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Analysis of genetic variation in the Chinese sturgeon, *Acipenser sinensis*: estimating the contribution of artificially produced larvae in a wild population

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Summary

Twenty-five sets of microsatellite primers developed from lake sturgeon (*Acipenser fulvescens*) and shovelnose sturgeon (*Scaphirhynchus platorynchus*) genomic DNA were tested on Chinese sturgeon, *Acipenser sinensis*. Ten sets of primers successfully produced resolvable amplicons, and four of these sets (Afu-39, Afu-54, Afu-68, and Spl-168) were used to analyze genetic variation in mature adults, juveniles, and a single family of Chinese sturgeon from the Yangtze River. Offspring from the single family were stocked prior to the juvenile sample being taken. Results from genotyping parents and offspring indicated that all four loci appear to be tetrasomic and all alleles appear to segregate among the offspring in a 1 : 1 (presence : absence) ratio. Neighbor-joining based on band-sharing in coupling with parentage analysis revealed that hatchery propagated individuals may comprise 5–10% of the juvenile population from a stocking of 30 000–60 000 larvae.

Introduction

The Chinese sturgeon, *Acipenser sinensis*, is anadromous, living mainly in the continental shelf of the Yellow Sea and the East China Sea and spawning in the upper Yangtze and Pearl River. After completion of the Gezhouba dam in 1981 in the Yichang section of the Yangtze, spawning of *A. sinensis* has been restricted to the middle and lower reaches of the Yangtze River. Although a new spawning ground has developed just below the dam (Hu et al., 1983, 1985), it is not large enough to accommodate all mature individuals (Kynard et al., 1992; Chang, 1999). Since 1984, hatchery larvae and juveniles have been released annually into the river for stock restoration (Fu et al., 1985; Xiao et al., 1999). Few studies have addressed the effects of stocking on recruitment and genetic diversity of this population. Moreover, late age to maturation (8 years to more than 26 years), extended intervals between spawning cycles (possibly more than 3 years), and the deep-water habitat of the Yangtze River make investigation of behavioral ecology, genetics, and population biology difficult (Deng et al., 1985; Wei et al., 1997; Chang, 1999).

Genetic research on *A. sinensis* has been limited to a few studies on allozymes (Zhang 1998), chromosomal numbers (Yu et al., 1989; Zhang et al., 1999a), mitochondrial DNA (Zhang, 1998; Zhang et al., 1999b) and randomly amplified polymorphic DNA (RAPD) (Zhang, 1998). However, low levels of genetic variation observed for these markers have proved non-informative.

Studies in a number of organisms have shown that variable number tandem repeat (VNTR) loci with simple sequence repeat (SSR) motifs, microsatellite loci, can offer high levels of allelic variation per locus (O'Connell and Wright, 1997). Microsatellite loci are abundant, distributed throughout the eukaryotic genome, and amplify easily by polymerase chain reaction (PCR) with length no larger than 300 base pairs (Tautz and Renz, 1984). Additionally, because microsatellite analysis is PCR based, non-lethal sampling of fin, blood, and skin can be employed. Also, primers designed for one species can often be used with other related species. For these reasons, these highly polymorphic markers are increasingly being used in different types of genetic studies, such as parentage analysis (Morin et al., 1994; Estoup et al., 1995; Colbourne et al., 1996), stock identification (Estoup et al., 1993; Norris et al., 1999; Brooker et al., 2000), and genome mapping (Dietrich et al., 1992; Jackson, 1995; Lee and Kocher, 1996).

In order to evaluate the impact of releasing hatchery-reared Chinese sturgeon larvae in the Yangtze River, microsatellite primers developed for lake sturgeon (May et al., 1997; Pyatskowit et al., 2001) and shovelnose sturgeon (McQuown et al., 2000) were used in preliminary studies on genetic variation and parentage analysis of *A. sinensis*.

Materials and methods

Samples

Twenty mature individuals of *A. sinensis* from 298 to 370 cm in total length (TL) were caught from the spawning ground below the Gezhouba Dam in autumn 1999 by the Institute of Chinese Sturgeon (ICS). Two of these individuals were crossed and 30 000 of their offspring with an average TL of 10 cm were released into the river on December 28, 1999. Another 30 000 offspring from an unknown number of parents were also released at the same time by the Yangtze River Fisheries Institute (YRFI). Forty juveniles, from 18.0 to 36.3 cm in TL, were collected and sampled in the estuary of the Yangtze River from April to June of 2000. In addition, 15 artificially propagated larvae and their parents were sampled in the ICS hatchery near the end of 1999. Portions of fins, ranging in size from 1 to 2 cm^2, of both adults and juveniles, as well as whole bodies of newly hatched larvae were sampled and stored in 100% ethanol and refrigerated at 4°C.

Amplification of microsatellite loci

Genomic DNA was extracted with a modified Cetyltrimethylammonium bromide (CTAB) protocol (Saghai-Maroof et al., 1984). Twenty-five sets of microsatellite primers were tested on randomly selected individuals of *A. sinensis*. Eleven sets were

developed for lake sturgeon (Afu-19, 22, 23, 34, 39, 54, 57, 58, 62, 68, and 69; May et al., 1997) and 14 were developed for shovelnose sturgeon (Spl-101, 102, 104, 105, 106, 113, 117, 123, 163, 168, 169, 170α, 173, and 176; McQuown et al., 2000). Amplifications were performed in a PE 2400 GeneAmp PCR System using a 20-μl reaction mixture. Each reaction mixture contained 1 unit of *Taq* DNA polymerase (Biostar), 0.6 μM of each primer, 10–15 ng template DNA, 150–175 μM dNTPs, 1.5 mM $MgCl_2$ and 2-μl of reaction buffer. Cycling conditions were as follows: denaturation at 94°C for 4 min; 35 cycles of 1 min at 94°C, 50 s at 58°C, and 50 s at 72°C; and a final extension at 72°C for 6 min. Following amplification, 10 μl of PCR product was mixed with 2 μl of loading dye and electrophoresed in a 4% Metaphor agarose gel (FMC Bioproducts) at 300 V for 2.5 h. After an initial 10 min loading run at 15°C, buffer (0.5 X TBE) temperature was maintained at no higher than 25°C with a coolant pump. Allele sizes were estimated in relation to the pBR322 /Msp I ladder (Promega). After electrophoresis, gels were stained with ethidium bromide and then photographed with an Ultraviolet Gel Document System (Biolab).

Statistical analysis

We followed the analytical approach of Samadi et al. (1999) where the similarity at each locus between two individuals was estimated by $S_{xy} = 2n_{xy}/(n_x + n_y)$ and n_{xy} is the number of shared bands between individuals x and y, and n_x and n_y are the number of bands in individuals x and y, respectively (Lynch, 1990). A genetic distance between two individuals was thus defined as $D_{xy} = 1 - S_{xy}$. An overall genetic distance between two individuals was estimated as the mean of D_{xy} over all loci.

The software RAPDPLOT (Black, 1998) was used to calculate the genetic distance between all pairs of individuals. The resulting genetic distance matrices were then used to build a phylogenetic tree using the neighbor-joining method (Saitou and Nei, 1987) as implemented by the software package PHYLIP version 3.5c (Felsenstein, 1994). Finally, TREEVIEW 1.5 (Page, 1996) was used to illustrate the tree.

Results

Applicability of primers

Among a total of 25 sets of microsatellite primers tested, ten sets reproducibly amplified Chinese sturgeon DNA. Five of the ten amplified microsatellites were developed from lake sturgeon (Afu-19, Afu-39, Afu-54, Afu-57, Afu-68), while the others were developed from shovelnose sturgeon (Spl-106, Spl-163, Spl-168, Spl-169, Spl-170α). While the five primers from lake sturgeon successfully amplified DNA of *A. sinensis*, only four loci were polymorphic with 4–11 alleles per locus. Afu-19 was monomorphic. All five shovelnose primers revealed polymorphic loci (PIC) in *A. sinensis*, with 5–10 alleles per locus. The amplified products of Spl-106, Spl-163 and Spl-170α showed stutter bands, and Afu-57 and Spl-169 displayed difficulties in replication.

Four microsatellites (Afu-39, Afu-54, Afu-68, Spl-168; Table 1) were chosen for use in further studies because of their high polymorphism, discrete bands, and reproducibility of amplicons within individuals and from parents to offspring. For each of these loci, more than two bands per locus with asymmetry in band intensities was usually observed, suggestive of four gene doses per locus (see Pyatskowit et al., 2001; McQuown et al., 2002). Repeated PCR assays and analysis of the fidelity of allelic transmission across generations confirmed the reproducibility of these patterns. With this suite of four loci, all 20 mature individuals sampled in 1999 could be discriminated from each other. The four microsatellites were then used to test for transmission of alleles from parents to offspring. All alleles were transmitted in a 1 : 1 (presence : absence) among the progeny.

Genetic variation

The level of genetic variation present in *A. sinensis* was estimated by the number of alleles and the D_{xy} distances between individuals. A total of 29 and 30 alleles were found among the mature individuals and the juvenile group, respectively, while only 15 total alleles were found in the parents and their offspring in the one family produced and stocked. The pairwise D_{xy} distances of the 20 mature individuals sampled from the section below the dam in 1999 ranged from 0.13 to 0.52 with a mean of 0.28, while the pairwise D_{xy} distances of the 40 juveniles collected in the estuary of the Yangtze River in 2000 ranged from 0.06 to 0.58 with a mean of 0.32. The 15 larvae and their two parents had pairwise D_{xy} distances ranging from 0.03 to 0.22 with a mean of 0.12.

Parentage analysis and effect of artificial releasing

The results of the neighbor-joining analysis of eight mature adults, 15 artificial offspring from Par02 (male) and Par10 (female), and 40 juveniles collected in the estuary of the Yangtze River in 2000 are shown in Fig. 1. All members of the test family appeared in a single cluster, along with two wild-caught juveniles (Juv23, Juv88). Comparison of the genotypes of the two juveniles with the genotypes of their parents revealed that all alleles in the two juveniles were found in at least one of the two parents (Fig. 2). These results suggest that

Table 1
Characteristics of the four microsatellite loci used in this study of Chinese sturgeon

Locus	GenBank Accession	Repeat motif in original clone	Allele size range (bp)	Total number of alleles	Alleles in parents	Alleles in juveniles	Alleles in offspring
Afu-39	U72734	$(GTT)_{10}$	116–137	4	3	4	2
Afu-54	U72735	$(GATA)_6$ $(GACA)_7$	160–204	5	5	4	2
Afu-68	U72739	$(GATA)_{13}$	110–180	12	11	12	4
Spl-168	AF276210	$(TATC)_{18}$	147–238	10	10	10	7
			Total alleles	31	29	30	15

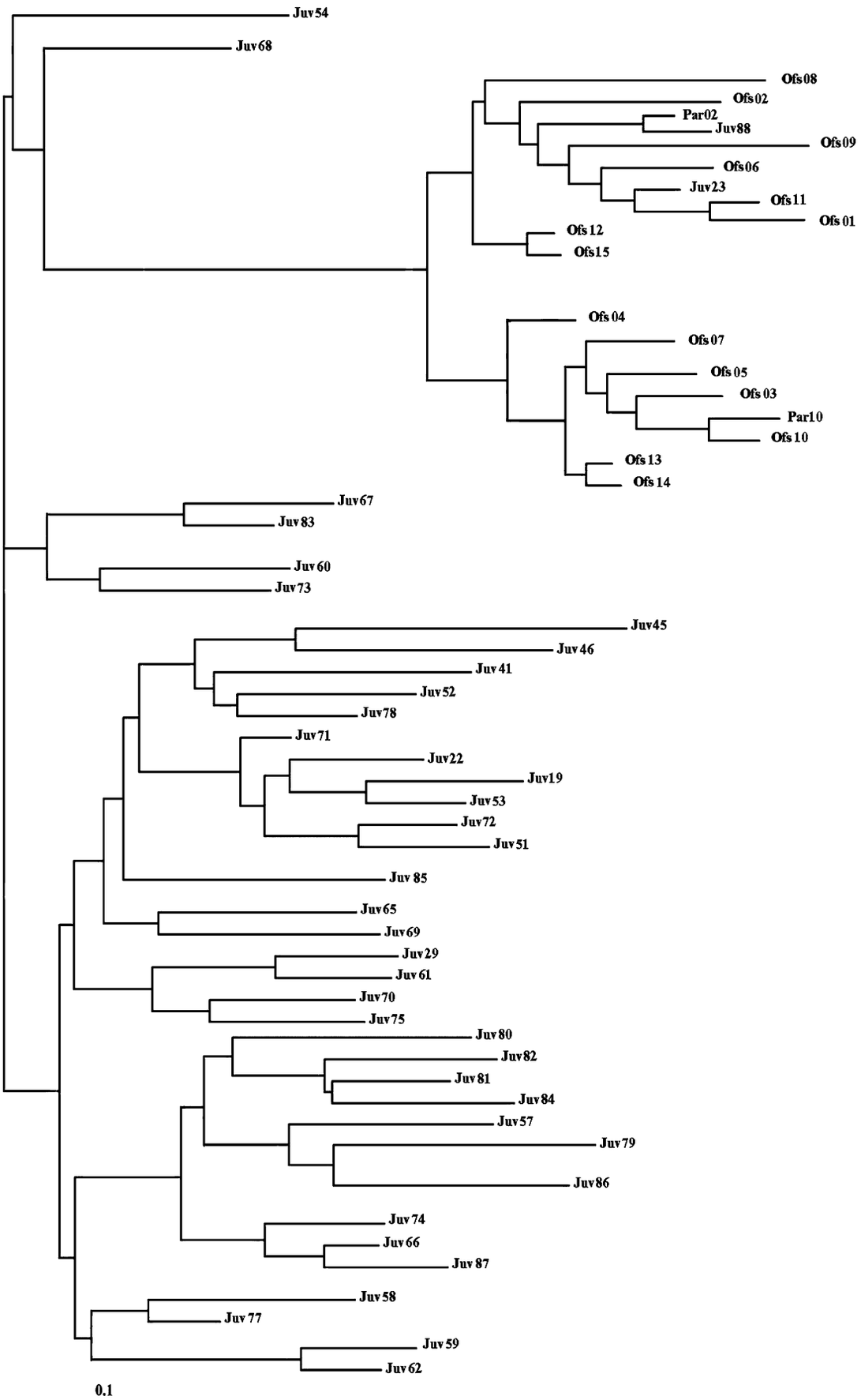

Fig. 1. Neighbor-joining phylogenetic tree based on pairwise D_{xy} distance derived from banding similarities for 40 juveniles (Juv) collected in the estuary of the river in 2000, eight spawners (Par) and 15 offspring (Ofs) of Par02 (male) and Par10 (female) sampled at ICS in 1999

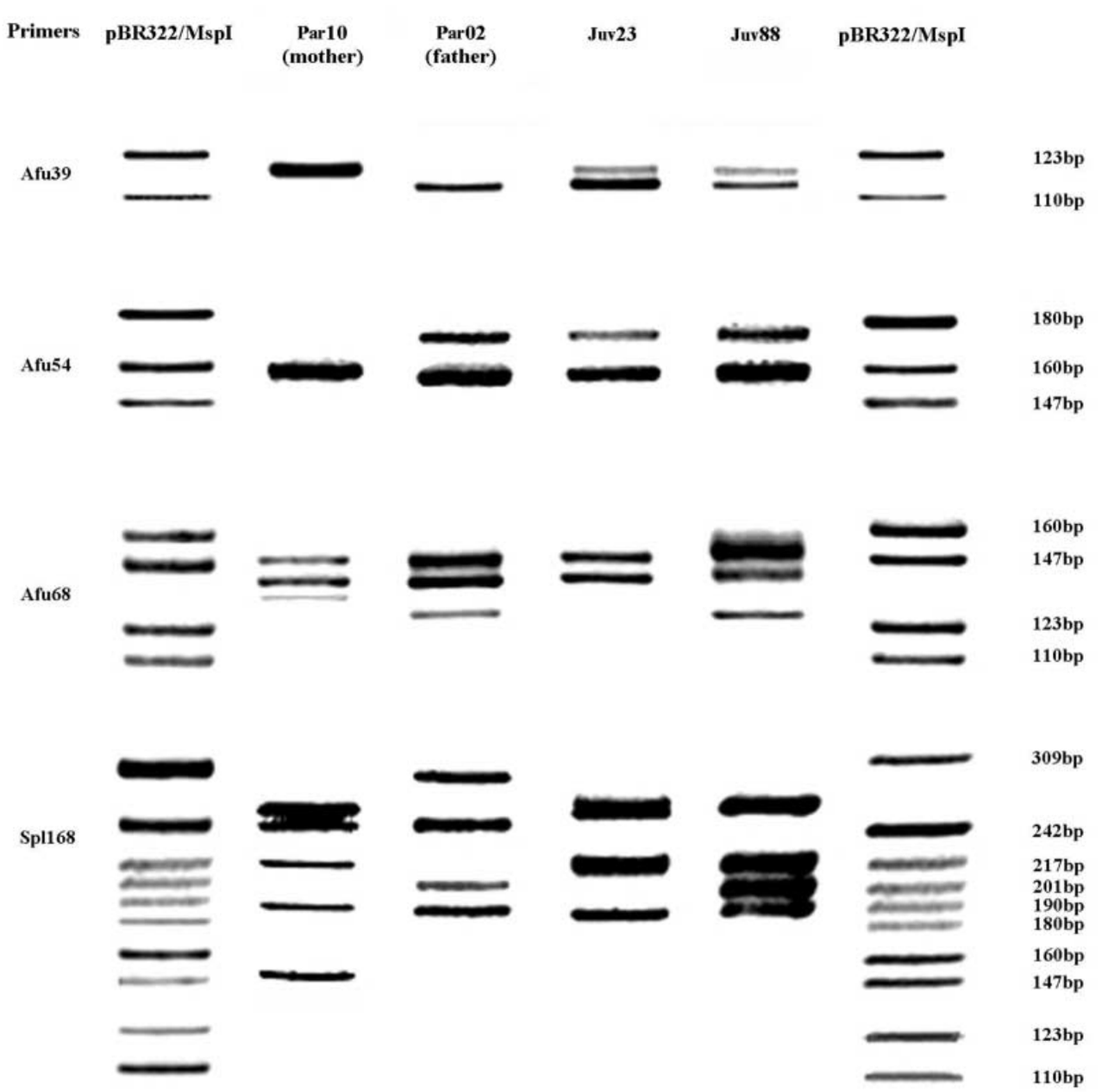

Fig. 2. Electrophoretic phenotypes of the two wild-caught juveniles (Juv23 and Juv88) found to associate genetically with the offspring of the cross made by ICS and their possible parents (Par02 and Par10) for the four microsatellite loci. The sized ladder pBR322/MspI is run on either side of the gel

the two individuals (5% of the total) were artificially propagated offspring from the single cross produced by ICS that had been stocked in late 1999.

Discussion

Of the 25 sets of microsatellite primers developed from lake sturgeon and shovelnose sturgeon, ten produced replicable amplicons in *A. sinensis*. These results suggest that there is evolutionary conservation of the flanking regions for these loci among related taxa. The cross-amplification between lake sturgeon, shovelnose sturgeon and *A. sinensis* is consistent with earlier findings that primers developed in one species often work in other related species (May et al., 1997; Davis and Strobeck, 1998; Smith et al., 1998).

Most individuals for the four loci we used exhibited banding patterns with more than two bands and asymmetry in band intensities (e.g. 3 : 1, 2 : 1 : 1, and 1 : 1 : 1 : 1). The multiple banding patterns observed at each locus are consistent with tetrasomy in *A. sinensis*, as has been shown for *A. fulvescens* (Pyatskowit et al., 2001; McQuown et al., 2002). Polyploidization may also play a role in the failure in amplification, as competitive hybridization with the non-homologous (homeologous) sets of chromosomes may lead to variable amplification and hence problems in replication within individuals or from parents to offspring.

The tetraploid derivative nature of the *A. sinensis* genome has important consequences for the study of population structure and parentage analysis. First, it is difficult to determine whether specific bands represent one, or more,

copies of the relevant allele, especially in individuals with one or two bands. Secondly, it is difficult to test for the occurrence of null alleles in *A. sinensis* from population data because it is difficult to determine the difference between 2 : 1 and 3 : 1 intensity ratios in two banded individuals. Thirdly, observed vs expected heterozygosity values are not possible to calculate, limiting the types of analyses can be performed (see also Samadi et al., 1999).

The population of *A. sinensis* in the Yangtze River has been reported as having low heterozygosity and a low percentage of PIC in a number of studies when examined using allozymes and RAPDs (Zhang, 1998). It has been suggested that there is little genetic diversity between spawning stock and juvenile stock. However, our results show that the mature individuals and the juveniles (primarily naturally produced) have the same number of alleles, suggesting the population has not lost very much genetic variation to date. This can be contrasted with the results of Norris et al. (1999) where they demonstrated that there was a considerable loss of rare alleles in a farmed Atlantic salmon (*Salmo salar*) population, while there was no significant difference in overall heterozygosity between farmed and wild strains.

With these highly polymorphic genetic markers, it is possible to distinguish the artificially and naturally propagated individuals among juvenile samples of Chinese sturgeon in the estuary of the Yangtze River. The parentage analysis revealed a low proportion (5%) of artificially propagated individuals among juveniles in the estuary of the Yangtze River. It should be noted that an equal number of artificially produced larvae from other crosses at the YRFI were also released at the same time (30 000 larvae from ICS and 30 000 larvae from YRFI). This would suggest that our two juveniles should be equaled by another two artificially produced larvae among the remaining 38 juveniles. As such, our best estimate would be a 10% representation of artificially produced fish among the juveniles that were sampled. The present rate of release of artificially produced larvae may not be adequate to maintain the survival of this population of *A. sinensis*, as the natural spawning area has been decreased. However, artificial breeding practices may inadvertently decrease the genetic variation of the *A. sinensis* population by breeding related individuals or by the use of small numbers of parents as broodstock. The high levels of variation in microsatellite loci make them very useful for the estimation of relatedness between potential breeding pairs, and more intensive breeding regimes based on these polymorphic markers should be employed to avoid loss of genetic variation within this population.

In conclusion, this study demonstrates the high levels of polymorphism in *A. sinensis* detectable with microsatellite primers developed from lake sturgeon and shovelnose sturgeon. Parentage analysis revealed a detectable proportion (5–10%) of artificially propagated individuals in the natural population of juveniles. Because of their codominant expression, high genetic variation and mutation rate, and the ability to use nonlethal sampling, microsatellite loci have the potential to be of great use in monitoring changes in genetic variation, parentage assignment and investigation of population structure, especially in threatened and endangered species.

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