Environ Biol Fish (2006) 77:79–86
DOI 10.1007/s10641-006-9056-x

ORIGINAL PAPER

Assessing genetic diversity of wild populations of Prenant's schizothoracin, *Schizothorax prenanti*, using AFLP markers

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Received: 31 October 2005 / Accepted: 3 April 2006 / Published online: 5 July 2006

Abstract Prenant's schizothoracin, *Schizothorax prenanti*, an endemic fish to China, has undergone a dramatic decline in numbers due to human impacts. We studied its genetic diversity in three tributaries of the Yangtze River: the Qingyi River, which has many hydropower dams, and the Dadu River and Muli River where many hydropower dams are being proposed. Using amplified fragment length polymorphism (AFLP), 621 loci were amplified with seven AFLP primer combinations in 45 individuals. The loci were highly polymorphic and heterozygous (87% polymorphism, 30% heterozygosity). The genetic distances within populations were large. The analysis of molecular variance demonstrated that most variation occurred within populations. The estimated fixation index (Φ_{st}) value averaged over all polymorphic loci across the three rivers was 0.0837, indicating a moderate genetic differentiation. The differentiations between populations were significant, and population structure was strong. The results suggested that China had wild populations of Prenant's schizothoracin with considerable genetic diversity in the Muli, Dadu and Qingyi rivers. The proposals to dam these rivers should take into account the importance of conserving their genetic quality.

Keywords Prenant's schizothoracin · Genetic diversity · AFLP · Upper reaches of the Yangtze River

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Introduction

Wild species must have available a pool of genetic diversity if they are to survive environmental pressures exceeding the limits of developmental plasticity. If this is not the case, extinction would appear inevitably (Frankel 1983). It is also crucial for endangered species to retain as much genetic variation as possible to enhance the chance for their recovery (Hedrick et al. 2000). The conservation of genetic diversity is important for the long-term interest of any species (Hamrick et al. 1991). Molecular markers are useful tools in the assessment of genetic diversity (Powell et al. 1996). Amplified fragment length polymorphism (AFLP) (Vos et al. 1995) depends on the reliability of RFLP and the high efficiency of PCR to amplify the digested genome DNA segment selectively, and is highly reliable for the assessment of genetic variation among and within

populations (Keiper and McConchie 2000). Compared with RAPD technique, AFLP has more stability and amplifies more loci so can be applied widely. Yue et al. (2002) reported AFLPs had higher power than RAPD for the detection of genetic diversity in populations of Asian arowana, *Scleropages formosus*. Mickett et al. (2003) argued that AFLP could resolve more loci and detect greater levels of polymorphism than isozyme analysis in channel catfish, *Ictalurus punctatus*. Some researchers have simplified, optimized and improved the AFLP protocol so that it was easily performed and had higher resolution to polymorphic loci (Habu et al. 1997, Suazo and Hall 1999).

Prenant's schizothoracin, *Schizothorax prenanti* (Tchang), is an important commercial fish distributed in upper reaches of the Yangtze River and its tributaries: lower reaches of the Jinsha River, Dadu River, Minjiang River, Wujiang River, Yalong River, Youshui River, and upper reaches of the Renhe River (Ding 1994). This species is very reputed in China because of its good taste, nutritional value and use in aquaculture. Due to over-fishing, water pollution, and construction of hydropower stations, natural resources of the fish have declined dramatically, and the size of individuals harvested is gradually becoming smaller. Therefore, investigations of the wild resources and genetic diversity, technologies of artificial propagation, and strategies of conserving and restoring the wild populations should be of top priority.

Former studies on Prenant's schizothoracin primarily focused on artificial propagation and breeding techniques, disease control, nutrient content analysis, and micro-examination of organs and histology. In terms of genetics, only Yu et al. (1987) have investigated the chromosomes of this species. The genetic diversity of this species is still unknown. The Yalong River, Dadu River and Qingyi River are the main areas inhabited by this species. In the Qingyi River many hydropower dams have been constructed, and in the Dadu River and Muli River where many hydropower dams are being proposed. We analyzed the genetic variation of Prenant's schizothoracin from these three rivers using the AFLP markers to determine how human impacts have affected the genetic diversity and quality of this species. It will provide reliable information for the protection and restoration of the wild resources, and will help in the selection of high quality individuals for artificial reproduction.

Materials and methods

Sample collecting

From June 2003 to June 2004, Prenant's schizothoracins were collected from Muli region of the Muli River (one branch of the Yalong River), Ebian and Shimian regions of the Dadu River, Duoying region of the Qingyi River, East River and West River (branches of the Baoxing River located in upper reaches of the Qingyi River) (Fig. 1). These three wild populations were named Muli population, Dadu population and Qingyi population in this study. All fish were stored in anhydrous ethanol immediately after capture.

DNA isolation

Twenty-two individuals from Qingyi population, thirteen from Dadu population and ten from Muli population were used in this study. About 100 mg of tissue from fins or muscle were sampled from each fish and DNA was isolated with Sambrook's method with some modifications (Sambrook et al. 1989). 0.8% agarose electrophoresis and a spectrophotometer were used to test the quality of the genome DNA.

AFLP analysis

AFLP was performed as described by Zabeau and Vos (1993) with some modifications. Eight primer combinations were used: E-AAC/M-CAC, E-AAC/M-CAG, E-AAC/M-CTA, E-AAC/M-CTC, E-AAG/M-CAG, E-AAG/M-CTG, E-ACT/M-CAA and E-AGG/M-CAA. AFLP products were analyzed on Sequi-Gen GT Nucleic Acid sequencer (BIO-RAD). The method of silver staining followed that of Sanguinetti et al. (1994) with some modifications. Gels were fixed for 10 min with a mixed solution of 10% ethanol and 0.5% acetic acid, and then stained for 30 min with

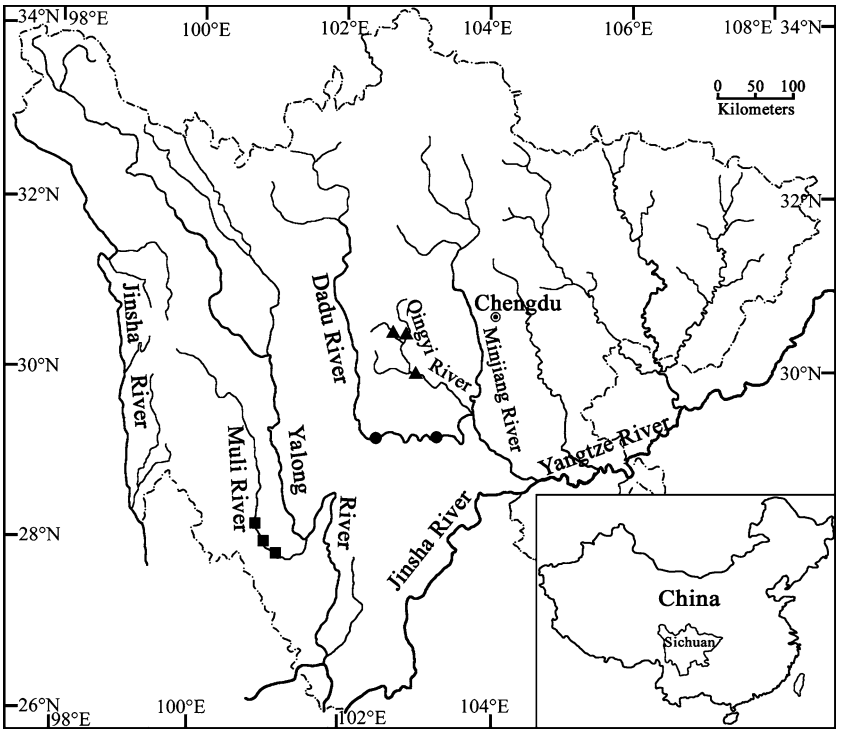

Fig. 1 Collecting locations of the three populations of *Schizothorax prenanti*
■ Muli population;
• Dadu population;
▲ Qingyi population

0.2 g/ml $AgNO_3$ solution. After being washed with distilled water the gels were developed in 1.75 g/ml NaOH solution with 0.4% formaldehyde until the bands noticeably appeared. Finally, the gels were washed with distilled water and air dried.

Data analysis

For the AFLP markers, bands were scored as 1 if present or 0 if absent, and the data were transferred to a binary (1/0) data matrix. TFPGA 1.3[1] was used to calculate the polymorphic loci, genetic distances, and average heterozygosities. The percentages of polymorphic loci were estimated based on the percent of loci not fixed for one allele. Genetic distances between populations were calculated by Nei (1978) unbiased distance and identity measures. Average heterozygosity estimates were calculated for each locus and then averaged over loci. Statistica 6.0[2] was used to test the difference in intrapopulation genetic distance and average heterozygosity between the populations. A dendrogram of the three populations was constructed based on UPGMA (unweighted pair-group method with arithmetic means) in TFPGA 1.3[1]. Population structure was evaluated using the analysis of molecular variance model (AMOVA) (Excoffier et al. 1992) in Arlequin version 3.01 program package (Excoffier et al. 2005). The overall molecular variance was partitioned into components corresponding to the divergence within and among populations. The fixation indices (Φ_{st}), analogue to the F_{st} of genetic variation, were calculated to assess the genetic divergence overall and between paired populations. The statistical significance of the total and pairwise fixation indices was estimated by comparing the observed distribution generated by 10,000 permutations. The gene flow estimates were derived using the equation $N_m = [(1/F_{st}) - 1]/4$.

[1] Miller, M.P. 1997. Tools for population genetic analysis (TFPGA) 1.3: A window program for the analysis of allozyme and population genetic data. Computer software distributed by the author.

[2] StatSoft Inc. 2001. STATISTICA (data analysis software system), version 6. www.statsoft.com.

Results

AFLP polymorphism

Selectively amplified results of seven primer combinations were analyzed except E-AAC/M-CAC due to its bad amplification effect. A total of 621 bands were identified for 45 individuals, and the average was 88.7 for each primer combination. The amplified bands of each primer combination were very abundant except the result of primer combination E-AAC/M-CAG (57 bands). The result of primer combination E-ACT/M-CAA (117 bands) was the largest (Fig. 2). Of the 621 loci, 87.12% (541 loci) were polymorphic, and the average was 77.3 loci for each primer combination.

The percentage of polymorphic loci was very high across populations. The population with the greatest percent polymorphism (77.13%) was the Dadu population, while percent polymorphism of the Qingyi population (74.72%) and Muli population (73.91%) were about equal (Table 1).

Genetic distance and heterozygosity

The average genetic distance between the Muli and Qingyi populations (0.0826) was the highest, followed by the distance between the Muli and Dadu populations (0.0516). The average genetic distance between the Qingyi and Dadu populations (0.0298) was the lowest.

The genetic distance among individuals within populations was 0.2048–0.5314 across all seven primer combinations, and the average was 0.3341 ± 0.0037 in Qingyi, 0.3764 ± 0.0123 in Muli, and 0.3725 ± 0.0075 in Dadu, respectively

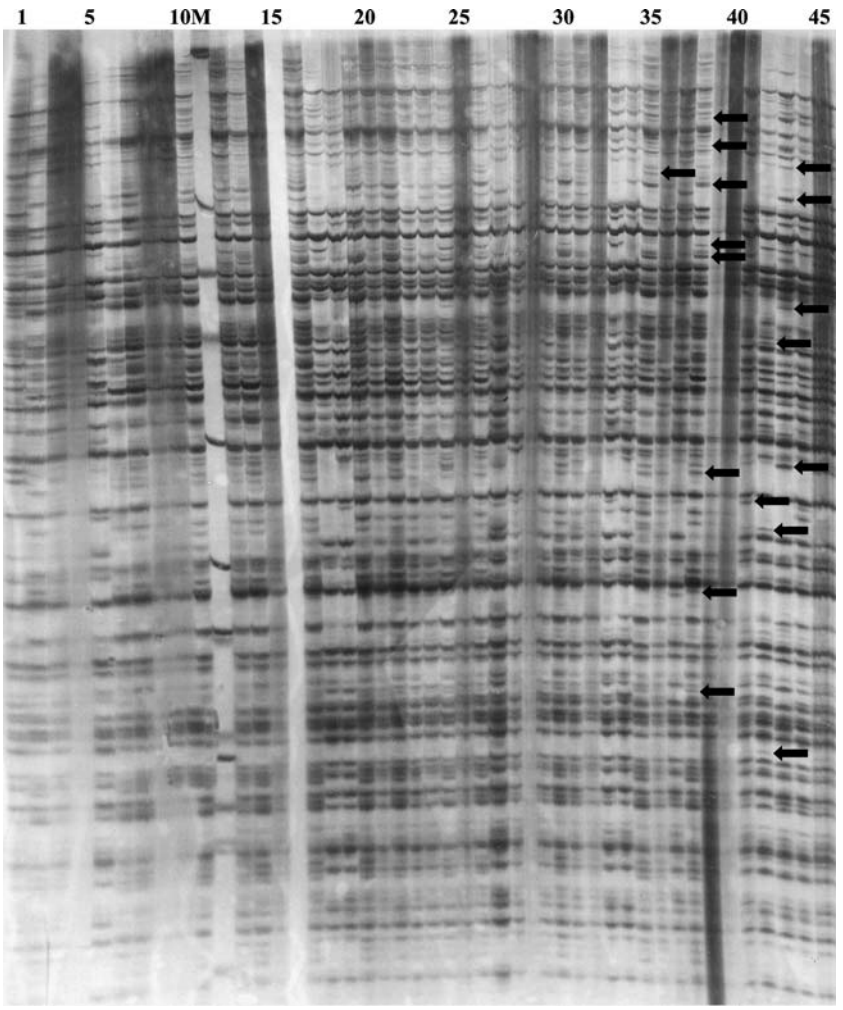

Fig. 2 Photograph of silver-stained gel showing amplified fragment length polymorphism with primer combination E-ACT/M-CAA. From the left to the right: Lane 1–3, 16–25 (Dadu population); Lane 4–10, 12–14 (Muli population); Lane 26–45 (Qingyi population); M (marker). Some highly polymorphic loci are indicated by arrows

Table 1 Percentage of polymorphic loci, average genetic distance within population and heterozygosity of the three populations of *Schizothorax prenanti*

Population	Percentage of polymorphic loci	Genetic distance within population (mean ± SE)	Heterozygosity (mean ± SE)	Sample number
Qingyi	74.72	0.3341 ± 0.0037	0.2558 ± 0.0076	22
Muli	73.91	0.3764 ± 0.0123	0.2645 ± 0.0078	10
Dadu	77.13	0.3725 ± 0.0075	0.2816 ± 0.0077	13
Total	87.12	0.3497 ± 0.0036	0.3016 ± 0.0045	45

(Table 1). The intrapopulation distance of Qingyi was lower than that of the other two populations (Newman–Keuls Test, $P < 0.001$). There was no significant difference of intrapopulation distance between Muli and Dadu populations (Newman–Keuls Test, $P = 0.7432$).

The average heterozygosity of all populations was 0.3016 ± 0.0045, with Qingyi, Muli and Dadu populations having values of 0.2558 ± 0.0076, 0.2645 ± 0.0078 and 0.2816 ± 0.0077, respectively (Table 1). The population with the greatest heterozygosity was Dadu, followed by Muli. The heterozygosity of Qingyi population was the lowest. The heterozygosity of Qingyi population significantly differed from that of Dadu population (Newman–Keuls Test, $P = 0.0434$). There was no significant difference of heterozygosities between populations Qingyi and Muli (Newman–Keuls Test, $P = 0.4112$), and Muli and Dadu (Newman–Keuls Test, $P = 0.1148$).

Population structure

The phylogenesis of the three populations was analyzed based on the amplified results across all seven primer combinations. The UPGMA dendrogram showed that the populations collected from the Dadu River (Ebian and Shimian) and Qingyi River (Duoying and Baoxing), which were geographically close and connected, could be clustered together. However, the Muli population, which was located far away from the others, was clustered into a separate branch (Fig. 3).

Analysis of molecular variance (AMOVA) was conducted to describe variance components of Prenant's schizothoracin populations. Most variance was observed to occur within populations

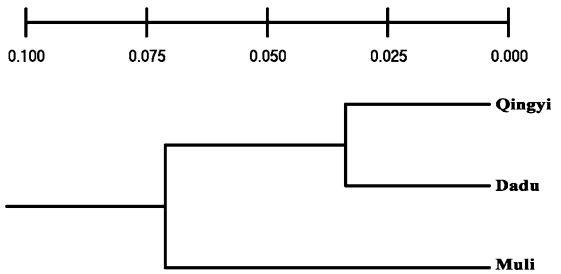

Fig. 3 UPGMA dendrogram of the three populations of *Schizothorax prenanti* based on AFLP markers

(91.63%), whereas variance among populations was only 8.37%.

The analysis of Φ_{st} on the three populations showed that there was significant differentiation among the populations ($\Phi_{st} = 0.0837$, $P < 0.0001$). Pairwise Φ_{st} showed that differentiations between populations Muli and Dadu, Muli and Qingyi, and Dadu and Qingyi were significant ($P < 0.0001$) (Table 2).

The gene flow among populations ranged from a low of 1.61 between Qingyi and Muli populations, and 3.11 between Dadu and Muli populations to a high of 5.14 between Dadu and Qingyi populations (Table 2). It can be seen that the gene interchange between Dadu and Qingyi populations occurred more frequently than among other populations.

Table 2 Fixation index (lower-left) and gene flow (upper-right) among the three populations of *Schizothorax prenanti*

Populations	Qingyi	Muli	Dadu
Qingyi		1.61	5.14
Muli	0.1345		3.11
Dadu	0.0464	0.0743	

Discussion

Genetic diversity among and within wild populations of Prenant's schizothoracin was analyzed using AFLP markers. The percentage of polymorphic loci was 87.12%, with populations from the Dadu River, Muli River, Qingyi River having values of 77.13%, 73.91% and 74.72%, respectively. The average genetic distance was 0.3341–0.3764 within populations, and the average heterozygosity was 0.3016. Compared with species previously reported (Wang et al. 2001, 2002; Zhang and Huang 2004; Zhang et al. 2004), the percentage of AFLP polymorphic loci in the three populations of Prenant's schizothoracin was very high. Compared with species in the same family, our results were very similar to wild *Gymnocypris przewalskii* (Chen et al. 2005), and higher than rock carp, *Procypris rabaudi* (Song et al. 2005). The average intrapopulation genetic distances were much higher than genetic distances among populations, and also higher than intrapopulation genetic distances of others species inferred from AFLP analysis (Wang et al. 2001, 2002; Song et al. 2005). The average heterozygosity of the three populations of Prenant's schizothoracin was higher compared to other species (Mickett et al. 2003; Zhang et al. 2004; Song et al. 2005). It could be concluded that the genetic diversity of these three populations was considerably high. The genetic varieties mainly existed within populations, which was in accordance with the result from AMOVA. Among the three populations, Dadu and Muli had higher intrapopulation genetic distance or heterozygosity than Qingyi. Prenant's schizothoracin in these two rivers had more genetic diversity. The considerable genetic diversity both in Muli and Dadu populations might be due to their remote living locations and lower fishing pressure compared to populations in other rivers. It should be noticed that the genetic diversity is also high in the population from the Qingyi River. It showed that human impacts have not affected the genetic diversity of this population. Yet, the over-fishing, water pollution, or construction of hydropower stations in the rivers has the potential to reduce genetic quality of Prenant's schizothoracin in the future. The proposals to dam these rivers should take into account the importance of conserving their genetic quality.

Mickett et al. (2003) suggested that the estimated F_{st} value (0.4456) of channel catfish indicated a high degree of genetic differentiation, and the 0.1763 indicated a moderate degree of genetic differentiation among populations. Yue et al. (2004) reported a moderate genetic differentiation in Asian arowana with an F_{st} of 0.047. The Φ_{st} value in the three populations of Prenant's schizothoracin was 0.0837, indicating a moderate genetic differentiation according to the results above. AMOVA showed that the differentiation among populations from Muli, Dadu and Qingyi were significant, indicating a strong population structure in the three rivers.

The three populations of Prenant's schizothoracin were collected from lower reaches of the Muli River, Ebian and Shimian regions of the Dadu River, Duoying region of the Qingyi River, and upper reaches of the Baoxing River (belonging to the Qingyi River system), respectively. The Muli population was far from the other two, while Dadu population and Qingyi population were geographically close and connected (Fig. 1). From analysis of the AFLP markers, the genetic distance between populations from Dadu and Qingyi was the lowest, while that between populations from Qingyi and Muli was the greatest. The estimation of gene flow showed that the populations from the Qingyi River and the Muli River seldom had gene flow, while gene flow among the populations from the Dadu River and Qingyi River occurred more frequently. According to UPGAMA analysis, the populations of Dadu River and Qingyi River could join together, while the Muli population was clustered in a separate branch. Therefore, the genetic difference of Prenant's schizothoracin among the different populations directly related to the distance of isolation and the connectivity level of the rivers they inhabited.

It is important that the genetic diversity of Prenant's schizothoracin is analyzed while its wild resources are still relatively abundant to ensure its protection. The results of genetic investigation will also be very useful for the development of artificial propagation and genetic improvement of the fish. Prenant's schizothoracin is a famous commercial fish in China, and is becoming an important cultural species. However, the artificial propagate technique is not successful enough and the propagate

scale is limited. Currently, the parental fish were primarily collected from the Qingyi River during artificial propagation. Our research revealed that wild Prenant's schizothoracin in its main distribution ranges of Qingyi, Dadu and Muli rivers had considerable genetic variety, especially the populations from the Muli and Dadu rivers. Due to declining wild resources, potential threats to the genetic quality of the Qingyi River population, and the high quality of heredity in populations from the Muli and Dadu rivers, parental fish for artificial propagation should be collected not only from the Qingyi River, but also from the Muli and Dadu River to ensure conservation of wild resources and genetic diversity in the future. Furthermore, the supply of offspring from hatcheries cannot meet the demand of the aquaculture market and consequentially, wild fry in the Dadu River has been harvested for culturing in the breeding season. This is negatively affecting the recruitment of the wild population in the Dadu River. In order to reduce the human pressure on wild resources of Prenant's schizothoracin, it is important that artificial propagation and rearing techniques be improved in an urgent manner. Thus, the protection of wild genetic diversity of this species will be possible.

Acknowledgements This work was funded by Science and Technology Bureau of Sichuan Province, China (No. 03JY029-071-2). We would like to thank Taiming Yan, Tianming Xie and Chunlin He for their help in sample collecting, and Fangdong Zou and Zen Zhang for their help during the experiment. We are grateful to Mart R. Gross, Aviva Patel, and two anonyms for their helpful comments on the manuscript, and Katherine Pieczora and Sean Clark for their help in English writing.

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