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Genetic Diversity of Farmed Chinese Soft-Shelled Turtle (*Pelodiscus sinensis*) Evaluated from Microsatellite Analysis

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Abstract: The Chinese soft shelled turtle (*Pelodiscus sinensis*) has been used as food and medicine for thousands of years in China. In the last 25 years, some large-scale *P. sinensis* farms have been established. However, little is known about the genetic variations in farmed broodstocks although, they are important in selective breeding. Researchers genotyped 336 individuals from seven stocks using eight polymorphic microsatellites to study genetic diversity and population structure of *P. sinensis*. In the seven stocks, a total of 120 alleles were detected at the eight loci. All seven stocks showed high allelic (A = 9.50-12.25; Ar = 9.25-12.50) and gene diversity (H_E = 0.73-0.79). But certain degree of inbreeding (inbreeding index franged from 0.07-0.23) was detected in all seven stocks. Both AMOVA and F_{ST} analysis revealed significant population structuring (F_{ST} = 0.114, $p<0.01$). The information about genetic diversity and population structure of the seven stocks would supply a basis for future genetic improvement of *P. sinensis* through selective and cross breeding.

Key words: Turtle, farm, diversity, breeding, broodstock, medicine, population

INTRODUCTION

The Chinese soft shelled turtle (*Pelodiscus sinensis*) belonging to the family Trionychidae is a freshwater turtle species (Bonin *et al*., 2006). It is found in China, Taiwan, Korea, Manchuria and North Vietnam (Hong *et al*., 2008) while its native range is not clear due to the long tradition of use as a food and subsequent spread by migrating people. It has been introduced to Japan, Malaysia, Singapore, Thailand, Timor, Batan islands, Guam, some of the Hawaiian islands and California (Brock, 1947; Kawagoshi *et al*., 2009; Haitao *et al*., 2008) and exchange of stocks among countries for culture also took place in past years.

Chinese people have used the Chinese soft shelled turtle as food and medicine for thousands of years (Hong *et al*., 2008; Haitao *et al*., 2008). In the last 25 years due to rapid increase of live standard, the demand for luxury items such as soft-shelled turtles has dramatically increased (Haitao *et al*., 2008). A corollary of this increased demand is that turtles command a high price and this has encouraged entrepreneurs to develop farms in China. Some large-scale freshwater turtle and tortoise farms in Guangxi, Guangdong and Hainan provinces were established in the 1980s and the number of new freshwater turtle farms increased steadily from 1990 onwards. In 2007, there were 1,499 officially recognized turtle farms in China (Haitao *et al*., 2008). According to a recent estimate, 303,076,700 and 124,849,800 individuals were cultured and sold, respectively in 2007. The revenue generated by selling the Chinese soft shelled turtle was 685,367,300 USD/year (Haitao *et al*., 2008). The farmed production is still increasing these years. However, it is difficult to know whether these productions are sustainable as there is no detailed breeding program, no any information about genetic diversity and population structure of any broodstock of soft-shelled turtles in China.

Microsatellites are an important tool for evaluating levels and patterns of genetic diversity (Goldstein and Schlotterer, 1999; Cai *et al*., 2011; Du *et al*., 2011; Yin *et al*., 2011) and have been used to study genetic diversity in a number of cultured fish species for breeding purposes (Liu and Cordes, 2004). Microsatellites have been extensively used to evaluate genetic diversity and structure of farmed food fish species such as grass carp, common carp (Bartfai *et al*., 2003), salmon (Norris *et al*., 1999), rainbow trout (Thrower *et al*., 2004), Asian seabass (Zhu *et al*., 2006), oyster (Carlsson *et al*., 2006) and tilapia (Rutten *et al*., 2004) leading to better understanding better management of hatchery and broodstocks.

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Although, a few microsatellites have been isolated from the Chinese soft shelled turtle (Li *et al*., 2010; Que *et al*., 2007), they have not been used to analyze genetic diversity and population structure yet. A few studies using dominant DNA markers such as RAPD and AFLP found that the genetic diversity of some populations was high (Liu *et al*., 2004). However, dominant DNA markers have their disadvantages in population genetic studies such as inconsistant polymorphic patterns (Liu and Cordes, 2004).

In this study, in order to obtain information about genetic variations and population structure of seven broodstocks of *P. sinensis* to start detailed breeding programs to improve growth performance and maintain genetic variation, researchers studied genetic diversity and population structure of these stocks using eight polymorphic microsatellites.

MATERIALS AND METHODS

Tissue samples and DNA extraction: Tissue samples of 336 adult turtle individuals from seven broodstocks were collected and stored in 75% ethanol until DNA extraction. Details about sample size, origin and breeding history are shown in Table 1. DNA was extracted using a method reported previously (Yue and Orban, 2005).

Genotyping of microsatellites: Eight primer pairs (Pse001-Pse007 and Pse010) were selected from published data (Li *et al*., 2010) to genotype 336 individuals from seven broodstocks. Primers labelled with a fluorescent dye (HEX, TET or 6-FAM) at the 5' end were used for PCR amplification. PCR was carried out on Eppendorf PCR machines. PCR reaction (25 μL) contained 20 ng of DNA, 1 unit of Taq polymerase (Roche), 1x Roche Taq PCR buffer containing 1.5 mM $MgCl_2$, 0.2 μM dNTPs and 200 nM of each primer. PCR was conducted using the following program: 2 min of denaturation at 94°C; 32 cycles of 30 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C and a final extension at 72°C for 10 min. PCR products were analyzed using an ABI3100 DNA sequencer (Applied Biosystems). Fragment sizes were analyzed with the GS 500 ROX standard using Software GeneMapper (Applied Biosystems).

Table 1: Detailed information about seven populations of the Chinese soft-shelled turtle used

Population	N	Origin	Breeding history
TH	44	Thaihu lake, China	Cultured F3 generation of 500 wild turtles collected from the lake in 1998
JP	52	Japan	F6 generation of 4000 cultured turtles imported from Japan in 1995 and 1997
TW	51	Taiwan, China	No record on breeding history
TD	45	Dongding lake, China	Cultured F5 generation of 300 wild turtles collected from the lake in 1999
WH	50	Yellow river, China	Directly collected from the wild in 2006
GX	46	Gianjiang river, China	Cultured F1 generation of 500 wild turtles collected from the river in 2007
WT	48	Qiantonjiang river, China	Cultured F1 generation of 300 wild turtles collected from the river in 2006

Data analysis: Allele number (A), allele frequencies, inbreeding index (f), observed (He) and expected (Ho) heterozygosities and pairwise linkage or Hardy-Weinberg disequilibrium were analyzed using GDA (Lewis and Zaykin, 2000). FSTAT 2.9.3 (Goudet, 1995) was applied to calculate Allelic Richness (A_R) which is a measure of allelic diversity to control for differences in the number of alleles among populations that differ in sample size. The calculation of F_{ST} and AMOVA was carried out using Arlequin 3.1 (Excoffier and Schneider, 2005). Phylogenetic trees were constructed using F_{ST} genetic distance based on Un-weighted Pair Group Methods with Arithmetic (UPGMA) averages, Neighbour-Joining (NJ) and Minimum Evolution (ME) Methods using Software MEGA 4.0 (Tamura *et al*., 2007).

Whether the seven stocks have experienced a recent bottleneck was tested under three Microsatellite Mutation Models TPM, IAM and SMM using Bottleneck 1.2.02 (Cornuet and Luikart, 1996). These methods implanted in Bottleneck test for the departure from mutation-drift equilibrium based on heterozygosity excess or deficiency. Allele frequency distribution of the microsatellite loci was also examined by using Bottleneck 1.2.02 for model shift which might indicate if a recent genetic bottleneck has occurred.

RESULTS

Microsatellites: All eight microsatellites were polymorphic with a total of 120 alleles detected in 336 individuals from the seven broodstocks (Table 2). The average allele number of the eight markers was 12/locus, ranging from six alleles for Pse006 to 23 alleles for Pse004. The expected heterozygosity was 0.84 ranging from 0.73 for Pse006-0.93 for Pse010 whereas the expected heterozygosity ranged from 0.57 for Pse006-0.73 for Pse004 with an average of 0.64.

Genetic variations in cultured and wild populations of the Chinese soft-shelled turtle: Table 2 shows Allelic diversity (*A* and *Ar*) gene diversity (H_O and H_E) and inbreeding index (f) of each locus in each of the seven broodstocks. Due to the slight difference of sample size in different stocks, researchers used Allelic richness (Ar) to compare allelic diversity in different stocks. All seven stocks showed high allelic and gene diversity. The broodstock TD showed the highest allelic richness (12.25) followed by TH (11.75), GX (11.37), WH (11.00), WT (10.87), JP (10.12) and TW (9.5). The expected

Table 2: Allele richness (Ar), observed (Ho) and expected (He) heterozygosity, inbreeding index (f) and p value for testing HWE (P) in seven broodstock of the Chinese soft-shell turtle*

Locus (n)	TH (44)	JP (52)	TW (51)	TD (45)	WH (50)	GX (46)	WT (48)
Pse001							
Ar	11.00	6.84	5.83	8.98	6.88	8.95	7.981
He	0.71	0.74	0.65	0.82	0.73	0.80	0.550
Ho	0.52	0.71	0.59	0.53	0.68	0.70	0.210
f	0.26	0.04	0.10	0.35	0.07	0.12	0.620
P	0.00	0.00	0.04	0.00	0.16	0.02	0.000
Pse002							
Ar	16.00	11.49	9.84	13.93	11.73	12.90	12.810
He	0.78	0.66	0.72	0.81	0.84	0.82	0.830
Ho	0.73	0.63	0.61	0.73	0.74	0.70	0.650
f	0.07	0.03	0.15	0.10	0.12	0.16	0.220
P	0.09	0.01	0.00	0.08	0.04	0.00	0.000
Pse003							
Ar	8.00	7.80	4.86	6.96	6.96	8.83	6.980
He	0.66	0.76	0.66	0.57	0.66	0.55	0.760
Ho	0.66	0.62	0.78	0.62	0.82	0.54	0.690
f	0.00	0.20	-0.20	-0.10	-0.25	0.01	0.090
P	1.00	0.17	0.07	0.08	0.46	0.18	0.470
Pse004							
Ar	12.00	12.47	12.54	20.88	11.72	19.82	14.740
He	0.88	0.85	0.84	0.93	0.83	0.93	0.880
Ho	0.70	0.73	0.65	0.78	0.70	0.80	0.780
f	0.20	0.14	0.23	0.16	0.16	0.13	0.120
P	0.00	0.03	0.02	0.79	0.41	0.01	0.000
Pse005							
Ar	1.00	5.00	7.71	12.93	14.49	14.82	6.830
He	0.57	0.65	0.75	0.78	0.71	0.85	0.650
Ho	0.45	0.33	0.53	0.62	0.56	0.97	0.330
f	0.21	0.50	0.30	0.21	0.22	-0.02	0.490
P	0.00	0.00	0.15	0.14	0.08	0.42	0.070
Pse006							
Ar	6.00	5.00	5.00	6.00	5.00	5.00	5.910
He	0.56	0.65	0.57	0.76	0.50	0.71	0.580
Ho	0.57	0.54	0.55	0.80	0.58	0.67	0.560
f	-0.01	0.17	0.04	-0.06	-0.16	0.05	0.320
P	0.19	0.02	0.88	0.96	0.76	0.00	0.540
Pse007							
Ar	12.00	12.62	11.68	8.95	12.86	7.94	11.750
He	0.74	0.89	0.82	0.73	0.86	0.66	0.810
Ho	0.73	0.71	0.71	0.56	0.82	0.56	0.600
f	0.01	0.20	0.14	0.25	0.04	0.15	0.260
P	0.62	0.00	0.00	0.00	0.01	0.09	0.020
Pse010							
Ar	18.00	17.44	16.51	18.91	11.79	11.95	18.620
He	0.91	0.90	0.83	0.93	0.89	0.83	0.830
Ho	0.86	0.60	0.53	0.76	0.70	0.63	0.750
f	0.06	0.34	0.37	0.19	0.21	0.24	0.100
P	0.02	0.00	0.00	0.00	0.00	0.00	0.080
Overall							
Ar	11.75	9.84	9.25	12.19	10.76	11.28	10.710
He	0.73	0.76	0.73	0.79	0.75	0.77	0.740
Ho	0.65	0.61	0.62	0.68	0.70	0.68	0.570
f	0.10	0.20	0.15	0.15	0.07	0.11	0.230
p<0.05	2/8	7/8	4/8	3/8	3/8	4/8	4/8

*Detailed information about populations in Table 1

heterozygosity was 0.73, 0.76, 0.73, 0.79, 0.75, 0.77 and 0.74 in stock TH, JP, TE, TD, WH, GX and WT, respectively. All stocks showed certain degree of inbreeding (f>0). The inbreeding index was highest (0.23) in the stock WT followed by JP (0.20), TW (0.15), TD (0.15), GX (0.11), TH (0.10) and WH (0.07). Deviation of HWE was seen at some loci in all stocks. Seven of eight loci were not conformed to HWE in JP sock, 4/8 in stock TW, GX and WT, 3/8 in TD and WH and 2/8 in TH. Bottleneck analysis revealed that no population displayed significant heterozygosity excess (p>0.05) suggesting no population has experienced a recent bottleneck.

Table 3: Pairwise genetic differentiation (F_{ST}) in seven broodstocks of the Chinese soft-shelled turtle*

Broodstocks	TH	JP	TW	TD	WH	GX	WT
TH	0.000	-	-	-	-	-	-
JP	0.147	0.000	-	-	-	-	-
TW	0.175	0.027	0.000	-	-	-	-
TD	0.128	0.132	0.116	0.000	-	-	-
WH	0.118	0.092	0.186	0.083	0.000	-	-
GX	0.144	0.140	0.126	0.053	0.093	0.000	-
WT	0.032	0.152	0.163	0.116	0.108	0.108	0

*All pairwise F_{ST} is statistically significant (p<0.05)

Table 4: Analysis of Molecular Variances (AMOVA) of microsatellites in seven broodstocks of the Chinese soft-shelled turtle*

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among stocks	6	243.02	0.391	11.4*
Within stocks	665	2018.25	2.035	88.6
Total	671	2261.29	3.426	100.0

*p<0.05

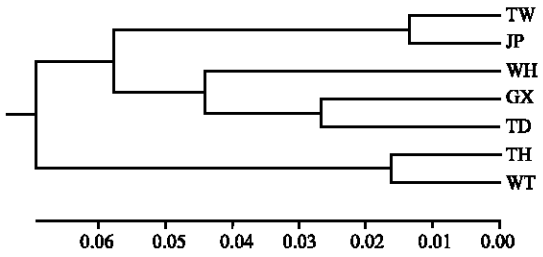

Fig. 1: A UPGMA-phylogenetic tree construed on the basis of microsatellite genotypes showing relationships among seven broodstocks of the Chinese soft-shelled turtle

Population structuring and relationships in the Chinese soft-shelled turtle: Table 3 shows the pairwise F_{ST} values in all seven broodstocks. F_{ST} values ranged from 0.032 (between TH and WT) to 0.186 (between TW and WH). The pair-wise genetic differentiation in all stocks was statistically significant (p<0.05). Phylogenetic trees were constructed using the F_{ST} distance matrix with UPGMA, NJ and ME.

Using all the three methods, the topology of the phylogenetic trees was identical. Figure 1 shows a tree based on UPGMA. TW stock and JP stock formed a cluster connecting a cluster formed by stocks WH, GX and TD whereas the stocks TH and WT formed another cluster. Analysis of Molecular Variances (AMOVA) of microsatellites revealed that the variation among stocks and within stocks was 11.4 and 88.6%, respectively (p<0.05) (Table 4).

DISCUSSION

Genetic variations in seven stocks: The average allele number (15 alleles/locus) per locus in the current study is high than that in freshwater fish species (Dewoody and Avise, 2000) and turtle species (Castellano *et al*., 2009; Escalona *et al*., 2009) suggesting high allelic diversity available in the seven *P. sinensis* broodstocks. However, allele number is related to the polymorphism of the markers used and the sample size (Goldstein and Schlotterer, 1999). Therefore, it is not a good parameter for indicating genetic diversity in broodstocks. Gene diversity (expected heterozygosity) is a better parameter for indicating genetic diversity.

In all 336 individuals in seven broodstocks, the gene diversity (0.84) was much higher that the average of freshwater fish and turtle species (Castellano *et al*., 2009; Dewoody and Avise, 2000; Escalona *et al*., 2009) indicating high genetic diversity exists in the seven broodstocks. In general, all brood stocks showed rather high allelic diversity (Ar = 9.25-12.25) and gene diversity (H_e = 0.73-0.79). The TD population showed the highest gene diversity followed by GX, JP, WH, WT, TH and TW cultured stocks. However, in a previous study on five populations of *P. sinensis* using RAPD (Liu *et al*., 2004) genetic diversity was highest in the TD wild population followed by TH and WH wild populations. This discrepancy may be caused by different nature of stocks (wild and cultured).

A number of previous studies on genetic diversity of wild and cultured populations of foodfish species showed that aquaculture influenced the genetic diversity substantially (Blake *et al*., 1997; Norris *et al*., 1999; Wang *et al*., 2008). Usually, the cultured stocks showed reduced genetic diversity as compared to wild stocks due to founder effect, genetic drift and inbreeding. An important finding of this study is that all the seven cultured stocks showed certain degree of inbreeding (f = 0.08-0.23) although, they contained high allelic diversity.

The high degree of inbreeding in the JP stock is understandable as this stock was established using eggs imported from Japan and it is already in F6 generation in captive breeding and the mating of related individuals is almost unavoidable in mass breeding (Taniguchi, 2003). While the high degree of inbreeding in the stock WT is somewhat surprise as it is only the F1 generation of wild caught turtles from Qiantanjiang river suggesting there might be some inbreeding in the wild stock in Qiantanjiang river or the founders of WT stock are genetically related.

Therefore, it is necessary to study the wild stock in Qiantanjiang river using microsatellites. The inbreeding in other five stocks might be related to founder effect, drift and selection pressure. Therefore, for future breeding of *P. sinensis* in each stock, the relatedness among individuals should be analyzed using polymorphic microsatellites. The genetic relatedness among individuals could be used to construct a phylogenetic tree to know the genetic relationships of all brooders (Yue *et al*., 2004). Such information is useful in setting up crosses among genetically unrelated individuals to avoid inbreeding.

Population relationships among seven soft-shelled turtle stocks: Both F_{ST} analysis and AMOVA showed significant genetic differentiation among the seven stocks. This kind of genetic differentiation is not a surprise as stocks were set up using wild caught turtles from different river systems. Geographic barriers among different river systems always hinder gene flow of freshwater turtles. Previous studies on freshwater turtle species showed that significant genetic structure between groups separated by distances of <30 km (Scribner *et al*., 1986).

Alternative explanations of the genetic differentiation among stocks involve genetic drift and selection pressure. In cultured populations due to small effective population size, random genetic drift and pressure of artificial selection, significant population structuring has been reported in a number of species (Hara and Sekino, 2003; Jackson *et al*., 2003; Norris *et al*., 1999; Wolfus *et al*., 1997; Xu *et al*., 2001). The close relationship between the JP and TW stocks could be explained by the recent exchange of genetic materials of *P. sinensis* between Japan and Taiwan (Sato, 2001) while the close genetic relationship between WT and TH as well as TD and GX may be related the close geographic distances. The close relationship between the stock WH and cluster of TD and GX can be not explained with the geographical distance. The WH stock was originated from the Yellow river which is >1000 km away from Gunaxi and Tongding lake where the stocks TD and GX were originated.

Therefore, human-mediated genetic exchange must be taken into account. In fact in the past years due to the rapid development of aquaculture of *P. sinensis* for food and medicine, exchange of genetic materials of *P. sinensis* among different locations took place frequently (Haitao *et al*., 2008). However, this kind of activity has not been recorded properly which may cause problems (mixed stocks from different populations) in breeding *P. sinensis*. Therefore, genotyping different stocks using DNA markers could help elucidate genetic relationships among stocks and facilitate to set up selective breeding

programs. The information of genetic relationships among populations is useful in setting up crosses between stocks to generated hybrid vigor. Certainly whether crosses between different stocks need to be testes by experiments.

Aquaculture of soft-shelled turtles for conservation of wild stocks: The consumption of *P. sinensis* was 124 millions of turtles/year in China in 2007 (Haitao *et al*., 2008). This increase has created a series of concerns relating to reduction of genetic variations of wild stocks. Catching of soft-shelled turtles from the wild is regarded as unsustainable and non-economical. Aquaculture and captive breeding have been successfully used for conservation of endangered species such as Asian arowana (Yue *et al*., 2004) and reduced the pressure of wild-caught individuals for human consumption. The studies revealed that genetic variation of 6 investigated stocks predisposes them to become a source of gametes for Chinese soft shell turtle aquaculture in addition, the stocks can be established as a source of stocking material for maintaining populations that are endangered.

CONCLUSION

Researchers have studied genetic diversity and population relationship in seven broodstocks of *P. sinensis* and demonstrated that that high allelic and gene diversity were available in these broodstocks. The high genetic diversity could be used in breeding programs, reducing the requirement of catching wild turtles for consumption. Thus, these broodstocks will also contribute to the conservation of wild stocks of *P. sinensis*.

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