

Comparative and evolutionary analysis in natural diploid and tetraploid weather loach *Misgurnus anguillicaudatus* based on cytochrome *b* sequence data in central China

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Abstract To obtain the phylogenetic relationship between diploid and tetraploid *Misgurnus anguillicaudatus*, the mitochondrial *cyt b* gene in the diploid and tetraploid weather loach were isolated and sequenced.

The DNA sequences were analyzed using MEGA 3.0 software to determine the phylogenetic relationship. Forty-five variable sites among *cyt b* gene sequences and 18 amino acid substitutions occurred within the diploid and tetraploid loaches as deduced from the nucleotide sequences analysis of the *cyt b* gene. The nucleotide pairwise distance between diploid and tetraploid loach ranged from 0.001 to 0.025. Phylogenetic analysis revealed evolutionary relationships between diploid and tetraploid loach. Our results indicated a significant difference between diploid and tetraploid loach about the *cyt b* gene. AMOVA analysis indicated that there were no significant genetic variations within diploid loaches ($F_{st}=0.2529$, $P>0.05$) and within tetraploid loaches ($F_{st}=0.0564$, $P>0.05$), neither. However, significant genetic differences were found between diploid and tetraploid loaches ($F_{st}=0.7634$, $P<0.05$). Thus, it is concluded that no reproductive isolation was found within the same cytotypes of different localities, but there was reproductive isolation between these two cytotypes. The diploid loach existed before the tetraploid loach in nature. The present study is the first to describe the phylogenetic relationships of natural polyploidy weather loach using mtDNA *cyt b* gene.

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Introduction

The weather loach, *Misgurnus anguillicaudatus* (Cobitidae, Cypriniformes), is an important freshwater fish species with both high economic and nutritional value throughout Asia. This omnivorous species inhabits the bottom of the water column and at times can be found in the sediment, feeding upon algae, bottom dwelling animals, detritus, and cladocerans. The weather loach has long been used in traditional Chinese medicine as a folk remedy for the treatment of hepatitis, osteomyelitis, carbuncles, inflammations and cancers. It has also been used to restore health from debilities caused by various pathogens and aging. Its attractive characteristics such as moderate size (10–20 g of body weight in marketable size), easy to culture, soft egg chorion, transparent embryos, fast embryonic development (24 h at 25°C), short generation time (3–4 months), and year-round spawning under controlled conditions make this loach suitable as a candidate for field and laboratory studies (Shao et al. 2005). It has been successfully used for several genetic manipulations, including ploidy manipulation, hybridization, and sex control.

Polyploidization is well known as an important evolutionary force in plants and indications of its importance in the evolution of animals are constantly accumulating (Soltis and Soltis 1995, 1999; Le Comber and Smith 2004; Slechtova et al. 2006). Examples of fish groups, in which changes in ploidy level have been already identified as key events in their evolution include Acipenseridae (Ludwig et al. 2001), Cyprinidae (Alves et al. 2001; David et al. 2003), Catostomidae (Ueno et al. 1988), and Salmonidae (Phillips and Ráb 2001; Crespi and Fulton 2004). Multiple origins of polyploidy were demonstrated in African barb (Tsigenopoulos et al. 2002) and Cobitidae (Janko et al. 2003). Weather loaches represent an interesting model to study the role of polyploidization in vertebrate evolution. Although the taxonomic relationship among these loaches has not yet been clarified, presence of taxonomically or genetically distinct loaches has been suggested. Cytogenetic studies have shown that the natural loaches in China are usually diploid ($2n=50$ chromosomes) and tetraploid ($2n=100$ chromosomes). To evaluate the origin of polyploidisation within weather loach and its contribution to its evolution, it is necessary to understand the phylogenetic relationships between the diploid and tetraploid species.

The mtDNA is being widely used across many groups of fishes, especially Cypriniformes, as a genetic marker in the analysis of genetic variation and for establishing phylogenetic relationships (Xiao et al. 2001). As a conserved protein-coding gene with relatively slow evolutionary rate, mitochondrial cytochrome *b* (*cyt b*) has been demonstrated to be useful for phylogenetic studies among closely related species (Tang et al. 2006). Many population genetic and phylogenetic studies have been conducted using *cyt b* gene sequences in fishes (Briolay et al. 1998; Song et al. 1998; He et al. 1999; Matsuo et al. 2001; Peng et al. 2004). It has been reported that restriction fragment length polymorphism analysis of mitochondrial DNA should be used to analyze genetic variation of Japanese loach (Khan et al. 2005). However, little work on the *cyt b* gene in the natural polyploid loach has been reported. There has been no research related to genetic differences and phylogenetic relationships between diploid and tetraploid loach.

The objectives of this study were to investigate the ploidy level distribution of weather loach collected from nine selected sites in central China and to analyze genetic diversity and phylogenetic relationships between diploid and tetraploid using *cyt b* gene sequence analyses to estimate the evolution of weather loach. To our knowledge, the present study is the first report to describe the phylogenetic relationships of natural polyploidy weather loach using mtDNA *cyt b* gene.

Material and methods

Specimen examination and ploidy determination

To survey the distribution and frequency of natural loach polyploids, six samples were collected from nine different geographic locations in central China (Fig. 1). The chromosome specimens belonging to *M. anguillicaudatus* were obtained from metaphase of kidney cells by in vivo injection of PHA and colchicin, hypatoic-air drying technique, and Giemsa staining (Quan et al. 2000; Zheng et al. 2005). The chromosome results were analyzed according to Levan et al (1964).

Total DNA extraction

The total DNA was extracted using scales or underlying muscle tissue following the description found in

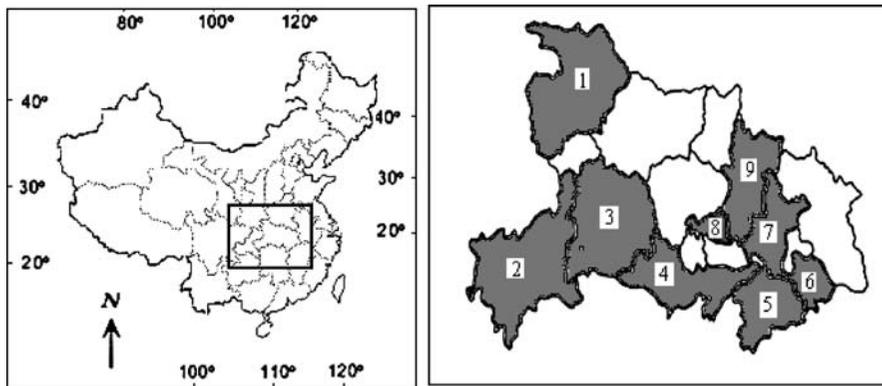


Fig. 1 Sampling localities of weather loach *M. anguillicaudatus* in central China (nine black areas indicating nine sampling sites): 1 Shiyang City (SY); 2 Enshi City (ES); 3 Zigui City

(ZG); 4 Shashi City (SS); 5 Chibi City (CB); 6 Yangxin City (YX); 7 Wuhan City (WH); 8 Tianmen City (TM); 9 Xiaogan City (XG)

Kocher et al. (1989) with some simplifications. The tissues from the sample fish were ground to powder in liquid nitrogen. Approximately 0.1 g of ground tissue was added to 700 µl of lysis buffer (50 mM Tris–HCl, pH 7.5, 50 mM EDTA, and 3% SDS) and digested by adding proteinase K (final concentration 100 µg ml⁻¹) and incubated at 50°C overnight. The supernatant was extracted once with chloroform/phenol (1:1). The nucleic acids were precipitated in a solution containing 10 µl of 3 M sodium acetate (pH 5.2) and 1 ml of absolute isopropanol. The resulting pellet was washed with cold 70% ethanol, dried, and re-suspended in 50 µl of distilled water.

PCR and sequencing

Two primers were used to amplify and sequence the *cyt b* gene of weather loach: L14724 (5'-GACTTGAAAA CCACCGTTG-3') and H15915 (5'-CTCCGATCTCCG GA- TTACAAGAC-3') (Xiao et al. 2001), synthesized by Shanghai Sangon Biological Engineering Technology and Service. PCR reactions were carried out in a 50 µl final volume containing 2.5 µl DNA, 5 µl 10×buffer, 0.5 µl dNTP, 3 µl of each primer, 1 µl Taq DNA, 4 µl Mg²⁺ and 31 µl H₂O.

The thermal cycling profile started with 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 45 s, extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The amplified DNA fragments were checked in 1% agarose gel and then purified for direct sequencing by an automatic DNA Sequencer (ABI PRISM 377, Perkin-Elmer) of Shanghai Sangon Biological Engineering Technology and Service.

Sequence analyses

The sequences were aligned using the CLUSTAL W (Thompson et al. 1994), and then were manually edited using the SEAVIEW (Galtier et al. 1996). Pairwise sequence comparisons to determine the distribution and amount of variation and the degree of saturation among codon position were performed using MEGA 3.0 (Kumar and Gadagkar 2001). Phylogenetic reconstructions were obtained using the neighbor-joining (NJ) and maximum parsimony (MP) trees. The reliability of the tree topology was assessed by 1000 bootstrap replications (Felsenstein 1985). Two diploid species *Paramisgurnus dabryanus* (PD) and *Botia supercilii* (BS) were used as out-groups in the phylogenetic analysis. AMOVA analysis was used to analyze the distribution of the molecular genetic variation among samples and their relationship with geographical or chromosome data (Excoffier et al. 1992).

Results

Ploidy level of loaches samples

After counting the chromosome number, only diploid and tetraploid weather loaches were found in the present study. No natural triploid loaches were detected in the present study. The number of chromosome in diploidy is 2n=50, and that of tetraploidy is 4n=100. The ploidy distribution of weather loach in each sampling site is presented in Table 1. Diploid loaches were found in ES city, YX city, TM city, SY city and ZG city, while tetraploid loaches were detected in the

Table 1 Ploidy distribution of *M. anguillicaudatus* in central China

Sampling location (abbr.)	Specimen no.	Ploidy level	Chromosome no.
Enshi (ES)	6	Diploidy	2n=50
Yangxin (YX)	6	Diploidy	2n=50
Tianmen (TM)	6	Diploidy	2n=50
Shiyan (SY)	6	Diploidy	2n=50
Zigui (ZG)	6	Diploidy	2n=50
Wuhan (WH)	6	Tetraploidy	4n=100
Shashi (SS)	6	Tetraploidy	4n=100
Xiaogan (XG)	6	Tetraploidy	4n=100
Chibi (CB)	6	Tetraploidy	4n=100

rest of sampling sites (WH city, SS city, XG city and CB city).

Base composition and variation

After alignment, 1,140 bp of the complete *cyt b* gene sequences were used for analysis. The average nucleotide composition for all sequences were $A=27.3\%$, $T=31.6\%$, $C=26.1\%$, and $G=15.0\%$ (Table 2). The content of $A+T$ (58.9%) was higher than that of $G+C$ (41.1%). In the triplet codons, the first position has even usage in four nucleotides; the second position has high content of T (41.0%) but low content of G (13.2%); the lowest G content (6.4%) was found in the third position (Table 3). There were 45 sites for all sequences, 23 of which were variable among diploid loaches and 14 among tetraploids. Base substitutions happened mostly at the third codon position. The average transition/transversion ratio

Table 2 Average base composition of mtDNA *cyt b* gene in *M. anguillicaudatus*

Sampling location (abbr.)	A (%)	T (%)	C (%)	G (%)	GeneBank accession no.
Enshi (ES)	27.2	31.7	26.1	15.1	EF424608
Yangxin (YX)	27.4	31.5	26.2	14.9	EF424604
Tianmen (TM)	27.5	31.4	26.3	14.8	EF376188
Shiyan (SY)	27.5	31.4	26.2	14.9	EF424605
Zigui (ZG)	27.5	31.6	26.1	14.8	EF424603
Wuhan (WH)	27.1	31.6	26	15.3	EF088651
Shashi (SS)	27.2	31.6	26.1	15.1	EF424606
Xiaogan (XG)	27.1	31.7	26	15.2	EF424607
Chibi (CB)	26.9	31.5	26.2	15.4	EF424602
AVERAGE	27.3	31.6	26.1	15.0	

across all the pairwise sequence comparisons was 0.9. Usually the level of transition bias less than 2.0 is considered that the sequence variation begins to saturation (Zhang and Ryder 1997; Zhou et al. 2001). Most of the variability among sequences was observed in the third codon position. First and second positions show very slight saturation, whereas substitutions at the third position are strongly saturated.

Amino acid variation

There were 380 amino acids deduced from the nucleotide sequences of the *cyt b* genes in both diploid and tetraploid loaches. Most of the nucleotide substitutions were located at the third codon position and were silent mutations. In addition, within 18 amino acid substitutions, 10 were in the diploids while 8 were in tetraploid loaches (Table 4).

Genetic distances and genetic variation

Among all loaches sequences, the nucleotide pairwise distance ranged from 0.001 to 0.378. The nucleotide pairwise distance between the tetraploid and diploid ranged from 0.001 to 0.025. The nucleotide pairwise distance between tetraploid and diploid was smaller than that between *M. anguillicaudatus* and the out-group species. Amino acid pairwise distances among these fish ranged from 0.003 to 0.359 (Table 5). Based on the AMOVA analysis, there were no significant genetic variations within diploid loaches ($F_{st}=0.2529$, $P>0.05$) and within tetraploid loaches ($F_{st}=0.0564$, $P>0.05$), neither. However, significant genetic differences were found between diploid and tetraploid loaches ($F_{st}=0.7634$, $P<0.05$).

Phylogenetic trees

NJ phylogenetic tree (Fig. 2) and MP phylogenetic tree (Fig. 3) of the *cyt b* genes from diploid and tetraploid *M. anguillicaudatus* in cypriniformes was reconstructed. The branches of two kinds of phylogenetic trees had the same topology and similar bootstrap probabilities. Phylogenetic analyses using NJ identified two major lineages supported by moderate to high bootstrap values: diploid and tetraploid loaches (51 and 99% bootstrap, respectively). In the MP tree, tetraploid (XG, CB, SS, WH) loaches were at the derivative clade, while diploid (TM, SY, YX, ES, ZG) loaches

Table 3 Base composition (%) at first, second, and third codon positions of *cyt b* gene in *M. anguillicaudatus*

Sampling location	First position				Second position				Third position			
	T	C	A	G	T	C	A	G	T	C	A	G
CB	26.3	23.9	23.9	25.8	41.1	25.8	20.0	13.2	26.8	28.9	36.8	7.4
SS	26.1	24.2	24.5	25.3	41.1	25.8	20.0	13.2	27.4	28.4	37.1	7.1
XG	26.3	23.9	23.9	25.8	41.1	25.8	20.0	13.2	27.4	28.4	37.4	6.8
WH	26.3	24.2	23.9	25.5	40.8	25.8	20.0	13.4	27.4	28.2	37.4	7.1
ZG	25.5	24.5	24.2	25.8	41.1	26.1	19.7	13.2	27.9	27.9	38.7	5.5
YX	25.8	24.5	24.2	25.5	41.1	25.8	19.7	13.4	27.4	28.4	38.2	6.1
SY	25.8	24.2	24.2	25.8	41.1	25.8	20.0	13.2	27.1	28.7	38.4	5.8
TM	25.8	24.5	23.9	25.8	41.1	25.8	20.0	13.2	27.1	28.4	38.4	5.8
ES	25.8	24.7	23.7	25.8	41.1	25.8	19.7	13.4	27.9	27.9	38.2	6.1

were at the basal clade of MP tree, which was similar to the NJ tree.

Discussion

Ploidy distribution

In the present study, only diploid and tetraploid loaches were detected. However, none of triploid loaches were observed. This result is in agreement with the former research (Yin et al. 2005) that there were probably only two cytotypes of loaches in China, including diploid and tetraploid loaches. The occurrence of natural polyploid individuals was firstly recognized by Ojima and Takai (1979) in Japan. Weather loach includes polyploid and unisexual biotypes in nature (Zhang and Arai 1999; Morishima et al. 2002; Yin et al. 2005) and appears to tolerate genomic changes by means of artificial chromosome manipulation from diploid to polyploid state (Arai 2001). However, in Japan, a relatively high frequency of diploid and triploid (3n=75) individuals

has been recorded in several places; out of approximately 40 localities examined for ploidy status by the measurement of relative DNA content with flow cytometry, but no tetraploid has been discovered in wild populations in Japan (Zhang and Arai 1999; Morishima et al. 2002). The actual origin of these polyploids is still unknown. Since the natural tetraploid with 100 chromosomes was reported to occur among the specimens from Yangtze River (Li et al. 1983), the tetraploid specimens are likely to originate from the Asian continent and thus are genetically different from common diploid loach in Japan. Probably due to the geographical differences between China and Japan, triploid loaches do not adapt to establish itself in China.

Sequence variation and gene flow

In the present study, among 1,140 nucleotides of the *cyt b* gene, the content of A+T (58.9%) was higher than that of C+G (41.1%), which fell within the range of GC content typical for vertebrates (Nei and Kumar 2000). Base compositional biases were found which

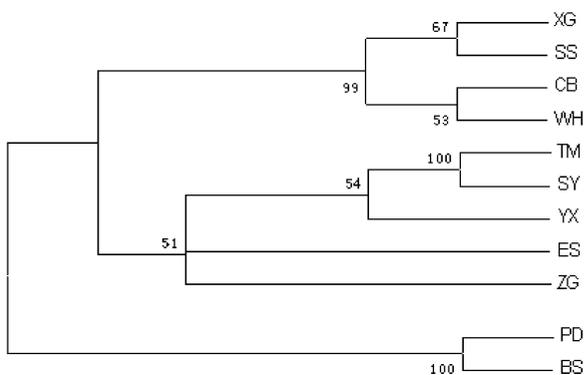
Table 4 Different amino acids among the deduced amino acid sequences of the mitochondrial *cyt b* gene

Amino acid sites	42	155	180	240	265	267	290	292	309	315	316	332	341	352	356	357	358	369
YX	I	Y	A	T	T	S	R	P	D	P	S	H	N	P	Y	P	I	G
TM	I	Y	A	T	T	S	R	P	H	P	S	H	N	T	C	P	I	G
SY	I	Y	A	T	T	S	R	P	H	P	S	H	N	T	C	H	I	G
ES	I	Y	A	A	T	S	R	S	D	P	G	P	D	P	Y	P	I	G
XG	I	Y	A	T	A	S	G	S	H	P	S	H	D	T	C	P	I	G
SS	I	H	T	T	A	S	G	S	H	P	S	H	D	T	C	H	I	A
CB	I	Y	A	T	A	P	G	S	H	P	S	H	D	T	C	P	I	G
WH	S	Y	A	T	A	S	G	S	H	T	T	H	D	T	C	P	I	G
ZG	I	Y	A	T	T	S	R	S	H	P	S	H	D	T	C	P	N	G

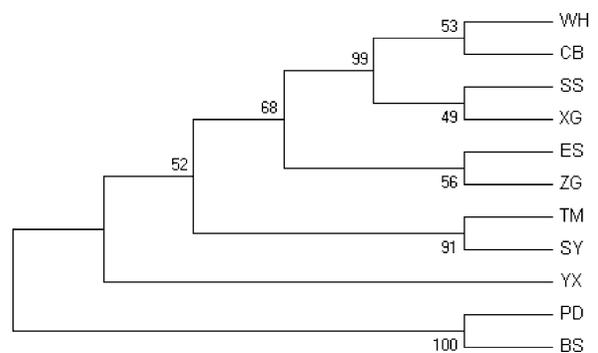
Table 5 The percent divergences of nucleotide sequences (below diagonal) and amino acid sequences (above diagonal) of the mitochondrial *cyt b* gene

Sampling location	YX	TM	SY	ES	PD	BS	XG	SS	CB	WH	ZG
YX		0.008	0.011	0.014	0.307	0.356	0.019	0.030	0.022	0.028	0.017
TM	0.008		0.003	0.022	0.307	0.356	0.011	0.022	0.014	0.019	0.008
SY	0.009	0.001		0.025	0.307	0.356	0.014	0.019	0.017	0.022	0.011
ES	0.011	0.017	0.018		0.309	0.359	0.022	0.033	0.025	0.028	0.019
PD	0.325	0.328	0.328	0.329		0.083	0.304	0.307	0.304	0.304	0.307
BS	0.371	0.372	0.372	0.378	0.200		0.354	0.356	0.354	0.354	0.356
XG	0.021	0.017	0.018	0.019	0.329	0.376		0.011	0.003	0.008	0.008
SS	0.024	0.019	0.018	0.022	0.331	0.378	0.004		0.014	0.019	0.019
CB	0.025	0.020	0.021	0.021	0.332	0.376	0.005	0.008		0.011	0.011
WH	0.025	0.020	0.021	0.021	0.330	0.378	0.005	0.008	0.007		0.017
ZG	0.015	0.011	0.011	0.010	0.328	0.375	0.013	0.016	0.015	0.015	

showed a strong bias against *G* in the *cyt b* gene of weather loaches, particularly at the third position. Our results fit well with Meyer (1993), who investigated the base compositional biases of several species of fishes and found that all species of fishes investigated showed a strong bias against *G* in the *cyt b* gene. The variable sites number (14) in tetraploid loaches is less than that of diploids (23). According to our results, tetraploid loaches were found to cluster in three sites close to WH city, but diploid loaches distributed sporadic in sampling sites. There was significant difference among diploid loaches from geographically distinct populations, while less difference observed among tetraploid loaches. The presence of genetically distinct populations in Chinese loaches has been suggested (Yin et al. 2005), but the results were fragmentary and inconclusive due to the small number of localities examined and specimens used. Khan

**Fig. 2** Neighbor-joining (NJ) tree of diploid and tetraploid *M. anguillicaudatus*: the number above branches is 1,000 bootstrapped value of NJ tree; *P. dabryanus* (PD) and *B. superciliosus* (BS) are two out-groups

and Arai (2000) conducted allozyme analyses in total 923 individuals collected from 44 populations all over Japan. They found that genetic differentiation among some groups was likely to be of inter-subspecies level, whereas divergence in other groups seemed to be a local-race level. Analyses of the genetic population structure in many fish species revealed that individuals from different areas of a lake are typically genetically structured into different local populations (Douglas et al. 1999; Garant et al. 2000; Gerlach et al. 2001). Genetic differentiation of local populations is caused, among other reasons, by environmental barriers and limited dispersal (Johnson and Gaines 1990), which restrict migration and therefore limits gene flow (Barluenga and Meyer 2005). However, in the present study, no intra-population variability was found. Many geographically distant populations frequently showed genetically close relationship. Such inconsistencies between genetic and geographical relationships might be

**Fig. 3** Maximum parsimony (MP) tree of diploid and tetraploid *M. anguillicaudatus*: the number above branches is 1,000 bootstrapped value of MP tree; *P. dabryanus* (PD) and *B. superciliosus* (BS) are two out-groups

due to insufficient number of samples or less sensitivities of analytical method (Khan et al. 2005). Based on the AMOVA analysis, there were no significant genetic variations within diploid loaches ($F_{st}=0.2529$, $P>0.05$) and within tetraploid loaches ($F_{st}=0.0564$, $P>0.05$). However, significant genetic differences were found between diploid and tetraploid loaches ($F_{st}=0.7634$, $P<0.05$). Thus, it is concluded that no reproductive isolation was found within the same cytotypes of different localities, but there was reproductive isolation between these two cytotypes.

Phylogenetic relationship

The phylogenetic relationships among branching lineages of weather loaches were resolved by *cyt b* sequences. In Figs. 2 and 3, the phylogenetic trees (NJ and MP) were both divided into two branches including diploid and tetraploid branches, which formed a monophyletic lineage in both analyses. The diploid branch in the basal clade of NJ and MP phylogenetic trees and tetraploid branches at the derivative clade of the trees indicate that diploids are earlier existing cytotype than tetraploids in nature. Among diploid and tetraploid loaches, the *cyt b* gene showed highly homology. High levels of substructuring between adjacent populations might be expected to facilitate speciation (Van and Turner 1997). Particularly in small isolated populations, drift, adaptation to local conditions or sexual selection will have important effects on gene frequencies. Therefore, one would expect that species with poor dispersal capabilities and high levels of substructuring exhibit high levels of intraspecific variation in some traits (Van and Turner 1997). This appears, however, not to be the case for stone loach, where no appreciable variation in morphological, physiological, or behavioral traits has been detected so far. Similar discordances between morphological and genetic differences have been documented in other fish taxa (Shaw et al. 2000; Wang et al. 2000; Taylor and Verheyen 2001). Therefore, high levels of genetic substructuring alone do not seem to be sufficient to promote high levels of speciation. This suggests that other species-specific factors might influence whether groups speciate or not, such as breeding behavior (Mayr 1984). This is the first report to describe the phylogenetic relationships of natural polyploidy weather loach using mtDNA *cyt b* gene, which provide a new insight on the evolution of two cytotypes. Analysis of

cyt b gene and elucidation of the variation of *cyt b* gene in different fish species prove that *cyt b* is a useful genetic marker to monitor the variations in the progeny of the crosses.

Conclusion

Based on our research, 45 variable sites among *cyt b* gene sequences and 18 amino acid substitution occurred within the tetraploid and diploid loaches which deduced from the nucleotide sequences of the *cyt b* genes. The nucleotide pairwise distance between diploid and tetraploid loach ranged from 0.001 to 0.025. Based on the AMOVA analysis, it is concluded that no reproductive isolation was found within the same cytotypes of different localities. But there was reproductive isolation between these two cytotypes. Phylogenetic analysis revealed an evolutionary relationship between diploid and tetraploid loaches. The diploid loach existed before tetraploid loaches in nature. This is the first report to describe the phylogenetic relationships of natural weather loach using mtDNA *cyt b* gene. To further understand genetic and cyto geographical relationships in weather loaches, the distribution of diploids and diploid–tetraploid mosaics needs to be investigated with more sensitive and reliable DNA marks.

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