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RESEARCH PAPER

Microsatellite Markers Reveal Genetic Differentiation of Chinese Dojo Loach *Misgurnus anguillicaudatus* in the Yangtze River Basin

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Abstract

The fish fauna in the Yangtze-based riparian ecosystem has been imperiled largely due to genetic degradation of populations. Regular genetic monitoring of the fish populations is required for an effective management and conservation. The genetic structure of Dojo loach, *Misgurnus anguillicaudatus* was investigated in twelve populations originating from the Yangtze River basin by using thirteen microsatellite loci. The number of alleles per locus varied between 2 and 8 with an average of 4.6 alleles per locus. Overall, low-to-moderate level of genetic diversity was observed in the loach populations. Significant deviations from Hardy-Wienberg equilibrium were observed in about 50% of the total locus-population combination tests. The AMOVA indicated that most of the variance existed among the individuals (90.50%) rather than among populations within groups (9.03%). Significant differentiation was found among the samples from scattered habitats with different connections to the

Yangtze River ($P<0.05$). The clustering of sample populations in UPGMA dendrogram followed their geographic distribution except for Zigui and Xiaogan which clustered against their geographical origin. The factors involved in genetic differentiation and shaping the existing patterns of population structure of the loach were discussed so as to provide guidelines for conservation strategies and management programs.

Keywords: population genetics, loach, Yangtze River, China, SSR

Introduction

During last decades, riparian ecosystems along major rivers have been reduced to a few scattered remnants due to anthropogenic hydrological alterations. Important feature of riparian ecosystems are water bodies which were originally connected to the river mainstream by annual floodings (Hänfling et al., 2004). In the Yangtze River of China, water regulations and large scale hydrological alterations have rendered many of such aquatic habitats virtually isolated (Wang et al., 2006; Xie et al., 2007). The hydrographic features leave a paramount impact on migration patterns and genetic differentiation of fish (Castric et al., 2001; Manel et al., 2003). The isolated nature of remnant populations makes inhabitant fish highly vulnerable to stochastic factors such as genetic drift and bottleneck. Furthermore, the small size of the remnant populations can lead to reduction in genetic variation, potentially leading to fixation of deleterious alleles and to lack of evolvability in response to environmental changes (Frankham et al., 2002). Therefore, marked genetic structure at neutral markers is expected particularly in non-migratory fish species inhabiting isolated freshwater habitats.

As to the loach *Misgurnus anguillicaudatus*, intensive studies have been conducted on chromosome set manipulation, polyploidy, gynogenesis and genetics (Arai, 2003). Genetic variation studies, carried out with allozyme markers on Japanese *M. anguillicaudatus* showed significant allele frequencies among wild populations (Khan & Arai, 2000). Genetic studies, conducted by Yang et al. (2009) using Cyt b marker in the Chinese loach, indicated no reproductive isolation among different loach population. The aforementioned studies cannot be relied upon due to drawbacks associated with biochemical and mitochondrial markers as suggested by Yang et al. (2009). The dwindling populations of Chinese loach in face of damming-induced fragmentation are required to be investigated comprehensively regarding genetic diversity and population configuration using robust and reliable markers. Due to spawning of cobitids in slow or stagnant waters and mosaic pattern of their habitats, gene flow among their populations is very difficult

(Bohlen, 2003; Kim & Park, 1995; Saito, 1990). Isolated spatial distribution can lead to genetic subdivision (Rundle & Nosil, 2005). Species with restricted dispersal capabilities show significantly greater population genetic structure. Dispersal of loach individuals may be conditioned by life-history and behavioral traits, as well as by geographical barriers imposed by the geomorphology of study area (Castric et al., 2001). In face of habitat destruction and dwindling loach populations (Beveridge et al., 1997; Li, 1999), evaluation of population genetic structure in widespread species is crucial for the development of conservation and management strategies (Dunham, 1999). Therefore, quantifying patterns of gene flow and genetic drift across both natural and man-made barriers to gene flow are important for determining their effects on population genetic structure (Kristy et al., 2007).

Microsatellites (SSR) have emerged as the most amenable neutral markers for population genetic studies. SSR markers characteristically exhibit extensive allelic variation with heterozygosity ranging from 4% to 90% (Liu & Cordes, 2004). These are mainstay of modern population genetics which provide contemporary estimates of migration, departure from panmixia, relatedness of individuals, demographic processes and phenomenon like inbreeding, gene flow, genetic drift and bottleneck. Moreover, abundant SSR loci have been characterized for loach which can be conveniently utilized for population studies on Chinese dojo loach. Despite population abundance of loach in lakes, streams, fields and ditches, it has never been meticulously investigated for demographic genetic differentiation in China. In face of rapidly disappearing natural habitats and increasing fragmentation of aquatic systems, SSR markers were employed to (i) evaluate the genetic variability from the perspective of genetic monitoring of the Chinese dojo loach in twelve scattered populations across the Yangtze river basin; (ii) test the alternative hypotheses of panmixia versus population subdivision within the Yangtze river basin; and (iii) analyze patterns of genetic differentiation in relation to geographic distribution.

Materials and Methods

Fish Sampling and DNA Extraction

A total of 293 adult loach samples were collected from twelve different localities across the Yangtze River basin in central China. The fish specimens were purchased from the markets close to the localities. All sample populations were located along either middle mainstream sub-basin or Hanjiang sub-basin of the Yangtze River: Enshi, Zigui, Jinzhou, Zhijiang, Xiantao, Chibi, Huangshi (middle mainstream sub-basin) and Shiyan, Xiangfan, Wuhan, Suizhou and Xiaogan (Hanjiang sub-basin). The populations were named after the initial letters of sampling localities (Figure 1). The fish samples were screened for ploidy level following to previously published protocol of Gao et al. (2007). We found some tetraploid individuals from Wuhan and Jinzhou. Since the majority of the sampled individuals were diploid, tetraploid individuals were discarded. The dorsal muscle tissues were isolated and stored at -20ºC. Total genomic DNA was extracted from small amounts (~0.2g) of frozen tissues by using traditional proteinase-K digestion and standard phenol/chloroform techniques described by Yue & Orban (2005) with slight modifications and visualized on 0.8% high melt agarose gel in TAE buffer.

Amplification of SSR Loci

Thirteen loach-specific polymorphic primers were utilized to amplify microsatellite loci of each individual. The PCR amplification was carried out in a 20µl reaction mixture; 0.8µl of each primer set (10µM), 0.4µl of dNTPs(10mM), 1.5µl $MgCl_2$ (20mM), 2.0µl of 10x PCR buffer (20mM), 0.4µl (2U/µl) *Taq* polymerase (Shanghai Sangon Biological Engineering Technology & Service Co. Ltd), and approximately 50ng of template DNA using gradient thermal cycler (Eppendorf 22331 Hamburg, Germany). The PCR cycles were as follows: five minutes at 94°C, 32 cycles of 1 minute at 94°C, 30

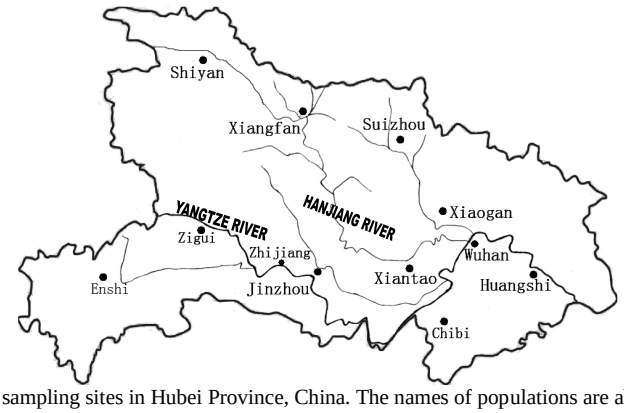

Figure 1. Hydrographic map of sampling sites in Hubei Province, China. The names of populations are abbreviated as: Enshi=ESH; Zigui=ZIG; Jinzhou=JZH; Zhijiang=ZJG; Xiantao=XNT; Chibi=CHB; Wuhan=WHN; Huangshi=HGS; Shiyan=SHN; Xiangfan=XGF; Suizhou=SZH and Xiaogan=XGN.

seconds at a primer-specific annealing temperature (Table 1), 1 minute at 72°C, and final elongation for 4 minutes at 72°C. Following PCR amplification, 5µl of the PCR product was mixed with 1µl of loading dye (deionized formamide containing 0.5% blue dextran). The PCR products were electrophoretically separated

Table 1. Individual microsatellite locus statistics for 12 populations of Dojo loach

	Parameter	XNT	JZH	HGS	XGF	SZH	ZJG	ESH	SHN	CHB	WHN	ZIG	XGN
Locus	N	20	26	24	25	21	25	27 30	24	23	25	23	
	N_a	2	3	3	3	3	3	2	2	3	2	3	3
	A_r	1.996	2.645	2.968	3.000	2.937	3.993	2.000	1.800	3.871	1.899	2.796	2.067
Mado11	H_o	0.3000	0.5500	0.7000	0.4000	0.4000	0.8000	0.6000	0.1000	0.5000	0.1667	0.4000	0.1333
	H_e	0.2684	0.4782	0.5316	0.3539	0.3579	0.6789	0.4421	0.1000	0.6703	0.1594	0.3526	0.1310
	f	-0.125	-0.155	-0.340	-0.167	-0.126	-0.108	-0.385	-0.000	0.286	-0.048	-0.143	-0.018
	N_a	4	4	4	3	3	3	4	4	4	3	3	4
	A_r	4.000	3.986	3.796	2.993	2.800	3.000	3.796	3.796	3.897	2.987	2.800	3.972
Mado21	H_o	0.4000	0.5000	0.3000	0.4000	0.3000	0.7000	0.6000	0.4000	0.3333	0.2500	0.2000	0.7333
	H_e	0.5579	0.7654	0.6053	0.4895	0.4158	0.6895	0.6053	0.6895	0.7283	0.6196	0.5421	0.7471
	f	0.243	0.352	0.518	0.191	0.289	-0.016	0.009	0.433	0.553	0.583	0.643	0.019
	N_a	4	4	3	3	4	3	4	3	4	4	4	3
	A_r	3.996	3.718	2.968	2.800	3.968	2.800	3.600	3.000	3.996	3.998	3.800	2.998
Mado31	H_o	0.9000	0.5000	0.8000	1.0000	1.0000	0.5000	0.6000	0.6000	0.7500	1.0000	0.9000	1.0000
	H_e	0.7632	0.6756	0.6158	0.5737	0.7368	0.4684	0.4895	0.6684	0.7536	0.7717	0.7105	0.6736
	f	-0.191	0.212	-0.321	-0.818	-0.384	-0.071	-0.241	0.107	-0.078	-0.308	-0.286	-0.510
	N_a	3	3	2	3	2	3	2	3	2	2	3	3
	A_r	2.968	2.679	1.968	1.968	1.968	2.800	2.000	3.000	1.999	1.667	2.996	2.994
Mac24	H_o	0.6000	0.2500	0.2000	0.4000	0.2000	0.6000	0.4000	0.7000	0.4167	0.0833	0.3000	0.9333
	H_e	0.4842	0.3115	0.1895	0.5421	0.1895	0.4684	0.3368	0.7000	0.3442	0.0833	0.5421	0.6253
	f	-0.256	0.201	-0.058	-0.058	-0.058	-0.301	-0.200	0.000	-0.222	0.000	0.460	-0.519
	N_a	5	5	4	4	2	4	4	5	5	3	4	5
	A_r	4.737	4.841	3.996	3.600	1.993	3.768	3.800	5.761	5.660	2.639	3.765	4.437
Mac37	H_o	0.9000	0.8500	1.0000	0.5000	0.2000	0.7000	0.9000	0.7000	0.6667	0.2500	0.5000	0.9333
	H_e	0.6684	0.7397	0.7526	0.4316	0.1895	0.6263	0.6474	0.7842	0.7754	0.3007	0.5000	0.7310
	f	-0.373	-0.186	-0.353	-0.241	-0.067	-0.125	-0.421	0.027	0.139	0.175	0.000	-0.289
	N_a	4	4	4	3	4	4	2	4	4	3	4	4
	A_r	3.778	3.679	3.968	2.800	3.568	3.993	1.800	3.768	3.972	2.899	3.568	3.582
Mac40	H_o	0.5000	0.6000	0.7000	0.6000	0.4000	0.4000	0.1000	0.4000	0.6667	0.4167	0.4000	0.5333
	H_e	0.6000	0.6603	0.7421	0.5632	0.3632	0.7000	0.1000	0.6263	0.7609	0.5399	0.3632	0.6161
	f	0.121	0.093	0.060	-0.069	-0.108	0.442	-0.000	0.374	0.129	0.236	-0.107	0.138
	N_a	2	2	2	2	2	2	2	2	2	2	2	2
	A_r	1.968	2.000	2.000	2.000	2.000	1.968	1.996	1.968	1.993	1.899	1.996	1.963
Mac44	H_o	0.2000	0.6000	0.8000	0.7000	0.4000	0.2000	0.3000	0.2000	0.3333	0.1667	0.3000	0.2667
	H_e	0.1895	0.4308	0.5053	0.4789	0.3368	0.1895	0.2684	0.1895	0.2899	0.1594	0.2684	0.2391
	f	-0.059	-0.407	-0.636	-0.500	-0.200	-0.059	-0.125	-0.059	-0.158	-0.047	-0.125	-0.120
	N_a	3	3	2	2	2	2	2	3	3	2	2	2
	A_r	2.800	2.819	2.000	2.000	2.000	1.800	1.800	2.996	2.666	1.999	2.000	2.000
Mac50	H_o	0.5000	0.1500	0.5000	0.4000	0.5000	0.1000	0.1000	0.4000	0.5833	0.4167	0.4000	0.6000
	H_e	0.8684	0.3833	0.3947	0.3368	0.3947	0.1000	0.1000	0.5421	0.4529	0.3442	0.3368	0.4805
	f	-0.071	0.615	-0.286	-0.200	-0.286	-0.000	-0.000	0.273	-0.305	-0.222	-0.200	-0.260
	N_a	2	3	2	4	2	2	1	3	2	2	3	4
	A_r	1.968	2.046	2.000	3.768	1.889	1.993	1.000	2.796	1.972	1.999	2.600	3.651
Mac462	H_o	0.2000	0.1500	0.1000	0.7000	0.1000	0.2000	0.0000	0.4000	0.2500	0.4167	0.2000	0.8667
	H_e	0.1895	0.1449	0.0950	0.5947	0.1000	0.1895	0.0000	0.3526	0.2283	0.3442	0.1947	0.6000
	f	-0.059	-0.036	-0.285	-0.188	0.000	-0.067	N.A.	-0.143	-0.100	-0.222	-0.028	-0.444
	N_a	5	6	5	7	5	7	5	5	4	4	5	4
Mac477	A_r	4.882	5.390	4.768	6.533	4.400	6.702	4.400	4.765	3.892	3.974	4.400	3.909
	H_o	0.9000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.7000	0.9167	0.9167	1.0000	1.0000
	H_e	0.7421	0.7936	0.7789	0.8263	0.6632	0.8579	0.6632	0.6684	0.6993	0.6848	0.6632	0.7356
	f	-0.196	-0.269	-0.374	-0.200	-0.551	-0.176	-0.551	-0.134	-0.330	-0.333	-0.551	-0.377
	N_a	4	6	5	7	7	4	5	6	7	5	6	7
	A_r	3.796	5.243	4.961	6.660	6.568	3.768	4.768	5.905	6.117	4.797	5.600	5.817
Mac547	H_o	0.5000	0.8500	0.8000	1.0000	1.0000	1.0000	0.9000	0.9000	0.8333	0.9167	1.0000	0.8000
	H_e	0.6053	0.7782	0.7842	0.8263	0.8684	0.6579	0.7632	0.8474	0.8080	0.7790	0.8316	0.8230
	f	0.181	-0.069	-0.000	-0.200	-0.161	-0.565	-0.191	-0.073	0.005	-0.180	-0.216	0.029
	N_a	3	3	2	3	2	2	2	2	3	3	4	2
	A_r	2.768	2.658	1.800	3.968	2.000	1.800	1.800	2.000	2.660	2.667	4.568	2.000
Mac574	H_o	0.3000	0.4500	0.1000	0.6000	0.8000	0.1000	0.1000	0.4000	0.4167	0.9167	1.0000	0.8000
	H_e	0.2789	0.3756	0.1000	0.4842	0.5053	0.1000	0.1000	0.3368	0.3587	0.5543	0.6474	0.4966
	f	-0.080	-0.218	-0.000	0.143	-0.636	-0.000	-0.000	-0.200	-0.170	-0.704	-0.475	-0.647
	N_a	5	6	3	4	2	6	4	6	5	3	1	4
Mac62	A_r	4.568	4.485	3.000	3.600	2.000	5.400	3.965	5.565	4.537	2.797	1.000	3.430
	H_o	0.8000	0.9000	1.0000	1.0000	1.0000	0.8000	0.8000	1.0000	0.7500	0.3333	0.0000	0.6000
	H_e	0.6158	0.6295	0.6789	0.6158	0.5263	0.7789	0.7211	0.7579	0.6920	0.3043	0.0000	0.4874
	f	-0.321	-0.422	-0.512	-0.682	-1.000	-0.028	-0.116	-0.343	-0.088	-0.100	N.A.	-0.241
Average N_a		3.5	4.0	3.2	3.7	3.1	3.5	3.0	3.7	3.7	2.9	3.4	3.6
Average H_o		0.5385	0.5654	0.6462	0.6692	0.5615	0.5462	0.4923	0.5308	0.5705	0.4808	0.5077	0.7077
Average H_e		0.4947	0.5513	0.5441	0.5478	0.4344	0.5004	0.4028	0.5587	0.5817	0.4342	0.4579	0.5682

N -Sample Size; N_a -Observed number of alleles per locus; A_r -Allelic Richness per locus and population; H_o -Observed heterozygosity; H_e -Expected heterozygosity; *f* -Coefficient of inbreeding

on 8% non-denaturing polyacrylamide gels and visualized by autoradiography using Alpha imager (Alpha Innotech, USA) (Maniatis et al., 1982). The sizes of alleles were estimated with reference to pUC18DNA/*MSP1* sequence ladder (Nelson et al. 1998). The number of individuals exhibiting known homozygous allele sizes was included in other gels to ensure that the estimated allele sizes were consistent among the gels.

Data Analyses

Various indices of genetic diversity for populations, e.g. number of alleles (N_a), allele frequency, observed heterozygosity (H_o), expected heterozygosity (H_e) (Nei, 1987), were calculated with FSTAT Ver.2.9.3.2 (Goudet, 2002). Linkage disequilibrium between all pairs of loci was tested using the procedure implemented by GENEPOP Ver. 1.2 (Raymond & Rousset, 1995b). The samples were then tested for deviation from Hardy-Weinberg equilibrium (HWE) across each locus using the Markov-chain random walk algorithm employed by ARLEQUIN, Ver. 2.000 (Schneider et al., 2000). The statistical significance of deviations from HWE was adjusted using a sequential Bonferroni correction (Rice, 1989) to maintain a within-sample type-I error rate of α=0.05 for each locus. Inbreeding coefficient (*f*) and level of population subdivision per population over loci were estimated by unbiased F-statistics (Weir & Cockerham, 1984) with FSTAT Ver.2.9.3.2 (Goudet, 2002).

Genetic structure was inferred by calculating Weir and Cockerham's (Weir and Cockerham, 1984) estimator of Wright's F_{ST} for all pairwise comparisons between sampling locations. The significance of the estimates of F_{ST} was assessed using 10,000 permutations. Hierarchical partition of genetic diversity was assessed by analysis of molecular variance (AMOVA) (Excoffier et al., 1992) using ARLEQUIN, Ver. 2.000. The software also was employed to calculate pairwise estimates of F_{ST} values and testing their significance by bootstrapping analysis (1000 replicates) for evaluating genetic differentiation between populations. Bayesian clustering methods were also applied to examine population genetic structure by using STRUCTURE V.2.2 (Pritchard et al. 2000). To determine the number of populations (K) within the complete data set, three independent simulations for K = 1–12 with 100,000 burn in iterations and 100,0000 MCMC data iterations were run. The number of populations (K) was estimated using the method described by (Evanno et al. 2005). Exact tests for population differentiation (Raymond & Rousset, 1995a) were conducted using TFPGA Ver. 1.3. The same software was used to construct UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram based on Nei's unbiased distance. We conducted a multivariate ordination to visualize the genetic relationships among populations by principal component analysis using the software PCAGEN (J. Goudet, unpublished; http://www.unil.ch/izea/softwares/pcagen.html).

Results

Genetic Variability

All thirteen microsatellite markers were applicable and polymorphic using the 0.95 allele frequency criterion. The results of of the microsatellite diversity indices are provided in Table 1. The number of alleles per locus varied between 2 and 8 with an average of 4.6 alleles per locus. The lowest observed values for allelic diversity (*Na* = 1.0; *Ar* = 1.0) were found in the ESH and ZIG whereas the highest values were found for ZJG (*Na* = 7.0; *Ar* = 6.7), XGF (*Na* = 7.0; *Ar* = 6.6) and SZH (*Na* = 7.0; *Ar* = 6.5) (Table 1). However, for a given locus, differences in allele number and allele size were found between the present data set and previously reported for *M. anguillicaudatus* (Table 2). The test for genotypic disequilibrium for each pair of the thirteen microsatellite loci for over all populations gave three significant values (P <0.05) out of 78 comparisons. After Bonferroni correction for multiple tests, none of the locus-pair combinations demonstrated significant linkage disequilibrium. Observed heterozygosity varied from below 0.38 at two loci (*Mac40* and *Mac50*) up to a maximum of 0.95 at *Mac477*; over all loci the average observed and expected heterozygosity was 0.57 and 0.56, respectively. The expected heterozygosity (H_e) per population was between 0.402 for ESH and 0.581 for CHB, and the observed heterozygosity (H_o) ranged between 0.480 for WHN and 0.707 for XGN. The observed heterozygosity at loci *Mado21, Mac24 and Mac37*over all populations were lower than the corresponding expected heterozygosity. The inbreeding coefficient showed little pattern at all loci except *Mado21* and *Mac40* which were consistently high.

Population Genetic Structure

The pairwise estimates of F_{ST} indicated the presence of moderate-to-significant genetic differentiation between the loach populations (Table 3). The highest level of differentiation was found as 0.229 and 0.227 in population pairs of ESH-SZH and ESH-WHN, respectively while the lowest F_{ST} value of 0.0168 was observed for JZH-CHB. Unbiased genetic distance among pair of populations showed considerable variation in magnitude (Table 3) but most were significant (P<0.05). Exact test for fitness to HWE indicated that samples from JZH, XGF, SZH, ZJG, SHN, ZIG and XGN showed significant departures (P<0.05) over all the loci. The locus *Mac547* showed highly significant (P<0.001) deviations for all sampling localities. Eight loci

Table 2. Characteristics of the 13 microsatellite Loci

Locus name	Core Sequence	Primer	Annealing Temperature ^{0}C	Allele size (bp)	N	DDBJ Accession #
[a]*Mado11*	(GA)5N16(GA)6N14(GA)8	F: AATGGTCTTTCTAAAGGTTG R: CAACATACTGAATCGATGAA	51.7	137-157	6	AB260955
[a]*Mado21*	(GA)5(GAAA)2(GA)12	F: TCAGATTATAGCAGCTCACG R: GAGGGCTCATTCATACTCTC	58.0	111-157	4	AB260960
[a]*Mado31*	(GA)2AT(GA)5(AG)7	F: TCTCTTTAAGGACAGTGAGG R: CACAGCTGCTTATTAGTGAG	55.80	102-125	4	AB260964
[b]*Mac24*	(CA)11	F:CAGACTGATGCTCTGACGTT R:TCATAACAGCACATCAGCAA	58.0	107-123	3	AB060178
[b]*Mac37*	(CA)15	F:GCAAGTACATGCTCATCCTT R:CACCTGCATTCCTTACATCT	55.80	81-103	5	AB060181
[b]*Mac40*	(CA)6-AA-(CA)9	F:GGCCTGGTACTAAATCACAA R:ATTTTTGGGTCCCACTCCGC	60.0	108-145	4	AB060183
[b]*Mac44*	(CA)10	F:GCCACACAGTTAAACTATGC R:TTTTTCGCCTCAGCTGCTAT	55.80	93-98	2	AB060184
[b]*Mac50*	(CA)10	F:TTCTGGATTTACTGTATCCA R:TCATCTCCTCACTCGTGATA	56.0	81-96	3	AB060182
[c]*Mac462*	(CA)33	F: CACTCAACTTCCATTTCCTG R: GTTTGTTTCTCCAGCAGAAC	55.80	215-270	4	AB303524
[c]*Mac477*	(CA)33	F:GCTGAGACTCTTTATGTCTCAC R:GCTATCAAGGGAACTGAATGG	58.0	93-155	8	AB303575
[c]*Mac547*	(GT)33	F: AGTGCTTGGATGTGTGGTTC R: AGTTCATCAGGCTGCGTAAG	58.0	165-255	8	AB303590
[c]*Mac574*	(GT)56	F: AGTTCATGCCTCCAAAG R: GTTTTCAGGCAGACCAA	53.0	115-173	4	AB303594
[c]*Mac612*	(GT)49	F: TAGCCACTAGAAGATGCTGA R: ATGTTCAAACTACCAGCTGT	56.0	147-219	8	AB303607

[a]- (Arias-Rodriguez et al., 2007); [b]- (Morishima et al., 2001); [c]- (Morishima et al., 2008)
N- number of alleles.
F- forward.
R-reverse.

Table 3. Pairwise Measures of Nei's Unbiased Genetic distance (above diagonal) and population differentiation (F_{ST}) (below diagonal) among population of the loach

	XNT	JZH	HGS	XGF	SZH	ZJG	ESH	SHN	CHB	WHN	ZIG	XGN
XNT	-	0.0391	0.0979	0.1132	0.1620	0.1062	0.1502	0.1260	0.0902	0.1521	0.1729	0.0844
JZH	0.0127	-	0.0442	0.1120	0.1625	0.0781	0.1184	0.1224	0.0592	0.1242	0.1513	0.1174
HGS	0.0596	0.0208	-	0.1658	0.2634	0.1029	0.1576	0.1973	0.0572	0.2269	0.2809	0.1368
XGF	0.0892	0.0614	0.1041	-	0.0582	0.1508	0.2382	0.1138	0.1967	0.0886	0.1009	0.1762
SZH	0.1434	0.1179	0.1955	0.0494	-	0.2071	0.2467	0.1449	0.2068	0.0500	0.0595	0.2297
ZJG	0.0678	0.0402	0.0693	0.1048	0.1666	-	0.0805	0.1675	0.0923	0.2353	0.2391	0.1435
ESH	0.1329	0.0918	0.1371	0.1908	0.2299	0.0732	-	0.1722	0.1165	0.2456	0.2281	0.2332
SHN	0.0735	0.0616	0.1123	0.0931	0.1135	0.0947	0.1325	-	0.1817	0.1220	0.0897	0.1460
CHB	0.0385	0.0168	0.0200	0.0988	0.1396	0.0425	0.0891	0.0799	-	0.1668	0.2228	0.1280
WHN	0.1232	0.0908	0.1723	0.0627	0.0435	0.1782	0.2275	0.0944	0.1158	-	0.0327	0.1656
ZIG	0.1393	0.1046	0.1967	0.0829	0.0556	0.1784	0.2094	0.0652	0.1398	0.0229	-	0.1955
XGN	0.0524	0.0712	0.0873	0.1151	0.1645	0.0931	0.1784	0.0815	0.0680	0.1268	0.1389	-

(*Mado21, Mado31, Mac24, Mac37, Mac477, Mac574, Mac612*) exhibited significant ($P<0.05$) deviations for all populations while highly significant deviation was observed at locus *Mac574* ($P<0.001$). For AMOVA, populations were categorized into two major geographical groups; the Hanjiang sub-basin group and the middle mainstream sub-basin group. The components of variance as calculated by AMOVA revealed that 90.53% genetic variation accounted within populations and 9.03% among the populations within groups (Table 4). The global fixation indices were 0.09472, 0.09072 and 0.00443 for F_{ST}, F_{SC} and F_{CT}, respectively. Over all populations, mean gene flow (Nm) estimated from $F_{ST} = 0.25(1 - F_{ST})$ F_{ST} was 1.63.

The most likely number of genetic clusters was determined by STRUCTURE analysis using admixture and allele frequency correlated models. The ΔK value indicates that K equals three (K = 3) is the optimal number of genetic clusters (Figure 2). At K = 3, three populations of mainstream sub-basin group (JZH, HGS and CHB) and one population (SHN) of Hanjiang sub-basin revealed an admixed structure (Figure 3). Populations; XGF, SZH, WHN, and ZIG (Hanjiang sub-basin) were differentiated in one genetic cluster from the rest XNT, XGN, ZJG and

Table 4. Hierarchal AMOVA analysis of the sample populations

Source of variation	d.f.	Sum of Squares	Variance components	Percentage of variation
Among groups	1	13.564	0.01666 Va	0.44
Among populations within groups	10	111.889	0.33942 Vb	9.03
Within populations	266	905.208	3.40304 Vc	90.53
Total	277	1030.66	3.75912	

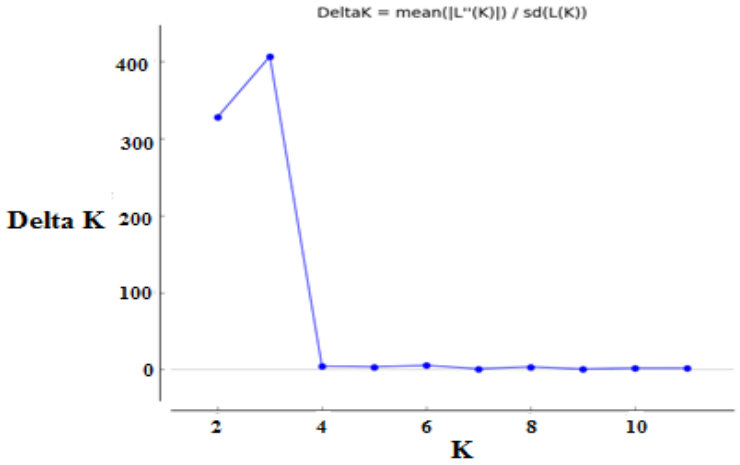

Figure 2. ΔK value estimated at the K ranged from 1 to 12.

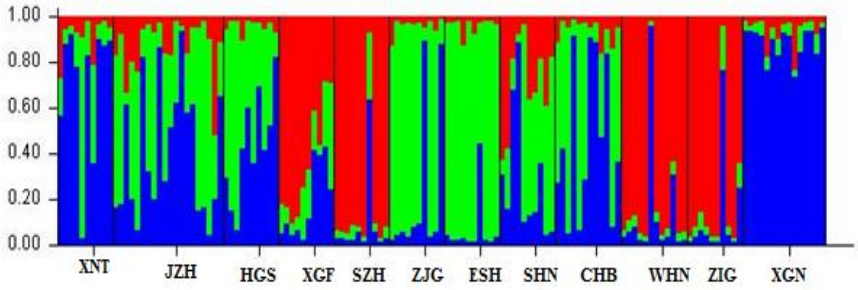

Figure 3. STRUCTURE analysis of 12 populations of Loach assigned to three genetic clusters (K = 3)

ESH (mainstream sub-basin) populations which showed two different genetic clusters. The pattern of principle component analysis was represented by a plot of scores based on allelic frequency from 12 loach populations (Figure 4). There were two informative axes in the principal component analysis (PCA), together explaining 53.5% of the total variance. The first principal component axis (PC1) accounted for 36.97% of the total genetic diversity, and the PC2 accounted for 16.53%. As shown in Fig.2, the PCA separated the populations into two groups: (1) Middle mainstream sub-basin group and (2) Hanjiang sub-basin group. The populations clustered following their demographic distribution except ZIG and XGN.

The UPGMA dendrogram depicting the underlying structure of the Nei,s distance matrix illustrates considerable differentiation between the populations and demonstrated two major clusters dividing the populations into two distinct geographic groups (Figure 5). It reveals that the observed genetic structure match with the current geographic configuration of populations except for the ZIG and XGN which clustered departing from their geographical origin.

Discussion

Despite intensive studies regarding atypical mode of reproduction, polyploidy and other genetic aspects of the species, data on population structure of Chinese dojo loach determined with microsatellite markers is deficient. The loci examined in the present study were originally characterized in Japanese strain but comparatively, our data showed differences in number and size of the alleles for the Chinese loach.

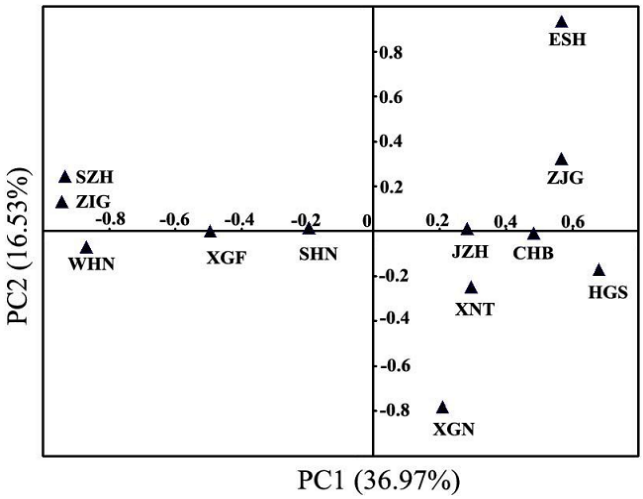

Figure 4. The principle component analysis showing the relationship between the loach populations sampled across two major sub-basin sectors of the Yangtze River

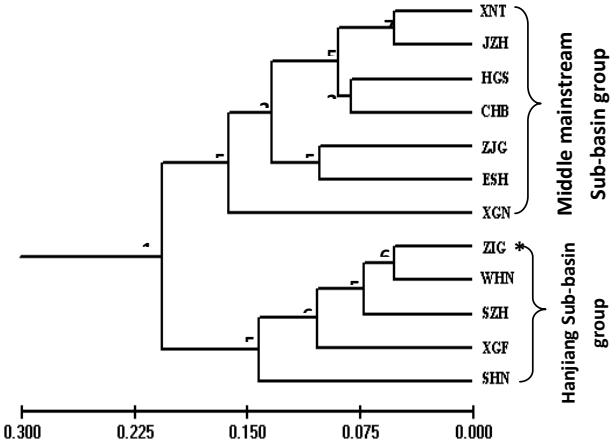

Figure 5. UPGMA dendrogram showing the genetic relationships among 12 dojo loach populations across middle- ower mainstream sub-basin and Hanjiang sub-basin of the Yangtze River; * the exceptional clustering of ZIG and GN populations, genetically departing from there geographical origin

Except for *Mac37, Mac40, Mac44 and Mac50*, all loci showed considerable variation in allele size while the allelic number was different in 50% loci. This discrepancy might have come out due to different strains and sampling. Compared with microsatellite diversity of Japanese strain (Arias-Rodriguez et al., 2007; Morishima et al., 2001; 2008), Chinese loach showed lower number of alleles at two loci, same at seven loci while higher at four loci used in present study (Table 2). With allozyme and RFLP markers, the Japanese loach showed mean heterozygosity values as 0.110 and 0.060, respectively (Khan &Arai, 2000; Khan et al., 2005). In the present study, moderate level of mean observed heterozygosity was found but it was significantly higher than that found in Japanese strain. Rare alleles were observed in a few microsatellite loci while most of the alleles were shared by all samples with various frequencies. However, the low-to-moderate observed heterozygosity may have reflected intra-population genetic variation of Chinese loach. The heterozygotes excess relative to HWE was observed in the JZH, XGF, SZH, ZJG, SHN, ZIG and XGN populations. Departures from HWE in natural populations of a wide range of fishes have been reported commonly (Castric et al., 2002; Yue et al., 2004). Such heterozygotes excess could be the possible outcome of. nonrandom sampling, (Castric et al., 2002) null alleles, a mixture of independent populations, non-random mating (Angel et al., 2006), fishing pressure (Bergh & Getz, 1989) or synergistic impact of aforementioned factors. The probability of Wahlund effect was rejected by the statistical analysis. There is no reason to expect that the observed variation in these microsatellite loci is a result of selective processes as the majority of microsatellite loci are

assumed to be selectively neutral. Fishing pressure and migration-drift disequilibrium cannot be denied, especially in face of reduced or no flooding in the Yangtze River after construction of dams (Fu et al., 2003). Due to hydrological alterations, Fu et al. (2003) expressed concern over spawning requirements of Chinese loach, likely to be affected by demographic changes. The construction of Gezhouba dam and three gorges dam (TGD) drastically reduced annual floods in central China with the immediate loss of habitat area and increased isolation of remaining habitat patches (Wu et al., 2008). The historic floods were causative agents of dispersal for nonmigratory aquatic species to mitigate the phenomena like genetic drift and bottleneck in isolated populations. The habitat fragmentation in the Yangtze River basin has rendered several other fish populations e.g. common carp and redfin culture, genetically differentiated with clear population structure (Wang et al., 2007, Liao et al., 2006). Draught is very common in loach habitats which, if prolonged, may leave disastrous effects on genetic structure of its populations. However, owing to cutaneous and intestinal respiration in species, loach populations are not so vulnerable to bottleneck and genetic drift as the fish can sustain life for about one month in mud (Park et al., 2003).

In the present study, significant genetic structuring was found among the studied populations. The F_{ST} = 0.299 was observed to be the highest value reported to date for the species. Pairwise comparisons (85%) of F_{ST} values showed significant differentiation among the populations (P<0.001). The population pairs (15%) with moderate values were interestingly located in same geographical sector which indicates ongoing restricted gene flow facilitated by unusual floodgate opening during flood season (Wang et al., 2006) or human mediated processes. Despite the frequent floods before the construction of dams which imply frequent gene flow among fragmented populations, the results suggest that dojo loach in the Yangtze River basin habitats are differentiated at a significant level. Our data reinforce the previously reported genetic differentiation between various populations of Japanese *M. anguillicaudatus* (Khan & Arai, 2000). Japanese loach was significantly differentiated into three geographical groups and this differentiation among different localities was suggested to be at inter-species or inter-subspecies level (Arai, 2003). In our study, maximum differentiation was demonstrated by ESH samples, particularly from those of WHN (middle mainstream sub-basin), SZH (Hanjiang Sub-basin) and ZIG. These results came up to the prediction based upon the demographic configuration of the populations. Geographical distance with physical and environmental barriers in between, may keep these populations reproductively isolated from each other. Moreover, reproductive habits of the loach, adaptability to local environment, nonmigratory behavior and sticky nature of eggs to local substrate (Tsui et al., 2004) limit the gene flow even between nearby populations of the loach. There were pronounced pairwise genetic distances between sampling locations. Nevertheless, higher genetic distances were observed between population pairs which are more geographically distantly located (for instance, between HGS and ZIG, WHN; between ESH and WHN, XGN). This agrees with population genetics theory which predicts that restricted gene flow would result in greater among-population divergence (Zattara & Premoli, 2005). Significant differentiation between ESH and ZIG in face of less geographic distance seems to be the outcome of bottleneck effect or genetic drift due to environmental interaction with local population. Genetic differentiation of local populations is caused, among other reasons, by physical barriers and limited dispersal (Johnson & Gaines, 1990), which restrict migration and hence limit gene flow (Barluenga & Meyer, 2005).

AMOVA results demonstrated that majority of the variance came from intra-population variations, and only 9.03% of total variance resulted from inter-population differentiation. Low but overall significant F_{ST} value (F_{ST} = 0.090, P < 0.001) showed a significant genetic structure for Chinese loach in middle-and-lower reaches of the Yangtze River basin. Based on Cyt b marker, Yang et al. (2009) reported no reproductive isolation among different populations in the middle reaches proximity of the Yangtze River basin. It is partially in line with our findings but not a reliable depiction of genetic structure of dojo loach due to drawbacks coherent with Cyt b marker for being single locus and uniparental with low rate of evolution (Birky et al., 1989). Moreover, small sample size, six individuals from each location, renders the interpretation doubtful.

The STRUCTURE and UPGMA analyses showed same pattern of clustering. The UPGMA dendrogram of the sample populations reveled clustering at the sub-basin level. There were two evident clusters, clearly dividing the examined populations into two groups following demographic pattern of distribution. The ZIG population, exceptionally, clustered with Hanjiang sub-basin group while XGN with middle mainstream sub-basin group, showing divergence from parent populations. The samples from ESH and ZJG clustered onto same branch. The within-group clustering pattern can logically be explained by limited dispersal either through anthropogenic intervention or occasional water currents in seasonal streams and canals across the populations within the sub-basin sector. On contrary, divergence between distant population groups of the Hanjiang sub-basin and middle mainstream sub-basin owed to hampered dispersal thus negating the gene flow across the whole middles reaches of the Yangtze River as reported by Yang et al. (2009).

Changing continuous habitat into several smaller spatially isolated remnants could alter both demographic and genetic factors (Tallmon et al., 2002). Despite claims about human-mediated fragmentation to be too recent to leave pronounced genetic effects (Sumner et al., 2004, Galeuchet et al., 2005), the impact of recent habitat fragmentation in terms of decreased genetic diversity and increased differentiation have been identified (Williams et al., 2003; Keller et al., 2001). Habitat fragmentation is expected to result in loss of genetic diversity in remnant patches through inbreeding or random process of genetic drift (Lande, 1999). Our results confirmed that fishes with limited dispersal capability are particularly vulnerable to the negative genetic impacts of habitat fragmentation (Tallmon et al., 2002).

A significant genetic structure for Chinese dojo loach from isolated populations across the Yangtze River basin was inferred from microsatellite markers. Due to historical genetic similarity, recent isolation of habitats (about 30 years), null alleles and sample sizes less than ideal may have lead the current population structure to not strictly comply with isolation by distance. However, the two major geographic sectors showed a spatial genetic structuring of loach following the current hydrographic configuration of the Yangtze River basin, clearly departing from panmixia. The spatial differentiation among the studied populations is suggestive of two distinct geographical groups of Chinese loach. The genetic diversity of Chinese loach populations in rapidly shrinking habitats needs be conserved through habitat management and genetic conservation strategies in isolated populations. In face of growing environmental perturbations, employment of highly polymorphic markers with exhaustive sampling over a wide geographic scale may disclose a fine-scale structure of loach populations.

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