

Detection of hybridization between two loach species (*Paramisgurnus dabryanus* and *Misgurnus anguillicaudatus*) in wild populations

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Abstract Artificial interspecific hybrids between large scale loach *P. dabryanus* and tetraploid pond loach *M. anguillicaudatus* (Cobitidae, Cypriniformes) are viable. To detect the occurrence of possible natural hybridization, genetic analyses by using microsatellite markers were performed for natural populations of large scale loach and pond loach, the reciprocal laboratory hybrids, and “supposed hybrids” with ambiguous morphology. The fertility of the artificial hybrids was also tested. At one diagnostic microsatellite (Mac50), one out of 20 “supposed hybrids” was identified to be F₁ hybrid between the two loach species because it had the same genotype as that of the laboratory hybrids. The triploid hybrids between the two species were confirmed to be female-sterile. The results show that rare hybridization has occurred between diploid large scale loach and tetraploid pond loach in nature although it may have little effect in genetic introgression. This study is helpful for fish

conservation and encourages further investigation on natural hybridization and introgression of loaches.

Keywords Loach · Natural hybridization · Artificial cross · Fertility · Microsatellite

Introduction

Hybridization has been documented as a widespread phenomenon among animals, which provides an opportunity to produce novel genetic variations (Allendorf et al. 2001; Seehausen 2004). Scribner et al. (2001) described 139 artificial or natural hybrids in freshwater fishes, of which 47 intergeneric hybrids were mostly observed in Cyprinidae. In practice, interspecific hybrid fishes have been artificially produced to increase economic profits (Bartley et al. 2000). Interspecific crosses in nature may lead to genetic introgression from one species to another and even to extinction of parental lineages if their hybrids are fertile (Verspoor and Hammar 1991; Epifanio and Philipp 2000). Hybridization between species has become an increasingly important issue in conservation biology.

Commercial farming for loaches has been plentiful in the past two decades, although the majority of the fisheries are harvested from natural populations. Large scale loach, *Paramisgurnus dabryanus*, is endemic to China, and distributed in middle and lower reaches of the Yangtze River, Pearl River and inland waters of

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Taiwan. Pond loach, *Misgurnus anguillicaudatus*, can be found in all areas of China except for Tibetan plateau, and is found in rivers, lakes, ponds, swamps and rice fields. These two loach species are found sympatrically in most areas with similar morphological characters and nearly overlapping spawning seasons (March to October). They were assigned to the same genus (*Misgurnus*) of the Cobitidae until large scale loach was nominated as a new genus *Paramisgurnus* in 1981, because a few distinct anatomic traits were recorded (Chen and Zhu 1984).

Pond loach in most areas of China has 100 chromosomes (Li et al. 1983), and artificial hybridization between large scale loach and pond loach can produce viable reciprocal hybrids (Zhao et al. 1999, 2002; Long et al. 2000). The hybrids were speculated to be sterile due to their remarkably different numbers of chromosomes (large scale loach=48 and pond loach=100) but data to support this is not available (Zhao et al. 1999). A suspicion has been raised as to whether or not hybridization between these closely-related loach species can occur in natural populations. There has been some evidence to support this suspicion (Yin et al. 2005).

Molecular evidence from diagnostic markers allows for the identification of potential hybrids in wild populations of teleosts. Microsatellites are particularly suitable because of they are codominant, taxon-specific, and can be used for detecting genome-wide processes such as hybridization and introgression (Gross et al. 2004; Hänfling et al. 2005). In the present study, we analyzed the natural populations of large scale loach, pond loach and phenotype-ambiguous individuals with microsatellite markers for detecting possible interspecific hybridization. The fertility of the artificial hybrids of the two loach species was also tested. The aims of the study were to expand our understanding of potential hybridization and introgression in freshwater fish, and to provide necessary genetic information for fish conservation.

Materials and methods

Sample collection and artificial crosses

Large scale loach and pond loach populations were collected from basins along the Yangtze River from Wuhan, Shashi and Poyang Lake areas, based on their

morphological characteristics as described by Yao (1989). Ambiguous individuals with intermediate phenotypic characteristics from both loach species were also collected as probable hybrids (H).

Mature females and males of the two loach species were sampled from local fish markets in Wuhan as candidates for artificial fertilization. A single intraperitoneal injection of human chorionic gonadotropin (HCG) with a dose of 6–8 IU/g body weight (BW) for females and 2 IU/g BW for males, was used for the preparation of artificially reciprocal and control crosses between the two loach species. All experiments were repeated three times, and fertilization (up to the gastrula stage), hatching (up to yolk sac absorption) and survival rates (up to feeding) were counted, respectively.

After hatching, 50 reciprocal hybrids from one replicate were continuously reared. They were initially cultivated in 50 l glass aquaria and then transferred to 1 m diameter fiberglass tanks until maturity, feeding on rotifers and subsequently commercial carp feed (39% crude protein).

Chromosome analyses

For all the reciprocal and control crosses, chromosome spreads were prepared from 20 embryos randomly sampled at the gastrula stage using the method described by Yu et al. (1989) with slight modifications. Forty clear metaphase plates on each slide were chosen to count the chromosome numbers by using an optical microscope under oil immersion at a magnification of 350 \times .

Genetic analyses with microsatellite DNA

Microsatellite markers from pond loach (Morishima et al. 2001) were used in this study. Five of the 15 microsatellite primers, namely Mac9, Mac15, Mac24, Mac45 and Mac50 were polymorphic and showed a Mendelian segregation in two full-sib families of large scale loach. Fourteen reciprocal hybrids were genotyped as the references of the artificial hybrids at these five loci. The polymorphic information of the five loci (Table 1) was based on 15 test individuals of each species (five individuals from each of the following three populations, Wuhan, Shashi and Poyang Lake, respectively).

The shared alleles were classified as typical for one species or another according to their frequencies, and

Table 1 Cross-species amplification in *Paramisgurnus dabryanus*, (*P. dabr*) using microsatellites from *Misgurnus anguillicaudatus* (*M. angu*)

Locus	DDBJ	Annealing temperature (°C)		Number of alleles		Allele size	
		<i>M. angu</i>	<i>P. dabr</i>	<i>M. angu</i>	<i>P. dabr</i>	<i>M. angu</i>	<i>P. dabr</i>
Mac9	AB060175	54	52	2	5	92–107	92–104
Mac15	AB060177	60	56	6	9	142–168	124–184
Mac24	AB060178	54	54	3	9	98–120	92–116
Mac45	AB060185	60	52	5	5	80–102	80–96
Mac50	AB060182	60	52	5	4	88–106	84–110

then each individual was assigned to a certain genotypic category based on the relative distribution of alleles of each species among the multi-locus genotypes (Hänfling et al. 2005; Ibarra et al. 2005). The five loci had largely overlapping allelic ranges between large scale loach and pond loach. Previous investigation on genetic diversity of these three populations (Wuhan, Shashi and Poyang Lake populations, 109 individuals) showed that the large scale loach had the typical alleles at Mac9, Mac45 and Mac50 but not at Mac24 and Mac15 (unpublished data). Mac9, Mac45 and Mac50 were also useful for classifying individuals when different typical alleles for the two loach species were observed. Therefore these three microsatellite loci were used for the analyses of pond loach and probably hybrids (20 individuals each population), together with our previous analyses of the large scale loach.

Genomic DNA was extracted from fin tissue or fry according to a traditional phenol-chloroform method. PCR was performed in a 12.5 μ l reaction mixture, which contained 1 \times buffer (with 1.5 mM Mg²⁺), 0.4U *Taq* polymerase, 100 μ M dNTP, 2.5 pmol primers, 50–100 ng template DNA, in the following conditions: an initial denaturation for 5 min at 94°C followed by 37 cycles of denaturation at 94°C for 40 s, annealing at 50–60°C for 35 s and extension at 72°C for 35 s, and a final extension at 72°C for 10 min. PCR products were separated by 8% polyacrylamide gels, and fragments were visualized by ethidium bromide staining and UV transillumination (GeneGenius, SYNGENE).

The software package FSTAT (Goudet 1995) was utilized to calculate the number of alleles, distributions of allele and genotype frequencies, and to test the linkage disequilibrium (LD) for the three loci in the wild populations of large scale loach, pond loach

and the probable hybrids, respectively. The program Micro-Checker v 2.2.3 (Van-Oosterhout et al. 2004) was used to detect null alleles that could exist in the microsatellite analyses.

Results

Cytology and morphology of artificial hybrids

There were no differences in mean fertilization, hatching and survival rates between the reciprocal and control crosses in the two loach species (data not shown). The chromosomal analyses showed a bulk of 74 pairs of chromosomes from the reciprocal crosses (Fig. 1), indicating that the offspring were hybrids rather than gynogenetic or androgenetic diploids of large scale loach ($2n=48$) or pond loach ($2n=100$). The reciprocal hybrids exhibited normal growth and had no significantly reduced survival rates compared to self-mating individuals within each species.

The progenies of large scale loach ♀ \times pond loach ♂ (PM) were more morphologically similar to pond loach rather than large scale loach. After being injected with HCG, 150–250 eggs were extruded from each of the two female progenies. The eggs were significantly smaller in size than those from normal females of large scale loach or pond loach. After these eggs were fertilized with sperms of large scale loach or pond loach, aberrant divisions were observed and no embryos survived to 24 h post-fertilization.

Genetic analyses of artificial hybrids

All the hybrids, either from PM (large scale loach ♀ \times pond loach ♂) or from MP (pond loach ♀ \times large scale loach ♂) groups, had heterozygous genotypes con-

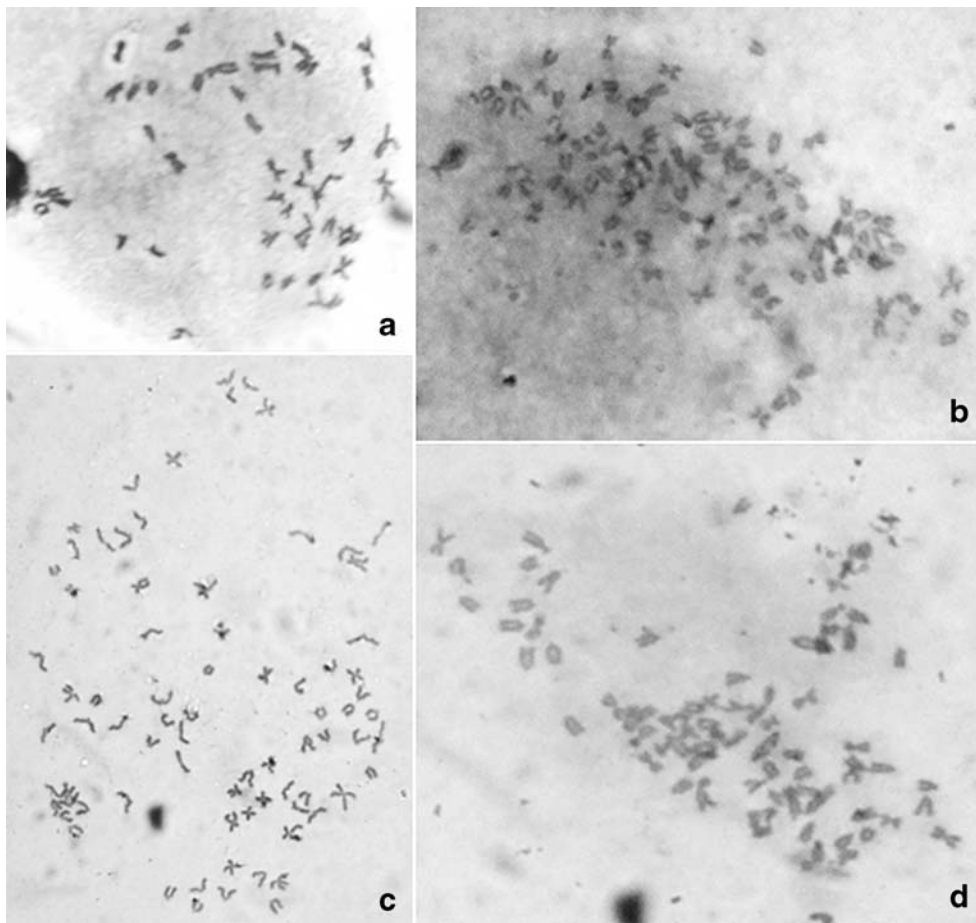


Fig. 1 The chromosomal set of the two loach species and their reciprocal hybrids. **a** *P. dabryanus*, $2n=48$; **b** *M. anguillicaudatus*, $4n=100$; **c** *P. dabryanus* ♀ × *M. anguillicaudatus* ♂, $3n=74$; **d** *M. anguillicaudatus* ♀ × *P. dabryanus* ♂, $3n=74$

taining alleles from both parents at Mac9, Mac15 and Mac45 (Fig. 2a). However, at the locus Mac24, all the reciprocal hybrids only possessed parental alleles from pond loach while the alleles from large scale

loach were absent (Fig. 2b). The paternal pattern of pond loach in the MP group at Mac24 exhibited a constant four-allele type, indicating a duplicated locus (Fig. 2b). At Mac50, the band intensity of all the

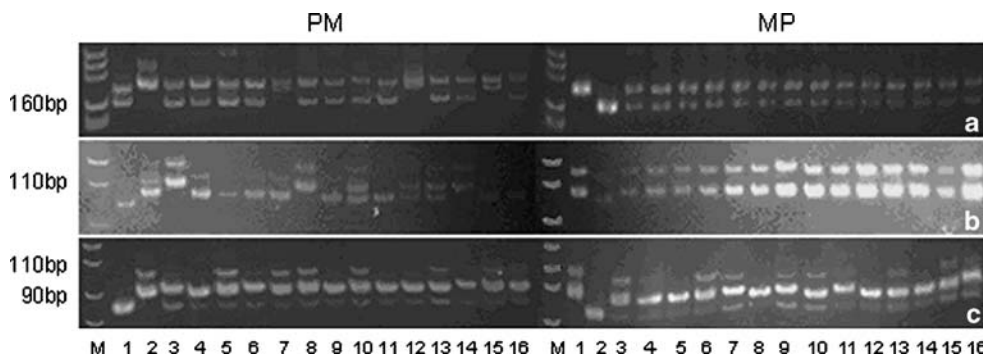


Fig. 2 Amplification profile for the F₁ reciprocal hybrids of *P. dabryanus* and *M. anguillicaudatus* at the microsatellite Mac15 (a), Mac24 (b), and Mac50 (c). *PM*: the laboratory hybrids of *P. dabryanus* ♀ × *M. anguillicaudatus* ♂, *MP*: laboratory hybrids of *M. anguillicaudatus* ♀ × *P. dabryanus* ♂; *M*: pBR322 DNA/*Msp* I molecular marker; 1: maternal parent; 2: paternal parent; 3–16: F₁ laboratory hybrids

Table 2 Genotypic segregations for three microsatellite loci in 20 individuals of the wild “supposed hybrids”

Supposed hybrids	Genotype		
	Mac9	Mac45	Mac50
H1	92/92	82/82	110/110
H2	92/92	82/96	84/90 ^a
H3	92/92	82/82	88/90/106 ^b
H4	92/92	82/92	90/92
H5	92/92	82/82	90/90
H6	92/92	82/82	90/96
H7	92/92	82/82	90/90
H8	92/104	82/82	88/90/106 ^b
H9	92/92	82/82	90/106
H10	92/92	82/82	90/90
H11	92/92	82/82	90/106
H12	92/92	82/82	90/106
H13	92/92	82/94	90/96/106 ^b
H14	92/92	82/82	90/90
H15	92/92	82/82	84/110
H16	92/92	82/82	90/90
H17	92/92	82/82	90/90
H18	92/92	82/82	90/96/106 ^b
H19	92/104	82/92	90/90
H20	92/92	82/92	90/90

^aF₁ hybrid genotype

^bIndividuals exhibiting three bands

reciprocal hybrids was stronger in pond loach than in large scale loach, and the three-band pattern of the hybrids maybe an indication of triploids (Fig. 2c).

Analyses of the wild populations using microsatellite DNA

Three microsatellite loci, Mac9, Mac15 and Mac45, were all in linkage equilibrium ($P > 0.05$) in natural populations of two reference species and probable hybrids (H). No null alleles were detected in the probable hybrids (H) at the three loci.

At Mac9 and Mac45, the two species held the same typical alleles e.g. Mac9-92 with 0.972 in large scale loach and with 0.925 in pond loach, and Mac45-82 with 0.894 in large scale loach and with 0.500 in pond loach, and provided little information for classifying 20 probable hybrids (H), though the other exclusive alleles of pond loach e.g. alleles Mac9-104, Mac45-92 and Mac45-94 were observed in five individuals (H₄, H₈, H₁₃, H₁₉ and H₂₀). At Mac50, the two loach species exhibited polymorphism with different alleles e.g. alleles 84 and 110 for large scale loach and alleles 88, 90, 92, 96 and 106 for pond loach, suggesting their potential use as diagnostic markers. Therefore, two of these individuals, H₁ and H₁₅, were assigned to large scale loach, and the others were assigned to pond loach (Table 2). However, a weakly amplified band with the same size as the diagnosed allele (Mac50-84) of the large scale loach attached to the bright diagnosed allele (Mac50-90) of the pond loach in individual H₂. This individual exhibited the same genotype as the laboratory reciprocal F₁ hybrids, and was identified as a natural F₁ hybrid (Fig. 3). Additionally, four individuals, H₃, H₈, H₁₃ and H₁₈, showed different three-band patterns at Mac50 (Table 2, Fig. 3).

Discussion

Generally, morphological examination was commonly used to identify large scale loach and pond loach hybrids, which was shown to be correct in most cases (Scribner et al. 2001). However, it could be misleading in cases where morphological measurements were used as the sole source of inference, particularly for hybrid individuals beyond the F₁ generation (Nuff and Smith 1979). The development of molecular markers helped to resolve this problem. In this study, from a total of 20 individuals of probable hybrids with ambiguous morphological traits, only one individual

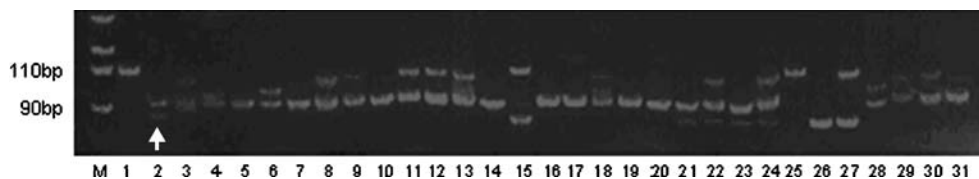


Fig. 3 Amplification profile for wild “supposed hybrids” (H) at microsatellite Mac50. M: pBR322 DNA/*Msp* I molecular marker; 1–20: individuals of the wild “supposed hybrids” (H₁–H₂₀), 21–24: four typical genotypes of the hybrids from the PM group or MP group, 25–27: typical genotypes of *P. dabryanus*,

28–31: typical genotypes of *M. anguillicaudatus*, the arrow indicated a “supposed hybrid” loach (H₂) which had the same genotype as the distinct genotype of some laboratory F₁ reciprocal hybrids

(H₂) was clearly identified as F₁ hybrid, and the others were assigned to be either large scale loach or pond loach by using a single diagnostic microsatellite locus. These results suggested that it was important to combine both morphologic and genetic data when identifying pure species and hybrids.

Thorgaard and Allendorf (1988) indicated that fish might be less susceptible to severe developmental incompatibilities than interspecific hybrids in other vertebrates under comparable levels of genetic divergence. In the present study, one out of 20 suspected individuals of the wild populations was identified as F₁ hybrid, suggesting natural hybridization between the two loach species occurred with a low frequency, probably due to spatial, temporal or behavioral isolating mechanisms (Scribner et al. 2001). Rare hybridization between pond loach and large scale loach was also observed in one of the wild individuals in Wuhan area, with 74 chromosomes (Yin et al. 2005). In the present study, the microsatellite genotype of natural F₁ hybrid showed a weak band from large scale loach allele and a strong band from pond loach allele, a pattern resembling that of the artificial hybrids. The similar phenomenon of amplification asymmetry was also observed in the hybridization between silver carp, *Hypophthalmichthys molitrix*, and bighead carp, *Aristichthys nobilis* (Mia et al. 2005), although it was noted that they should be recorded with caution to avoid incorrect scoring of unknown stutter bands. More diagnostic microsatellite loci or other molecular markers of the two species are required to identify hybridization events in natural populations in the future.

In fish, great variations in the fertility of F₁ hybrids have been reported (Nikoljukin 1971). The artificial F₁ hybrids of large scale loach ♀ × pond loach ♂ appeared to be female-sterile. However, we could not deduce the conclusion that all F₁ hybrids were sterile as the male hybrids were not tested. Atlantic salmon, *Salmo salar*, and brown trout, *Salmo trutta*, also differed in terms of chromosome numbers (2n=58 and 80, respectively), and 0.1 to 13% hybridization rates between two species in wild populations were observed (Verspoor 1988; Jansson et al. 1991; Garcia-Vazquez et al. 2001). The females of these F₁ hybrids were backcrossed with both parental species in artificial conditions, suggesting that they were actually fertile to various degrees (Garcia-Vazquez et al. 2004). A similar case was observed in another fish species

e.g. sturgeons (Nikoljukin 1971). In this study, gene introgression may have occurred due to transmission of whole exotic chromosomes or chromosome sections (Verspoor and Hammar 1991). However, in interspecific, intergeneric and even in more remote crossings, reciprocal and backcrossed hybrids may vary significantly in viability or fertility because of the cytogenetic compatibility (Nikoljukin 1971; Gui et al. 1993; Garcia-Vazquez et al. 2004).

A historical event of genome duplication from diploidy (2n=50) to tetraploidy (100 chromosomes) has been proposed for pond loach in most areas of China including Wuhan (Li et al. 1983). This was confirmed by the amplification pattern of four bands of pond loach at Mac24 in the MP group. The controlled laboratory hybrids in the present study were cytogenetically deduced to be triploids (3n=74), which was consistent with their three-banded patterns at Mac50. Four individuals from the probable hybrids, with other three-band patterns at Mac50, might be an indicator of triploid hybrids of pond loach (Table 2, Fig. 3). Natural triploids of pond loach were found in Japan (Oshima et al. 2005). The triploid males were generally sterile and showed little potential to produce haploid sperm, while the triploid females were fertile and laid a large triploid egg and a normal-size haploid egg (Matsubara et al. 1995; Oshima et al. 2005). Viable reciprocal diploid and triploid hybrids between mud loach, *Misgurnus mizolepis* (2n=48) and pond loach, *M. anguillicaudatus* (2n=50) were also recorded in South Korea (Kim et al. 1995), in which diploid hybrids (2n=49) were fertile, and triploid hybrids (2n=74), with two sets of pond loach and one set of mud loach chromosomes, were sterile in both sexes (Nam et al. 2004). The triploid hybrids of the two loach species were female-sterile. However, introgressions would be possible if the hybrids between large scale loach and diploid pond loach were fertile, because the F₁ hybrids might backcross to their parental generation as shown in other loaches (Kim et al. 1995; Nam et al. 2004). Diploid pond loaches were also reported in some areas of China (Yu et al. 1989).

In summary, the results of the present study demonstrated that rare hybridization occurred between large scale loach and tetraploid pond loach, although there may be little danger in genetic introgression due to their sterile offspring. For the purposes of fish ecology and conservation, further studies on the fertility of F₁ hybrids, occurrence of natural hybridization and

possible introgressions between large scale loach and diploid pond loach in China are necessary by using controlled matings and additional molecular markers.

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