

## Phylogeographic Structure of Lenok (*Brachymystax lenok* Pallas) (Salmoninae, Salmonidae) Populations in Water Systems of Eastern China, Inferred from Mitochondrial DNA Sequences

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**Ying-Zhe Xia, Yi-Yu Chen, and Yan Sheng (2006)** Phylogeographic structure of lenok (*Brachymystax lenok* Pallas) (Salmoninae, Salmonidae) populations in water systems of eastern China, inferred from mitochondrial DNA sequences. *Zoological Studies* 45(2): 190-200. Salmonid fishes represent a model system for addressing a wide range of biogeographic, evolutionary, and conservation questions. Studies on the genetic structure and phylogeographic pattern of *Brachymystax lenok* Pallas (Salmoninae, Salmonidae) populations are important for addressing the systematics, evolution, and effective conservation of the species. Partial sequences of the mitochondrial control region (835 bp) and cytochrome (cyt) *b* (1069 bp) were obtained by PCR amplification from 71 *B. lenok* individuals from 7 populations in the river systems of northern China. Analysis of molecular variance indicated that a high proportion of the total genetic variance was distributed among regions, supporting strong geographic structuring of mtDNA polymorphism. Phylogenetic analysis was conducted using Neighbor-joining (NJ), maximum likelihood (ML), and Bayesian approaches, based on the combined control region and cyt *b* sequences. Seventeen haplotypes were assigned to 3 clades that were related to geographic regions. No shared haplotypes were found among regions. The pattern of phylogenetic discontinuity, which is associated with spatial separation, is a result of both historical (long-term, zoogeographic barriers to gene flow) and contemporary (limited dispersal and gene flow capabilities) components. Based on our results, together with geological age data, we inferred that after entering the Amur River drainage, lenoks spread southwardly along inland drainages and hypothesize the dispersal route of the species in the water systems of eastern China. We further propose that each of the 3 evolutionarily distinct groups of lenok populations should be protected in order to avoid the loss of biodiversity. It is highly recommended that management efforts focus on riverine conservation, and avoid translocations among populations of different regions.  
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**Key words:** Phylogeography, Mitochondrial DNA, Control region, Cytochrome *b*, *Brachymystax lenok*.

Salmonid fishes are economically important worldwide. Some species have long been of great interest due to their commercial and recreational value (Crespi and Fulton 2004). In recent years, they are becoming increasingly important as model systems for addressing a wide range of biogeographic, evolutionary, and conservation questions (Bermingham and Avise 1986, Bernatchez and Wilson 1998, Wilson and Bernatchez 1998,

Ramsden et al. 2003, Cortey et al. 2004, Utter 2004, Ah-King et al. 2005, Gum et al. 2005, McLean et al. 2005, Tonteri et al. 2005) together with the rise of molecular phylogeography (Avise et al. 1987, Avise 2000). These studies are being carried out at different geographic scales and in different regions. The scales can be as large as the Holarctic, covering a species' distribution (Brunner et al. 2001), or as small as a few basins

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(Asplund et al. 2004), or even within basins (Weiss et al. 2002). The regions are not evenly distributed. Most such studies have been carried out in Europe (Cortey et al. 2004, Ah-King et al. 2005, McLean et al. 2005) and North America (Stamford and Taylor 2004, McLean et al. 2005), whereas fewer studies are being conducted in Asia, in comparison with other geographic regions (McCusker et al. 2000, Brunner et al. 2001). Some research on salmonid fishes in Siberia has been accomplished (Froufe et al. 2003) but similar studies in other Asian regions are urgently needed.

*Brachymystax lenok* Pallas (Salmoninae, Salmonidae) is distributed throughout eastern Siberia and portions of northern Mongolia, China, and Korea (Institute of Hydrobiology et al. 1993). Due to its wide distribution, great adaptability, and restriction to freshwater habitats, as well as the complexity of its morphological and ecological traits, *B. lenok* is an ideal model organism to study the geographic distribution of genetic variation and the mechanisms that create and maintain these patterns in eastern Asia. However, populations of the species are currently declining through overexploitation, environmental pollution, and other causes, and it has been listed as a threatened species (Wang 1998, Yang and Wei 1999). To protect and make full use of the natural resources of the species, controlled reproduction is now in progress (Liu et al. 2000, Zhang 2003). Understanding the extent of phylogenetic differentiation among extant populations is important for efficient conservation and management of the lenok. However, information about *B. lenok* is still very scarce (Froufe et al. 2004). Results of former taxonomic and biogeographic studies have produced some controversy, such as on specific and subspecific classifications (Kifa 1976, Song and Fang 1984, Qin and Wang 1989, Mina 1992, Alekseyev 1994, Alekseyev 2003, Ma et al. 2005), and on the origin and evolution of the species (Gao 1980, Xie 1981, Li 1984,

Alekseyev 1986, Mina 1991, Shed'ko et al. 1996 2001, Froufe et al. 2003, Alekseyev 2004). Therefore, comprehensive studies on the genetic structure and phylogeographic patterns of lenok populations based on molecular techniques are important for understanding lenok systematics and evolution, and for developing effective conservation of the species.

Due to the rapid pace of mtDNA nucleotide substitution, and the special mode of maternal non-recombining mtDNA inheritance, mtDNA has become an effective molecular marker for phylogeographic analysis at the intraspecific level (Avice 1987), and has been widely used in studies of salmonid fishes (McCusker et al. 2000, Brunner et al. 2001, Weiss et al. 2002, Asplund et al. 2004, Stamford and Taylor 2004). In the present study, we first characterized the genetic and phylogeographic structure among 71 *B. lenok* individuals from 7 populations in river systems of northern China using sequences of the mitochondrial control region (D-loop) and of cytochrome *b* (cyt *b*). Then we discuss the mechanism for the formation of the present structure. Based on the phylogeographic structure of lenoks together with geological age data, we tested 2 hypotheses on the dispersal route of the species in water systems of eastern China. Finally, we provide some conservation implications for protecting the evolutionary potential and genetic diversity of the species.

## MATERIALS AND METHODS

### Sampling and DNA extractions

*Brachymystax lenok* is found in rivers of the Amur River basin (including the Erguna River, Humahe, Amur River, Nenjiang, Wusuli River, Songhua River, and Suifenhe), rivers from the Changbai Mountains (including the Yalu River and

**Table 1.** Population abbreviations, locality, sample size, and geographical coordinates for *Brachymystax lenok* individuals

Population	Locality	<i>n</i>	Latitude	Longitude
WN	Woniuhe, Amur River	10	50°05'N	127°51'E
SF	Suifenhe, Amur River	8	43°48'N	131°12'E
SH	Songhua River, Amur River	9	50°33'N	125°49'E
YL	Yalu River, Changbai Mountains	12	42°00'N	127°22'E
TM	Tumen River, Changbai Mountains	14	42°05'N	129°00'E
YE	Yellow River, old Yellow River	10	34°24'N	108°14'E
TL	Tuligenhe, old Yellow River	8	42°14'N	116°42'E

Tumen River), headwaters of the Haihe and Luanhe, tributaries of the Yellow River (the rivers around Taibai Mountain including the Heihe, Xushuihe, and Taibaihe), and the Erqisi River in northern Xinjiang Province. *Brachymystax lenok* populations are continuously distributed in water systems of eastern China (Gao 1980). Seventy-one individuals were sampled from 7 different populations chosen from north to south in 3 geographic regions (the Amur River, Changbai Mountains, and old Yellow River) in China. Exact sampling localities and sample sizes are presented in table 1 and figure 1. Ethanol-preserved muscle tissues were transported back to the laboratory. Total DNA was obtained from digestion of 100 mg of minced tissue in 1 ml of buffer (50 mM Tris (pH 8.0), 100 mM EDTA (pH 8.0), 1% SDS, and 0.6% proteinase K) for 16 h at 37°C with constant gentle mixing. The solution was sequentially extracted once each with 1 volume of phenol and phenolchloro-alcohol (24: 1). Total DNA was recovered by overnight precipitation in 2.5 volumes of absolute ethanol, and centrifugation at 13,000 rpm for 30 min. DNA was resuspended in 100-300  $\mu$ l of sterile, deionized water and frozen at -20°C until amplification.

### PCR amplification and sequencing

The mitochondrial control region was amplified by polymerase chain reaction (PCR) using the primers *M13/t-pro*: TGT AAA ACG ACG GCC AGT CCC AAA GCT AAG ATT CTA AA (an *M13* universal forward primer followed by *t-pro*; Shedlock et al. 1992), and *s-phe*: GCT TTA GTT AAG CTA CG (Nielsen et al. 1994). Reactions of 50  $\mu$ l in total volume contained 0.2 mM dNTPs, 1.5  $\mu$ M each of the light- and heavy-strand primers, 2.5 units of *Taq* DNA polymerase (Promega), 10x *Taq* DNA polymerase buffer, and 3  $\mu$ l of template DNA. PCR amplifications were performed in a PTC-200 thermocycler (MJ Research, Watertown, MA, USA) under the following conditions: 94°C for 5 mins; 40 cycles of 94°C for 30 s, 49°C for 30 s, and 72°C for 1 min; with a final extension at 72°C for 5 mins.

The *cyt b* region was amplified using the same PCR reagents and quantities as for the D-loop but with 2 primers we designed: S2UP (AAC CAC CGT TGT TAT TCA) and S2DOWN (GCT CAT TGG AGG GCT TTA). PCR cycles were performed in a PTC-200 thermocycler (MJ Research) under the following conditions: 94°C for 5 mins; 40 cycles of 94°C for 1 min, 56°C for 1

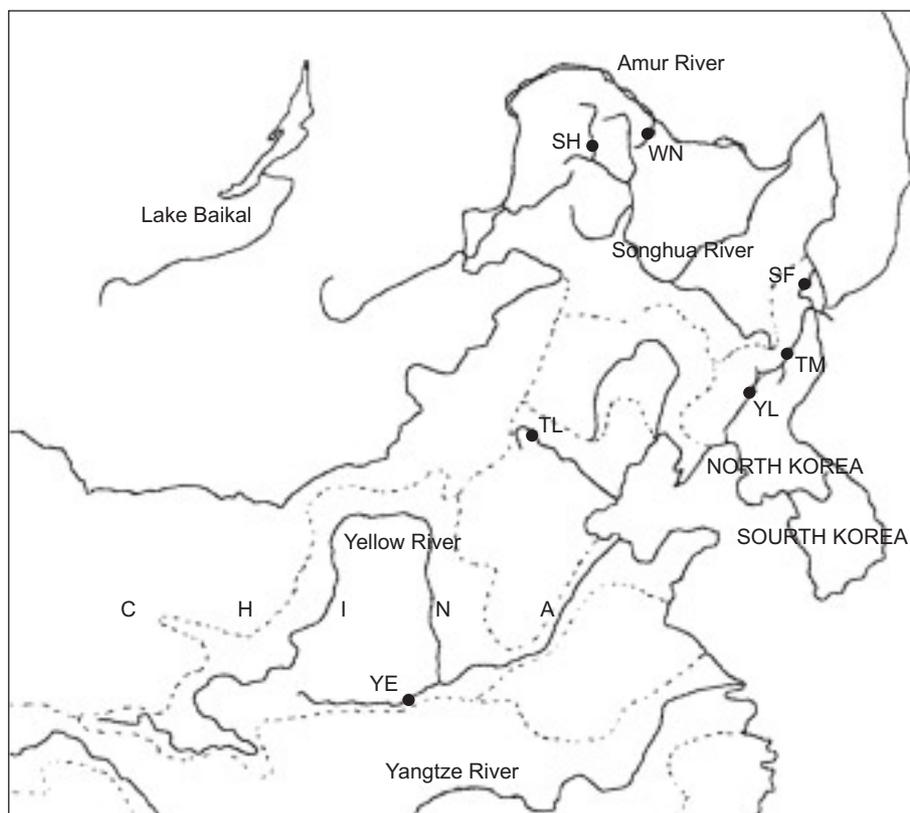


Fig. 1. Map showing *Brachymystax lenok* populations analyzed in this study. See table 1 for the population abbreviations.



(BPML) obtained after 100 pseudoreplicates, using the previous ML parameters and TBR branch swapping. We conducted Bayesian analyses using MrBayes 3.0b (Huelsenbeck and Ronquist 2001). The inference was conducted using the GTR + I +  $\Gamma$  model (Yang 1994); the data matrix was partitioned "by\_condon", starting trees were random, and phylogenetic constraints were not used. One million generations of Markov chain Monte Carlo (MCMC) were run; every 100th topology was sampled resulting in 10,000 trees. We discarded the first 1000 trees obtained before the Markov chain reached convergence, meaning that stationarity was empirically determined to have occurred by the 1000th tree. The posterior probability of the phylogenetic and tree topologies were then determined from the remaining 9000 trees. This procedure was repeated 2 times resulting in 18,000 trees. Majority rule consensus trees were constructed from these remaining trees using the "sumt" option in MrBayes 3.0b. All phylogenetic analyses were carried out on the combined control region and *cyt b* sequences using PAUP\* 4.0b10 (Swofford 2002).

## RESULTS

For each of the 71 lenok individuals, 835 bp of the mtDNA sequence from the 5' end of the control region was obtained. In total, 43 (5.1%) nucleotide positions were polymorphic including 33 parsimony informative positions and 10 singleton polymorphic positions, defining a total of 15 haplotypes (GenBank accession nos.: AY960113, and DQ017066-DQ017079; Table 2). No haplotype was shared among different populations. The pairwise sequence divergences among the 15 haplotypes are shown in table 3. The overall average sequence divergence was 1.93%. The average base composition was 32.1% A, 17.5% C, 18.4% G, and 32.06% T. The A+T content (64.1%) was much higher than the G+C content (35.9%). The analysis of molecular variance (Table 4) indicated that a high proportion of the total variance was distributed among regions (63.55%), whereas 24.17% and 12.28% occurred among populations within regions and within populations, respectively. Estimates of genetic differentiation between populations, using *F*-statistics, are given in table 5. Populations SF, TM, and YE showed high levels of genetic differentiation from all other populations.

The 5' end of the *cyt b* mtDNA region (1069 bp) was amplified from 70 lenok individuals. In

total, 106 (9.9%) nucleotide sites were polymorphic including 72 parsimony informative sites and 34 singleton polymorphic sites. The average base composition was 24.3% A, 30.4% C, 15.1% G, and 30.2% T. The G+C content (60.6%) was much higher than the A+T content (39.4%). Thirteen haplotypes were obtained, and no haplotype was shared among different populations (GenBank accession nos.: DQ086216-DQ086218, DQ086220, DQ086222, DQ086223, and DQ086225-DQ086232; Table 2). The pairwise sequence divergences among the 13 haplotypes are shown in table 3. The overall average sequence divergence was 3.77%. The analysis of molecular variance among populations was performed as previously presented for the control region (Table 4). Again, the proportion of genetic variance among regions was large and significant ( $p < 0.01$ ). Pairwise population differentiation revealed by the *cyt b* sequences were similar to those revealed by the control region sequences, with a high level of differentiation between populations SF and TM.

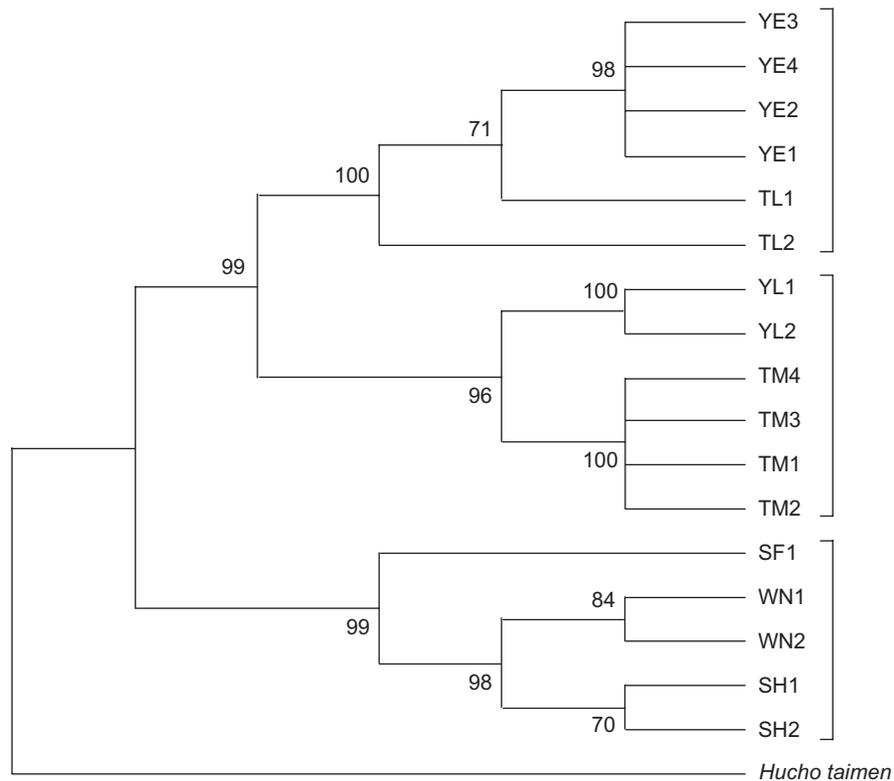
NJ analysis resulted in a molecular phylogenetic tree with high bootstrap values at most nodes (Fig. 2). Seventeen haplotypes were assigned to 3 clades that were related to geographic regions, hereafter referred to as the Amur River, Changbai Mountains, and old Yellow River regions. Trees constructed using the ML and Bayesian methods (not shown) were very similar to the NJ tree. High bootstrap values and posterior probabilities were found at most nodes of the consensus trees resulting from the ML and Bayesian analyses.

## DISCUSSION

### Population structure and phylogeographic pattern

In this study, analysis of molecular variance indicated that a high proportion of the total genetic variance was distributed among regions, supporting strong geographic structuring of mtDNA polymorphism. Phylogenetic analyses assigned haplotypes to 3 lineages that are related to geographic regions. No shared haplotypes were found among regions. The present-day pattern of phylogenetic discontinuity, which is associated with spatial separation, is a result of both historical (such as long-term zoogeographic barriers to gene flow) and contemporary components (such as limited dispersal and gene flow capabilities) (Avise et al. 1987,





**Fig. 2.** Neighbor-joining tree based on the combined control region and cyt *b* sequences. Seventeen haplotypes were assigned to 3 clades, which are the Amur River, Changbai Mountains, and old Yellow River. Numbers at the nodes are bootstrap values computed by 1000 replications using Kimura's 2-parameter distance. *Hucho taimen* was used as the outgroup taxon.

**Table 4.** Analysis of molecular variance (AMOVA) for *Brachymystax lenok* populations from 3 geographic regions (the Amur River, Changbai Mountains, and old Yellow River)

Molecular marker	Source of variation	Total variance (%)	<i>p</i> value
D-loop	Among regions	63.55	< 0.001
	Among populations within regions	24.17	< 0.001
	Within populations	12.28	< 0.001
Cytochrome <i>b</i>	Among regions	54.92	< 0.01
	Among populations within regions	41.26	< 0.001
	Within populations	3.82	< 0.001

**Table 5.** Pairwise  $F_{ST}$  values for the control region (above the diagonal) and cytochrome *b* sequences (below the diagonal) and *p* values of the *Brachymystax lenok* populations

FST	WN	SF	SH	YL	TM	YE	TL
WN	-	0.9811*	0.3684	0.9938	0.9925*	0.7035	0.9838
SF	0.7103*	-	0.7125*	1.0000*	1.0000**	0.7799**	1.0000*
SH	0.2789	0.5237*	-	0.9684	0.9628*	0.6928	0.9216
YL	0.9076*	0.9143*	0.6822	-	0.8265*	0.7487	1.0000
TM	0.7947*	0.9688**	0.7822**	0.6548	-	0.7830*	1.0000*
YE	0.9499*	0.9432*	0.7758*	0.9287*	0.8812**	-	0.5161
TL	0.8513	0.9019*	0.7143	0.7147	0.8990***	0.6486	-

\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

ability to achieve trans-basin dispersal when hydrological corridors provided suitable paths for this cold-tolerant species. Extensive sampling and further analyses are needed to clarify this point.

### Population dispersal and molecular clock

Pleistocene biogeographic events have traditionally been ascribed a major role in promoting speciation and in sculpting the present-day diversity and distributions of vertebrate taxa (Avice and Walker 1998). A central tenet in phylogeography is that contemporary lineages living in or near formerly glaciated areas can be traced to ancestral lineages from glacial refugia (Avice et al. 1987). The distributions of contemporary lenok populations were found to be in concordance with the Quaternary remnants of glaciation, which suggests that the postglacial climate offered suitable living conditions for lenok populations (Gao 1980). A widely accepted point of view is that *B. lenok* originated from the Eurasian north frigid zone and dispersed from Siberia to the water systems in northern China with the advent of Pleistocene glaciations (Gao 1980, Xie 1981, Li 1984). Yet the dispersal routes of the lenok are unknown. There are 2 hypotheses: (1) after entering the Amur River drainage, lenoks spread southwardly along inland drainages (Xie 1981); and (2) after entering the Amur River drainage, lenoks spread southwardly along the coastline. Lenok populations dispersed from the Sea of Japan to the Yellow Sea or to the southeastern East China Sea during the penultimate glaciation or the last glaciation. When the climate turned warm, the range of lenoks was reduced in the north, while a few individuals colonized and settled in montane rivulets, which were the ancestors of the contemporary *B. lenok* populations distributed in the headwaters of the Weihe, Chaobaihe, Lunhe, and Hunhe (Li 1984). According to our results, we believe the 2nd viewpoint about the dispersal route of lenoks is questionable. Because of global climate changes during the Pleistocene, major glaciations occurred 4 times in China (Li 1990). With the advance of glaciation, a large amount of freshwater was enclosed in the ice sheets, which resulted in the rapid lowering of sea levels. In the late Pleistocene, the sea level of the East China Sea declined as much 150-160 m on the edge of continental shelf (Zhu et al. 1979). At that time, the Sea of Japan, Bohai Gulf, Yellow Sea, and East China Sea were at the edge of the continent, where freshwater low-lying areas and swamps

were distributed. Exchanges of fishes were possible due to the mixture of close drainages in those regions. First, both the Suifenhe and Tumen River flow into the Sea of Japan, and the entrances to the sea of the 2 rivers are no more than 80 km apart. If the 2nd viewpoint is correct, exchange of the lenok between the 2 water systems would have been likely. As a result, population SF should be closely related to population TM. However, the 2 populations were sorted in different clades on the phylogenetic tree. Population SF was closer to the Amur populations than population TM, which together with population YL, constituted a different clade. Second, 1 mtDNA control region sequence (downloaded from GenBank; accession no.: AF125519) of a lenok individual from Hanjiang, Korea, was used in the phylogenetic analysis in combination with the control region we sequenced. The Hanjiang lenok individual did not form a clade with the coastal population TM, but rather with populations TL and YE. Finally, if the 2nd dispersal route were correct, we could deduce that *B. lenok* should have spread into the rivers of Japan along the Sea of Japan. But it has virtually not been reported from any of the islands of Japan (Tomiyama 1998).

Based on the phylogeographic structure of the lenoks, together with geological age data, we suggest that the dispersal route of the species in the water systems of eastern China was as follows. With the advent of Pleistocene glaciations, *B. lenok* dispersed from Siberia into the Amur River drainage and Nenjiang drainage in northern China (Gao 1980, Xie 1981). Then the lenok populations in the Amur River basin diffused to the old Yellow River basin, which consists of the Tuligenhe, Weihe, and Hanjiang (Liu 1958). With the uplift of the Xiaoxing-anling (Lin et al. 1999), lenok populations in the Amur River drainage became separated from those in the old Yellow River basin. After the occurrence of marine transgression, the tributaries of the old Yellow River basin successively became isolated (Chen 2003, Shi and Yu 2003). *Brachymystax lenok* populations evolved separately under the respective different environments.

A widely used divergence time for mtDNA in bony fishes is 2% sequence divergence between lineages per million years (my) (Bermingham and Avice 1986, Grewe et al. 1990). According to this molecular clock of 2%/my, the haplotypes found in the Amur River basin may have diverged from the old Yellow River basin haplotypes 2 million years ago (mya), which corresponds to the geological

age of the uplift of the Xiaoxing-anling (Lin et al. 1999). The haplotypes found in the Changbai Mountain area may have diverged from the old Yellow River basin haplotypes 1.5 mya. The haplotypes found in the Yalu River may have diverged from the Tumen River haplotypes 1.2 mya, which corresponds to the geological age of the uplift of the Changbai Mountains (Li 1990).

### Conservation implications

In Siberia, lenoks are known to occur in 2 relatively distinct morphological forms (blunt-snouted lenoks and sharp-snouted lenoks) (Kifa 1976). The 2 forms are found sympatrically in some regions and allopatrically in other regions (Alekseyev 1994, Alekseyev et al. 2003). In sympatric regions, the blunt-snouted lenok is more restricted in its distribution to high-order mountain tributaries and to upstream lakes (Alekseyev et al. 2003). Taxonomically the 2 forms are viewed either as 1 complex species, *B. lenok*, represented by 2 infraspecies (Mina 1991), or as 2 nominal species, *B. lenok* and *B. tumensis* (Shed'ko 2001). The taxonomy of lenok populations in China is still controversial. Li (1966) proposed that the lenok populations in the rivers around Taibai Mountain in the Qinling Mountains should be identified as a subspecies, *B. lenok tsinlingensis*, which is in contrast to the views of Song and Fang (1984), Gao (1980), and Qin (1989). Ma et al. (2005) reported that 2 species of lenok are found in the Amur basin: *B. tumensis*, a blunt-snouted lenok, and *B. lenok*, a sharp-snouted lenok. In our studies, the lenok populations in the Amur basin and the Changbai Mountain regions do not distinctly differ in morphology, whereas the lenok populations in the tributaries of the Yellow River are closer to the blunt-snouted lenok form than to the sharp-snouted lenok form.

The conservation implications of our findings are clear. Failure to recognize the 3 evolutionarily distinct groups of lenok populations could unwittingly lead to the loss of biodiversity (Soltis and Gitzendanner 1999). Considering the divergences in morphology and distribution, it is likely that some genetic information remains to be uncovered among the populations of the 3 regions. Therefore, it is highly recommended that management efforts focus on riverine conservation, and avoid translocations of populations among different regions. In the practice of developing controlled reproduction to increase the population resources, hybridization stemming from translocation among

different regions could severely limit the adaptive potential and unique evolutionary trajectories of these groups.

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