Genetic Analysis of *Scleropages formosus* Golden Asian Arowana Using Microsatellite DNA

Xidong Mu

Pearl River Fisheries Research Institute, Chinese Fishery Academy of Sciences

Xilang, Fangcun district, Guangzhou 510380, China

Tel: 86-20-2217-5232   E-mail: muxd1019@126.com

Yinchang Hu (Corresponding author)

Pearl River Fisheries Research Institute, Chinese Fishery Academy of Sciences

Xilang, Fangcun district, Guangzhou 510380, China

Tel: 86-20-8161-6088   E-mail: huyc2@163.com

Xuejie Wang

Pearl River Fisheries Research Institute, Chinese Fishery Academy of Sciences

Xilang, Fangcun district, Guangzhou 510380, China

Tel: 86-20-8161-7952   E-mail: aqwang@163.com

Xiaohui Li

Pearl River Fisheries Research Institute, Chinese Fishery Academy of Sciences

Xilang, Fangcun district, Guangzhou, 510380, China

Tel: 86-20-2217-5232   E-mail: lxh152706@126.com

Hongmei Song

Pearl River Fisheries Research Institute, Chinese Fishery Academy of Sciences

Xilang, Fangcun district, Guangzhou, China 510380, China

Tel: 86-20-2217-5232   E-mail: shm1227@126.com

Jianren Luo

Pearl River Fisheries Research Institute, Chinese Fishery Academy of Sciences

Xilang, Fangcun district, Guangzhou, 510380, China

Tel: 86-20-8161-6133   E-mail: fishlo@163.net

Abstract

Asian arowana (*Scleropages formosus*) is a highly endangered fish species listed in Appendix 1 of CITES since1980.In order to explore the population genetic structure of golden Asian arowanas *Scleropages formosus* from the breeding population, of 26 fish sampled were genotyped at twenty microsatellite loci. The average allele number of 14 polymorphic microsatellites was 2.94 loci. The average observed heterozygosity was 0.446 ranging from 0.143 to 1.00, and the gene diversity was quite high (0.78).All these data suggest that middle level of genetic diversity existed in the golden Asian arowana population. Data showed the population somewhat departed from HWE, such as excessive and deficient heterozygote numbers.AMOVA analysis offered evidence of a weak genetic differentiation with 0.51% variation between samples.

Keywords: Asian arowana, Genetic diversity, Osteoglossidae

1. Introduction

Asian Arowana (*Scleropages formosus*), commonly known as the Dragonfish, belong to an ancient family of fishes, the Osteoglossidae, which literally means bony-tongue. Several types of *S. formosus* with different color patterns inhabit separate regions of Southeast Asia (Borneo,Sumatra,and Indochina) that were probably connected through freshwater habitats during the Pleistocene glacial ages(Goh,1999).It is acquired a special status in Japan and some East Asian countries as a very popular but extremely expensive aquarium fish. Due to its popularity and great demand, Asian Arowanas have been fiercely hunted in its native habitat for profits, leading to its inclusion among species threatened with extinction of the population of these fish in the wild listed in CITES appendix I since 1980(Dawes, 1999).However there has been not any systematic work or genetic assessments about Asian arowana population.

Due to their exceptional variability and relative ease of scoring, microsatellites are now generally considered the most powerful genetic markers. Microsatellites, or simple sequence repeats (SSR), have many attributes making them excellent for scientific studies, such as abundant polymorphisms, codominant heredity and easy detection. These features provide the foundation for their successful application in a wide range of fundamental and applied fields of biology and medicine, including forensics, molecular epidemiology,parasitology,population and conservation genetics, genetic mapping and genetic dissection of complex traits(Chistiakov,2006;Waldbieser,2001;Triantafyllidist,2002).In this study, the highly variable microsatellites of green Asian arowana provide a perspective on the diploid nuclear structure of golden arowana and provide reference for further genetic study of this species.

2. Material and methods

2.1 Samples and DNA isolation

Fin clips of twenty-sixth breeding adult(>six years) golden Asian arowanas samples from Guang zhou tiny-lake aquatic organism technology co.,ltd housed which were originated from Malaysia collected in 2001,were collected, and kept in absolute ethanol. Genomic DNA was extracted from a golden Asian arowana individual using a simple and cost-effective method (Yue, 2001).DNA was suspended to be used as the template DNA in polymerase chain reactions.

2.2 PCR amplification

Twenty microsatellite primers described (Yue,1999) were used to amplify the genomic DNA of golden Asian arowana.Details of all microsatellite loci and PCR condition are given (Note 1).PCR amplification for microsatellites was performed on a MWG-BIOTECH thermal cycler in a total volume of 20μl. Reactions contained 10×PCR buffer 2.0μl, MgCl$_2$ (25 mmol/L) 1.0μldNTPs (10mmol/L each) 0.4μl, each primer (10μmol/L) 1.0μl, *Taq* DNA polymerase 0.15U, and DNA template 50ng. Amplification was carried out using 4 min of initial denaturation followed by 30 cycles of 30s of denaturation at 94°C,30s annealing at the temperature detailed in table 1 and 30s extension at 72°C with a final extension period of 7 min at 72°C. PCR products were run on a 4% (w/v) 1xTBE horizontal metaphor agarose gel at 250 V for 2 hours.

2.3 Data analysis

The bands of electrophoretic patterns were ana1yzed using Alpha Ease FC soft and artificial adjustment. The number of alleles, efective number of alleles, observed heterozygosity (*Ho*), expected heterozygosity (*He*) was computed from the microsatellite genotype data using Pop-gene (version3.2) software. The Polymorphism Information Content (*PIC*) was calculated with the formula as follow (Botstein, 1980):

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2p_i^2 p_j^2$$

where n was the number of alleles at one locus; Pi and Pj were the frequencies of the ith and jth alleles at one locus, j=i+1. Departure from Hardy-Weinberg equilibrium (heterozygote deficiency or excess) was calculated for each locus

and each population with GENEPOP v.4.0 (Rousset, 2008) according to Weir and Cockerham's FIS (Weir, 1984), and was tested using the Markov chain method (10000 dememorization steps, 1000 batches, and 5000 iterations) to obtain unbiased estimates of the exact p value.Arlequin software version 3.11 was used to do analysis of molecular variance (AMOVA) (Excoffier, 1992).

3. Results

A total of 20 novel microsatellites were characterized using 26 golden Asian arowana individuals.17 primer pairs (85%) produced clear and stable bands from the fish after optimizing the PCR conditions,16(80%) were polymorphic. All others were abandoned as they were unable to produce specific, clear, stable or polymorphic bands (Note 3).For instance, SSRLY8 only produced one allele. The total number of alleles from the 16 primer pairs in golden Asian arowana was 47, ranging from 2 to 5 with locus D33,D38 and D72 being the mostpolymorphic with 5 alleles, and markersSSRLY1,SSRLY3,SSRLY4, SSRLY9, SSRLY9, SSRLY10,D42,D85 and D92 being the least polymorphic, displaying only two alleles. The average allele number of the 16 polymorphic markers was 2.94 loci.

Heterozygosity (*H*), or gene diversity, can highlight the genetic variations of many loci in a population. It is thus considered to be a suitable parameter to estimate the genetic variation of a population. The observed heterozygosity (*Ho*) ranged from 0.1429 to 1.00 (Note 2).Expected heterozygosity (*He*) varied between 0.1429 in D42 and 0.8242 in D72. Mean intrapopulation diversity was different among loci, both in terms of number of alleles per locus and heterozygosity values (Note 2).

The polymorphic information content (*PIC*) is an index for analysis of the polymorphism of an amplified product. The *PIC* of the sixteen loci in golden Asian arowana population ranged from 0.124 to 0.865, and the average *PIC* was 0.432.According to the protocol of Botstein (1980).The *PIC* was over 0.432 in all cases, except with SSRLY6, D33, D38, D72 and D85 (*PIC*>0.5), meaning that these locus were middling polymorphic and could be used to calculate the genetic diversity of golden Asian arowana population, which indicates medium genetic diversity in golden Asian arowana population.

Significant departures from Hardy-Weinberg equilibrium were tested using the parameter d. Heterozygote excess was found at loci SSRLY1, SSRLY2,SSRLY10,SSRLY11,SSRLY12,D33,D38,D42 and D92 in golden Asian arowana population.(*d*<0). (Note 2)

AMOVA analysis with Arlequin software showed faint differentiation with 0.51% variation in population. This could be caused by gene flow between populations, because few migrants per generation are sufficient to eliminate genetic evidence of stock structure, and that is common in marine species (Waples, 1998).

4. Discussion

Ne, *Ho*, *He* and *PIC* are parameters of genetic variations which were 2.94, 0.446, 0.4945 and 0.432, respectively.Data showed the levels of genetic diversity were relatively middle in the population, compared to previously published surveys of population variability. Both the average observed heterozygosity (*Ho*) and average expected heterozygosity (*He*) were 0.446, 0.4945 and was higher than the mean heterozygosity of 13 species of fresh water fish calculated (DeWoody, 2001). Meanwhile, our results were relatively lower to the report (Yue, 2006), which analyzed the genetic population structure of a red Asian Arowana population with microsatellite markers and found *H*e was 0.51~0.95, which suggest that high level of genetic diversity

The Hardy-Weinberg departure value (*d*) is a fixed index, describing the departure of a locus in a population from Hardy Weinberg equilibrium (HWE).It suggests the population is close to HWE when the value of *d* is closer to zero. *d*>0 means the population lacks heterozygotes whereas *d*<0 indicates heterozygote excess. The value ranges between -1 and 1.But there's no recognized standard. As shown (Note 2), nine loci with heterozygosity deficiency were deviated the HWE severely in gold population. Though Artificial selection, population substructuring,shortage of samples,mutation,low levels of polymorphism and inbreeding may lead to deviations from HWE,the presence of inbreeding caused by over-fishing and environmental could provide a reasonable explanation for the lack of agreement with HWE in the populations we analyzed. According to geological habitat that several of arowana strains diverged from late Pliocene to middle Pleistocene, gold Asian Arowana lived in Bukit Merah Lake of Malaysian and Sumatra of Indonesia (Dawes, 1999) and all the arowana strains are highly endemic to certain river systems and no hybridization could have occurred in hatcheries (Yue, 2000).On the other hand, Asian Arowana were fiercely hunted in its native habitat, causing declination of the population of these fish in the wild. So overfishing and habitat deterioration diminish the population size, and lead to reproductive isolation of unattached groups, which led to decreasing gene flow among the populations.

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Table 1. The amplification conditions and results of 20 microsatellite primers in *Scleropages formosus* Asian Arowana

Primer	Primer sequences(5`→3`)	Product size(bp)	Tm(°C)
SSRLY1	F:GAATGCTTAAAGTGGCAGTGAA R: TAACACAGGGCGTAAGGCCAG	174-216	55
SSRLY2	F:GCTTAAACCCATTACAGACAGG R:AAAGTGGTTTTGCATGAAGAAA	171-229	55
SSRLY3	F:AAGGGAGCAGCAGTTAGGTAGCAG R:AGAGGAAATGTTAATTCACCACGG	196-246	55
SSRLY4	F:GACTGGCGTCCCGTCCTG R:TATGCTCTTTCCCATTGACACTAA	224-256	57
SSRLY5	F:CTTGCGCCCTGTGTTGC R:TTACCAGCAGAAAAGGCCTT	127-176	57
SSRLY6	F:GTGTCAGTATAGTGAATCTGTAG R:ATCTCATTATGCTGCCATTGTCA	154-196	57
SSRLY7	F:GTTTGTCCCTCCATGCACTGAGAG R:CCAACAAAACCATGTGGCAATCAC	154-223	52
SSRLY8	F:AGCACCCTGTTACTGGAAGAGA R:TTCTCAAAGCAAAAGCATCACACT	236-292	55
SSRLY9	F:TATTACCATGCGCCCAGCACAC R:AGTCCTGCTTCTGGCTCACCCA	130-138	52
SSRLY10	F:AGCTGACACTTTGAAGCACT R:AAGAGTCGCTGAATTAGCAC	217-238	52
SSRLY11	F:CAGTGGTTGCACACTTACAG R:TATTTCATCATGCCGACTTT	194-244	55
SSRLY12	F:GTTTCTTCTAGGTGCTCTGGTTTC R:GGATGAGTGACCCAGTGTAAGTAG	223-294	55
D11	F:TGGTTTCCACCTACAGTCCAAAGA R:GTTACGAGTACTGGCCCAATGG	154-170	50
D33	F:GTTCTTCTAGGTGCCGCGTTTC R:CTACTTACACTGGGTCACTCATCC	190-216	50
D38	F:TTGGGGTCATGCCACTGG R:CAATAAATACCAAACAGGGAACC	179-216	53
D42	F:AGGAACATCACTGACAACACT R:TGGACTAACTAGGAGCACAT	145-201	50
D72	F:AGCAGGTTAATTTGGAGACT R:CGACCCTGTATGGGACAAG	105-144	55
D85	F:GTTCCACAGGGGCTGAGAAAAT R:GAGGACGGAACAAAAGCATTGG	140-166	50
D92	F:AGTCGCACACCACCACCTCAG R:TCAGCGATAACCCCACACCT	190-220	50
D94	F:CAGCAGCACTGACACGGGTTCG R:TCGCAGGCTGATTAAAGGTGTG	194-246	50

Table 2. Allele frequencies, heterozygosity and polymorphism information content

Locus	Allele frequencies					No.of allele	*Ho/He*	*PIC*	D-value
	P1	P2	P3	P4	P5				
SSRLY1	0.6429	0.357				2	0.7143 (0.4945)	0.353	-0.5556
SSRLY2	0.7857	0.142	0.071			3	0.4286 (0.3846)	0.332	-0.2000
SSRLY3	0.6429	0.357				2	0.4286 (0.4945)	0.354	0.0667
SSRLY4	0.7143	0.285				2	0.2857 (0.4396)	0.324	0.3000
SSRLY6	0.143	0.429	0.286	0.143		5	0.2857 (0.7473)	0.653	0.5882
SSRLY9	0.7143	0.286				2	0.2857 (0.4396)	0.325	0.3000
SSRLY10	0.7143	0.286				2	0.5714 (0.4396)	0.325	-0.4000
SSRLY11	0.7857	0.214				2	0.4286 (0.3626)	0.280	-0.2727
SSRLY12	0.071	0.071	0.857			3	0.2857 (0.2747)	0.247	-0.1200
D11	0.0714	0.785	0.143			3	0.1429 (0.3846)	0.326	0.6000
D33	0.0714	0.142	0.286	0.143	0.357	5	0.8571 (0.8022)	0.733	-0.1507
D38	0.0714	0.142	0.286	0.357	0.143	5	1.0000 (0.8022)	0.715	-0.3425
D42	0.0714	0.928				2	0.1429 (0.1429)	0.124	-0.0769
D72	0.0714	0.285	0.286	0.143	0.214	5	0.7143 (0.8242)	0.746	0.0667
D85	0.2143	0.285				2	0.2857 (0.4396)	0.865	0.3000
D92	0.8571	0.142				2	0.2857 (0.4396)	0.215	-0.1667
mean						2.94	0.446 (0.4945)	0.432	
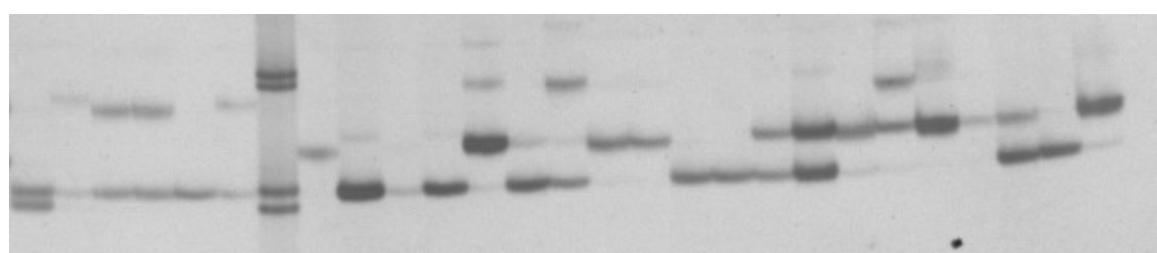
Figure 1. Electrophoregram of microsatellite primer D33 amplified in Golden Asian arowana