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Historical Demography of an Endangered Salamander, *Ranodon sibiricus* (Amphibia, Urodela, Hynobiidae): A Reassessment

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Chinese populations of the endangered Siberian salamander *Ranodon sibiricus* are reported to have diverged only about 120 years ago, and to have the lowest genetic diversity of any amphibian. However, these conclusions require verification, as the main range of the species is in Kazakhstan. Moreover, the generation time used for estimating divergence time has a weak ground. In order to clarify these problems, we investigated the molecular phylogenetic relationship and historical demography of the species covering its whole distribution range using the mitochondrial DNA region reported for Chinese population (1072 bp sequences of the control region), while conducting skeletochronological analysis to estimate accurate generation time. As a result, the range expansion was estimated at 88,000–50,000 YA, based on the generation time of 6–10 years. Degree of intraspecific genetic differentiation is actually very small, but, as a single species, is not so small as had been reported for Chinese population alone.

Key words: *Ranodon sibiricus*, biogeography, mitochondrial DNA, control region, age determination, salamander

INTRODUCTION

Ranodon is a monotypic hynobiid genus represented by *R. sibiricus* Kessler, 1866, which occurs only on the Dzungarian Alatau Mountains extending from southeastern Kazakhstan to northwestern Xinjiang Uyghur Autonomous Region of China (Fig. 1, species range < 500 km^2: Kuzmin et al. [1998]). This species from Central Asia and *Paradactylodon* from the Middle East are reported to form a clade, which is greatly differentiated from the remaining hynobiid genera from Eurasia (Zhang et al., 2006).

Ranodon sibiricus is semiaquatic and inhabits cool mountain brooks and small rivers in coniferous forests or subalpine meadows at 1400–3200 m asl (Brushko and Dujsebayeva, 2009; Wang and Bai, 2000; Wang et al., 2006a, b). Adults and juveniles hide themselves under the stones in shallow water or among grass or debris on the shore during the day, and forage under the water and on the land at night. The breeding season ranges from April to August, and the larvae hatched between late June and late August metamorphose probably in the next year (Paraskiv, 1953; Kuzmin, 2012).

The distribution of the species appears to be severely restricted by the presence of stable running waters originating from glaciers, which encompass abundant prey fauna all

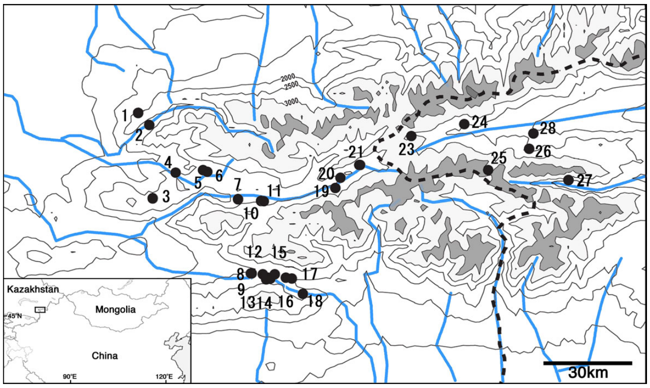

Fig. 1. Map of the Dzungarian Alatau showing sampling localities of *Ranodon sibiricus* (Sample 22 not shown because of lack of detailed locality information). The coarsely dotted area > 3500 m asl and finely dotted area > 3000 m asl. Contour lines are drawn at every 500 m.

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year round (Wang and Bai, 2000). Thus, as was hypothesized by Chen et al. (2012), the populations of this salamander are thought to have moved up to high altitudinal valleys and to have been genetically isolated in the interglacial period, but to have moved to lower altitudes where gene flows occurred during the glacial period.

Because of its local population decline and its small and fragmented habitats, its conservation has drawn great attention (Yuan and Wang, 2009). Desiccation of streams, forest degradation due to overgrazing, and illegal poaching for traditional medicine, continue to threaten this species. Presently, the species is listed on the RDB of IUCN as Endangered (IUCN, 2014), and has been legally protected both in Kazakhstan and China. In order to establish management measures for conserving this endangered salamander, several studies using ecological approaches, including ecological niche modelling (Dujsebayeva, 2013; Dujsebayeva et al., 2014; Malakhov and Dujsebayeva, 2014) have been conducted recently.

However, population genetic studies on this species are scarce, and only the report by Chen et al. (2012) is available at present. They examined intraspecific mitochondrial DNA (mtDNA) and nuclear DNA (microsatellite) variations of the species in China, and obtained an extremely low genetic diversity that indicates extraordinary recent expansion within China. However, their conclusion requires confirmation in considering the whole evolutionary history of the species, because they studied only Chinese populations, which are significantly limited in the species distribution range. Furthermore, the generation time used for estimating time of expansion (five years) was based on a value incorrectly cited from Wang and Bai (2000), who only noted larval periods as about three years, and did not report any maturing age or generation time.

Taking this situation into consideration, we studied mtDNA variation of *R. sibiricus* from Kazakhstan, which is the main distribution range of the species. We combined our data with those from Chinese reports and explored the intraspecific variation of *R. sibiricus* as covering the whole distribution range of the species. We also conducted skeletochronological age estimation on the females from Kazakhstan, and used the data obtained to estimate the diversification time of the populations. From these approaches we could obtain more concrete insights into the phylogeographic structure of this endangered species.

MATERIALS AND METHODS

Sampling, preparation of DNA, PCR, and DNA sequencing

Because of strict regulation, we could collect only digits of limited number of individuals. After collection, we measured snout–vent length (SVL: from the tip of the snout to the anterior margin of the cloaca), checked sex (see below), and cut digits. The salamanders were released at the site of collection immediately after this procedure. As a result, we could collect fingers and toes of 65 specimens of *R. sibiricus* from 22 sampling localities in Kazakhstan covering its entire distributional range in the country (Fig. 1, Table 1). Digits were first preserved in 99% ethanol for DNA analyses, and then kept in 10% formalin for skeletochronology (see below). Total DNA was extracted from ethanol-preserved tissues of fingers and toes using standard phenol-chloroform extraction procedures (Hillis et al., 1996). The fragment containing the control region (CR) was amplified by PCR using combinations of forward (F) Rano_CRF1 (5′-GCTTAACTAAAGCATTGGTCTTG-3′) and reverse (R) Rano_CRR2 (5′-ATAAAATTTTAATTAATAATAAGGC-3′). The reaction conditions were initial heating at 94°C for 4 min; 33 cycles of 94°C (30 sec), 51°C (30 sec), and 72°C (1.5 min); and a final extension at 72°C for 7 min. Cycle sequencing reactions were done using an ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and then sequencing was performed on ABI 3130 automatic sequencers using primers described above and two internal primers, Rano_CRF2 (5′-TAGAGTGACTGTCTTTTAACTTGAAC-3′) and Rano_CRR1 (5′-CTACAGATGAGGGCAGACTCAGTT-3′). The obtained sequences were deposited in GenBank under accession numbers LC031504–LC031517. Alignment of data from all individuals was performed using the Clustal option in the BioEdit software (Hall, 1999).

Haplotype network analysis and genetic diversity

In the subsequent analyses, we added sequence data of 123 specimens from six sampling localities in China deposited in GenBank to our own data (Fig. 1, Table 1). Intraspecific gene genealogies were inferred using TCS version 1.21 (Clement et al., 2000), which implements the statistical parsimony method of Templeton et al. (1992). Connections among haplotypes within the network were justified by 95% parsimony criterion. We calculated genetic distances (uncorrected p-distance) for pairwise combinations of haplotypes using MEGA, version 4 (Tamura et al., 2007). Haplotype and nucleotide diversities were calculated by Arlequin 3.0 (Excoffier and Schneider, 2005).

Demographic analyses

Demographic parameters were estimated based on mismatch distribution analysis of pairwise difference (Rogers and Harpending, 1992). The fitness of the observed data to a model of either sudden population expansion (Rogers and Harpending, 1992) or spatial expansion (Schneider and Excoffier, 1999; Excoffier, 2004) was tested using Arlequin 3.0 (Excoffier and Schneider, 2005) for the entire populations. In addition to mismatch distribution analysis, we conducted Fu's test of neutrality (Fu, 1997) for the entire population. Significant negative values of Fu's statistics were interpreted as a signature of demographic expansion. Statistics were calculated with Arlequin 3.0 and their significances were assessed through 10,000 simulations.

To explore the geographical origins of this species, geographical trends in genetic diversity were examined using landscape shape interpolation analysis implemented in the software Alleles In Space (AIS: Miller, 2005). The sample 22 without detailed locality information in Kazakhstan was not used in this analysis. The software required the description of sampling localities in XY coordinates with an arbitrary scale. We simply converted the latitude–longitude coordinates into decimal degree. Using the geographic coordinates and DNA sequences, we initially generated a Delaunay triangulation-based connectivity network of sampling localities and the relative genetic distances amongst them, which inversely decreased with increasing geographic distance as seen in dark (higher genetic distances) and light (lower genetic distances) colors. We set the grid size as 0.1 latitude or longitude degree and set the distant weight value $\alpha = 10$. Populations distributed in the areas that have served as refugia during interglacial periods are generally assumed to contain more genetically distant alleles than those in areas that have been colonized in post-interglacial periods. We expect that areas marked by darker color in this analysis thus indicate higher genetic diversity and represent sites where refugia were located.

Skeletochronological analyses

In order to estimate the divergence time in maternal mtDNA samples, we need to know the correct ages of adult females. We therefore used skeletochronology (Castanet and Smirina, 1990; Smirina, 1994) for determining the ages of females in the wild.

Table 1. Samples for mtDNA analysis and haplotypes obtained. Abbreviation in the locality from China were those in Chen et al. (2012). A = adult (unsexed), E = egg, L = larva, J = juvenile, S = subadult, M = adult male, F = adult female, U = unknown.

Sample N	Locality	N and Sex/age	Latitude	Longitude	Altitude (m)	Haplotype N (N of individuals other than 1)	Source
Kazakhstan							
1	Balykty River	3A, 2J, 2L	45.00056	78.88107	1828	CR02(7)	This study
2	Kora River	3L	44.96167	78.92139	1560	CR01, CR06, CR07	This study
3	Cherkassay River	4L	44.74972	78.93667	2054	CR01, CR02, CR14(2)	This study
4	Shimbulak River B	2L	44.81869	79.03289	2253	CR12, CR14	This study
5	Oysaz River	2M	44.83033	79.14842	2036	CR01, CR13	This study
6	Shimbulak River A	2L	44.82194	79.16658	2206	CR01, CR14	This study
7	Ermensay River	3U	44.74250	79.28639	2007	CR01(3)	This study
8	Karagaily River	L, S	44.52333	79.34611	1864	CR01(2)	This study
9	Karassu River	2S	44.52333	79.34750	1850	CR02, CR08	This study
10	Ermenssay River B	L, M	44.73686	79.37142	2363	CR01(2)	This study
11	Ermenssay River A	J	44.73656	79.38361	2254	CR14	This study
12	Keskenterek River A	2S	44.51944	79.39278	2045	CR02, CR05	This study
13	Keskenterek River B	4A, 2S	44.50222	79.40556	2212	CR01(2), CR02(3), CR11	This study
14	Borokhudzir River A	5A	44.51056	79.42167	2112	CR01(2), CR02(3)	This study
15	Borokhudzir River B	2E	44.51778	79.44167	2083	CR01(2)	This study
16	Kereysu River	E	44.51300	79.48653	2080	CR09	This study
17	Aktasty River	2L	44.50972	79.51278	2070	CR04, CR14	This study
18	Sarlytan River	3A, F, 2M	44.46653	79.55975	2018	CR01(2), CR02(2), CR10(2)	This study
19	Kazan River C	L, M, 2S	44.77522	79.69311	2519	CR02(3), CR15	This study
20	Kazan River B	L, M, S	44.80244	79.71428	2377–2500	CR01(2), CR14	This study
21	Kazan River A	2A, S	44.84608	79.79328	2508	CR01, CR02(2)	This study
22	Unknown	U	–	–	–	CR02	This study
China							
23	Zemaike (JMK)	20U	44.92972	80.00500	2800	CR01(20)	Chen et al. (2012)
24	Sarbastou (SAR)	20U	44.96639	80.22611	2465	CR01(17), CR02(3)	Chen et al. (2012)
25	Aksai (AKS)	23U	44.83139	80.32389	3200	CR01(20), CR02(2), CR03	Chen et al. (2012)
26	Sulbeijin (SLBZ1#)	20U	44.89083	80.49583	2041	CR01(16), CR02(4)	Chen et al. (2012)
27	Sulbeijin (SLBZ2#)	20U	44.83361	80.65278	2250	CR01(11), CR02(9)	Chen et al. (2012)
28	Sulbeijin (SLBZ)	20U	44.93444	80.51194	2100–2300	CR01(10), CR02(10)	Chen et al. (2012)

We collected six larvae, three unsexed juveniles, and 19 adult females from the Borokhudzir River A (Locality 14) and B (Locality 15) in Kazakhstan (Table 1, Fig. 1). Sex determination was made by observation on gravid condition (ripe eggs visible through skin in matured females in the breeding season), presence/absence of a tubercle at the anterior edge of the ventral slit (present only in matured males and not in females), leg and head robustness (more robust in males than females), tail height and length (tails are relatively higher and longer in males than in females), caudal fin fold appearance (females have weakly expressed caudal fin folds in general; during the reproductive period the male caudal fin fold is much higher and with fewer undulations than in females) (Kuzmin and Thiesmeier, 2001; Brushko and Dujsebayeva, 2009; our observations). Sexual differences are more pronounced during the reproductive period. Validity of these sexing methods was confirmed by dissection of preserved specimens (18 males and nine females: Supplementary Text S1).

We prepared cross-sections (ca. 25–30 μm thick) of formalin-preserved digits using a freezing microtome and stained them with hematoxylin (Mayer's acid hemalum), basically following Misawa and Matsui (1999). Under a light microscope at 400 X magnifications, we chose sections from the central regions of the diaphysis and counted the number of lines of arrested growth (LAGs). We preliminary compared the number of LAGs counted in cross-sections of finger, toe, and femur of an adult specimen of *R. sibiricus* and confirmed no difference in the number among the bones.

Estimation of times of population expansion

The time of population expansion was estimated from the formula $t = \tau/2\ \mu$ (Excoffier and Schneider, 2005), where t is the time of population expansion, μ is the mutation rate per generation per locus. The τ value was estimated through the mismatch distribution analysis by Arlequin 3.0. We first adopted a substitution rate of 1.28% sequence divergence per MY in hynobiids for the CR region. Although this rate was originally reported for the mitochondrial genome not including CR region (Weisrock et al., 2001), the value has been pertinently applied to CR and/or cyt b genes and provided reasonable estimates of divergence times in several hynobiids (Matsui et al., 2007, 2008; Yoshikawa et al., 2008). We then obtained the mutation rate of 0.1984% per lineage that was derived from the following formulae: 1.28% (mutation rate of cytochrome b in Weisrock et al., 2001) × 0.31 (ratio of CR/cyt b mutation rates given by Matsui et al., 2007) × 1/2 (per lineage). We estimated generation times of *R. sibiricus* by applying the mean, youngest, and oldest ages of mature female specimens obtained from skeletochronology, because the times of generation in this species are hardly clarified due to its multiple breeding through the long life.

RESULTS

Sequence variation and haplotype network

We determined sequences of 1072-bp of the CR for all 65 samples from Kazakhstan. After adding the sequences of 123 samples from China in the GenBank, alignment of mtDNA sequences revealed a total of 15 haplotypes in this species, of which 14 were found in Kazakhstan and three in China (Table 1). The most frequently observed haplotype in Kazakhstan was CR02 (24 samples), followed by CR01 (22). Twelve haplotypes (CR04–15) were found only in Kazakhstan, while one (CR03) was only in China. Of 1072-bp sites, 18 sites were polymorphic, but 16 were singletons and only two were parsimony informative. Haplotype and nucleotide diversities for entire populations were 0.5439 ± 0.0312 (SD) and 0.0010 ± 0.0007, respectively. Haplotype diversity in China (0.3671 ± 0.0420) was smaller than in Kazakhstan (0.7457 ± 0.0359), but nucleotide diversity overlapped between them (0.0006 ± 0.0001 in China and 0.0015 ± 0.0010 in Kazakhstan).

Statistical parsimony network constructed by TCS connected all observed haplotypes with five missing haplotypes (Fig. 2). All haplotypes were connected within two mutations from either one of two major haplotypes. Uncorrected p-distances between haplotypes were very small, varying from 0.09 to 0.47%.

Historical demography

The mismatch distribution for total samples showed a unimodal distribution (Fig. 3). The distribution was not deviated significantly from either the sudden expansion model (sum of squared deviation [SSD] = 0.029 [*P* = 0.107], Harpending's raggedness index [Hrag] = 0.103 [*P* = 0.153]) or the spatial expansion model (SSD = 0.022 [*P* = 0.221], Hrag = 0.103 [*P* = 0.292]). In Fu's test of neutrality, a significantly negative value was observed (Fs = –7.51365, *P* < 0.05).

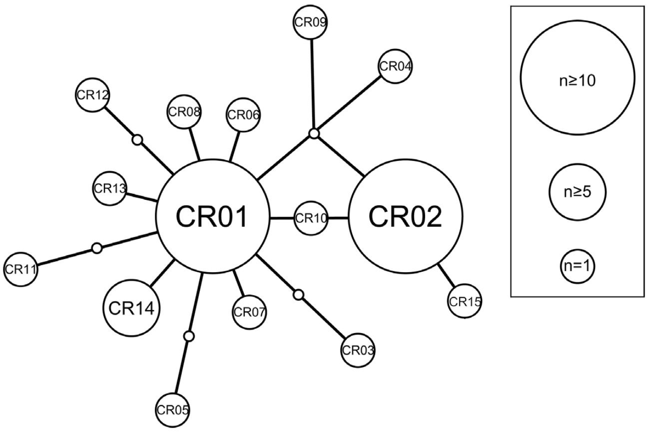

Fig. 2. Statistical parsimony network based on 1072 bp of CR from Kazakhstan and China by TCS. Small circles: missing haplotype.

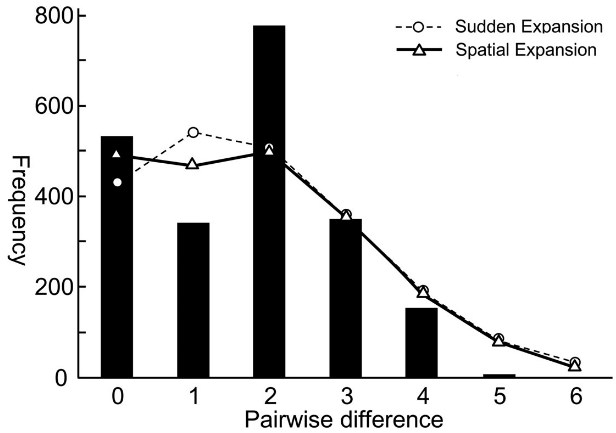

Fig. 3. Mismatch distributions of pairwise sequence differences between individuals. The observed data (bars) fitted to models of sudden population expansion (circles) or spatial expansion (triangles).

Landscape shape interpolation analysis revealed that high genetic diversity was found in Kazakhstan but not in China. In Kazakhstan, five areas showed higher diversity (R1–5 in Fig. 4) and could be regarded as refugia at the time of population decline. These areas include regions with higher altitudes than the present distribution (Figs. 1 and 4).

Skeletochronology

We did not find any difference in the number of LAGs between fingers and toes from the same individuals obtained in the field. We obtained LAG data for all the individuals examined, except for one female with substantial resorption. In several other genera of Hynobiidae, LAGs are known to be formed in a season when bone has low growth rate (*Hynobius*: Misawa and Matsui, 1999; Ento and Matsui, 2002; Matsuki and Matsui, 2009; *Salamandrella*: Hasumi, 2010), we thus assumed the number of LAGs as the number

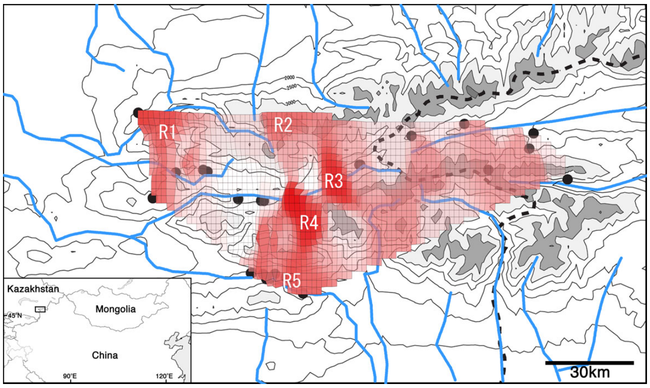

Fig. 4. Results of a genetic landscape interpolation analysis. R1–5 indicate five refugia. The grid size set at 0.1 latitude or longitude degree and the distant weight value set as α = 10.

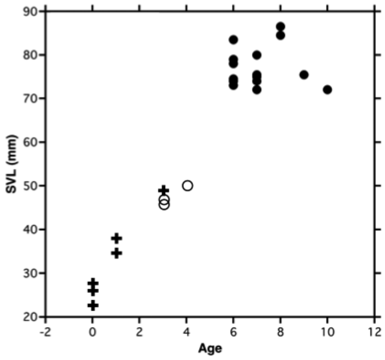

Fig. 5. Growth and maturation of *Ranodon sibiricus* from Kazakhstan. Cross: larva, open circle: juvenile, closed circle: mature female.

Table 2. The time of expansion of *Ranodon sibiricus* under sudden-expansion and spatial-expansion models estimated for average, minimum, and maximum generation time of females. Values in parentheses are 95% CI.

generation time	Time of expansion (year)	
	sudden expansion [$\tau = 2.258$]	spatial expansion [$\tau = 2.140$]
7 years (average)	75,833.2 (5507.8–144,781.7)	71,870.3 (12,829.2–132,254.7)
6 years (minimum)	88,472.1 (6425.8–168,911.9)	83,848.6 (14,967.4–154,297.2)
10 years (maximum)	53,083.2 (3855.5–101,347.2)	50,309.2 (8980.4–92,578.3)

of overwinterings individuals experienced. Three fully-grown larvae with SVL ranging 22.0–28.0 mm, two with 34.5 and 38.0 mm, and one with 48.0 mm showed one, two, and three LAGs, respectively, suggesting one LAG formation after one overwintering (Fig. 5). Because a new LAG is thought to be formed up to several months after hibernation (Ento and Matsui, 2002), we carefully examined the specimens collected in early summer and ascertained the absence of a new LAG at the outer margin of the bone at this season.

Based on the number of LAGs and life history information noted above, we estimated the age of all samples examined (Fig. 5). Adult females had the mean age of seven years, and matured six years old at ca. 70 mm in minimal SVL. The oldest individual was estimated as 10 years old.

Times of population expansion

The estimated times of expansion in *R. sibiricus* only slightly differed between sudden expansion and spatial expansion models, but varied depending on the generation times (ages of breeding adults) of females assumed (Table 2). At the minimum generation time (six years: the youngest age of breeding adults), the times of expansion were estimated to be 88,472.1 years ago (YA; 95% CI 6425.8–168,911.9) and 83,848.6 YA (14,967.4–154,297.2) for sudden expansion and spatial expansion models, respectively, whereas at the maximum generation time (10 years: the oldest age of breeding adults), the estimated times were 53,083.2 YA (3855.5–101,347.2) and 50,309.2 YA (8980.4–92,578.3), respectively. At the average generation time (seven years), the estimated times were 75,833.2 YA (5507.8–144,781.7) and 71,870.3 YA (12,829.2–132,254.7), respectively, and were closer to the estimations from the minimum generation time. When our generation times were applied to Chinese populations, the times of expansion were estimated to be 122,991.1 (0–178,393.9) YA and 81,615.3 (0–226,939.9) YA for sudden expansion and spatial expansion models, respectively, at the minimum generation time (six years). At the maximum generation time (10 years), they were 73,794.6 (0–107,036.3) YA and 48,969.2 (0–136,163.9) YA, respectively, and at the average generation time of seven years, the times were estimated to be 105,420.9 (0–152,909.0) YA and 69,956.0 (0–194,519.9) YA.

DISCUSSION

Degree of genetic variation

The degree of genetic differentiation was extremely low in *R. sibiricus* as reported by Chen et al. (2012). However, the mean nucleotide diversity for its whole distribution range was 0.10%, which was nearly twice lager than that for Chinese populations (0.06%) reported by Chen et al. (2012) and recalculated herein. Using this value for Chinese populations, Chen et al. (2012) noted *R. sibiricus* to be least variable genetically in all the amphibians investigated by that time. However, as shown here, the degree of genetic variation of a given species varies depending on the sampling scale, and in order to compare the degree of variation among species, we must confirm whether the sampling for a given species is substantial.

It seems clear that the genetic variation is higher in Kazakhstan than China, and that this difference in the degree of genetic diversity must be caused by the different demographic history in each area (see below). Even so, it is certain that the genetic variation of the species from the whole range of its distribution is still low, as reported by Chen et al. (2012).

Age estimation and life history

Antipenkova (1982) estimated the ages of *R. sibiricus* from Kazakhstan to be 4–12 years old by skeletochronological method. The individuals with the total length of 170 mm were estimated as 11–12 years old. However, the age at maturation was not clarified in that report. Chen et al. (2012) applied a generation time of five years in the molecular dating of Chinese populations, based on Wang and Bai (2000), who only noted the larval period to be three years. Thus, the age at maturation of female *R. sibiricus* has never been clarified before the present study.

The minimum age of female maturation in *R. sibiricus* was estimated at six years in this study. Information on comparable data are meager, but in the genus *Hynobius*, some montane, lotic-breeders mature at seven to eight years (Misawa and Matsui, 1999; Nishikawa and Matsui, 2014), while lentic-breeders from lower altitudes at three to four years (Ento and Matsui, 2002; Kusano et al., 2006). Thus *R. sibiricus* is more similar to montane, lotic-breeding *Hynobius*, as expected by their resemblance in habitats. However, *R. sibiricus* is semiaquatic throughout its life but the species of *Hynobius* are terrestrial after metamorphosis. Further, the hibernation period is also different among species, which affects growth and maturation of the salamanders. *Ranodon sibiricus* has been thought to hibernate for nearly half a year or even longer, from the end of September –late October till April or later when the snow and ice in the wetlands become to thaw (Paraskiv, 1953; Kuzmin and Thiesmeier, 2001; Kuzmin, 2012), while the species of *Hynobius* do not hibernate so long. At the moment we can simply conclude that the age of female maturation in *R. sibiricus* is not unique among hynobiids, hence our estimation is not odd.

Historical demography

The present study revealed much higher haplotype diversities in Kazakhstan than in China, although their nucleotide diversities overlapped. The genetic landscape interpolation analysis suggested the presence of several refugia only in Kazakhstan. In China, the salamander occurs only in one river basin (Borotala River), but in Kazakhstan it is found in several basins that were formed by intermountain depressions on the Dzhungarian Mountains (Potapova et al., 1991; Didenko-Kislitsina, 2001, 2006). Probably, such geological property provided the several refugia in Kazakhstan.

Based on the molecular data and generation time, the expansion time of all the extant populations is estimated from about 88,000 to 50,000 years ago, which includes the first stage of the Würm glaciation (80,000–50,000 YA) in the Tien Shan and Dzhungarian Mountains (Grosswald et al., 1994; Vilesov, 2008; Vilesov et al., 2013). In order to compare the estimated time of expansion between the present study and Chen et al. (2012) that examined only Chinese populations, we recalculated the minimal time of expansion in Chinese populations at the maximum generation time of 10 years and under the spatial expansion model using our generation times. The value was 48969 YA, much larger than that (120 YA) by Chen et al. (2012).

Because of warmer temperatures in the Riss-Würm interglacial period (Fig. 6B), the salamanders escaped to higher altitude habitats. They had much limited distribution range before the Würm glaciation. Probably the individuals that moved over the Koksu-Borotala watershed from the refugia near the border and down to Chinese territory established the present Chinese populations. A possibility of such migration can be kept by the lower hypsometric level of the Koksu-Borotala watershed uplifted maximally to ca. 3000 m asl at that time, as opposed to 3400 at present (Vilesov, 2008; Perevozov, 2010), a significant increase of moisture of climate in a range of 40–50°N latitude (Velichko, 1989) and a rise of the forest belt (Serebryanny et al., 1980); see Fig. 6B). However, subsequent Würm glaciation induced a migration of the salamander to lower altitudes (due to sudden expansion or spatial expansion), where the extended glacial cover may have created an abundant cool streams and wetland suitable for *R. sibiricus* (Fig. 6C). The present range must be risen up from the glacial period (Fig. 6D).

The past range of *R. sibiricus* before the Würm period is not well documented. The fossils assigned to *R. sibiricus* were recorded from the Upper Pliocene (2.5–3.2 MYA) of the Zaili Alatau Range, Northern Tien-Shan Mountains, Kazakhstan (Averianov and Tjutkova, 1995), which area is ca. 180 km far from the present distribution range. The past range of the species must have been much wider than the present one, but the distribution range repeatedly experienced shrinkage and expansion until now.

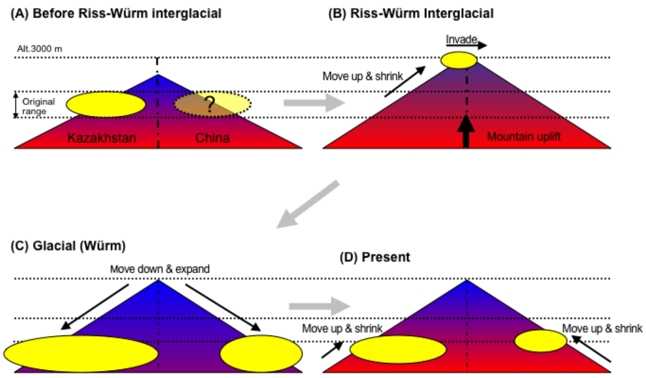

Fig. 6. Diagram showing estimated demography and fluctuation of population size in *Ranodon sibiricus*. Yellow (gray in the printed version) circles indicate distributional range of the salamander. Relative temperature is shown as warm to cold (light to dark in the printed version) colors.

From the result of present molecular analyses, the Chinese territory was inhabited with populations of *R. sibiricus* in Riss-Würm interglacial period. Only a single unique haplotype (CR03) was found in China, which has two-steps difference from the nearest main haplotype of CR01. We estimated that this unique haplotype appeared after invasion into Chinese territory. Nevertheless, we cannot completely rule out the possibility that the salamanders also occurred in China before the invasion from Kazakhstan at the Riss-Würm interglacial. However, much drier and cooler conditions in mountain foothills in Xinjiang, China than Kazakhstan (Aubekerov and Gorbunov, 1999; Abuduvaili and Toropov, 2005) at the Riss glaciation (ca 250,000–150,000 YA and ca 140,000–100,000 YA with the first stage of maximal development in the Dzungarian Alatau [Vilesov et al., 2013]), may have forced the extinction of the Chinese population.

It is difficult for now to detect the reasons why the species could survive only in the Dzungarian Alatau. However, because *R. sibiricus* is a semiaquatic salamander and requires a stable water level for recruitment and surrounding wet habitats for obtaining sufficient animal prey, these environmental conditions may have been best maintained in the present distributional range.

Conservational implication

Results of the present study and the fossil record indicate that *R. sibiricus* once greatly decreased its distribution, but thereafter expanded the range to some extent. Although the genetic variation was very low in Chinese populations, we found one unique haplotype in one of them. In order to protect the genetic diversity of this threatened species, we need to first pay attention to populations with unique variation.

Our skeletochronological analysis indicates the minimal female maturing age to be six years old. Thus we propose to monitor the population of this species at least six years in order to trace the complete recruitment of the generations.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

KN, DT, and AT collected sample and prepared DNA samples. NY and AT performed PCR and sequencing and conducted molecular phylogenetic and population genetics analyses. KN, DT, MM, NY, and AT wrote the paper. All authors read and approved the final manuscript.

SUPPLEMENTARY MATERIALS

A supplementary material for this article is available online (URL: http://www.bioone.org/doi/suppl/10.2108/zs160099).

Supplementary Text S1. Specimens examined for sex determination and maturation.

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