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Intraspecific Genetic Differentiation of the Siberian Newt (*Salamandrella keyserlingii*, Amphibia, Caudata) and the Cryptic Species *S. schrenckii* from Southeastern Russia

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Abstract—The nucleotide sequences of the mitochondrial cytochrome *b* gene in the Siberian newt *Salamandrella keyserlingii* Dybowski 1870 from the populations of the Ural Mountains, Magadan oblast, Chukchi Peninsula, Sakhalin Island, and Primorskii krai are analyzed. It is shown that in most populations studied (except for Primorskii krai), a low geographic variation in morphological characters corresponds to a low level of genetic variation (0.38% in the combined sample from the Magadan, Sakhalin, Chukchi, and Ural populations). Different scenarios for the origin of the genetically and morphologically homogeneous hyperpopulation are discussed, taking into account the obvious lack of genetic exchange between the marginal populations of the range. They involve the rapid formation of the species range in the Holocene, which followed its gradual development in the Pleistocene; unidirectional stabilizing selection within the entire range; the maintenance of variation at a stable level by mixing of the population during the dispersal of the young and, possibly, by group fertilization. The population inhabiting the Primorskii krai, despite minor morphological differences from other populations, is characterized by a high level of mtDNA divergence (9.8–11.6%) and considerable intrapopulation variation (1.86%). In view of the data obtained, it appears feasible to restore for *Salamandrella* from Primorskii krai the name *S. schrenckii* (Strauch, 1870), which was a junior synonym for *S. keyserlingii.* Based on the mtDNA sequences, the times of emergence for *S. keyserlingii* and *S. schrenckii* are dated 490 ka and 2.4 Ma, respectively. These should be considered two species of different ages which diverged from a common stem approximately 14 Ma, rather than a descendant and its ancestor.

INTRODUCTION

The Siberian newt (*Salamandrella keyserlingii* Dybowski 1870) occupies an extensive range from the tundra to the steppe and from the Pacific Ocean to northwestern European Russia. Morphometric studies have revealed a low geographic variation in the morphological characters of this species. Although Siberian newts from the Yekaterinburg and Yakutsk regions and Primorye differ in their body proportions and plastic characteristics, the extent of the differences is too low to classify them as separate taxa. The available data allow only the conclusion that the Far East Siberian newt has some distinctive features (Ostashko, 1981). Borkin (1994) analyzed variation in eight parameters (including the lengths of the body and tail, the relative length and width of the head, the ratio of limb measurements, and the number of digits and grooves on the body sides) in population samples from the Yekaterinburg region, Yakutia, Transbaikalia, Sakhalin Island, and Primorskii krai (Primorye). He also concluded that, "taking into account all the characteristics considered, it seems that Siberian newts from the southeastern areas of the range differ to a greater extent than the others…" (Borkin, 1994, p. 79).

Basarukin and Borkin (1984) proposed the probable taxonomic significance of the nonspiral shape of the spawn, the absence of nuptial behavior, and some other features distinctive of the Siberian newt population from Primorye. However, referring to unpublished data of G.P. Sapozhnikov, Borkin (1994) subsequently indicated that "some ecological distinctions of *S. keyserlingii* from Primorye are less pronounced than was previously believed" (Borkin, 1994, p. 80). At the same time, the idea of substantial differences of this population from other populations of *S. keyserlingii* has recently been corroborated by Vorob'eva *et al.* (1999) based on the development of elements of the larval locomotor system, by Litvinchuk *et al.* (2001) based on the genome size, and by Litvinchuk and Borkin (2003) based on the number of vertebrae and costal grooves. On this basis, Litvinchuk *et al.* (2004) proposed that Siberian newts from Primorye should be regarded as a separate subspecies, *Salamandrella keyserlingii tridac-*

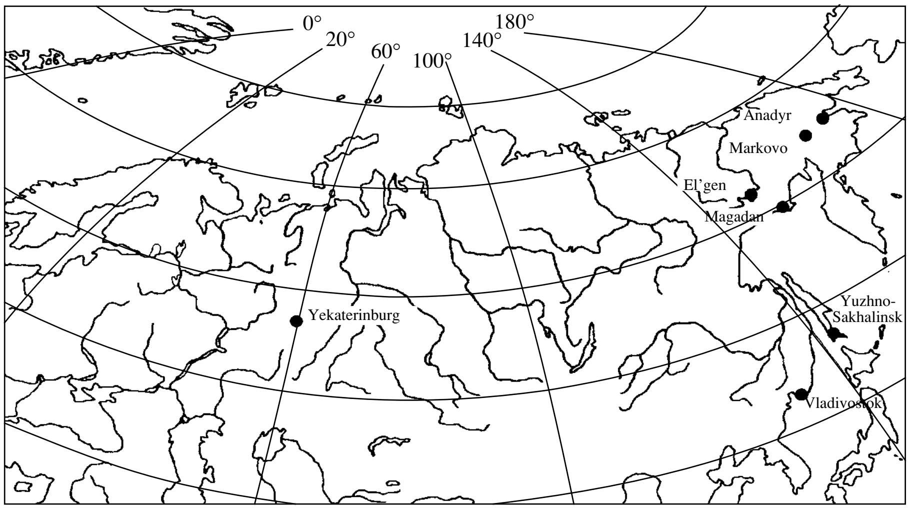

Fig. 1. Geographic location of the populations examined in the study.

tyla Nikolsky 1906.[1] Kuzmin and Maslova (2003) showed the genetic specificity of the Siberian newt from Primorye and, discussing its Latin name, proposed to designate it *Salamandrella keyserlingii tridactyla* Nikolsky 1905 in the case of its subspecies rank or *Salamandrella tridactyla* Nikolsky 1905 in the case of species rank, i.e., in the presence of reproductive isolation.

The challenge to explain the surprising morphological uniformity of *S. keyserlingii* throughout its extremely large range, combined with the taxonomic independence of animals from southeastern Russia, required the application of molecular genetic methods. A low geographic variation in the phenotype can mask a significant genetic polymorphism and even the existence of cryptic species (Kryukov and Suzuki, 2000; Borkin *et al.*, 2001; Zink *et al.*, 2002a; Khalturin *et al.*, 2003). Therefore, we investigated genetic variation in *S. keyserlingii* over its wide range by analyzing polymorphism in the nucleotide sequences of the cytochrome *b* gene in mitochondrial DNA (mtDNA). As is well known, mtDNA is characterized by maternal inheritance and lack of recombination; it displays a high level of variation, providing, therefore, important molecular data on the evolution of different animals, including amphibians (Avise, 1994; Garsia-Paris *et al.*, 2000; Steinfartz *et al.*, 2000; Riberon *et al.*, 2000).

MATERIALS AND METHODS

We analyzed samples of *Salamandrella* (tissues of adults, larvae, and embryos frozen or preserved in 70% alcohol) from the following regions (Fig. 1):

(1) southern Sakhalin Island, Lake Tunaicha, 9 specimens;

(2) vicinity of Vladivostok (Fig. 2a), 28 specimens, including 2 specimens from the vicinity of the Bogatinskoe Reservoir, 7 from the Malaya Sedanka River valley, 8 from the Botanical Garden, 5 from the Bol'shaya Sedanka River valley, and 6 from the vicinity of Lazurnaya Bay;

(3) northeastern Asia (Fig. 2b), 41 specimens: Chukchi Peninsula (2 specimens from the village of Markovo and 1 from the town of Anadyr); Kolyma River Basin (5 specimens from the vicinity of the village of El'gen); Ola, Oira, and Yana rivers (Pacific Basin, west and east of Magadan), lakes nearby Magadan (at the top of the Kamennyi Venets Hill, Staritskogo Peninsula), 33 specimens;

and (4) vicinity of Yekaterinburg, 8 specimens.

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 deproteination in a phenol–chloroform mixture. The quality and quantity of DNA were estimated by electrophoresis in 1% agarose gel followed by gel staining with ethidium bromide and examination under UV light.

The cytochrome *b* gene was amplified as two overlapping DNA fragments by polymerase chain reaction (PCR) with the pairs of primers MVZ15L and MVZ18H, and MVZ25L and ControlWH proposed by Goebel *et al.* (1999). Amplification included 40 cycles at the following temperatures: 94°C for 60 s, 46°C for 90 s, and 72°C for 90 s. The PCR products were puri-

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[1] Here and below, dates are given according to the authors cited.

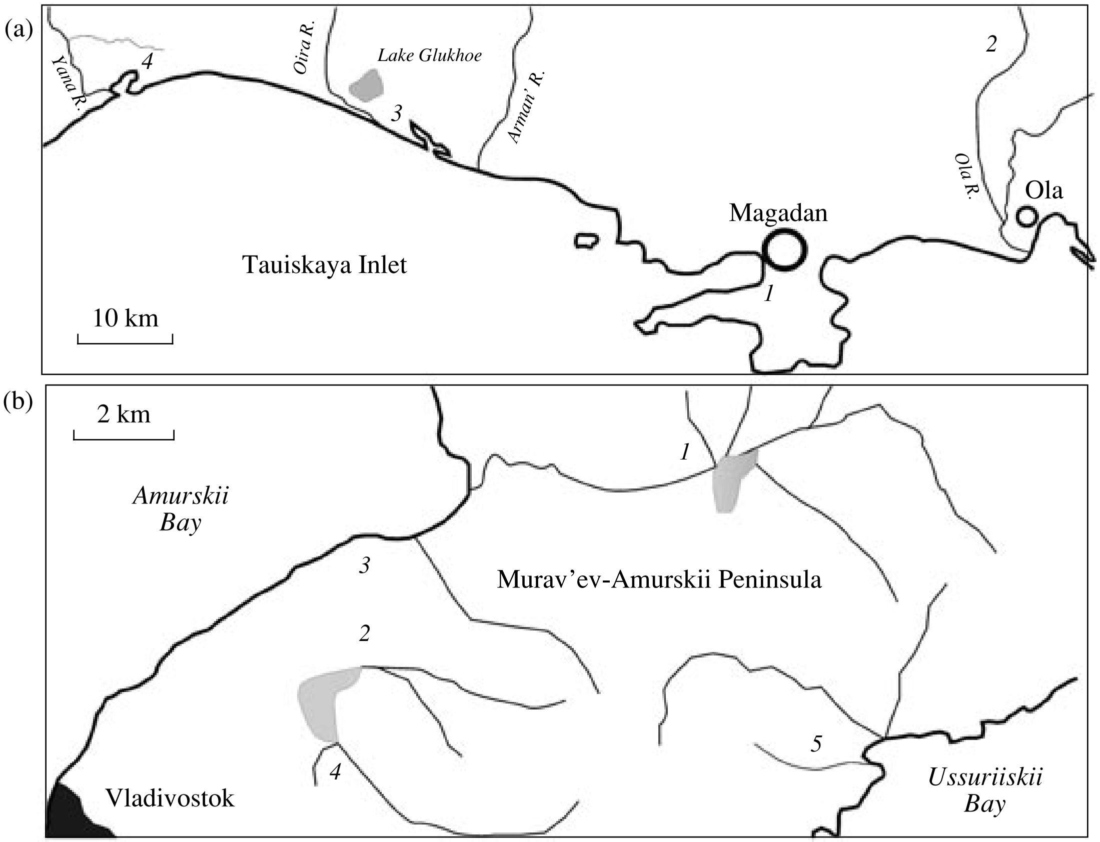


Fig. 2. Sampling sites: (a) in the vicinity of Magadan: (*1*) Lake Venechnoe, (*2*) vicinity of the village of Gadlya, (*3*) vicinity of Lake Glukhoe, and (*4*) left bank of the Yana River near the mouth; (b) on the Muraveva-Amurskogo Peninsula in the vicinity of Vladivostok: (*1*) near the Bogatinskoe Reservoir, (*2*) floodplain of the Malaya Sedanka River, (*3*) Botanical Garden of the Far East Division of the Russian Academy of Sciences, (*4*) the Bol'shaya Sedanka River valley, and (*5*) creek near Lazurnaya Bay.

fied by ultrafiltration in Microcon 100 columns (Amicon, United States).

Fragments of the cytochrome *b* gene were sequenced in an ABI PrismTM 377 automatic sequencer using a BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, United States) and primers MVZ15L and MVZ25L. The sequences obtained were aligned and analyzed using the Sequence Navigator program (Applied Biosystems), and phylogenetic analysis was performed using the MEGA 2.1 program package (Kumar *et al.*, 2001). Pairwise genetic distances (*p* distances) between individual DNA sequences were calculated based on the number of nucleotide substitutions per base pair. The time of divergence and evolutionary age of the mtDNA lineages were estimated on the assumption that the rate of substitutions in the cytochrome *b* gene was 0.77% of divergence (for transitions and transversions) per million years (Caccone *et al.*, 1997). This value was obtained by calibrating the "molecular clock" based on paleontological data on the time of divergence between species of the genus *Euproctus* (Caccone *et al.*, 1997). Furthermore, this value agrees with the rate of divergence for this gene (ranging from 0.47 to 1% per million years) in a number of species of the family Salamandridae (for reviews, see Tan and Wake, 1995; Caccone *et al.*, 1997; Riberon *et al.*, 2000).

The neighbor-joining (NJ) method (Saitou and Nei, 1987), unweighted pair group method with arithmetic averages (UPGMA), and maximum parsimony (MP) method (Nei, 1987) were used to construct phylogenetic trees. The data from GenBank (http://www.ncbi.nlm.nih.gov/entrez) on the nucleotide sequences of the cytochrome *b* gene (a 743-bp fragment) in members of different genera of the family Hynobiidae were used for comparative analysis. Three species of the genus *Euproctus* from the family Salamandridae were used as outgroups (Table 1).

The sequences of the cytochrome *b* gene obtained in this study for the 86 Siberian newts were deposited in the GenBank (accession nos. AY701904–AY701989).

RESULTS

The nucleotide sequences of a 825-bp fragment of the mitochondrial cytochrome *b* gene between positions 14214 and 15308 (according to the numbering in the complete mitochondrial genome of *Ranodon sibiri-*

Table 1. Taxa from the GenBank included in phylogenetic analysis

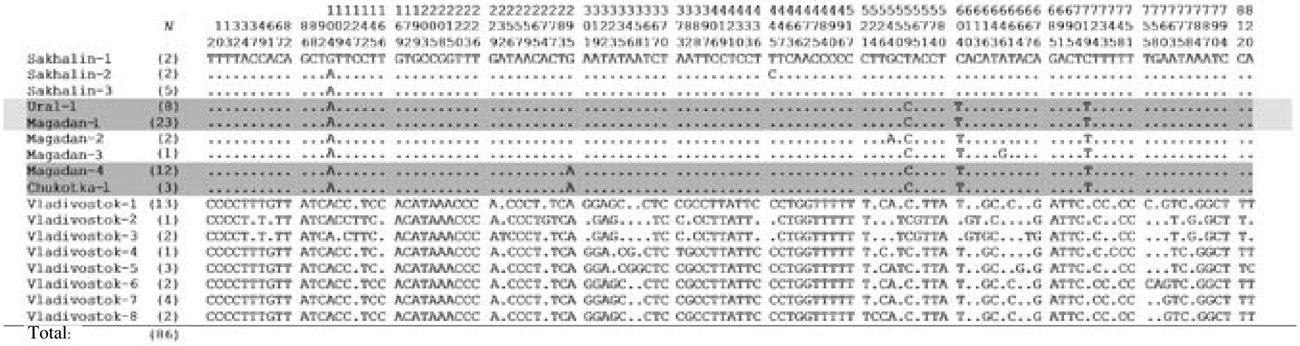
Taxon	*N*	GenBank accession no.	Reference
Batrachuperus tibetanus	13	AY028756, AY028757, AY028760, AY028761, AY028764–AY028772	Fu *et al*., 2001
B. yenyuanensis	2	AY028762, AY028763	Fu *et al*., 2001
B. taibaiensis	2	AY028758, AY028759	Fu *et al*., 2001
B. karlschmidti	2	AY028754, AY028755	Fu *et al*., 2001
B. pinchonii	4	AY028749–AY028753	Fu *et al*., 2001
B. londongensis	2	AY028747, AY028748	Fu *et al*., 2001
Pseudohynobius tsinpaensis	1	AY372535	Li, unpublished
P. flavomaculatus	2	AY028773, AY028774	Fu *et al*., 2001
Hynobius yiwuensis	7	AY300153–AY300159	Fu *et al*., unpublished
H. amjiensis	2	AY300151, AY300152	Fu *et al*., unpublished
H. leechii	1	AY300150	Fu *et al*., unpublished
Liua shihi	1	AY028775	Fu *et al*., 2001
Ranodon sibiricus	1	AJ419960	Zhang *et al*., 2003
Euproctus platycephalus	1	U55947	Caccone *et al*., 1997
E. montanus	1	U55946	Caccone *et al*., 1997
E. asper	1	U55945	Caccone *et al*., 1997

Note: *N* is the number of sequences.

cus, see Zhang *et al*., 2003) were determined in 86 Siberian newts from five regions. The total sample included 15 variants (haplotypes or mitotypes) of the gene differing in 112 polymorphic positions, of which 102 were phylogenetically informative (i.e., occurred in more than one mtDNA haplotype). The majority of mutations (99) are transitions, while transversions are far less numerous (13 positions). There are 19 and 1 mutations in the first and second codon positions, respectively. There are 9 positions where mutations result in amino acid substitutions. Thus, most mutations are synonymous (Fig. 3).

The most frequent haplotypes in the samples examined were designated Magadan-1 (recorded in 23 Siberian newts from the Magadan sample) and Ural-1 (the same haplotype revealed in all specimens from the Ural sample). Another haplotype observed in 12 out of 38 specimens from the Magadan sample (Magadan-4) was also detected in all specimens from the Chukchi Peninsula (haplotype Chukotka-1). The most frequent haplotype of the Primorye sample (Vladivostok-1) was revealed in 14 out of 28 specimens (Fig. 3).

The distribution of haplotypes of the cytochrome *b* gene manifests obvious geographic differentiation, as is evident from the NJ phylogenetic tree constructed on



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                             1111111 1112222222 2222222222 3333333333 3333444444 4444444445 5555555555 6666666666 6667777777 7777777777 88
                  N     113334668 8890022446 6790001222 2355567789 0122345667 7889012333 4466778991 2224556778 0111446667 8990123445 5566778899 12
                       2032479172 6824947256 9293585036 9267954735 1923568170 3287691036 5736254067 1464095140 4036361476 5154943581 5803584704 20
Sakhalin-1      (2)    TTTTACCACA GCTGTTCCTT GTGCCGGTTT GATAACACTG AATATAATCT AATTCCTCCT TTCAACCCCC CTTGCTACCT CACATATACA GACTCTTTTT TGAATAAATC CA
Sakhalin-2      (2)    .......... ...A...... .......... .......... .......... .......... C......... .......... .......... .......... .......... ..
Sakhalin-3      (5)    .......... ...A...... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..
Ural-1          (8)    .......... ...A...... .......... .......... .......... .......... .......... .....C.... T......... ....T..... .......... ..
Magadan-1      (23)    .......... ...A...... .......... .......... .......... .......... .......... .....C.... T......... ....T..... .......... ..
Magadan-2       (2)    .......... ...A...... .......... .......... .......... .......... .......... ...A.C.... T......... ....T..... .......... ..
Magadan-3       (1)    .......... ...A...... .......... .......... .......... .......... .......... .....C.... T....G.... ....T..... .......... ..
Magadan-4      (12)    .......... ...A...... .......... .........A .......... .......... .......... .....C.... T......... ....T..... .......... ..
Chukotka-1      (3)    .......... ...A...... .......... .........A .......... .......... .......... .....C.... T......... ....T..... .......... ..
Vladivostok-1  (13)    CCCCTTTGTT ATCACC.TCC ACATAAACCC A.CCCT.TCA GGAGC..CTC CGCCTTATTC CCTGGTTTTT T.CA.C.TTA T..GC.C..G ATTC.CC.CC C.GTC.GGCT TT
Vladivostok-2   (1)    CCCCT.T.TT ATCACCTTC. ACATAAACCC A.CCCTGTCA .GAG....TC C.CCTTATT. .CTGGTTTTT T...TCGTTA .GT.C....G ATTC.C..CC ...T.G.GCT T.
Vladivostok-3   (2)    CCCCT.T.TT ATCA.CTTC. ACATAAACCC ATCCCT.TCA .GAG....TC C.CCTTATT. .CTGGTTTTT T...TCGTTA .GTGC...TG ATTC.C..CC ...T.G.GCT T.
Vladivostok-4   (1)    CCCCTTTGTT ATCACC.TC. ACATAAACCC A.CCCT.TCA GGA.CG.CTC TGCCTTATTC CCTGGTTTTT T.C.TC.TTA T..GC....G ATTC.C.CCC ...TC.GGCT TT
Vladivostok-5   (3)    CCCCTTTGTT ATCACC.TC. ACATAAACCC A.CCCT.TCA GGA.CGGCTC CGCCTTATTC CCTGGTTTTT T.CATC.TTA T..GC..G.G ATTC.C..CC ...TC.GGCT TC
Vladivostok-6   (2)    CCCCTTTGTT ATCACC.TCC ACATAAACCC A.CCCT.TCA GGAGC..CTC CGCCTTATTC CCTGGTTTTT T.CA.C.TTA T..GC.C..G ATTC.CC.CC CAGTC.GGCT TT
Vladivostok-7   (4)    CCCCTTTGTT ATCACC.TCC ACATAAACCC A.CCCT.TCA GGAGC..CTC CGCCTTATTC CCTGGTTTTT T.CA.C.TTA T..GC.C..G ATTC.CC.CC ..GTC.GGCT TT
Vladivostok-8   (2)    CCCCTTTGTT ATCACC.TCC ACATAAACCC A.CCCT.TCA GGAGC..CTC CGCCTTATTC CCTGGTTTTT TCCA.C.TTA T..GC.C..G ATTC.CC.CC ..GTC.GGCT TT
Total:         (86)
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Fig. 3. Haplotypes of the mitochondrial cytochrome *b* gene of *Salamandrella keyserlingii*: positions of variable nucleotides are shown in comparison with the nucleotide sequence of Sakhalin-1 (*N* is the number of individuals with a certain haplotype; identical haplotypes found in different local groups are highlighted.)

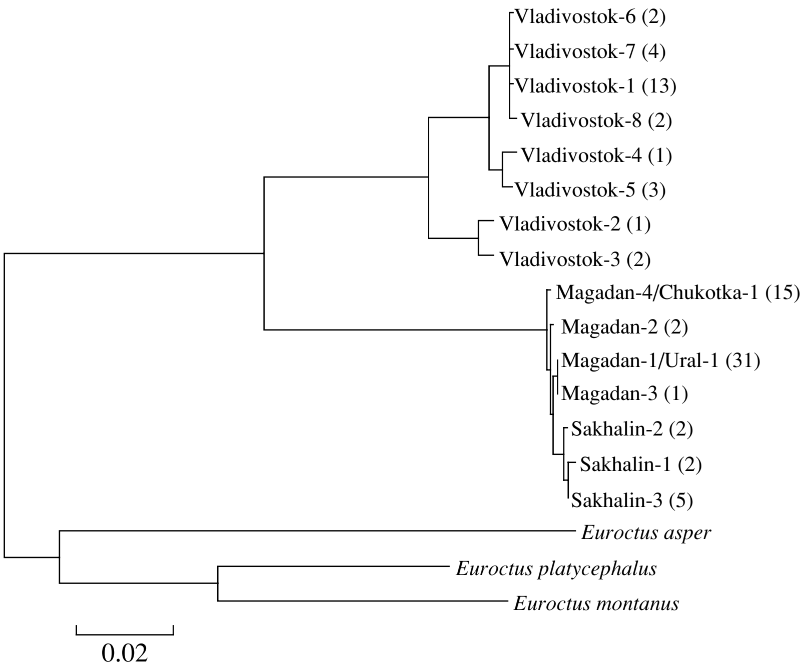

Fig. 4. NJ tree of *p*-distances between haplotypes of the cytochrome *b* gene in Siberian newts (for designations of haplotypes, see Fig. 3; members of the genus *Euproctus* are used as outgroups; the number of amphibians with a certain haplotype is shown in parentheses).

the basis of both transitions and transversions (Fig. 4). Two large monophyletic clusters are distinct—the first contains only mtDNA variants from Primorye, while the second includes mtDNA variants found in other local samples, i.e., those from the Ural, Sakhalin, Magadan, and Chukchi populations. The Sakhalin mtDNA variants in the second cluster are segregated and form an individual second-order cluster. The mitochondrial variants from Primorye are also clearly heterogeneous; the Vladivostok-2 and Vladivostok-3 variants are distinctly segregated from the others. It should be emphasized that the phylogenetic trees constructed by different methods (i.e., UPGMA or MP) have the same topology.

The pairwise genetic distances between the nucleotide sequences of the cytochrome *b* gene variants range from 0.1 to 11.6%, that is, from 1 to 96 nucleotide substitutions (Table 2). Siberian newts from Primorye make the main contribution to these values; thus, their mtDNA variants differ from those in other samples by 81–96 substitutions (9.8–11.6%). The intrapopulation variation in Siberian newts from Primorye (1.86%) is ten times that in other local populations, i.e., 0.16% in the Sakhalin sample and 0.18% in the combined Magadan, Chukotka, and Ural sample (Table 3). Moreover, in the first-order clusters, the degrees of intragroup differentiation of the mtDNA variants recorded in Primorye are 0.84 and 0.61% (Fig. 4); i.e., this differentiation is also substantially higher than in other local populations of *S. keyserlingii.*

DISCUSSION

The above analysis has shown that all the populations studied (from the Urals, Magadan oblast, Chukchi Peninsula, and Sakhalin Island), except for the population from Primorye, combine insignificant geographic variation in morphological characters with a low level of genetic variation in the mitochondrial cytochrome *b* gene. Thus, *S. keyserlingii* is a genetically uniform species throughout its vast range (except for Primorye). Only the Sakhalin variants of mtDNA form a separate subcluster. At present, it is premature to discuss the taxonomic significance of this subcluster because of the limited sample size and the relatively small differences from the other samples (0.54%, Table 3). Note that a separate species, *Salamandrella cristata* Anderson, 1917, was described from southern Sakhalin; however, it was subsequently synonymized with *S. keyserlingii* (Borkin, 1994). Therefore, it is desirable to use a larger sample from this area, because the nine spawn samples examined come from the same water body, but, nonetheless, belong to three different variants of the cytochrome *b* gene. It may well be that the Siberian newt population from southern Sakhalin differs from the continental populations to an even greater extent than is presently thought.

The insignificant morphological specificity of *Salamandrella* from Primorye concealed the fact that this population is characterized by considerable genetic differences (10.8%) from all the other populations studied. To reveal the probable taxonomic significance of these differences, we performed a comparative phylo-

Table 2. Differences between haplotypes of the mitochondrial cytochrome *b* gene of the Siberian newt expressed as the number of nucleotide substitutions (below the diagonal) and genetic distances, % (above the diagonal)

Haplotypes of cytochrome *b* gene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Sakhalin-1	–	0.2	0.1	0.5	0.6	0.6	0.6	11.5	9.9	11.6	11.4	11.0	10.1	11.5	11.3
Sakhalin-2	2	–	0.1	0.5	0.6	0.6	0.6	11.3	9.9	11.4	11.2	10.8	10.1	11.3	11.0
Sakhalin-3	1	1	–	0.4	0.5	0.5	0.5	11.4	9.8	11.5	11.3	10.9	9.9	11.4	11.2
Magadan-1/Ural-1	4	4	3	–	0.1	0.1	0.1	11.3	9.9	11.4	11.2	10.8	10.1	11.3	11.0
Magadan-2	5	5	4	1	–	0.2	0.2	11.2	10.1	11.3	11.0	10.9	10.2	11.2	10.9
Magadan-3	5	5	4	1	2	–	0.2	11.4	10.1	11.5	11.3	10.9	10.2	11.4	11.2
Magadan-4/Chukotka-1	5	5	4	1	2	2	–	11.2	9.8	11.3	11.0	10.7	9.9	11.2	10.9
Vladivostok-1	95	93	94	93	92	94	92	–	3.3	0.1	0.1	1.3	3.4	0.2	1.3
Vladivostok-2	82	82	81	82	83	83	81	27	–	3.4	3.2	2.9	0.6	3.3	3.0
Vladivostok-6	96	94	95	94	93	95	93	1	28	–	0.2	1.5	3.5	0.4	1.5
Vladivostok-7	94	92	93	92	91	93	91	1	26	2	–	1.2	3.3	0.1	1.2
Vladivostok-4	91	89	90	89	90	90	88	11	24	12	10	–	3.0	1.3	0.7
Vladivostok-3	83	83	82	83	84	84	82	28	5	29	27	25	–	3.4	3.2
Vladivostok-8	95	93	94	93	92	94	92	2	27	3	1	11	28	–	1.3
Vladivostok-5	93	91	92	91	90	92	90	11	25	12	10	6	26	11	–

genetic analysis of the nucleotide sequences of the cytochrome *b* gene in 13 species belonging to different genera of the family Hynobiidae (Table 4). The interspecific distances within the genus *Batrachuperus* (with six species included in the comparison) are 4.4–9.3%; those in the genera *Pseudohynobius* (two species) and *Hynobius* (three species) are 15.5 and 10.7–12.7%, respectively. The interspecific distances in the genus *Euproctus* (three species) from the family Salamandridae (used as an outgroup in our analysis) are even greater, 10.7–21%. Thus, the distance of 10.8% between two clusters of the *Salamandrella* samples examined is in the range of values characteristic of interspecific differences in several genera of the family Hynobiidae and in the genus *Euproctus*.

These results support the conclusion of our predecessors concerning the specificity of *Salamandrella* from Primorye, which was based on differences in morphology, biology, and genome size (Ostashko, 1981; Basarukin and Borkin, 1984; Borkin, 1994; Vorob'eva *et al*., 1999; Litvinchuk *et al*., 2001, 2004; Litvinchuk and Borkin, 2003; Kuzmin and Maslova, 2003). All these data in the aggregate strongly suggest that *Salamandrella* from Primorye is a separate species that should be named *S. schrenckii* (Strauch, 1870) (Berman *et al*., 2005). This name was previously regarded as a senior synonym of *S. keyserlingii* (see review, Borkin, 1994), which was proposed by A. Strauch 35 years earlier than the name *Salamandrella keyserlingii* var. *tridactyla* introduced by Nikol'skii (1905). In the following text, this author indicates that the establishment of this form is tentative:

"The Zoological Museum of the Imperial Academy of Sciences has sent me for examination one specimen of this species from Vladivostok; the forelimbs of this animal have only three digits, which are equally developed; the third of the four digits present on normal limbs is absent. Based on this sole specimen, it is impossible to conclude whether it represents a particular variety or an anomaly; if this feature proves sufficient for distinguishing a variety, I will name it var. *tridactyla*" (Nikol'skii, 1905, p. 491).

Subsequent studies of the Siberian newt have shown that the proportion of animals with tridactylous forelimbs averages 17%, increasing in Primorye to 37%; nevertheless, these individuals were justly considered to be abnormal (Borkin, 1994) and were not segregated in a separate taxon.

Table 3. Local differentiation (on the diagonal) and interregional differentiation (below the diagonal) of Siberian newt mtDNA (genetic distances, %)

Region	1	2	3	4
Sakhalin	0.16			
Magadan, Ural, Chukchi Peninsula	0.54	0.18		
Primorye-1*	11.27	11.13	0.84	
Primorye-2**	9.96	10.03	3.23	0.61

* Primorye-1 combines mtDNA haplotypes Vladivostok-2 and Vladivostok-3.

** Primorye-2 combines other mtDNA haplotypes from Primorye.

Table 4. Genetic distances (%) between species of the families Hynobiidae and Salamandridae based on divergence in the mitochondrial cytochrome *b* gene sequence

No.	Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	*Batrachuperus tibetanus*																	
2	*B. yenyuaensis*	7.3																
3	*B. taibaiensis*	9.0	8.6															
4	*B. karlschmidti*	7.6	7.7	7.6														
5	*B. pinchonii*	7.1	6.0	8.3	4.4													
6	*B. londongensis*	7.8	7.8	8.9	9.3	8.8												
7	*Pseudohynobius tsinpaensis*	17.0	15.2	17.9	18.1	17.0	14.5											
8	*P. flavomaculatus*	16.0	14.4	16.1	17.0	15.8	14.6	15.5										
9	*Hynobius yiwuensis*	14.6	14.4	15.9	16.3	15.2	15.1	17.4	18.1									
10	*H. amjiensis*	16.1	16.3	17.1	16.9	16.4	16.5	18.9	18.0	11.1								
11	*H. leechii*	15.9	16.0	15.6	15.7	16.0	16.6	17.6	18.8	10.7	12.7							
12	*Salamandrella keyserlingii*-1	16.7	16.1	15.7	17.0	16.8	15.9	16.8	16.9	16.2	16.9	15.5						
13	*S. keyserlingii*-2	16.4	15.9	15.7	16.6	16.3	15.6	17.2	19.3	16.8	17.8	16.2	11.0					
14	*Liua shihi*	16.8	16.6	16.7	18.9	17.6	16.1	11.9	18.1	18.3	19.4	18.0	18.8	18.8				
15	*Ranodon sibiricus*	15.4	15.0	14.8	17.3	16.3	14.3	18.0	16.8	16.4	20.0	18.0	16.4	14.9	17.2			
16	*Euproctus platycephalus*	21.2	19.7	21.8	21.5	21.1	20.5	22.9	23.0	21.6	21.5	18.9	20.2	19.4	21.8	20.2		
17	*E. montanus*	20.9	20.5	20.8	21.9	21.9	20.7	25.0	22.4	22.1	21.5	21.0	20.8	20.8	23.4	21.1	10.7	
18	*E. asper*	24.7	24.6	25.7	25.0	24.8	24.4	23.7	23.9	21.7	23.3	24.9	23.6	21.7	25.4	22.0	17.7	21.0

Note: *Salamandrella keyserlingii*-1 is a combined sample from the Ural, Magadan, Chukchi Peninsula, and Sakhalin populations; *Salamandrella keyserlingii*-2 is the population from Primorye.

The name *S. schrenckii* was introduced earlier to designate normal animals; therefore, it should be prefered to *S. tridactyla*, which could have been used if the Siberian newt from Primorye was advanced in taxonomic rank (relative to the subspecies *Salamandrella keyserlingii tridactyla*) (Litvinchuk *et al*., 2004).

It should also be emphasized that the data obtained in this study corroborate the generic rank of *Salamandrella*, which was separated from *Hynobius*. Table 4 shows that the differences between either cluster of *Salamandrella* and 13 species of other genera of the family Hynobiidae (used for comparison) range from 15.5 to 18.8%, while the corresponding values for species of the genera *Pseudohynobius*, *Hynobius, Batrachuperus, Ranodon*, and *Liua* are 14.4–18.1, 14.4–20, 14.3–18.9, 20, and 19.4%, respectively. Naturally, species of the genus *Euproctus*, which are members of a different family, differ from the same 13 species to a greater extent (18.9–25.7%).

The detached position of *Salamandrella* as a separate genus is clearly seen in the maximum parsimony tree with local bootstrap probabilities (Fig. 5). The genus *Salamandrella* forms a monophyletic cluster in the tree, with a high bootstrap value (92%). The two groups of mtDNA variants which correspond to the species *Salamandrella keyserlingii* and *S. schrenckii*, are differentiated within this cluster with an even higher bootstrap value (99%).

Supplement to the Description of Salamandrella shrenckii

Salamandrella schrenckii (Strauch, 1870), sp. dist. Schrenck's Siberian newt.

Isodactylium schrenckii Strauch, 1870: p. 56.

Salamandrella keyserlingii var. *tridactyla* Nikolsky, 1905: p. 491.

Salamandrella keyserlingii var. *kalinowskiana* Dybowski, 1928: p. 1080.

Salamandrella keyserlingii tridactyla: Litvinchuk *et al*., 2004, p. 282.

Salamandrella schrenckii: Berman *et al*., 2005.

Lectotype. Specimen labeled "no. 115. *Isodactylium schrenckii* Str. Agdeki ad Ussuri. Dr. L. v. Schrenck. 1855," "no. 115. Trdet. O. Gumilevskii. *Hynobius keyserlingii* (Dyb). Ogdeki Ussuri 1855. Leg.: Schrenck." The lectotype is housed at the Zoological Institute of the Russian Academy of Sciences (ZIN); it comes from the vicinity of the village of Agdeki[2] (= Kukolevo, Khabarovsk krai), situated on the Ussuri River south of the Podkhorenok River mouth (less than 20 km from the

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[2] In the map with Schrenck's route given by Strauch (1870), the settlement is designated Agdiki; in the original label of the type specimen, no. 115 (and in the old registration book, see below) it is named Agdeki, while in the label with the identification of Gumilevskii, it is named Ogdeki. The village of Kukalevo is named in some maps as Kukelevo.

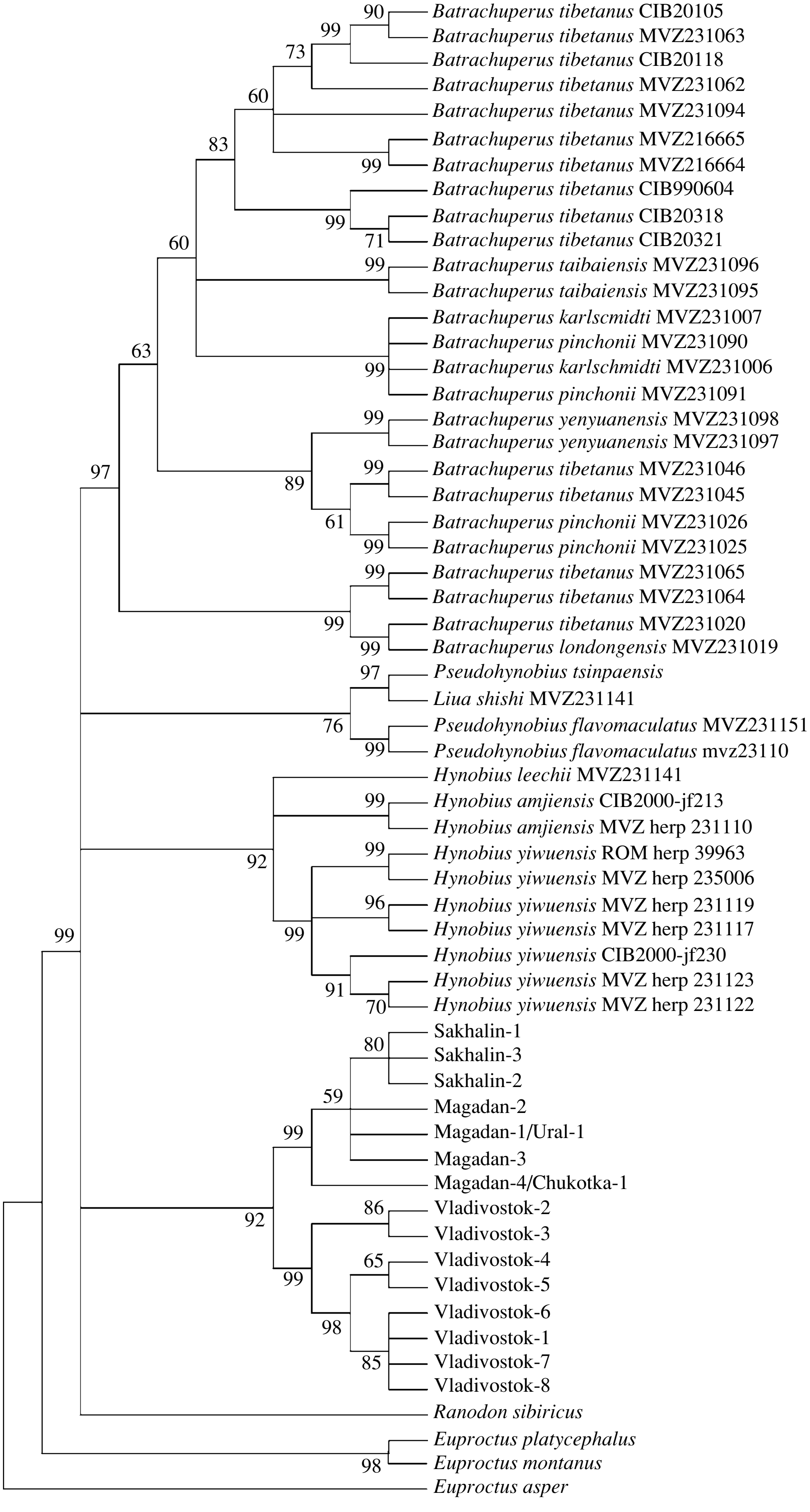

Fig. 5. Phylogenetic relationships of some species of the families Hynobiidae and Salamandridae based on the nucleotide sequences of the cytochrome *b* gene (maximum parsimony tree). Numbers at nodes are bootstrap values. Members of the genus *Euproctus* are used as outgroups.

railroad station Dormidontovka); designated by Berman *et al.* (2005).

Strauch described the species based on several specimens. The number of specimens or information from their labels were absent, only the following data were provided: "Habitat. Ost-Sibirien, am Ussuri, an der Schilka und am Baikal-See" (Strauch, 1870, p. 57). The old registration book of the Department of Herpetology of ZIN contains the following data on 12 specimens listed under the name "*Isodactylium schrenckii* Str.:"

nos. 110 and 111: Kunstkamer;

nos. 112 (2 specimens), 113, and 114: fl. Schilka, Popoff, 1854;

no. 115: Agdeki ad Ussuri, N 187, Dr. L. v. Schrenck, 1855;

no. 116 (2 specimens): Sibiria orient., Radde;

no. 117: Fl. Schilka, Maack, 1855;

no. 118 (marked "var."): Lac Baikal, Maack, 1855;

no. 119 (marked "var."): Des. Kirgis.??, Motschulsky.

All these specimens were collected before 1870, and their labels are not in conflict with the points of capture indicated in the original description (see below); therefore, it is reasonable to suppose that all of them were included in the type series. The specimens stored at the Cabinet of Curiosities (Kunstkamer) were collected before 1830 and, probably, beyond the area of Primorye or Khabarovsk krai, because these regions were investigated later. G.I. Radde collected materials near Lake Baikal in 1855, in eastern Transbaikalia in 1856, and in the Amur River basin, in the area of the present-day Jewish Autonomous Region, in 1856 to 1858 (Ruzskii, 1937). Motchoulski (1844) traveled to Lake Baikal and western Transbaikalia in 1839 and 1840, and his specimen was most likely collected there, rather than in the steppes of Kazakhstan (Desertis Kirgisorum), as is specified in the registration book (with two question marks). Out of these specimens, nos. 111–113, 115 (designated above as the lectotype), and 116 are currently kept in the collection of ZIN. It is hardly probable that specimens from Lake Baikal and Shilka belong to *S. schrenckii*, because *S. keyserlingii* was originally described from the Baikal Region.

As indicated above, all the specimens analyzed by us from Primorye (28 individuals) are assigned on the basis of mtDNA to *S. schrenckii.* Therefore, we refer the data on *Salamandrella* from this region to this species.[3]

Salamandrella schrenckii differs from *S. keyserlingii* in the following features:

(1) in a different (nonoverlapping) set of haplotypes (11.6% of the mtDNA distance);

(2) in genome size; *Salamandrella* from the vicinity of Vladivostok significantly differs in the amount of nuclear DNA (by 3.1%) from those collected in Yakutia, Nizhni Novgorod, Tomsk, Kamchatka, and Sakhalin oblasts (Litvinchuk *et al*., 2001);

(3) in the average number of vertebrae and costal grooves; in *Salamandrella* from Primorye, these values are less than 16.5 and less than 11.5, respectively, but they are greater in *Salamandrella* from other areas of the range (Ostashko, 1981; Litvinchuk and Borkin, 2003);

(4) in certain developmental characteristics of limbs and other elements of the larval locomotor system, which are basically reduced to heterochronies (Vorob'eva *et al*., 1999). However, the authors of the paper cited believe that all differences are of a cenogenetic nature and are mostly associated with different conditions of larval development in the Ural region (relatively warm water bodies) and Primorye (cold mountain creeks);[4]

(5) in the earlier completion of metamorphosis and emergence on land (Sapozhnikov, 1990). This point requires additional study, because *Salamandrella* larvae have not been examined in detail in other southern regions;

(6) in the shape and dimensions of spawns; they are not only spiral (as in all other parts of the range), but also straight, bag-shaped (Korotkov, 1977; Chernichko, 1982; Regel', personal communication, cited from Basarukin and Borkin, 1984; Sapozhnikov, personal communication, cited from Borkin, 1994). Bag-shaped spawns of the thoroughly investigated *Salamandrella* from the Ural population form 1–4 coils (Sytina *et al*., 1987), while those of *S. schrenckii* form only 0.5–1.5 coils; in Primorye, bags are on average half as long (86.0 ± 1.0 mm versus 180 ± 5.1) and 1.5 times narrower in cross-section area than in the Ural population (G.P. Sapozhnikov, personal communication). Sapozhnikov believes that the number of coils in spawn depends basically on the bag length; the lengths of bags with 0.5, 1, 1.5 coils average 75.0 ± 2.4, 87.9 ± 2.6, and 106.4 ± 5.5 mm, respectively; in Primorye, the degree of coiling is independent of the number of embryos. As regards the fecundity of *Salamandrella* from Primorye, contradictory data have been reported: according to Bromlei *et al.* (1977), the average number of embryos in a spawn is 140–160; according to Korotkov (1977), it is up to 132; and Sapozhnikov (personal communication) believes that it is 106.2 ± 1.4. These differences apparently result from

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[3] During the preparation of this paper, N.E. Dokuchaev collected five additional *Salamandrella* specimens in the vicinity of the village of Georgievka (southern Khabarovsk krai, the middle reaches of the Kiya River, a right tributary of the Ussuri River), which is situated less than 50 km from the village of Kukalevo (about 25 km to the north by latitude), i.e., approximately within the *terra typica* of *Salamandrella schrenckii.* All of them were referred to this species based on their mtDNA sequences.

[4] All other published and original data on *Salamandrella* from Primorye, which are discussed in the present paper, concern amphibians and their spawn collected in small stagnant water bodies only.

making measurements of spawns in water bodies differing in the age and size structure of the population, rather than from artifacts.

In the Ussuriiskii Nature Reserve, spawn in the shape of straight bag prevails: such bags accounted for three fourths of the 212 spawn samples examined by G.P. Sapozhnikov, ranging from 50 to 97% in particular water bodies (personal communication). In places, only straight bags are recorded in the reserve (S.M. Lyapkov, personal communication, cited from Ishchenko *et al.*, 1995; Kuzmin and Maslova, 2003). Straight spawn also occurs southeast of Khabarovsk, in the region named after Lazo near the village of Sidim (N.E. Dokuchaev, personal communication) approximately 100 km northeast of the type region in the upper reaches of the Nemta River, a right tributary of the Amur River. In the vicinity of Komsomolsk-na-Amure, only usual spiral spawn of *S. keyserlingii* is observed, while straight spawn has not been recorded (V.A. Mutin, personal communication). In contrast, in central Yakutia "…spawn of Siberian newts in the shape of relatively small conical bags was repeatedly observed" (Belimov and Sedalishchev, 1983, p. 42) in the Zarechnaya group of regions (V.T. Sedalishchev, personal communication). Unfortunately, at present, it is impossible to compare in detail the shape of straight spawn from Primorye and Yakutia. Isolated findings of straight spawn were reported from the Ural Mountains (Ishchenko *et al.*, 1995). Thus, the ranges of variation in the majority of spawn characteristics of the two species also overlap.[5] At present, the sole character distinguishing the spawn of *S. schrenckii* is its considerably smaller size, namely, the length and thickness of bags. This character should also be verified, however.

Another feature that was usually thought to distinguish *Salamandrella* from Primorye is the absence of nuptial behavior of the kind observed in *S. keyserlingii* (Korotkov, 1977; Basarukin and Borkin, 1984; Borkin, 1994). However, G.P. Sapozhnikov observed well-manifested courtship rituals performed by males, which were identical to those described by us in the upper reaches of the Kolyma River. This researcher believes that the nuptial behavior of males in Primorye is rarely observed in the daytime, but their activity probably increases at night (Sapozhnikov, personal communication). In the water bodies containing only straight spawn (undoubtedly belonging to *S. schrenckii*), we observed only weak manifestations of male nuptial behavior, which did not differ from that in *S. keyserlingii.* The presence of unique nuptial behavior resembling a "round dance" in *Salamandrella* from Primorye, which was described by Yu.B. Pukinskii (cited from Grigor'ev, 1981), should also be checked. Note that, in Primorye, constant and temporary creeks are used by *Salamandrella* for reproduction more frequently than in other parts of the range (Kuzmin, 1999).

According to the molecular data obtained by us, *S. schrenckii* is divided into two subclusters with a fairly large genetic distance between them: on average, 3.2%, with a maximum of 3.5% (Table 2). Note that the two haplotypes occur together. Such a great difference formally corresponds to the subspecies rank; however, comparative data on the intraspecific structure in members of various genera of the family Hynobiidae are as yet absent.

On Factors Accounting for Insignificant Geographic Variation in the Siberian Newt

The low level of genetic variation in *S. keyserlingii* from different parts of the vast species range with different natural conditions requires explanation. The geographic populations examined display such a great genetic uniformity that they appear to form a single hyperpopulation (except for the above-mentioned *Salamandrella* from Sakhalin). This phenomenon is surprising, as it is impossible to propose a mechanism for efficient gene exchange between marginal populations which could have provided for this uniformity.

The simplest way to explain the insignificant geographic variation (both morphological and genetic) is to attribute it to late formation of the present-day range, possibly, during one of the most humid periods of the Holocene. The genetic uniformity of the hyperpopulation suggests the rapid distribution of one haplotype. A similar phenomenon was described using molecular genetic data in the three-toed (Zink *et al.*, 2002b) and great spotted woodpeckers (Zink *et al.*, 2002a), which colonized Eurasia in the postglacial period along with the restoration of forests. It is evident that *Salamandrella* has a much more restricted migration ability (compared to birds), since it is confined to water bodies during reproductive period and to moist habitats at other stages of the life cycle. The outstanding (for amphibians) ability to live at low summer temperatures (which is evident from the successful existence of this species in the southern tundra subzone) suggests that abundant moistening or, more precisely, the presence of many small water bodies of different types, rather than heat supply, could be the factor promoting the expansion of the Siberian newt. Note that in northeastern Asia, interfluves with elevations of up to 1000 m above sea level, covered with forests or mountain tundra, do not prevent its expansion (Berman and Sapozhnikov, 1994).

In principle, not only rapid, but also gradual formation of the range was possible even during the cold phases of the Pleistocene. This was probably facilitated by the outstanding cold hardiness of the Siberian newt: during wintering, it survives temperatures as low as –40°C (Berman *et al.*, 1984). This is not in conflict with the age of this species, which was estimated by us as 490 ka

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[5] During the preparation of this paper, we analyzed mtDNA of 22 spawn samples collected in the Ussuriiskii Nature Reserve. All of them were shown to belong to *S. schrenckii*, irrespective of the degree of coiling. No correlation between the spawn shape and the mtDNA haplotype was revealed.

based on mtDNA variation. However, the conclusion concerning fast and recent expansion of the species is supported by the undoubted absence of gene exchange between marginal populations during recent times (see above).

Berman (2003) examined the biological and ecological mechanisms providing for the maintenance of variation at the same level in different local populations in order to find another explanation for the genetic uniformity combined with low morphological variation in *S. keyserlingii* throughout the continental part of its range (Berman, 2003). The extremely low level of geographic variation within such an extensive range seems extraordinary, since environmental conditions in its extreme zones differ greatly and, hence, the abiotic component of natural selection must be well-manifested. In spring, when ambient temperatures increase above zero, the conditions in different natural zones become more or less similar and the impact of the climate is alleviated. In summer and autumn, however, this impact must be significant because of differences in the sum of temperatures and their biocenotic consequences. The young of the year are especially sensitive to climatic conditions: their growth rate, body size, and other parameters strongly depend on the period of time between their emergence on land and the onset of the cold season, which ranges from a few days in cold years in the northern taiga (in the upper reaches of the Kolyma River) to 1.5–2 months in the southern taiga (Novosibirsk) and forest–steppes (the Baraba region).

There are also other, ordinary factors responsible for the heterogeneity of populations, for example, variation in conditions of animal growth, development, reproduction, etc., in different aquatic and terrestrial habitats. The initial stages of the divergence of local populations (Ishchenko, 1989; *Siberian Newt…*, 1995) are short-term, probably accomplished within the life span of one generation. Initial differentiation remains insignificant, however, as the individuality of populations is smoothed because of group fertilization of females,[6] and wide, fan-shaped migrations of the young, which result in constant intermixing. Both factors apparently provide for assimilation of deviant varieties, thus maintaining the morphological standard of the population and, eventually, restricting geographic variation (Berman, 2003).

It is intuitively clear that the absence of geographic variation is a consequence of unidirectional stabilizing selection throughout the species range. Selection may be directed toward the maintenance of adaptation to winter climatic conditions, which are extremely severe in almost all parts of the vast range of the Siberian newt. Due to the specific features of the Siberian High (with the values of January isotherms decreasing from west to east rather than from north to south), differences in winter temperatures are leveled off from the tundra to the steppe, irrespective of natural zonality (Alfimov, 2005). The most severe conditions are observed at the southern boundary of the range in Siberia, where low ambient temperatures are combined with the absence of snow cover (Berman, 2003). Unfortunately, we cannot yet propose a concrete mechanism of natural selection.

CONCLUSIONS[7]

Thus, the results of this study confirm that wide Palearctic species ranges have a specific phylogeographic structure in which populations from the southeast clearly stand out against a background of general genetic uniformity of virtually all other populations. A similar situation was previously described for the carrion crow *Corvus corone*, in which southeastern populations (from Primorye and southern Sakhalin) were distinguished by a special mtDNA haplotype (Kryukov and Suzuki, 2000). Moreover, a similar division of the range was revealed in the great spotted woodpecker *Dendrocopos major*: one mtDNA cluster comprised variants characteristic of the southeastern area of the range (including Sakhalin, Primorye, and Hokkaido), while the other included those of other areas of the range in northern Eurasia (Zink *et al*., 2002a). Based on the sequences of two mitochondrial genes of the magpie *Pica pica*, its range was also divided into two parts, with the tentative boundary between them lying along the Amur River (Kryukov *et al*., 2004). Increased variability of mtDNA in the population from Primorye was also revealed in the course of wide geographic studies on variation in the mouse *Apodemus peninsulae* (Serizawa *et al*., 2002). The situation with *Salamandrella* and the other examples discussed in this paper is obviously accounted for by the same factor. Primorye is a refugium well-known for its highly stable natural conditions, at least, in the Late Quaternary; unlike other areas of the boreal Palearctic, it did not suffer from catastrophic breaks in development such as those produced by glaciations (Kolesnikov, 1969; Nazarenko, 1990). Hence, it is not surprising that the age of *S. schrenckii* is much greater than that of *S. keyserlingii* calculated by the same method. It is estimated as 2.4 Ma, which suggests that *S. schrenckii* is a native inhabitant of Primorye. We believe that the existence of two haplotypes in *S. schrenckii*, the greatest genetic distance between which is 3.5% (see above), is also associated with the early emergence of this species.

Although the age estimates for the two *Salamandrella* species are tentative and approximate, it is possible to conclude, taking into account only the magnitude

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[6] The possibility of group fertilization in *S. keyserlingii* was put into doubt by Savel'ev *et al.* (1993).

[7] During the preparation of this paper, the sample of mtDNA sequences of *Salamandrella schrenckii* and *S. keyserlingii* increased approximately twofold (from 86 to 160 specimens) due to analysis of animals from new areas (southern Primorye, southern Khabarovsk krai, the lower reaches of the Kolyma River, the upper reaches of the Indigirka River, and the vicinity of Tomsk) and additional experiments with mtDNA variants described above. The data obtained confirm the conclusions made in this paper.

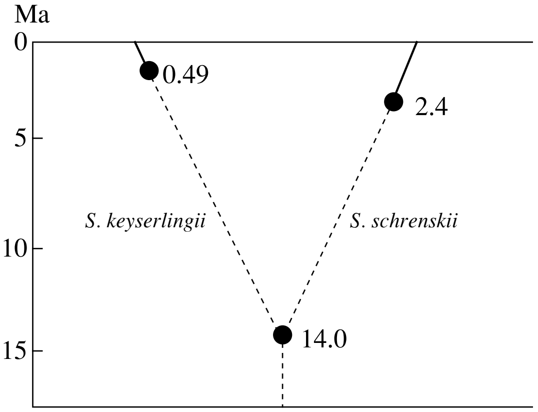

Fig. 6. Relations of ages and time of divergence of two *Salamandrella* species.

of values and their ratios, that *S. keyserlingii* and *S. schrenckii* are two relatively young species of different ages, the ancestral lineages of which diverged from a common stem approximately 14 Ma (estimated according to the calibration accepted in this study), rather than a descendant and its ancestor. The scheme of relationships and the time of the divergence of these species are shown in Fig. 6. These estimates appear to be quite credible, since the available data on the divergence of caudate amphibians within a genus (in particular, in the genus *Euproctus* used here as an outgroup) are similar to ours (Tan and Wake, 1995; Caccone *et al.*, 1997; Riberon *et al.*, 2000).

The high level of genetic differences between *Salamandrella* from Primorye and from all other regions strongly suggest the existence of two species. However, they are difficult to distinguish morphologically. At present, it is impossible to identify each individual without employing molecular genetic methods, which provides a basis for applying the term "cryptic species" to *Salamandrella schrenckii.* In this connection, it is important to outline the range of *S. schrenckii* and, in particular, its northern boundary, which is the zone of contact between the two species. To date, the spread of *S. schrenckii* to the north has been delimited by two points in the vicinities of the villages of Georgievka and Sidim in Khabarovsk krai, which are located several dozens of kilometers south and north of the 48th parallel.

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