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Assignment of parentage by microsatellite analysis in the endangered *Brachymystax lenok tsinlingensis* (Salmonidae)

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ABSTRACT: We evaluated the use of microsatellite markers to identify parentage in *Brachymystax lenok tsinlingensis* Li, 1966, an endangered salmonid. Computer simulations showed that when no parent information was available, or when information was only available from 1 parent, then exclusion probabilities ranged from 23.6 to 45.1% and from 25.9 to 52.4%, respectively; combined exclusion probabilities for the 8 loci investigated were 97.7 and 98.4%, respectively. However, a breeding experiment with known parental and filial information resulted in 91.28% of progeny being exclusively assigned to their correct parent pair. Our data demonstrate that microsatellite-based parentage assignment is a reliable means with which to obtain information pertaining to genealogical relationships and could therefore benefit genetic conservation of this species.

KEY WORDS: *Brachymystax lenok tsinlingensis* · Microsatellites · Parentage assignment · Inbreeding depression

INTRODUCTION

The genus *Brachymystax* includes 4 species: *B. lenok*, *B. savinovi*, *B. tumensis*, and *B. tsinlingensis* (*B. lenok tsinlingensis* Li, 1966), which are landlocked coldwater salmonid fishes. They are thought to originate from Siberia and were introduced to the Yellow Sea and the Bohai Sea as a result of glacial advances during quaternary glaciation (Li 1984). Migration to the sea was prevented by deglaciation, which led to sealed off distributaries and isolated local populations. Consequently, fish from this genus are not distributed contiguously in mountain streams, leading to long-term isolation in northern China (Sun & Fang 1984).

Unfortunately, populations of *B. lenok tsinlingensis* have been rapidly declining as a result of overexploitation, environmental pollution, and habitat fragmentation (Zhao & Zhang 2009, Froese & Pauly 2015). The species is listed as 'vulnerable' in the China Red Data Book of Endangered Animals, and is classified as a second-class state-protected wild animal in China (Yue & Chen 1998). Since 2008, *B. lenok tsinlingensis* has received increasing levels of attention for conservation via artificial propagation programs for the sole purpose of restocking the natural population to a sustainable level.

In order to minimize the deleterious effects of inbreeding, however, it is vital to understand the genetic background of the fish before considering artificial propagation. Currently, genetic evaluation of relatedness for broodstock management in captivity has been studied for some salmonid species, e.g. *Oncorhynchus nerka* (Kozfkay et al. 2008, Kali-

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nowski et al. 2012) and *Hucho taimen* (Zhang et al. 2010). However, genetic studies of *B. lenok tsinlingensis* are limited to a number of investigations relating to the mitochondrial genome and general phylogeny (Liu et al. 2015, Yu & Kwak 2015). In order to maintain genetic diversity, optimal survival, and growth traits, the most appropriate practice is to isolate and raise the progeny of different family groups in separate tanks until they are large enough to be tagged physically. However, this approach takes up too much space and is labor intensive. Moreover, it introduces environmental effects common to full-sibling groups that can confound genetic effects when estimating breeding values and other phenotypic and genetic parameters (Herbinger et al. 1999). Parentage inference using highly polymorphic codominant genetic markers, such as microsatellites (also called simple sequence repeats, or SSRs), is becoming a widely used tool to assess genetic relationship in selective breeding and population conservation for aquaculture as a way to identify and maintain pedigrees (Munkres et al. 2007, Harrison et al. 2014, Luo et al. 2014a,b). The use of a microsatellite-based parentage assignment system would allow progeny from different families to be communally stocked and retrospectively assigned to family of origin.

The present study was conducted to assess the potential for microsatellite markers previously developed for *B. lenok tsinlingensis* (Wang et al. 2015) to assign correct parentage. First, computer simulations were used to predict how many loci were required to provide a satisfactory rate of assignment success. We then used known parent-progeny relationships to test the power of selected loci to correctly assign parentage under hatchery conditions.

MATERIALS AND METHODS

Mating strategy and rearing

All broodstock were reared at the Shitouhe River Reserve (Shanxi Province, China). A small piece of dorsal fin was collected from all *Brachymystax lenok tsinlingensis* breeders. Broodstock was composed of 10 females (F) and 30 males (M), and artificial propagation was carried out with a ratio of 1 F: 3 M. Fertilized eggs from each full-sib family were hatched in individual polycarbonate tanks (0.5 × 0.5 × 0.3 m, 75 l capacity) containing water from mountain streams that had been pre-treated by precipitation and filtration in a sedimentation pond. Temperature was maintained at 11 ± 1.5°C (mean ± SD). After hatching, fingerlings were separately sampled from each family, and the sample size depended on the availability of offspring in each family. In total, 172 individuals were collected from all families.

DNA extraction and microsatellite analysis

Genomic DNA was extracted from fins (parents) or whole bodies (offspring) by using an established salt-extraction method with slight modifications (Aljanabi & Martinez 1997). A total of 172 progeny and 40 broodstock were genotyped with 8 polymorphic microsatellite loci (Wang et al. 2015). After a preliminary verification by PCR-PAGE, these loci were found to be highly polymorphic and were steadily amplified with clear bands from DNA obtained from *B. lenok tsinlingensis*. Details of the selected microsatellite loci are listed in Table 1. Each forward primer was labeled with carboxyfluorescein (FAM), carboxy-X-rhodamine (ROX), or hexachloro fluorescein (HEX). All PCR amplifications were carried out in a 10 µl volume containing 1 µl of 10× buffer (with Mg^{2+}) for *Taq* DNA polymerase, 100 µM dNTP, 0.5 µl primer pairs,

Table 1. Microsatellite makers and primers used for the analysis of DNA in. Ta: amplification temperature used for PCR; FAM: carboxyfluorescein; HEX: hexachloro-fluorescein; ROX: carboxy-X-rhodamine

Locus	Sequence (5′–3′)	Ta (°C)	Repeats	Size (bp)	Label
BLT23	F: AATGCTTATTCACGCGAGGT R: ACACACAGCTTGGGACACAG	59	$(AGTT)_{13}$	237–253	HEX
BLT31	F: TGGATGGGTGTTACAAGCAA R: CAGATCTTGAGACAAAGAGCCA	56	$(GTTA)_{10}$	161–177	FAM
BLT21	F: CATTAATCCATCCAACCATGC R: ACATCCCTGCCTTCGAAAC	60	$(TCAT)_{10}$	129–149	ROX
BLT5	F: TTGAAGTTGCCTTCTGGTCC R: GGCCACACATGCAAAACAT	60	$(TAA)_{11}$	164–176	FAM
BLT10	F: GACAACAGCTACAGGGCACA R: GACCTGGCTCTGGGTGATAG	60	$(ATC)_{12}$	239–521	FAM
Hbl668	F: AATGAAACCAGCTCATTGCC R: CAAGTCCTTCCAAATGGTCC	58	$(TG)_{14}$	171–189	ROX
BLT101	F: TGTAATGTCACACACGCACG R: CCAGACCAGAGGGACTTCAA	60	$(CA)_{13}$	171–189	HEX
Hbl116	F: TGAACAGACACTCACACAGGC R: GTGTTTCAGCTGCTGCGTT	59	$(TG)_{11}$	160–170	HEX

1 U *Taq* DNA polymerase, and 50 ng genomic DNA. The PCR program involved 5 min at 95°C, followed by 30 cycles of 30 s at 94°C, 30 s at optimized annealing temperature (Table 1), 30 s at 72°C, and a final extension period at 72°C for 8 min.

Computer simulation analyses

A computer simulation was carried out to examine the feasibility of parentage analysis and to estimate the number of loci required. In the simulation, we assumed that markers were in linkage equilibrium, and there were no mutations or transmission errors between parents and progeny. Computer simulations were conducted using the CERVUS 3.0 program (Kalinowski et al. 2007). The term Excl 1 probability represented the power of each locus, and the combined probability over loci to exclude a false candidate parent from a family when the genotypes of the offspring were unknown, while the Excl 2 probability was used when the genotype of 1 parent was known. For these simulations, it was assumed that the proposed breeding program consisted of mating 10 dams to any 1 of 50 candidate sires.

Parentage identification with known parental and filial information

In order to evaluate the reliability of the 8 microsatellite markers in a real breeding program, an experiment was conducted in which both parental and filial information was known. The most likely candidate parent had the highest log-of-odds score. The parameters for this simulation were 10 000 replication cycles and a pool of 40 candidate parents that were genotyped. In order to determine the number of mismatches between known mothers and their offspring, an initial computer simulation was performed with the typing error rate set at 1%.

RESULTS

Computed simulation

CERVUS computer simulations revealed that exclusion probabilities per locus ranged from 0.24 to 0.48 when no parental information was known (Excl 1), and from 0.26 to 0.52 when information for 1 parent was known (Excl 2). Combined probabilities of exclusion in the hypothetical population calculated for 8 polymorphic loci are shown in Fig. 1. Computer simulations demonstrated that at least 6 loci were required to assign 95% of progeny to both parents and 8 loci to assign 98% of progeny to both parents with acceptable confidence (greater than 95% of trials).

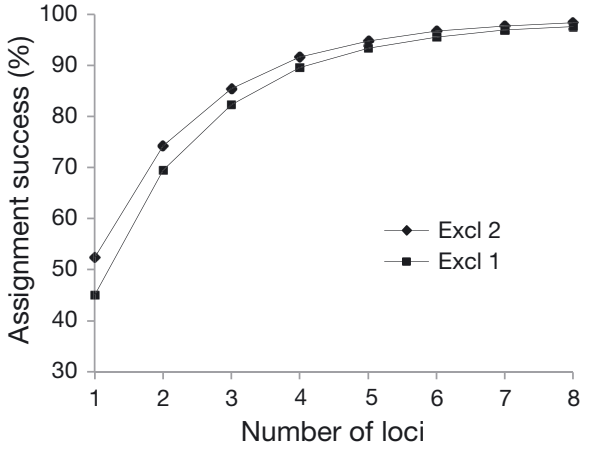

Fig. 1. Combined probabilities of exclusion derived from simulation studies and calculated over 8 selected polymorphic loci in *Brachymystax lenok tsinlingensis*, analyzed when no parental information was known (Excl 1) and when information for 1 parent was known (Excl 2)

Parentage identification with known parental and filial information

The number of alleles detected from these 8 microsatellite loci ranged from 4 to 7 (Table 2). Mean values of expected heterozygosity and polymorphic information content were 0.69 and 0.61, respectively.

Table 2. Probabilities of exclusion based either on the genotype of no known parentage (Excl 1) or when 1 parent was known (Excl 2) for 172 offspring of *Brachymystax lenok tsinlingensis* at 8 microsatellite loci. *k*: number of alleles; H_e: expected heterozygosity; H_o: observed heterozygosity; PIC: polymorphic information content; HW: Hardy-Weinberg equilibrium

Locus	*k*	H_e	H_o	PIC	Excl 1	Excl 2	HW	Null frequency
BLT23	5	0.710	0.689	0.702	0.422	0.437	0.058	+0.000
BLT31	7	0.783	0.774	0.725	0.451	0.524	0.115	+0.017
BLT21	5	0.767	0.715	0.712	0.444	0.459	0.256	−0.006
BLT5	6	0.772	0.784	0.720	0.412	0.427	0.564	+0.000
BLT10	4	0.562	0.523	0.515	0.325	0.365	0.652	+0.000
Hbl101	5	0.687	0.664	0.642	0.366	0.378	0.254	−0.009
Hbl668	4	0.539	0.492	0.458	0.312	0.325	0.024	+0.054
Hbl116	6	0.679	0.645	0.613	0.236	0.259	0.231	+0.000
Average	5.25	0.687	0.661	0.636	0.371	0.397		

Comparing mating design records and parentage assignment data determined by the 8 microsatellite markers, 96.5% and 93.6% of individuals were exclusively assigned to their true dam and sire, respectively. All but 15 of 172 offspring were exclusively assigned to their parent pair (assignment success rate was 91.3%).

DISCUSSION

Many factors, including the number of microsatellite loci, their levels of polymorphism, and the number of potential pairings, affect the precision of assignment of an individual to a parental pair (Norris et al. 2000, Matson et al. 2008). In this study, the mean number of alleles was smaller than that reported for other fishes (Munkres et al. 2007, Li et al. 2010, López et al. 2012, Luo et al. 2014a). As a consequence, in our simulations, at least 6 microsatellite markers were needed to achieve 95% assignment success when there was information available regarding the parents. One possible explanation for this could be that a gradual reduction of genetic diversity has occurred in this species owing to inbreeding caused by several generations of domestication and artificial breeding (Estoup et al. 1998, Wang et al. 2015).

While previous computer simulations have provided important knowledge with regard to key parameters affecting parentage identification (Bernatchez & Duchesne 2000, Norris et al. 2000), detailed experimental evaluation is still needed for individual species. In our study, 212 individuals (172 offspring and 40 parents) were genotyped for 8 selected microsatellite loci. These data demonstrated that this approach is a reliable method for parentage assignment and determining genealogical relationships in *Brachymystax lenok tsinlingensis*.

The real assignment success of the selected microsatellite markers to correctly assign parentage was lower than that obtained by computer simulations. Literature suggests that the presence of null alleles may represent a key source of errors in parentage assignment (Marshall et al. 1998, Li et al. 2010, Luo et al. 2014a). Four of the 8 selected loci presented null alleles, and the null allele frequency was 5.43% for locus Hbl668 (Table 2). Marshall et al. (1998) considered that pedigree determination was likely to be compromised if null allele frequencies exceeded 5%. Thus, it was considered that this may have exerted at least some influence upon the accuracy of the assignments. Another reason for the lower assignment success observed in the real scenarios rather than the computer simulations may have been due to the existence of genotyping errors relating to allele mismatches at 1 or 2 loci (BLT31, Hbl668, or Hbl101). This accounted for an average of 8.33% at the 3 loci, a value higher than that reported for most other species of fish (Hoffman & Amos 2005, Castro et al. 2007, Luo et al. 2014a). Thus, when choosing markers for parentage assignment, besides polymorphism and null allele frequency, the quality of the allelic pattern should also be taken into consideration since loci with a high number of stutter bands (or shadow bands) are often associated with high typing error rates (Estoup et al. 1998, López et al. 2012).

In conclusion, the microsatellite-based parentage assignment approach established here can replace physical tagging in order to obtain information regarding genealogical relationships among individuals over multiple generations, and thus to avoid inbreeding in stocks of *B. lenok tsinlingensis*. Consequently, this method represents a powerful tool for managing breeding programs and conserving the genetic resources of this endangered fish in the future.

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