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Conservation genetics assessment and phylogenetic relationships of critically endangered *Hucho bleekeri* in China

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Summary

Hucho bleekeri is a critically endangered salmonid fish found in the Yangtze River drainage in China. In this study, the genetic diversity of a small population (n = 43) was first assessed with partial mitochondrial DNA sequences (D-loop region and a cytochrome *b* gene [*CYTB*] gene fragment) and 15 microsatellite markers. Low levels of nucleotide diversity (*Pi*) were demonstrated in the *H. bleekeri* population based on the two mitochondrial DNA markers. The number of haplotypes (*h*) and the haplotype diversity (*Hd*) in the D-loop region (12 haplotypes and *Hd* = 0.8208) were higher than in the *CYTB* gene fragment (three haplotypes and *Hd* = 0.0941). The number of microsatellite alleles (*Na*) ranged from 2 to 13 in these individuals. The mean observed heterozygosity (*Ho*) and the expected heterozygosity (*He*) were 0.59719 and 0.44735, respectively. Analysis of molecular variance showed that the degree of differentiation in the population was low (F_{ST} = 0.04041) and the coefficient of inbreeding (F_{IS}) was negative, indicating no obvious evidence of inbreeding in this population. A demographic assessment suggested that this species expanded a long time ago, but has suffered great losses in recent years. A molecular phylogenetic analysis clearly indicated that *H. bleekeri* is not introgressed by *Brachymystax lenok tsinlingensis*. The baseline population genetic information supplied by this study will be vital in monitoring this highly threatened species.

Introduction

Hucho bleekeri, also known as Sichuan Taimen or Chuanshan Taimen, is a freshwater salmonid fish endemic to the Yangtze River drainage in China and a glacial relict, with the lowest-latitude distribution of any Eurasian salmonid (Du et al., 2014). Widely distributed in several provinces, including Sichuan, Qinghai, and Shanxi provinces until the 1960s (Hu et al., 2008), both its population size and geographic range, however, have shown a significant and continuous decline, bringing it close to extinction in recent years (Shen et al., 2006). The population is predicted to be in continuous decline at a rate of at least 20% over its next two generations (34 years) (Peter, 2013), thus it was added as 'endangered' in 1998 to the China Red Data Book of Endangered Animals (Yue and Chen, 1998) and to the International Union for Conservation of Nature (IUCN) Red List as 'critically endangered' (CR) in 2012 (Song, 2012). This severe decline seems predominantly attributable to anthropogenic pressures, such as overfishing, incomplete regulation of fisheries, and habitat destruction (hydroelectric projects and water pollution), with few populations remaining (Hu et al., 2008). Importantly, the ability of this species to adapt to changing habitat conditions is weaker than that of other taimens, which may also have contributed markedly to the decline in its population size (Wang et al., 2009). Many measures have been taken to protect this endangered species, including artificial rearing and prohibition of fishing. In the 21st century, despite occasional reports of the unexpected capture of one or two individuals in the Markehe River in Qinghai Province (Shen et al., 2006), *H. bleekeri* has disappeared without a trace in most of its usual distribution areas in Shanxi and Sichuan provinces. Fortunately, 19 adult individuals were captured accidentally in September 2012 from the Taibai River in the Qinling Region, Shanxi Province (Du et al., 2014), with more individuals then collected. Recently, the controlled reproduction of this species has been successful, and breakthrough technology suggests that the protection of this fish has entered a new phase.

Ecosystem diversity, species richness, and genetic diversity are the three major aspects of biodiversity, all warranting equal attention in assessing conservation strategies (Sivaramakrishnan et al., 2014). Population genetic structure and genetic diversity are important considerations in conservation genetics because the genetic diversity present in a population is the result of its long-term evolution, and can restrict its survival in and adaption to new environments. Low genetic diversity caused by habitat degradation or lack of species connectivity may cause random genetic drift, elevated inbreeding, and reduced levels of gene flow (Young et al., 1996), resulting in the loss of genes and/or the accumulation of harmful genes, and the increasing isolation of populations. Therefore, the development and implementation of reasonable conservation strategies for rare and endangered species must be based on the genetic assessment of the species.

Microsatellite markers and mitochondrial DNA (mtDNA) are reliable molecular tools with which to evaluate genetic diversity, and are especially useful for identifying endangered

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populations (Farah et al., 2011; Gary et al., 2015). The genetic diversity of some other *Hucho* species has been successfully analyzed with molecular markers (Geist et al., 2009; Kuang et al., 2009; Liu et al., 2011; Weiss et al., 2011; Skurikhina et al., 2013). Because of the endangered status of *H. bleekeri* and our current understanding of the need to conserve genetic diversity, in this study we first investigated the levels of genetic diversity, the population structure, and the phylogenetic relationships of a small wild population of *H. bleekeri* (n = 43 individuals) in the Taibai River, to identify conservation strategies that will ensure population enhancement and controlled breeding. We analyzed mtDNA sequences (D-loop region and cytochrome *b* gene [*CYTB*] fragment) and microsatellite markers isolated in a previous study.

Materials and methods

Sample collection and DNA extraction

Forty-three fin tissue samples of *H. bleekeri* were collected from the Taibai River in Baoji City, Shanxi Province, China (N33°50′25.40″; E107°14′02.02″) (Fig. 1). The small sample size reflects the restrictions on the collecting of this critically endangered species. The fin clips were stored in 75% ethanol. The total genomic DNA of *H. bleekeri* was extracted with the rapid salt-extraction method (Salah and Iciar, 1997).

mtDNA fragment amplification and sequencing

The D-loop region and *CYTB* gene were amplified with PCR. The primers are shown in Table 1. PCR was performed in a total volume of 25 μl containing the components: 1× PCR buffer, 50–100 ng of genomic DNA, 0.25 μM each primer, 200 μM dNTPs, 0.5 mM $MgCl_2$, and 0.25 U of *Taq* DNA polymerase (TaKaRa Bio, Dalian, China). The PCR cycling parameters consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, annealing at the appropriate temperature (Table 1) for 30 s, and extension at 72°C for 60 s, with a final extension at 72°C for 10 min. The PCR products were purified with the E.Z.N.A.™ Gel Extraction Kit (Omega Biotek, Atlanta, GA) and sequenced directly on an ABI 3730XL automated sequencer. The sequences were assembled with LASERGENE (SeqMan) v. 7 (Clewley and Arnold, 1997) and deposited in GenBank under accession numbers KR021391–KR021398, KR021400–KR021402, and KR073000–KR073003. We also obtained mtDNA sequences from GenBank: (i) *H. bleekeri* (KF908853) (Zhang et al., 2014a), (ii) *H. bleekeri* (HM804473, HQ317211, and HQ317212) (Wang et al., 2011a), (iii) *H. bleekeri* (FJ597623) (Qi et al., 2009), (iv) *H. taimen* (HQ897271) (Wang et al., 2011b), (v) *H. taimen* (KJ711550) (Balakirev et al., 2013), (vi) *H. hucho* (KM588351) (Zhang et al., 2014b), (vii) *H. perryi* (NC_021651) (Shedko et al., 2014), (viii) *H. perryi* (AP013048) (Campbell et al., 2013), (ix) *Brachymystax lenok* (JQ686730) and *B. lenok tsinlingensis* (JQ686731) (Si et al., 2012), and (x) *Oncorhynchus mykiss* (DQ288268) (Brown et al., 2006), which was used as the outgroup.

Microsatellite marker amplification and analysis

A set of 15 microsatellite primers (Hbl4-10, BLT25, Hbl10-16, Hbl14-29, BLT4, BLT16, Hbl6-240, Hbl2-2, Hbl7-290, Hbl12-23, Hbl15-31, BLT20, Hbl3-5, Hbl8-163, and BLT19), developed in a previous study (Wang et al., 2015), were used for the genetic analysis. The forward primer of each pair was

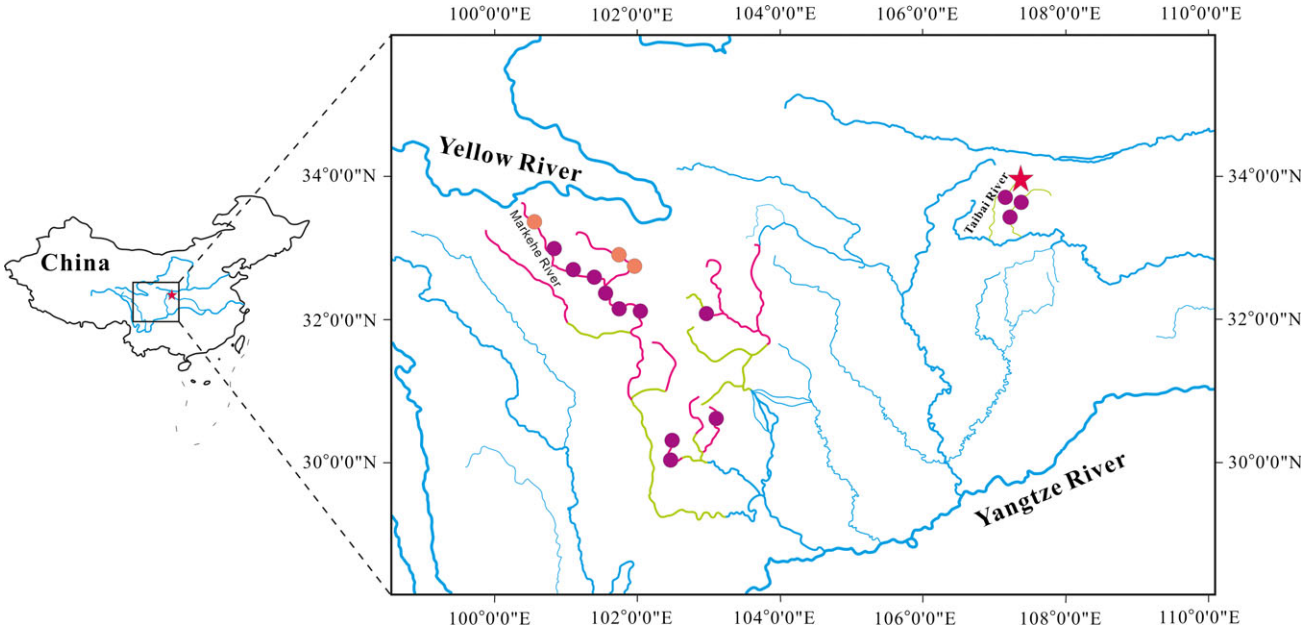

Fig. 1. Map of the Yangtze River drainage and distribution area of *H. bleekeri* in China. Red pentagram = study sampling site (N33°50′25.40″; E107°14′02.02″); Orange dots = sampling sites between years 2000–2010; Purple dots = areas of this species found in years 1980–2000; Pink lines = rivers with this species in years 1960–1980; Green and pink lines = distribution area of *H. bleekeri* before 1960s

Table 1
mtDNA primer sequences of *Hucho bleekeri*

Primers	Primer sequence (5′–3′)	Tm (°C)	Fragment length (bp)
D-loop-F	GTCTTGTAATCCGGAGGCCGGAG	58	1180
D-loop-R	CCATCTTAACAGCTTCAGTG		
Cytb-F	ATGACTTGAAAAACCACCGT	54	1119
Cytb-R	CTCTGGCGCTGAGCTACTAG		

labeled with a fluorescent dye (HEX or FAM) (Table 2). The microsatellite loci were amplified using the optimized reaction system and conditions described above. The PCR products labeled with one of two different fluorescent markers, which were more than 20 bases in length, were mixed together, and then analyzed on an ABI 3730XL automated sequencer. The alleles were scored using the GENEMARKER software v. 1.5 (Applied Biosystems).

Genetic diversity analysis

The mtDNA sequences were aligned with the CLUSTALX v. 2.0 software (Larkin et al., 2007). The DNASP v. 5 program (Librado and Rozas, 2009) was used to calculate the number of polymorphic sites (*S*), number of haplotypes (*h*), haplotype diversity (*Hd*), nucleotide diversity (*Pi*), and the average number of nucleotide differences (*k*).

The ARLEQUIN v. 3.5 program (Excoffier and Lischer, 2010) was used to analyze the microsatellite data, assessing the genetic diversity at the intrapopulation level. Several parameters were calculated, including the number of alleles (*Na*), observed heterozygosity (*Ho*), expected heterozygosity (*He*), departure from Hardy–Weinberg equilibrium (HWE) for all pairs of loci (with the corresponding P values), and the coefficient of inbreeding (F_{IS}). Genotypic linkage disequilibrium was estimated pairwise between the loci using the GENEPOP v. 4 program (Raimond and Rousset, 1995). We set the number of dememorizations to 1000, the number of batches to 100, and the iterations per batch to 1000.

Demographic assessment and population structure

To assess the demography of the total population, we used mtDNA markers to test the selective neutrality of the total population using the infinite sites model with Tajima *D* (Tajima, 1989) and Fu's *Fs* (Fu, 1997) with the ARLEQUIN program. We also determined the modified Garza–Williamson index (*M*) (Garza and Williamson, 2001) for each locus, calculated on the basis of the microsatellite data with the ARLEQUIN program.

The BOTTLENECK v. 1.2.02 software (Piry et al., 1999) was used to detect genetic bottleneck signatures, and the following tests were run: (i) sign test (Cornuet and Luikart, 1996),

Table 2
Microsatellite primer sequences of *H. bleekeri*

Locus	Dye	Primer sequences (5′–3′)	Tm (°C)	Accession no.
Hbl4-10	HEX	F: GACAACAGCTACAGGGCACA R: GACCTGGCTCTGGGTGATAG	56	KM385541
BLT25	FAM	F: AATGAAACCAGCTCATTGCC R: CAAGTCCTTCCAAATGGTCC	56	KM051921
Hbl10-16	HEX	F: TTCCTCTCCTCCTCCTCCAT R: GACTTCGGGGATGGCTCTAT	56	KM385547
Hbl14-29	HEX	F: GAGGTGCACGTGTTCAAAGA R: TGGTTAAGACCAAGACCAACG	55	KM385551
BLT4	FAM	F: TGAACAGACACTCACACAGGC R: GTGTTTCAGCTGCTGCGTT	55	KM051900
BLT16	HEX	F: ATCCAGTCAATAACCGCTGG R: CCTCGAGAAACTCGGTTGTT	55	KM051912
Hbl6-240	FAM	F: GGGGAATGCAGTTGAAATGT R: ATGGCATGTGTGGTGTGTTT	55	KM385543
Hbl2-2	HEX	F: TCTAGTGCTGCATGTCTGCC R: AGGCTCACATTGGCTCGTAT	57	KM385539
Hbl7-290	FAM	F: ACTCTGATCGCTACCTGGGG R: TCTGTTCGGACTGCTACATCA	56	KM385544
Hbl12-23	HEX	F: AATGCTTATTCACGCGAGGT R: ACACACAGCTTGGGACACAG	56	KM385549
Hbl15-31	FAM	F: GAGCTGGCTTGGTTGGTTAG R: GCACCAGTCTTCTTTTTCGG	57	KM385552
BLT20	HEX	F: CCGTACTGCCTAGCAACACA R: GGCTGTTTTCACAGAAAGGC	56	KM051916
Hbl3-5	FAM	F: TTGAAGTTGCCTTCTGGTCC R: GGCCACACATGCAAAACAT	58	KM385540
Hbl8-163	HEX	F: GACTGGTGAGTCACAGGCAA R: GCCTAGAGTGAGACCGATGC	60	KM385545
BLT19	FAM	F: GTTCCTCTCTGTCCCCTTCC R: AAACACCATGGAACTCGACC	55	KM051915

and (ii) the allele frequency distribution test (mode-shift test; Luikart et al., 1998). The first test was run to check whether the number of loci with excess heterozygotes was significantly higher than would be expected by chance alone, and accommodates three mutational models: the infinite alleles model (I.A.M.), the stepwise mutation model (S.M.M.) and the two-phase model (T.P.M., with 90% S.M.M. and 10% I.A.M.).

The degree of genetic structure analyzed with mtDNA markers was determined by examining an unrooted phylogenetic network using the median-joining method, with the NETWORK v. 4.613 software (http://fluxus-engineering.com). STRUCTURE v. 2.3 (Pritchard et al., 2000) was used for the Bayesian clustering of the 43 individuals based on the microsatellite data. Allelic frequencies were used to analyze the dataset without prior population information, using a number of clusters (K) ranging from 1 to 7 for 10 000 burn-in periods and 10 000 repetitions, and the criterion proposed by Evanno et al. (2005) was used to estimate the appropriate K value.

Phylogenetic portrait

A neighbor-joining (NJ) phylogenetic tree and a maximum-likelihood (ML) tree were constructed from the mtDNA markers with the MEGA v. 5 program (Tamura et al., 2011). The NJ analysis was performed with the Kimura two-parameter model and the ML analysis with the Tamura–Nei model. The node support for the NJ and ML trees was assessed with 1000 bootstrap pseudoreplicates.

Results

Genetic diversity

As shown in Table 3, the sequences of the D-loop region and the *CYTB* gene fragment in the mtDNA were 1180 and 1119 bp, respectively. There were 10 polymorphic sites in the D-loop region but no singleton variable site, and two polymorphic sites in the *CYTB* gene, with two singleton variable sites. Both the number of haplotypes (h) and the haplotype diversity (Hd) of the D-loop region (12 haplotypes and $Hd = 0.8208$) were higher than those of the *CYTB* gene fragment (three haplotypes and $Hd = 0.0941$). The nucleotide diversity (Pi) of both markers was very low: 0.00159 for D-loop region and 0.00009 for the *CYTB* gene. The average numbers of nucleotide differences (k) were also low: 1.871 for the D-loop and 0.095 for the *CYTB* gene.

In the microsatellite analysis, we found a total of 77 alleles in the *H. bleekeri* population, ranging from 2 (Hbl10-16) to 13 (Hbl8-163) per locus (Table 4). The calculated Ho varied from 0.04651 to 1.00000, with a mean of 0.59719, whereas He ranged from 0.09111 to 0.67387, with a mean of 0.44735. Most of the 15 microsatellite loci deviated significantly from HWE ($P < 0.05$), except Hbl2-2. The coefficient of inbreeding (F_{IS}) ranged from −0.90487 to 0.49398, with a mean of −0.34033, and was not significant ($P > 0.05$). Significant ($P < 0.05$) linkage disequilibrium was not detected in any loci combinations.

Demographic assessment and population structure

Tajima's neutrality test calculated with the mtDNA markers produced values of −0.64500 ($P > 0.05$) for the D-loop region and −1.48214 ($P < 0.05$) for the *CYTB* gene. Similar results were also derived with Fu's Fs test: −2.91103 for the D-loop region ($P > 0.05$) and −2.79487 for the *CYTB* gene ($P < 0.05$). The different values for these two mtDNA markers can be explained by their different rates of evolution.

The modified Garza–Williamson index (M) across all 15 microsatellite loci ranged from 0.07018 to 0.37500, with a mean of 0.18653, indicative of a large proportion of missing alleles (Table 4). Garza and Williamson (2001) showed, given the appropriate mutational model, datasets with seven or more loci that show $M < 0.68$ can be assumed to have experienced a reduction in population size.

The microsatellite marker analysis performed with the BOTTLENECK program detected no distortion of allele frequencies with the mode-shift test (the population showed an L-shaped distribution). However, significant excess heterozygosity was observed when the T.P.M. and S.M.M. models were used, both of which showed 12 heterozygosity-deficient loci and three loci with excess heterozygosity ($P < 0.05$), lending support to the possibility that demographic factors interfere in the genetic structure of this population (Table 4).

We examined 43 individuals and identified three haplotypes among the *CYTB* sequences and 12 haplotypes among the D-loop sequences, which differed in their nucleotides (Fig. 2). Haplotype Hbl-CYTBb01 was the major haplotype of the *CYTB* sequence, which occurred in 41 individuals, accounting for 95% of the total samples, whereas haplotypes Hbl-CYTB03 and Hbl-CYTB05 both occurred in one individual. Hbldloop43 was the major haplotype of the D-loop sequences, accounting for 34% of all haplotypes, whereas the proportions of Hbldloop02, Hbldloop30, Hbldloop03, and Hbldloop19 were 24, 13, 5, and 5%, respectively. Seven other haplotypes were identified, all in one individual. We used 1119 bp of the *CYTB* gene sequence and 1180 bp of the D-loop sequence for the haplotype analysis.

Table 3
Parameters calculated by mtDNA markers

mtDNA Markers	Number of sites	S	h	Hd	Pi	k	D	Fs
D-loop region	1180	10	12	0.8208	0.00159	1.871	−0.64500	−2.91103
Cyt *b* gene	1119	2	3	0.0941	0.00009	0.095	−1.48214*	−2.79487*

S, number of polymorphic sites; h, number of haplotypes; Hd, haplotype diversity; Pi, nucleotide diversity; k, the average number of nucleotide differences; D, Tajima's neutrality test; Fs, Fu's Fs test.
*Significant value ($P < 0.05$).

Table 4
Detection results for all microsatellite loci of *H. bleekeri*

Locus	*Na*	*Ho*	*He*	F_{IS}	P–HWE	*M*	P–Bottleneck analysis		
							I.A.M.	T.P.M.	S.M.M.
Hbl4-10	6	0.93023	0.62052	−0.50808	0.00000	0.37500	0.4816	0.1565	0.0688
BLT25	5	0.48837	0.54008	0.09677	0.03351	0.11628	0.4658	0.1517	0.0786
Hbl10-16	2	0.95349	0.50588	−0.90487	0.00000	0.20000	0.0135	0.0160	0.0166
Hbl14-29	5	0.74419	0.52531	−0.42373	0.00035	0.20000	0.4302	0.1328	0.0606
BLT4	6	0.95349	0.61231	−0.56759	0.00000	0.31579	0.3417	0.3084	0.1853
BLT16	5	0.79070	0.55404	−0.43446	0.00000	0.21739	0.4926	0.1808	0.0851
Hbl6-240	3	0.20930	0.26566	0.21414	0.00897	0.20000	0.3991	0.1775	0.1139
Hbl2-2	5	0.46512	0.53844	0.13758	0.34716	0.09615	0.3293	0.0640	0.0178
Hbl7-290	4	0.04651	0.09111	0.49245	0.00061	0.07018	0.0302	0.0018	0.0004
Hbl12-23	5	0.04651	0.09138	0.49398	0.00038	0.08197	0.0018	0.0002	0.0000
Hbl15-31	4	1.00000	0.67387	−0.49256	0.00000	0.23529	0.0567	0.1619	0.2215
BLT20	3	0.95349	0.51737	−0.86162	0.00000	0.20000	0.1943	0.3541	0.4245
Hbl3-5	4	0.18605	0.19371	0.04000	0.01938	0.30769	0.1118	0.0144	0.0036
Hbl8-163	13	0.23810	0.41853	0.43409	0.00000	0.08075	0.0004	0.0000	0.0000
BLT19	7	0.95238	0.56196	−0.70922	0.00000	0.10145	0.2376	0.0226	0.0060
Mean	5.133	0.59719	0.44735	−0.34033	–	0.18653	0.5164	0.0030	0.0029

Na, number of alleles; *Ho*, observed heterozygosity; *He*, expected heterozygosity; P–HWE, P value of Hardy–Weinberg equilibrium; *M*, Garza–Williamson modified index; P–Bottleneck analysis, P value of Bottleneck analysis under three different models of mutation.

To identify the ancestral source populations, we ran the STRUCTURE program on the entire dataset with increasing values of *K*. The most probable *K* value (number of clusters) inferred from lnP (*D*) was $K = 2$, thus the population was divided into two clusters (Fig. 3). When we divided the samples into two groups according to the Structure analysis, group 1 contained only nine individuals, and group 2 contained the other 34 individuals. The fixation index (F_{ST}) between the two groups, assessed with microsatellite markers, was 0.04041 when the ARLEQUIN program was used.

Phylogenetic portrait

Phylogenetic trees were constructed using NJ and ML analyses based on the haplotypes of the mtDNA markers (D-loop region and *CYTB* gene). The tree topologies generated with the different methods were similar and were supported by high posterior probability or bootstrap values at the main nodes (Fig. 4). The interrelationships among the members of the genus *Hucho* matched their published taxonomy, and *H. perryi* was shown once again to be more closely related to *Oncorhynchus mykiss* on these trees. The *H. bleekeri* specimens examined in this study were most closely related to *H. taimen*, and then to *H. hucho*, forming a clade with these members of the genus *Hucho*, which clustered in turn with *B. lenok* and *B. lenok tsinlingensis*.

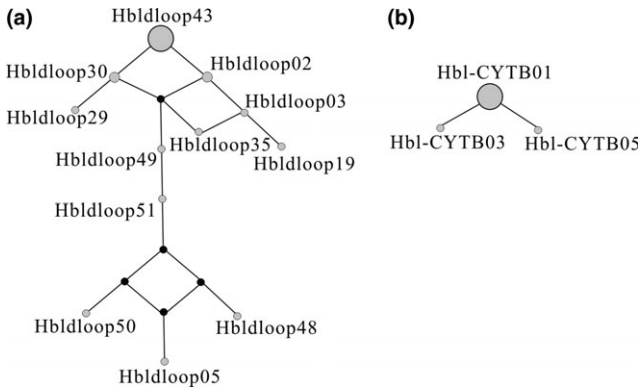

Fig. 2. Median-joining networks created by two mtDNA markers for all *Hucho bleekeri* haplotypes found in samples: (a) Network based on D-loop region with 12 haplotypes; (b) Network based on Cyt *b* gene sequences with three haplotypes. Black dots = potential mutation site

Discussion

The loss of genetic diversity in natural populations can have many causes, including reductions in the effective population size (Vrijenhoek, 1998). Freshwater fish are particularly susceptible to biodiversity loss because they often exist naturally in small, fragmented populations vulnerable to habitat degradation, pollution, and the introduction of exotic species (Hughes et al., 2012). In recent years, the genetic diversity of the genus *Hucho* has been evaluated with microsatellite markers (Kuang et al., 2009; Liu et al., 2011), mtDNA sequence (Geist et al., 2009; Weiss et al., 2011), and a restriction fragment length polymorphism (Skurikhina et al., 2013). Zhang et al. (2014a) found that the number of repeats in the mitochondrial DNA control region (four) in *H. bleekeri* sampled from the Taibai River differed significantly from the 6–7 tandem repeats in the fish sampled from the Markehe River.

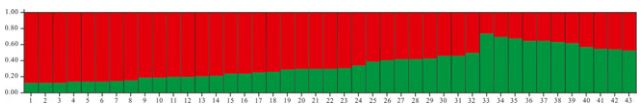

Fig. 3. Results of Bayesian analysis (STRUCTURE) of *Hucho bleekeri* samples from Taibai River, based on $K = 2$. Each column represents a different individual; colors represent probability membership coefficient per individual for each genetic cluster

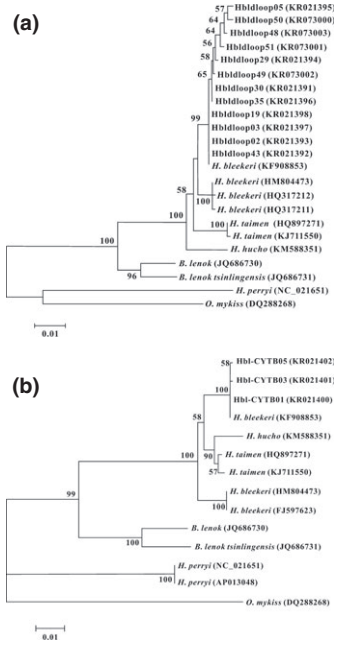

Fig. 4. Phylogenetic trees based on different fragments of mitochondrial DNA sequences: (a) Neighbor-Joining (NJ) Phylogenetic tree based on D-loop region, (b) Neighbor-Joining (NJ) Phylogenetic tree based on Cyt *b* gene. In parentheses = sequences downloaded from GenBank

As in that study, we identified four tandem repeats in all D-loop sequences in this study, confirming the genetic differences between populations sampled from different sites. The divergence between *H. bleekeri* populations sampled from different sites, in the Taibai River (our study) and Markehe River (Qi et al., 2009; Wang et al., 2011a), was apparent in the phylogenetic analysis (Fig. 4). There were also more haplotypes among the D-loop sequences than among the *CYTB* gene sequences, and *Hd* and *Pi* differed greatly between the results obtained with the D-loop-region marker and the *CYTB* gene marker, which may be attributable to the different rates of nucleotide substitution throughout the mtDNA. The main variations in the control region were in the polyT sequence and the 82-bp tandem repeat region. Combining the results for the mtDNA sequencing and microsatellite marker analyses, we have demonstrated that this wild population of *H. bleekeri* has a relatively low genetic diversity, reflected in the limited number of closely-related mtDNA haplotypes and low *He*, which was similar to or lower than values reported for other endangered taimens (Kuang et al., 2009; Liu et al., 2011; Weiss et al., 2011).

The majority of microsatellite loci deviated seriously from HWE ($P < 0.05$) in response to the population size, suggesting that both the genotypes and gene frequencies fluctuated continuously. This phenomenon can be mainly interpreted as an effect of sampling error (the restricted population size), predominantly arising from the degree to which this species is endangered and the difficulty in capturing the fish. Although the results of the neutrality test suggest that *H. bleekeri* experienced a population expansion during the long history of its evolution, this population exhibit excess heterozygosity in the T.P.M. and S.M.M. models of the bottleneck test, indicating recent reductions in its effective population size. We infer from the results of the demographic analyses that this species expanded continuously during its evolution, and was distributed extensively throughout its history. However, it suffered extreme devastation and the population declined rapidly for a variety of reasons, probably including those of human activity.

In the *F*-statistic analysis, the mean F_{IS} calculated from the microsatellite data was negative and not significant ($F_{IS} = -0.34033$, $P > 0.05$), and the observed heterozygosity ($Ho = 0.59719$) was greater than the expected heterozygosity ($He = 0.44735$), suggesting no loss of heterozygosity. F_{ST} indicates the genetic differentiation among groups, and although the Structure analysis showed two weak clusters in the population, AMOVA showed that the F_{ST} between these two clusters was low ($F_{ST} = 0.04041$), clearly indicating that there is no genetic divergence in this *H. bleekeri* population ($F_{ST} < 0.05$) (Weir and Cockerham, 1984).

Two species from different genera of salmonid fish are distributed discontinuously in the Qinling Region. One is *H. bleekeri* and the other is *B. lenok tsinlingensis*, which share the same ecological niche. Similarly, *H. taimen* and *B. lenok* are sympatric in many parts of their distributions. Recently, researchers have detected hybridization and mtDNA introgression between *H. taimen* and *B. lenok*, an introgression attributed to artificial hybridization (Wang et al., 2011c; Balakirev et al., 2013). The present study has shown a clear divergence between the members of the genus *Hucho* and the two species of *Brachymystax* in a phylogenetic analysis, which indicated that no *H. bleekeri* samples were introgressed by *B. lenok* or *B. lenok tsinlingensis*. We also found that *H. perryi* is closely related to *O. mykiss*, in the genus *Oncorhynchus*, which is consistent with the findings of Alexis et al. (2012).

In summary, by analyzing partial mitochondrial DNA sequences (D-loop region and *CYTB* gene fragment) and 15 microsatellite markers, we have shown that a low level of genetic diversity remains in this *H. bleekeri* population, which implies that further action is urgently required to protect this threatened species in its remaining area of distribution.

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