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ISSR Markers as a Tool for Assessing Genetic Diversity in the Chinese Alligator (*Alligator sinensis*)

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Abstract Eight different inter simple sequence repeat (ISSR) markers were used as tools to investigate genetic variability and population differentiation in the Chinese alligator, *Alligator sinensis,* in this study. Eleven polymorphic bands (17.2%) out of a total of 64 were generated from 110 individuals in three populations. Analysis of molecular variation showed that most of the genetic variation (98.0%) occurred within the populations. Dendrogram relationship based on Nei's unbiased genetic diversity illustrated that two breeding populations were genetically closely related. The *Nm* value of the study was 4.520, suggesting that high levels of gene flow existed and no differentiation appeared in the populations. In a reconstructed Neighbor-Joining tree, the haplotypes coming from the same populations did not gather as a class, suggesting the three populations had no apparent geographic pattern. This study shows that ISSR markers could be well applied as a feasible tool to assess genetic diversity in Chinese alligator individuals.

Keywords inter simple sequence repeat (ISSR), genetic diversity, Chinese alligator, endangered species

1. Introduction

The Chinese alligator (*Alligator sinensis*) is a critically endangered species endemic to China and currently categorized as Accessory I in CITES (Convention on International Trade in Endangered Species). Genetic variability of this relict species is obviously essential for genetic management of the captive alligators and development of a release program. In 1999, the results from the research conducted at the Anhui Research Center of Chinese Alligator Reproduction (ARCCAR) indicated that inbreeding depression could occur in captive populations (Wu *et al*., 1999). The genetic status of wild and captive populations was studied using RAPDs (Wu *et al*., 2002), mtDNA D-loop sequencing (Wang *et al*., 2003), AFLP (Wang *et al*., 2006), microsatellite (Huang and Wang, 2004; Wu *et al*., 2007; Jing *et al*., 2009; Zhu *et al*., 2009), and the MHC gene (Shi *et al*., 2004; Liu *et al*., 2007). These studies displayed little genetic variation across populations, and failed to reveal enough information about genetic variation and population structure of these populations of this species. Therefore, more sensitive methods are required to reveal more polymorphic loci.

The first study employing inter simple sequence repeats (ISSRs) was published in 1994 (Zietkiewicz *et al.*, 1994). The techniques are nearly identical to RAPD techniques except that ISSR primer sequences are designed from microsatellite regions and it demands fewer experimental steps. This method provides genomic information for a range of applications, and it is widely used in population genetic studies (Behura, 2006). This technique also has been applied widely in plants, notably in the conservation of rare species (Kothera *et al*., 2007). It is clear that ISSR markers have great potential for studying natural populations (Wolfe *et al.*, 1998). In recent years, it has been used for some animals. Luque *et al*. (2002) showed that the amplification of ISSRs was possible and demonstrated their applicability in studying intra- and inter-specific variation in some Noctuid populations. Hundsdoerfer and Wink (2006)

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tested whether the distribution of phenotypes reflected a genealogical division of *Hyles tithymali tithymali* using ISSR-PCR. Seven different ISSR markers have been tested as a tool for population discrimination and genetic variations among *Plutella xylostella* (L.) populations (Roux *et al*., 2007). Hoffman *et al*. (2006) investigated the role of selection in the maintenance of a dorsal colour polymorphism in natural populations of the northern leopard frog, *Rana pipiens*. Maltagliati *et al*. (2006) used this technique to obtain species-specific molecular markers for the cyprinodontiform fish *Valencia hispanica*, *Valencia letourneuxi* and *Aphanius fasciatus*, with the aims to assess the effectiveness of ISSRs in discriminating the three species and to identify tissues of two unidentified fish suspected to belong to one of the three above species by comparing ISSR genotypes. Guicking *et al*. (2006) compared mitochondrial cytochrome b gene sequences and genomic ISSR-PCR fingerprints from Mallorcan and mainland European viperine snakes. Identical or nearly identical haplotypes and very similar ISSR-PCR profiles provided strong evidence that *Natrix maura* arrived only recently to Mallorca. A phylogeographic analysis of eight species complexes of European reptiles was performed using different molecular methods (mainly cytochrome b sequences and ISSR) (Joger *et al*., 2007).

Salima *et al*. (2009) used ISSR markers to study the genetic status of the crocodile, *Crocodylus acutus*. However, the technique has never been applied to the Chinese alligator, *A. sinensis*. In this study, we analyzed genetic diversity of Chinese alligator using ISSR markers, aiming to further assess the genetic diversity and obtain more DNA markers with higher resolution for genetic management of this alligator.

2. Materials and Methods

2.1 Samples A total of 110 individuals were sampled, of which 80 came from ARCCAR (referred to as XZ), 10 from another breeding population in Changxing County, Zhejiang (CX), and the rest from wild population (WP) (Table 1). Blood was collected without injury from a caudal vein by one-off injectors and was added directly to 1/7 volume of 0.5 M EDTA or ACD (including 0.48% citric acid, 1.32% citrate sodium and 1.47% glucose). The blood samples were preserved in liquid nitrogen until storage at -80°C (Wu *et al*., 2002; Wang *et al*., 2003). The blood samples of wild animals had been collected for many years before from field investigations.

2.2 DNA extraction DNA extraction followed a conventional phenol/chloroform procedure (Sambrook and Russel, 2001), and genomic DNA was dissolved with ddH_2O. The extracted DNA was examined on 1% agarose gels stained with 10 mg/ml ethidium bromide, and stored at -20°C for further use.

2.3 PCR procedure In this study, eight different primers (Table 2) were finally used for ISSR analysis. There were four primers coming from the study on *Crocodylus acutus* by Salima *et al*. (2009), and others coming from the study on *Trachidermus fasciatus* by Xu *et al*. (2009).

ISSR amplification was performed in a 25 μL volume containing 30 ng genome DNA, 2.5 μL 10 × PCR buffer (Sangon in Shanghai), 2 μL 25 mM $MgCl_2$ (Sangon in Shanghai), 1 μL 25 mM dNTP (Sangon in Shanghai), 2 μL 10 mM primer (synthesized by Genscript in Nanjing) and 1 U *Taq* DNA polymerase (Sangon in Shanghai). The amplification reaction consisted of an initial denaturation step at 94°C for 5 min, and then followed by 35 cycles at 94°C for 45 s, at 51–55°C for 45 s (primer annealing), and at 72°C for 1 min (primer extension). A final extension at 72°C for 10 min was incorporated, followed by cooling to 4°C until recovery of the samples. For electrophoresis, 5 μL amplified products mixed with 3 μL loading buffer were layered on a 1.5% agarose gel using 0.5 × TBE at 100 V for 1 h. The bands were detected with ethidium bromide (EB) under UV light (Bio-Vision 3000, Vilbert-Lourmat).

2.4 Statistic analysis ISSR amplified fragments, with the same mobility according to molecular weight (bp), were scored manually for band presence (1) or absence (0). Data recording followed the three principles: 1) Only the easily recognizable bands can be recorded, and the obscure bands are excluded; 2) the bands that cannot be precisely identified should be excluded; and 3) the bands with the same mobility but different intensity should not be treated as the same bands (Weising *et al*., 2005).

Genetic diversity within and among populations was measured as the percentage of polymorphic bands, Nei's gene diversity (Nei, 1973), Shannon's index, and Nei's unbiased genetic distance, all of which were measured suing program POPGENE, v. 1.32 (Yeh *et al*., 1999).

Analyses of molecular variance (AMOVA) were performed using Arlequin3.1 (Exeoffier *et al*., 2005) to assess genotypic variations across all the populations studied. These analyses, apart from partitioning of total genetic variation into within-group and among-group variation components, provided a measure of intergroup genetic distance as the proportion of the total variation residing between populations. Dendrogram by NJ (neighbor-joining) method was calculated through MEGA

v. 4 (Tamura *et al*., 2007).

3. Results

Samples from the three populations were amplified with the 8 selected ISSR primers. In total, 64 clear and stable bands were obtained after PCR amplification, of which 11 were polymorphic. The genetic diversity of the three populations is given in Table 3**.** Nei's (1973) gene diversity (*He*) and Shannon index (*I*) (Pearson correlation is 0.996, $P < 0.01$), and effective number of alleles (*Na*) and the percentage of polymorphic loci (*PPB*) (Pearson correlation is 1, $P < 0.01$), were basically positively correlated. The genetic diversity of WP was relatively high, and the *PPB*, *He* and *I* of WP were 17.2%, 0.056 and 0.085, respectively. Followed by XZ and CX, CX had the lowest genetic diversity among the populations, that is, $He = 0.008$, $I = 0.013$, $PPB = 3.1\%$.

Twenty haplotypes were defined from the 110 individuals, and their distribution among the three populations is shown in Table 4. Total gene diversity (*Ht*) and gene diversity within population (*Hs*) were 0.031 and 0.035, respectively. The coefficient of gene differentiation (*Gst*) among populations was 0.099, and generation number (*Nm*) was 4.520. The result indicates that 98.0% of the variation existed within a population ($P = 0.092$), and 2.0% of the variation among the populations (Table 5). Genetic distance between XZ and CX was the smallest (0.002), and the closest relationship between XZ and WP was 0.003, and that between WP and CX was the largest (0.009). WP showed the most diverse from the other two. The dendrogram based on genetic distance (Figure 1) showed that XZ and CX were clustered firstly, and then WP was clustered with them.

NJ trees constructed from 20 haplotypes are shown in Figure 2. The haplotypes coming from a same population, for example, XZ or WP, did not gather together. There were some haplotypes from the same population which were found having a relatively large genetic distance.

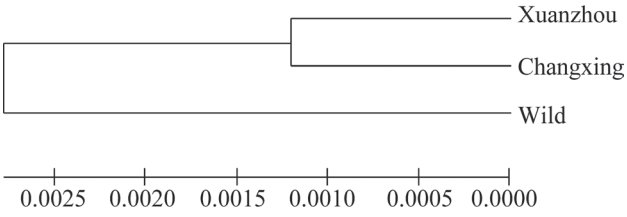

Figure 1 Dendrogram relationship of three populations of Chinese alligator.

Table 1 Samples of Chinese alligator used for this study.

Population	Population size	Sources	No.	Sample
Wild population (WP)	About 120 (Thorbjarnarson *et al*., 2002)	Xuanzhou, Anhui	20	Blood
Captive population in Xuanzhou (XZ)	About 10000 (Wu *et al*., 1999)	ARCCAR	80	Blood
Captive population in Changxing (CX)	About 450 (Xu *et al*., 2005)	Changxing, Zhejiang	10	Blood

Table 2 ISSR primers used in this study and some summary results.

ISSR No.	Primer sequence	No. of bands scored	No. of polymorphic bands	Percentage of polymorphic bands (*PPB*) (%)
ISSR1	CACACACACACACARY [a]	8	2	25
ISSR2	ACAACAACAACAACABDB [b]	7	1	14.3
ISSR3	BDBACAACAACAACAACA	11	3	27.3
ISSR4	GACAGACAGACAGACAWB [c]	9	1	11.1
ISSR5	ATGATGATGATGATGATG	8	1	12.5
ISSR6	AGAGAGAGAGAGAGAGGA	8	1	12.5
ISSR7	AGAGAGAGAGAGAGAGGT	6	1	16.7
ISSR8	AGAGAGAGAGAGAGAGCT	7	1	14.3

[a]: R:A/G; Y:T/C; [b]: B:C/G/T; D:A/G/T; [c]: W:A/T.

Table 3 Statistical analysis of genetic diversity of 3 populations.

Population	Observed No. of alleles (*Na*)	Effective No. of alleles (*Ne*)	Nei's (1973) gene diversity (*He*)	Shannon's information index (*I*)	No. of polymorphic bands	Percentage of polymorphic bands (*PPB*)
XZ	1.156 ± 0.366 [a]	1.040 ± 0.114	0.030 ± 0.083	0.050 ± 0.136	10	15.6
CX	1.031 ± 0.175	1.014 ± 0.096	0.008 ± 0.055	0.013 ± 0.082	2	3.1
WP	1.172 ± 0.380	1.093 ± 0.237	0.056 ± 0.138	0.085 ± 0.202	11	17.2
Total	1.172 ± 0.380	1.047 ± 0.127	0.034 ± 0.090	0.057 ± 0.145	11	17.2

[a]: Values behind the "±" are standard deviations.

Table 4 The distribution of haplotypes among 3 populations.

Haplotype	Populations		
	XZ	CX	WP
Hap1	65	8	6
Hap2	1		
Hap3	1		
Hap4	3		1
Hap5	2		
Hap6	1		
Hap7	2		2
Hap8	1		
Hap9	1	1	1
Hap10	1		1
Hap11	1		
Hap12	1		
Hap13		1	2
Hap14			1
Hap15			1
Hap16			1
Hap17			1
Hap18			1
Hap19			1
Hap20			1

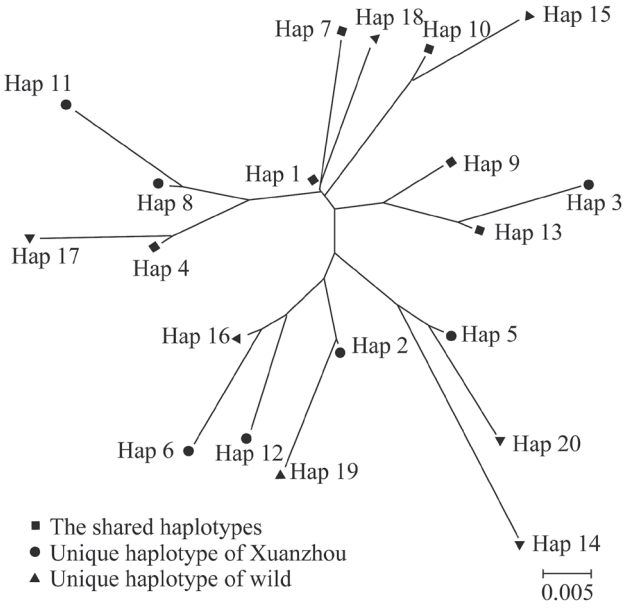

Figure 2 Neighbor-Joining (NJ) tree constructed from 20 haplotypes.

4. Discussion

Population fixation index (*Fst*) represents the genetic differentiation among populations, ranging from 0 to 1. The larger the *Fst* value, the higher the degree of differentiation among populations (Carlos *et al*., 2007). Gene flow (*Nm)* values were grouped into 3 categories: high (≥ 1.0), intermediate, and low (≤ 0.249) (Govindajaru, 1989). In this study, *Fst* was very low and *Nm* very high, indicating a lack of isolation among populations. Furthermore, genetic diversity across all the populations was revealed by AMOVA (Table 5), the variance components within population were higher than those among populations, and the difference among the three populations was not significant ($P > 0.05$). This indicated that the genetic difference did not occur within populations. The NJ tree of haplotypes (Figure 2) indicates that the three populations had not any apparent geographical pattern. The stock of two captive populations of Chinese alligator was recently coming from wild populations, which is only dated back from the 1970s and 1980s. So, each population had not yet reached significant levels of genetic isolation and differentiation.

We also compared the genetic diversity of the Chinese alligator populations based on different molecular markers (Table 6). The result showed that AFLP and microsatellite were better, but the step of these two methods was tedious and the cost was high. Compared

Table 5 Analysis of molecular variance (AMOVA) within/among populations of Chinese alligator from 3 populations using 8 ISSR markers.

Source of variation	Degrees of freedom (d. f.)	Sum of squares	Variance components	Percentage of variation	Fixation index (*Fst*)	*P* - value
Among populations	2	1.069	0.007Va	2		
Within populations	107	38.413	0.359Vb	98		
Total	109	39.482	0.366		0.02	0.092 ± 0.009

Va: Varinance components among populations; Vb: Varinance components within population.

Table 6 Comparison of genetic diversity of Chinese alligator based on some molecular markers.

	RAPD (Wu *et al*., 2002)	AFLP (Wang *et al*., 2006)	ISSR (This study)	Microsatellite
PPB (Percentage of polymorphic loci)	*PPB*	*PPB*	*PPB*	*PIC*
PIC (Polymorphism information content)	10.88%	58.50%	17.20%	32.7% (Huang and Wang, 2004) 40.7% (Zhu *et al*., 2009)
Average genetic similarity	0.9894 ± 0.0055	0.9195 ± 0.1011	0.9531 ± 0.0313	0.910 (Xu *et al*., 2005)
Fst		0.011	0.02	

to these above, our results using ISSR indicated that it is a sufficiently informative and powerful method to use ISSR for estimating the genetic diversity in the Chinese alligator. This study provides a significant insight into genetic diversity. The information and data on genetic variability obtained from this study might be a potential source for rejuvenation in Chinese alligator. Our results also confirm that the ISSR marker is a feasible tool for the assessment of genetic diversity in Chinese alligator.

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