutions per position in pairwise comparisons. To estimate the divergence time and evolutionary age of mtDNA lineages, we used the number of mutations in the cytochrome *b* gene, assuming that 0.77% divergence (for transitions and transversions) corresponds to evolutionary changes arising over 1 Myr [2].

Phylogenetic trees were constructed by the neighbor-joining (NJ) [3] algorithm, unweighted pair group method with arithmetic averages (UPGMA), and the maximum parsimony (MP) algorithm [4], using the MEGA 2.1 [5] and Network 4.5 [6] software packages. Median networks were constructed by the median-joining (MJ) algorithm, using the Network 4.5 package. Optimal trees were sought using the MP Calculation option. The extent of mtDNA divergence was estimated using the ρ distance, which corresponded to the mean distance from the ancestral haplotype to all, including hypothetical, derivative haplotypes median vectors) [6].

The estimates of mtDNA diversity, mtDNA divergence, and demographic parameters characterizing the effective population size and time-related changes in population size were obtained using the Arlequin 3.01 software package [7]. Interpopulation differentiation was characterized by F_{ST}, which was computed via analysis of molecular variance (AMOVA) with the use of the Arlequin 3.01 package.

The molecular clock hypothesis was checked by the Tajima test [8], which estimates the evolution rate

in pairs of DNA sequences relative to an outgroup DNA sequence (MEGA 2.1 package). As an outgroup, we used the cytochrome *b* gene sequence of Semirechensk salamander *Ranodon sibiricus* (accession no. NC 004021). For comparisons, nucleotide sequences of the cytochrome *b* gene (a 743-bp region) of several genera of the family Hynobiidae were extracted from GenBank (www.ncbi.nih.gov/entrez). The nucleotide sequences established in this work for the *S. schrenckii* cytochrome *b* gene region under study were deposited in GenBank under accession nos. EU567390–EU567451.

RESULTS AND DISCUSSION

The 825-bp region (14 228–15 322 according to the full-length *Salamandrella keyserlingii* mitochondrial genome [9]) of the cytochrome *b* gene was sequenced in 167 Schrenck newts from various localities of Primorye and the Khabarovsk region. In total, 62 haplotypes of the cytochrome *b* gene were observed in our sample. The haplotypes were due to polymorphism at 94 positions, of which 53 were phylogenetically informative (they were found in more than one mtDNA haplotype) (Fig. 2). The majority of mutations (86) were transitions; transversions were only observed in 11 positions. Mutations in ten positions led to amino acid substitutions, but only three of these mutations were informative. Thus, most mutations were synonymous. The nucleotide composition of the *S. schrenckii* region under study was biased towards higher T and A contents (33.5 and 28.5%, respectively; the C and G contents were 23.9 and 14.5%, respectively), as characteristic of animal mitochondrial genomes [10].

Haplotypes Vl1 and Vl46 were most common in the *S. schrenckii* sample, occurring in 23 and 12 newts, respectively (Fig. 2). More than 50% of the haplotypes (34) were found in single newts. Phylogenetic analysis of the cytochrome *b* gene haplotypes revealed two distinct clusters with a high bootstrap support, 97% for cluster 1 and 82% for cluster 2 (Fig. 3). The same topology was observed for the phylogenetic trees constructed with different algorithms (NJ, UPGMA, and MP, including MJ analysis), testifying to its high significance (data not shown). Each cluster had its own substructure. Cluster 1 included three subclusters (1a, 1b, and 1c), each having a bootstrap support of more than 70%. The substructure of cluster 2 was less distinct. Cluster 2 included only a few subclusters with a bootstrap support of more than 70% (Fig. 3).

The phylogeography of *S. schrenckii* also reflected the structured character of its mitochondrial gene pool. Analysis of the mtDNA haplotype distribution revealed a geographical subdivision of clusters 1 and 2 (Fig. 3). Newts of cluster 2 (northern) occupied the northern Sikhote-Alin region and adjacent territories, including the Ussuri and Amur valleys (Fig. 1, collections sites 1–22 and, additionally, 26, 27, 32, and 33). Newts of cluster 1 (southern) occupied the southern Sikhote-Alin region and adjacent territories (collection sites 23–35), which corresponded to the southern and central parts of the *S. schrenckii* species range. The subclusters isolated within large clusters 1 and 2 included mtDNA haplotypes that showed mostly a local distribution, suggesting high interpopulation differences.

Table 1. Genetic differences between *S. schrenckii* populations

Population	1	2	3	4
(1) South Primorye	0	1.61	0.43	0.30
(2) Upper Ussuri	0.237	0	0.58	0.30
(3) Lower Ussuri	0.539	0.464	0	3.40
(4) Amur	0.623	0.621	0.128	0

Note: Values below the diagonal are interpopulation F_{ST}-distances (all differences are significant at $P < 0.01$); values at the top of the diagonal are M values, which estimate the exchange of migrants [11] ($M = Nm$, where N is the effective size of a population and m is the migration rate).

To study this issue in more detail, we grouped the collection sites into four conventional regions (regional groups): (1) southern Primorye, (2) the basin of the upper and central stretches of the Ussuri River, (3) the basin of the lower Ussuri River, and (4) the basin of the right tributaries of the Amur. AMOVA [7] revealed high between-group differentiation (Table 1). The between-group differences accounted for 50% of the total variance (F_{ST}= 0.501, $P = 0$). The distribution of pairwise interpopulation F_{ST} differences indicated distinct differentiation of the southern and northern geographical populations of *S. schrenckii*, since F_{ST} differences between populations (1) and (2) or (3) and (4) were substantially lower than between populations (2) and (3) (Table 1). Likewise, the estimates of *M*, which characterizes the contribution of migrations into the population structure [11], showed that gene exchange between neighbor populations was intense in the southern and northern regions of the species range, but not in its central part.

Although the *S. schrenckii* samples corresponding to clusters 1 and 2 were approximately equal in size (N = 80 and 87, respectively), they substantially differed in many genetic parameters (Table 2). The divergence (d) of the nucleotide sequences of the cytochrome *b* gene was 0.8 and 1.0% within clusters 1 and 2, respectively, and 3% between the clusters, which agreed with our previous estimates of mtDNA divergence in *S. schrenckii* [1]. A greater difference between clusters 1 and 2 was observed for diversity h, which is based on the mtDNA haplotype frequencies in populations. Cluster 2 had twice as many haplo-

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                          111111111 1111112222 2222222333 3333333333 4444444555 5555566666 6666666667 7777777777 7777888
                 133445677 9000123456 6789990345 6778899022 3444556778 3344889223 4566600111 3334466661 1334556667 7888002
                6047692103 7349879516 9070392270 5470625123 5136581362 6945470469 4023545036 1470314785 6395580395 8147580

#Vl1  (23)  ACTTATCGTC TAACACACTC AGTATAAACC TATCTTAGAG CAAATACCAG CAACTGTTCG ACACATTACG TCACCCACCT TCTTCGGTTC AAGGTTT
#Vl2  (8)   ...C...A.. .....T...T .......... .G.....A.. T.....T..A T..T....T. GT..GC.GTA .....T.... .T..T.A..T G.A...A
#Vl3  (2)   ...C...A.. ...T.T...T .......T.. .......A.. T.....T..A T..T....T. GT..GC.GT. .....T.T.. .T..T.A..T G.A...A
#Vl4  (1)   .......... .........T .......... .........A ...G...T.. .......... GT........ .....T.... .T.CT.A... ......
#Vl5  (3)   .......... .........T .......... .........A ...G.G.... .......... .T........ .....TG... .T..T.A... ......C
#Vl6  (2)   .......... .......... .......... .......... .......... .......... .......... .......... .....A.... .......
#Vl7  (6)   .......... .......... .......... .......... .......... .......... .......... .......... ....T..... .......
#Vl8  (2)   .......... .......... .......... .......... .......... .......C.. .......... .......... ....T..... .......
#Vl9  (1)   .......... .........T .......... .........A ...G...... .......... .T........ .....T...C .T..T.A... .......
#Vl10 (7)   .......... .........T .......... .........A ...G...... .......... .TT....... .....T.... .T..T.A... .......
#Vl11 (2)   .......... .......T.. .......... .......... .......... ....C..... .......... .....T..T. ....T..... .......
#Vl12 (1)   .......... .......... .......... .......... .......... .......... .......... .....T.... ....T..... .......
#Vl13 (7)   .T........ .......... .......... .......... .......... .......... .......... .......... ....T..... .......
#Vl14 (2)   .......... .......... .......... .......... .......... .....A.... .......... .......... .......... .......
#Vl15 (2)   .......... .......... .......... .......... .......... .....A.... .......... ..G..T.... ....T..... .......
#Vl16 (3)   G..C..TA.. ..G......T .......... .......A.. T...C.T..A T..T..C.T. GT..GC.G.. ....T.... .T..T.A..T G.A...A
#Vl17 (1)   G..C..TA.. .........T .....G.... .......A.. T........A T..TC...T. GT..GC.G.. .....T.... .T..T.A..T .CA...A
#Vl18 (1)   ...C..TA.T .........T .......... .....CGA.. T.....T..A T..T....T. GT..CC.G.. .....T.... .TC.T.A..T G.AA..A
#Vl19 (2)   ...C..TA.. ..G......T .......... .......A.. T...C.T..A T..T..C.T. GT..GC.G.. .....T.... .T..T.A..T G.A...A
#Vl20 (1)   .......A.. .....T...T .......... .......A.. T.....T..A T..T....T. GT...C.GT. .....T.... .T..T.A..T G.A...A
#Vl21 (1)   ...C...A.. .....T...T .......... ...T...A.. T.....T..A T..T....T. GT..GC.GT. .....T.... .T..T.A..T G.A...A
#Vl22 (1)   ...C...A.. .....T...T .......... ........AG. T.....T..A T..T....T. GT..GC.GT. .....T.... .T..T.A..T G.A...A
#Vl23 (1)   ...C..TA.. .........T .......... C...G..A.. T.....T..A T..TC...T. GT..GC.G.. ....TT.... .T..T.A..T .CA...A
#Vl24 (1)   ...C..TA.. .........T .....G.... .......A.. T.........A T..TC...T. GT..GCCG.. .....T.... .T..T.A..T .CA...A
#Vl25 (8)   ...C..TA.. ..G......T .......... .......A.. T.....T..A T..T..C.TA GT..GC.G.. .....T.... .T..T.A..T G.A...A
#Vl26 (1)   ...C..TA.. .........T .......... .......A.. T.....T..A T.GTC...T. GT..GC.G.. .....T.... .T..T.A..T .CA...A
#Vl27 (2)   ...C...A.. A.....G..T T.......... .......A.. T.....T..A T..T....T. GT..GC.G.. .....T.... .T..T.A.CT G.A..AA
#Vl28 (3)   ...C...A.. A.....G..T T.......... .......A.. T.....T..A T..T....T. GT..GC.G.. .....T.... .T..T.A..T G.A..AA
#Vl29 (1)   ...C...A.. .....T...T ......G... .G.....A.. T.....T..A T..T....T. GT...C.GT. ....?????? ?????????? ???????
#Vl30 (2)   ...C..TA.. .........T .....G.... .......A.. T.........A T..TC...T. GT..GC.G.. .....T.... .T..T.A..T .CA...A
#Vl31 (1)   ...C...A.. .....T...T .......... .......A.. T.G...T..A T..T....T. GT...C.GT. .....T.... .T..T.A..T G.A...A
#Vl32 (1)   ...C...A.. .....T...T .......... .G.....A.. T.....T..A T..T....T. GT...C.GT. .....T.... .T..T.A..T G.A...A
#Vl33 (1)   ...C...A.. .....T...T ...T...... ..C....A.. T.....T..A T..T.A..T. GT..GC.G.. C....T.... .T..T.A.CT G.AA..A
#Vl34 (1)   ...C...A.. .....T...T .A........ .......A.. T.....T..A T..T....T. GT..GC.GT. .....T.... .T..T.A..T G.....A
#Vl35 (1)   ...C...A.. .....T...T .......... .......A.. T.....T..A TG.T....T. GT...C.GT. .....T.... .T..T.AC.T G.A...A
#Vl36 (2)   ...C..TA.. ..G......T ...T...... .......A.. T.....T..A T..T..C.T. GT..GC.G.. ...T.T.... .T..T.A..T G.A...A
#Vl37 (1)   ...C.CTA.. .........T .......... .......A.. T.....T..A T..TC...T. GT..GC.G.. ....TT.... .T..T.A..T .CA...A
#Vl38 (1)   ...C..TA.. .........T ..C....... .......A.. T.....T..A T..TC...T. GT..GC.G.. .....T.... .T..T.A..T .CA...A
#Vl39 (1)   ...C..TA.. .........CT .......... .......A.. T.....T..A T..TC...T. GT..GC.G.. .....T.... .T..T.A..T .CA...A
#Vl40 (2)   ...C..TA.. .........T .......... .......A.. T.....T..A T..TC...T. GT..GC.G.. .....T.... .T..T.A..T .CA...A
#Vl41 (4)   ...C...A.. .G...T...T ...T...... ..C....A.. T.....T..A T..T.A..T. GT..GC.G.. C....T.... .T..T.A..T G.A...A
#Vl42 (1)   ...C...A.. .....T...T .......... .......A.. T.....T..A TG.T....T. GT...C.GT. .....T.... .T..T.A..T G.A...A
#Vl43 (1)   ...C...A.. ......G..T T.......... .......A.. TG....T..A T..T....T. GT..GC.G.. ....?????? ?????????? ???????
#Vl44 (1)   ...C...A.. ......G..T T.......... .......A.. T.....TA.A T..T....T. GT..GC.G.. ....?????? ?????????? ???????
#Vl45 (4)   ...C...A.. ......G..T T.....G... .......A.. T.....T..A T..T....T. GT..GC.G.. .....T.... .TC.T.A..T G.....A
#Vl46 (12)  ...C...A.. ......G..T T.......... .......A.. T.....T..A T..T....T. GT..GC.G.. .....T.... .T..T.A..T G.A...A
#Vl47 (1)   ...C...AC. ......G..T T.......... .......A.. T.....T..A T..T....T. GT..GC.G.. .....T.... .T..T.A..T G.A...A
#Vl48 (2)   ??....T... .........T .........T .........A ......T... ........T. .T.T...... .....T.... .T..T.A... .......
#Vl49 (1)   ??CC...A.. .....T...T ...TC...T. ..C....A.. T.....T..A T..T.A..T. GT..GC.G.. C....T.... .T..T.A..T G.A...A
#Vl50 (1)   ??....T... ..G......T .........T .........A ......T... ........T. .T........ .....T.... .T..T.A... .......
#Vl51 (4)   ??.CG.TA.. ..G......T .......... .......A.. T.....T..A T..T..C.T. GT...GC.G.. .....T.... .T..T.A..T G.A...A
#Vl52 (6)   ??........ .........T .......... .........A ...G...... .......... GT........ .....T.... .T..T.A... .......
#Vl53 (1)   ??........ .........T .......... .G.......A ...G...... .......... GT........ .....T.... .T..T.A... .......
#Vl54 (1)   ??........ .......... .......... .......... .......... .......... .......... .....T...C ....T..... .......
#Vl55 (9)   ??....T... .........T .........T .........A ......T... ........T. .T........ .....T.... .T..T.A... .......
#Vl56 (1)   ??....T... .........T .........T .........A ......T... ........T. .T........ .T...T.... .T..T.A... .......
#Vl57 (2)   ??.C...A.. .....T...T .......... .......A.. T.....T... T..T....T. GT..GC.G.. .....T.... CT..T.A..T G.A...A
#Vl58 (1)   ...C...A.. .....T...T .......... .......A.. T.....T.GA T..T....T. GT..GC.GT. .....T.... .T..T.A..T G.A...A
#Vl59 (1)   ??.C...A.. .....T...T .......... .......A.. T.....T..A T..T....T. GT..GC.GT. .....T.... .T..T.A..T G.A...A
#Vl60 (1)   ??.C..TA.. ....G....T ..C....... .......A.. T.....T..A T..TC...T. GT..GC.G.. .....T.... .T..T.A..T .CA...A
#Vl61 (1)   ??....TA.. .........T .......... .......A.. T.....T..A T..TC...T. GT..GC.G.. .....T.... .T..T.A..T .CA.C.A
#Vl62 (1)   ??.C..TA.. .........T .......... .......A.. T.....T..A ...TC...T. GT..GC.G.. .....T.... .T..T.A..T .CA...A
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Fig. 2. Haplotypes of the mitochondrial cytochrome *b* gene in *S. schrenckii.* Variable nucleotide positions were identified against the sequence of haplotype Vl1 (EU567390). Nucleotide position 1 corresponds to position 14 228 of the *S. keyserlingii* full-length mitochondrial genome (NC008082, [9]). The number of newts with the given haplotype is given in parentheses. Unidentified nucleotides are indicated with (?).

types (*k*) and polymorphic positions (*s*) as cluster 1. Similar results were obtained for population indices θ_S and θ_K, which have been estimated from the number of polymorphic positions and haplotypes in populations. Since θ_K reflects the effective size of a population, our results indicated that clusters 1 and 2 substantially differed in effective size, which was almost four times greater in cluster 2 (Table 2). The haplotypes of this cluster displayed a unimodal distribution of pairwise nucleotide differences (Fig. 4), suggesting

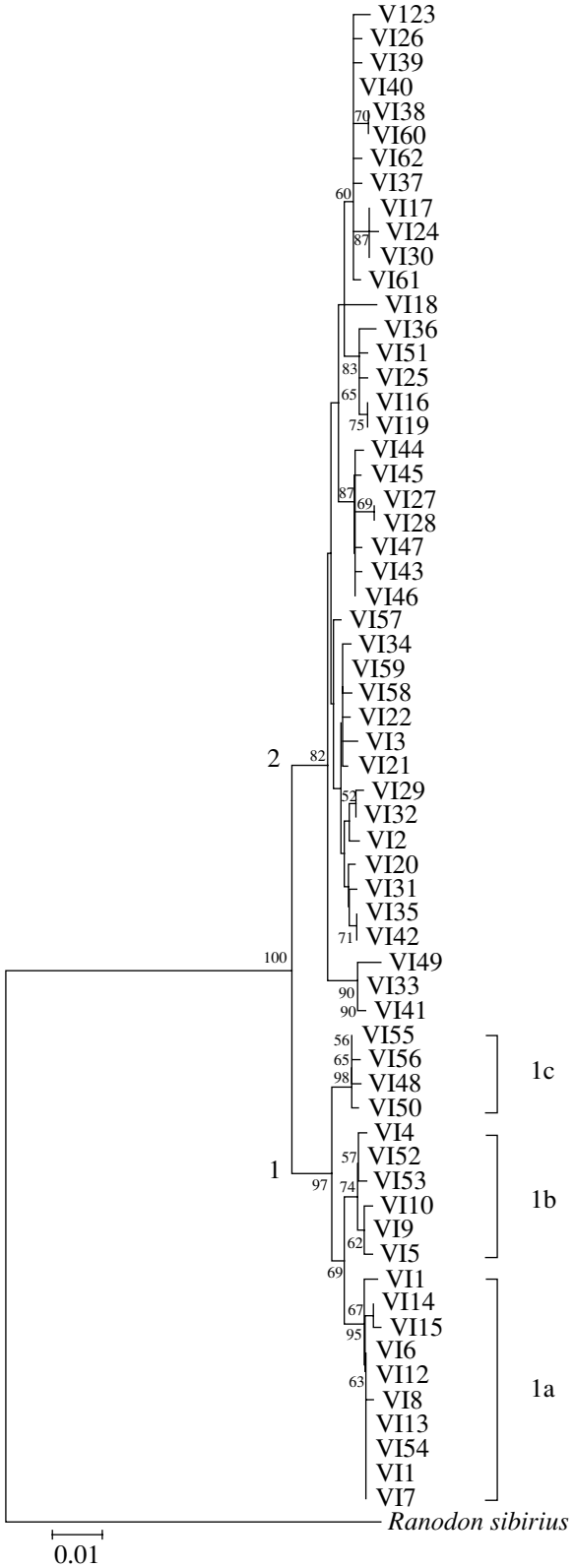

Fig. 3. NJ dendrogram of ρ-distances between cytochrome *b* gene haplotypes in *S. schrenckii.* Haplotypes are designated as in Fig. 2. Bootstrap indices (~60%) are indicated. The nucleotide sequence of the *R. sibiricus* cytochrome *b* gene was used as an outgroup. The main phylogenetic clusters (1 and 2) and their subclusters are shown.

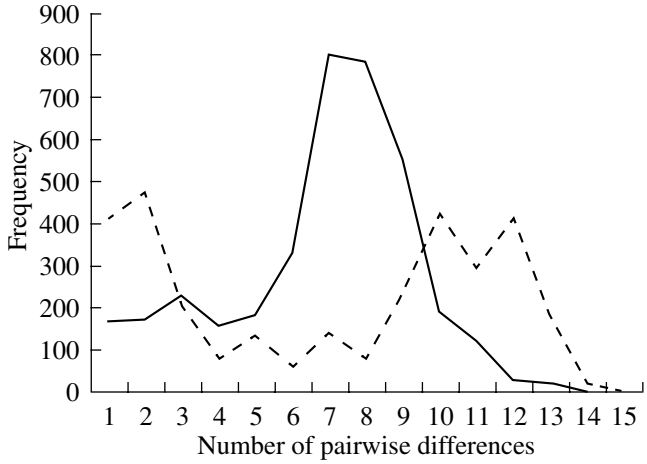

Fig. 4. Distribution of pairwise nucleotide differences between cytochrome *b* gene sequences in *S. schrenckii* clusters 1 (dashed line) and 2 (solid line).

recent demographic expansion or a series of expansions with a high migration rate between neighbor groups [12–14]. In contrast, a multimodal distribution of pairwise nucleotide differences was observed for cluster 1, suggesting demographic equilibrium; i.e., the population size did not grow exponentially.

We tested the two demographic expansion models and found that the character of mtDNA diversity corresponded to that expected from the models with a high significance only for cluster 2. The spatial expansion model assumes that the population size initially grows in a limited region and then the region rapidly increases owing to the high migration rate [14]. Fu's F_S test for selective neutrality [15] also suggested an exponential growth of the population size for cluster 2, yielding a significant negative F_S value only for this cluster (2 (F_S = –24.7, P = 0). D-statistics (Tajima's test [16]) were also high for cluster 2, but did not reach the 5% significance level. It should be noted that, when haplotypes were analyzed by regional groups, the significance of F_S-statistics increased northwards, from nonsignificant values (P = 0.9 and 0.3) in populations (1) and (2) to highly significant values (P = 0.016 and 0.002) in populations (3) and (4). This result confirmed that demographic expansion took place only in the northern part of the *S. schrenckii* species range, where cluster 2 haplotypes were mostly observed.

It is known that deviations from neutrality are caused not only by demographic factors, but also by heterogeneity of mutation rates in the DNA region under study [8]. We checked the molecular clock hypothesis by Tajima's test [8] and found no heterogeneity of mutation rates in comparisons of the two *S. schrenckii* clusters with the outgroup (*R. sibiricus*) or the two newt species (*S. schrenckii* and *S. keyserlingii*) with *R. sibiricus* ($P > 0.2$ in all cases). Thus, the

Table 2. Parameters of mtDNA diversity in *S. schrenckii* clusters 1 and 2

Genetic parameter	Cluster 1	Cluster 2
Sample size *n*	80	87
Total haplotypes *k*	20	42
Total polymorphic positions *s*	31	53
Diversity *h*	0.88 ± 0.02	0.96 ± 0.01
Mean number of pairwise nucleotide differences *i*	6.04 ± 2.91	5.82 ± 2.81
Distance ρ	5.68 ± 1.55	4.31 ± 0.86
Divergence *d*	0.008 ± 0.002	0.010 ± 0.002
Population parameter θ_S	6.2	10.3
Population parameter θ_K	8.2	31.3
Demographic parameters (rapid expansion model) τ, θ_0–θ_1	10.92, 0–11.0 (P = 0.45)	6.86, 0–60.6 (P = 0)
Demographic parameters (spatial expansion model) τ, θ_0, M	7.87, 3.29, 1.96 (P = 0.9)	6.72, 0.016, 25.4 (P = 0)
D (Tajima's test)	–0.11 (P = 0.5)	–1.41 (P = 0.07)
F_S (Fu's test)	–1.55 (P = 0.33)	–24.69 (P = 0)
Distribution of pairwise nucleotide differences	multimodal	unimodal

Note: *h* is the mtDNA haplotype diversity [4]; τ is the expansion time corresponding to the number of pairwise differences in the period of a maximal growth in population size; $\theta_0$and θ_1 are the mutation parameters at the initial and final steps of the increase in population size, respectively; and M is the parameter that characterizes the interpopulation exchange of migrants. The significance of differences (P) is given in parentheses. See text for the definitions of the other parameters.

data on mtDNA variation proved suitable for dating the population events by the molecular clock.

The question arises as to the origin of the two *S. schrenckii* groups (clusters), but the results of the phylogenetic analysis of nucleotide sequences are insufficient for a reliable conclusion on a more ancient origin of one of the groups. The clusters both converge to one common ancestor and have similar levels of within-cluster divergence. A difference between the clusters was detected by analyzing the distribution of pairwise nucleotide differences: the mean number of pairwise differences in cluster 1 (i = 6.0) was somewhat higher than in cluster 2 (i = 5.8, Table 2). The difference was better seen in the analysis of the median networks of mtDNA haplotypes (data not shown). We computed the ρ-distance, which estimates the mean distance from the founder haplotype to all derivative haplotypes in terms of the number of mutations, and found that cluster 1 was more diverse (ρ = 5.7) than cluster 2 (ρ = 4.3). Assuming that the mutation rate of the cytochrome *b* gene is 0.77% of divergence (for transitions and transversions) per 1 Myr [2], we estimated the evolutionary ages of clusters 1 and 2 at 895000 ± 244000 and 680000 ± 135000 years, respectively.

Interesting results were obtained by analyzing the distribution of amino acid substitutions in the *S. schrenckii* cytochrome *b* region under study. As already noted, only three amino acid substitutions were phylogenetically informative. One of them, Ile43Met, was observed in two related haplotypes, Vl27 and Vl28 (Fig. 3). Substitution Ile46Val was found in both of the clusters, occurring in haplotype Vl50, which belongs to subcluster 1c, and in haplotypes Vl16, Vl19, Vl25, Vl36, and Vl51, which form a separate subcluster (bootstrap index 83%) within cluster 2. Analysis of the cytochrome *b* amino acid sequences of other species of the family Hynobiidae (GenBank and published data [1, 9, 17]) detected the Ile46Val polymorphism in *S. keyserlingii, Liua* sp., and *Pseudohynobius* sp., indicating that the substitution arose independently several times in the course of Hynobiidae evolution.

The third amino acid substitution, Ile119Val, was similarly detected in several species of the genera *Hynobius* and *Salamandrella* (Table 3). However, between-species comparisons of the corresponding cytochrome *b* region revealed Ile119 in most species of the families Hynobiidae and Salamandridae (Table 3). This finding testifies that Ile119 is an ancestral variant. This variant was characteristic of some cluster 1 haplotypes: subclusters 1b and 1c (Fig. 1, collection sites 23–33) had Ile119, while subcluster 1a (collection sites 30 and 32–35) and all northern *S. schrenckii* groups carried the Ile119Val substitution. It seems that the Ile119Val substitution independently arose in the two *S. schrenckii* clusters. However, the presence of the ancestral variant Ile119 in southern subclusters 1b and 1c suggests their more

Table 3. Amino acid difference in cytochrome *b* in representatives of the families Hynobiidae and Salamandridae

Taxon	Amino acid position		
	118	119	120
Euproctus (3 species)	Val	Ile	Leu
Batrachuperus (6 species)	Ile	Ile	Leu
Pseudohynobius (2 species)	Ile	Ile	Leu
Hynobius (7 species)	Val	Ile	Leu
Hynobius (6 species)	Val	**Val**	Leu
Pachyhynobius shangchengensis	Val	Ile	Leu
Onychodactylus fischeri	Val	Ile	Leu
Liua shihi	Ile	Ile	Leu
Ranodon sibiricus	Ile	Ile	Leu
Salamandrella keyserlingii	Val	Ile	Leu
Salamandrella keyserlingii	Val	**Val**	Leu
Salamandrella schrenckii (subclusters 1b and 1c)	Val	Ile	Leu
Salamandrella schrenckii (subcluster 1a and cluster 2)	Val	**Val**	Leu

Note: Val in position 119 is in bold. Data were extracted from GenBank and published works [1, 2, 9, 17].

ancient origin as compared to the northern *S. schrenckii* group.

Our results are the first to provide insight into the molecular evolution of *S. schrenckii* and its intraspecific structure. We used the data on the nucleotide sequence variation of the cytochrome *b* gene to demarcate the *S. schrenckii* species range, which is now possible only by molecular genetic methods. The *S. schrenckii* range proved to include a substantial part of southeastern Russia. The high mtDNA variation at the intraspecific and interpopulation levels indicates that *S. schrenckii* genetic diversity formed during a long period of time and that its structure was affected by many factors. Analyses of the mtDNA and cytochrome *b* amino acid sequence variations showed that the modern *S. schrenckii* species range was probably colonized from south Primorye northwards, since southern *S. schrenckii* populations carry an ancient amino acid sequence variant (Ile119) and display higher pairwise nucleotide differences. At the same time, genetic demographic data indicate that populations of the northern part of the species range (cluster 2) experienced a substantial increase in size in the course of their evolution and that their expansion (in particular, southward) is likely to continue. High interpopulation differentiation suggests a low migration activity for *S. schrenckii* and/or prolonged isolation periods for its evolution.

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