ELSEVIER

Biological Conservation 106 (2002) 435–441

BIOLOGICAL CONSERVATION

www.elsevier.com/locate/biocon

Short communication

Genetic variation in captive population of chinese alligator, *Alligator sinensis*, revealed by random amplified polymorphic DNA (RAPD)

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Received 8 July 2001; received in revised form 10 September 2001; accepted 26 September 2001

Abstract

Chinese alligator (*Alligator sinensis*) is an endemic species in China. The likely extinction of it in the wild has been recognised. To prevent this species becoming extinct, the Anhui Research Centre of Chinese Alligator Reproduction (ARCCAR) was established in Xuanzhou, Anhui Province in 1979, where has been established the largest captive population of Chinese alligator (XZSP) in the world. Another farm (CXSP) was established by villagers in Changxin, Zhejiang Province. The results of an investigation of the two captive subpopulation structures by genetic analysis are presented in this paper. We examined the genetic variation in the two captive subpopulations using RAPDs. Thirty-one random primers were selected among 199 random primers screened. A total of 193 reproducible RAPD fragments were scored among 43 individuals, of which 21 (10.88%) were polymorphic. The genetic distances between 43 individuals ranged from 0 to 0.0376 with average of 0.0104 ± 0.0055 S.E. The genetic similarity in CXSP (0.9948 ± 0.0029 S.E.) was higher than that in XZSP (0.9894 ± 0.0055 S.E.). The founder effect is a possible explanation for very low genetic variation in CXSP. Analysis of the RAPD data showed that the mean phenotypic band frequencies of each polymorphic loci was 0.6656 ± 0.3730 S.E. The lowest phenotypic band frequency (0.0233) was found in four of those polymorphic loci. There was no genetic difference between the two subpopulations ($D_{ij} = 0.0009$). According to the dendrogram and the distribution of polymorphic fragments in two subpopulations, CXSP originated genetically from XZSP. This paper summarises a preliminary research on genetic structure in populations of Chinese alligator. Although there is higher genetic similary (0.9896 ± 0.0055 S.E.) in captive population of *A. sinensis*, we did not determine whether or not loss of genetic variation had occurred in relation to a wild control population. The data of malformed offspring will be collected carefully, and wild samples be added to set up a control population in future study. © 2002 Published by Elsevier Science Ltd.

Keywords: *Alligator sinensis*; RAPD; Genetic variation; Ex sito conservation

1. Introduction

A major concern among conservation biologists is loss of genetic diversity in small or captive populations through genetic drift and inbreeding (Lande, 1988). Because of their restricted population size, inbreeding is virtually unavoidable in captive populations (Pray et al., 1994). It was reported by Ralls and Ballou (1983) that juvenile mortality increased dramatically with inbreeding in 41 of the 44 species surveyed.

Chinese alligator is one of the most endangered of 23 species of crocodiles. Only a single population, of no more than 200 individuals, remains in the wild at Xuanzhou, Anhui Province in China (Thorbjarnarson and Wang, 1999). A continuing decline of population size is inferred based on the influence of human activities (Huang, 1982; Huang et al., 1986; Chen, 1990). To save this species, captive propagation programs were established, which consist of two captive subpopulations at Xuanzhou and Changxin breeding farms. The first crocodile farm, The Anhui Research center of Chinese alligator Reproduction (ARCCAR), is the largest captive subpopulation of this alligator (XZSP), and was

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0006-3207/02/$ - see front matter © 2002 Published by Elsevier Science Ltd.
PII: S0006-3207(01)00249-X

set up at Xuanzhou, Anhui Province in 1979. Two hundred and twelve alligators were brought from the wild as foundation stock for ARCCAR, and subsequently raised in a natural pond (Webb and Vernon, 1992), where the survivous (about 60–70 individuals) remain today. From then on, about 9000 individuals including first (F_1) and second filial generation (F_2) were bred in ARCCAR (Wu and Chen et al., 1999). Another captive subpopulation (CXSP) was established at Changxin, Zhejiang Province. No alligators had been found living in the wild there since 1982. However, four parental alligators from the wild produced 150–200 individuals in this breeding farm (Webb and Vernon, 1992).

The molecular approach has proved an increasingly valuable tool in the identification of animal genetic variation. Genetic markers, such as random amplified polymorphic DNA (RAPD), can be applied to rapid testing and assessing genetic structure of many individuals in a population (Gordon, 1998; Neveu et al., 1998), owing to relatively cheapness, limited quantities of DNA and simple method of acquiring data on variation in genomic DNA (Welsh and McClelland, 1990; Hadrys et al., 1992), Although RAPDs may result in unreproducible amplified fragments (Ellsworth et al., 1993), it has been also widely used to detect the genetic variation of animals, such as the black tiger prawn (*Penaeus monodon*) (Tassanskajon et al., 1997), guppy fish (*Poecilia reticulata*) (Foo et al., 1995), Blanding's turtle (*Emydoidea blandingii*) (Mockford et al., 1999), guinea fowl (*Numida meleagris*) (Sharma et al., 1998), koalas (*Phascolarctos cinereus*) in both captive and wild population (Fowler et al., 1998a,b). Sika deer (*Cervus nippon*) (Tamate et al., 1995), and so on, because the reproducible majority of RAPD bands could be acquired by following a strictly standardized protocol (Hadrys et al., 1992; Cooper, 2000).

Although captive propagation has continued for more than 20 years, genetic information on Chinese alligators has not been clarified. This paper is a preliminary research on genetic structure in the population of Chinese alligator. The purpose of the present study is to detemine genetic variation between two captive subpopulations and within subpopulations of Chinese alligator.

2. Materials and methods

2.1. Samples

Forty-three individuals were studied. Thirty-three of them were from Xuanzhou subpopulation (XZSP) including three generations: parent generation, P ($n=3$). F_1 ($n=15$) and F_2 ($n=15$). Parent generations from the wild were reared in two isolated rearing ponds. But only three samples were involved. The other 10 samples were from another breeding colony in Changxin (CXSP).

2.2. Sample collection and genomic DNA extraction

Blood was collected without injury from a caudal vein by one-off injectors and was added directly to 1/7 volume of 0.5M EDTA or ACD (including 0.48% citric acid, 1.32% citrate sodium and 1.47% glucose). These were preserved in liquid nitrogen until storage at -80 °C. Genomic DNA was isolated from red cells by lysis buffer and standard proteinase K digestion, followed by phenol/chloroform extraction and ethanol precipitation.

2.3. RAPD-PCR and primers screening

PCR amplifications had final concentrations of 50 mM KCl, 10 mM Tris–HCl pH 8.3, 2 mM $MgCl_2$, 100 μM of each dNTP,15 ng random primer, 1 unit *Taq* DNA polymerase and 25–50 ng DNA templates. Thermocycling parameters were 95 °C for 2 min, followed by 40 cycles of 94 °C for 1 min, 37 °C for 1 min and 72 °C for 2 min. After amplification, the PCR products were separated in 1.5% agarose TBE gels containing 0.5 μg/ml ethidium bromide at 1.5V/cm for 3 h to ensure even migration of the samples and standards. Following electrophoresis, the gels were scanned with GDS-7600 Gel Documentation System. Samples with the same primer were run on a single gel to reduce between-gel variance in the interpretation of the results.

Two samples of each subpopulations (total four samples) were used to screen all 199 random 10-mer primers in order to test amplification profiles for readability and reproducibility. Thirty-five primers producing reproducible bands were chosen to obtain RAPD profiles for all individuals, and repeatability test samples were included in each amplification reaction. The distinct RAPD products of polymorphic primers were electrophoresed twice to ensure that bands were not artefactual. The bands that were not reproducible were excluded from the analyses. Not all individuals were amplified clear bands in four primers. So, 31 primers were used to analyse. Amplification products were scored as discrete, binary states (present/absent) for each individual. The faint and undistinguished bands by eyes were ignored as genetic markers.

2.4. Statistical analysis

DNA fragments generated by RAPD were scored as discrete characters. The presence of fragment is "1", and the absence of fragment is "0". We determined coefficients of similarity (S) from profiles of individual RAPD DNA bands. Similarity between individuals is calculated as:

$$S_{(x,y)} = 2Nxy/(Nx + Ny)$$

(Nei and Li, 1979)

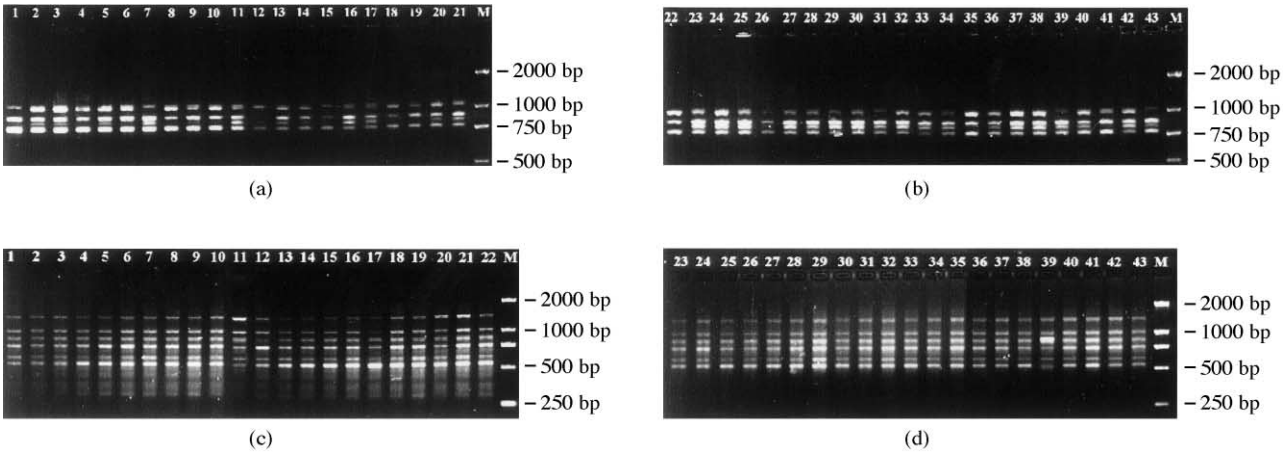

Fig. 1. The fingerprints generated by primer H12 (above) and F04 (below). 1–3: individuals of parental generation from wild in XZSP; 4–22, 33–43: individuals of filial generation in XZSP; 23–32: individuals in CXSP.

In the above formula, N_x and N_y, respectively, stand for the number of RAPD bands in x and y individuals, and N_{xy} is the number of bands shared by both individuals. The similarity index was caculated using Spss 8.0. Based on values of $S_{(x,y)}$, genetic distances (D) between individuls can be defined as:

$$D = 1 - S_{(x,y)}$$

The results can be analysed by MEGA, and the dendrogram is constructed by UPGMA. Mean bandsharing similarity indices both between individuals within a subpopulation (S_x or S_y) and between subpopulations (S_{xy}) were calculated for all possible comparisons according to the method of Lynch (1991). Similarity value between the two subpopulations was estimated transformed to account for the within-subpopulation variation using the formula:

$$S = S_{xy}/\sqrt{S_{xx} \times S_{yy}}$$

Similarity value was then converted to distance with the formula

$$D_{xy} = -\ln S$$

3. Results

3.1. The polymorphic analysis among 43 individuals

It was clear that almost all of the alligators shared a high percentage of fragments for each of the RAPD-PCR amplification. Although 31 selected random primers produced 193 bands in total, only eight of these selected primers produced 21 polymorphic bands from 43 individuals analysed (Fig. 1). One hundred and eighty-nine bands were identified in CXSP. The percentage of polymorphic bands was 10.88%. The phenotypic band frequencies of polymorphic loci were listed in Table 1, and ranged from 0.0233 to 0.9767 at an average of 0.6656 ± 0.3730 with the lowest at 0.0233 in combined population. The other 172 bands were monomorphic with the percentage of 89.12. The genetic distances between 43 individuals ranged from 0 to 0.0376 at an average of 0.0104 ± 0.0055 S.E.

The dendrogram of 43 individuals was showed as Fig. 2. In the dendrogram, CXSP individuals were not clustered closely together, however, there were a number of cases, in which CXSP and XZSP animals were clustered together, such as C9906, C9909, X9929 and X9931; C9910 and Xf29920; C9904 and X9933.

3.2. The polymorphic analysis and genetic distance between two subpopulations

Only four primers produced six polymorphic bands among 10 individuals in CXSP, and all the eight primers produced 21 bands in XZSP. The percentage of polymorphic bands was separately 3.11 and 10.88% in the two subpopulations. The average dominant frequences of polymorphic bands was 0.7000 ± 0.2098 S.E. (ranged from 0.4000 to 0.9000) in CXSP, and 0.6479 ± 0.3716 (ranged from 0.0303 to 0.9697) in XZSP. The genetic distance between the two subpopulations is only 0.0009 (Table 1).

4. Discussion

4.1. Between-subpopulation variation

Although XZSP and CXSP are separated, the RAPD data presented here reveal no genetic differentiation between them ($D_{ij} = 0.0009$). This may be attributed to

Table 1
The phenotypic band frequencies of polymorphic loci in separate subpopulations and combined population

Primers	Total bands	Polymorphic bands	The phenotypic band frequencies of polymorphic loci (Fr) in subpopualtions		
			Fr in XZSP	Fr in CXSP	Fr in combined population
G04	2	G04-1	0.8788	0.9000	0.8837
G17	5	G17-1	0.0303	–	0.0233
		G17-2	0.9697	–	0.9767
		G17-3	0.0303	–	0.0233
		G17-4	0.0303	–	0.0233
H03	7	H03-1	0.7879	–	0.8372
H12	4	H12-1	0.6667	–	0.7442
H18	13	H18-1	0.8182	0.7000	0.7907
		H18-2	0.9394	–	0.9535
J01	5	J01-1	0.0303	–	0.0233
		J01-2	0.8788	0.9000	0.8837
		J01-3	0.6970	0.8000	0.7209
M04	5	M04-1	0.9697	–	0.9767
		M04-2	0.9697	–	0.9767
		M04-3	0.9697	–	0.9767
		M04-4	0.9697	–	0.9767
N14	8	N14-1	0.9697	–	0.9767
		N14-2	0.9394	–	0.9535
		N14-3	0.3030	0.5000	0.3488
		N14-4	0.2727	0.4000	0.3023
		N14-5	0.4848	–	0.6047
Average	6.125±3.314	2.6250±1.5980	0.6479±0.3716	0.7000±0.2098	0.6656±0.3730

recent isolation from each other. Surveys in the 1950s indicated that the range of Chinese alligator shrunk to the area of junction of Jiangxi, Anhui and Zhejiang Provinces (Chen, 1990; Webb and Vernon, 1992). Gene flow may exist between XZSP and CXSP. In the dendrogram (Fig. 2), all the individuals from CXSP were divided into several clusters mixed with individuals from XZSP instead of forming a isolated cluster. Consequently, CXSP should be considered a part of XZSP. The historic gene flow was confirmed between the two subpopulations. This indicated that there was genetically only one population of Chinese alligator in the world, and that CXSP originated genetically from XZSP. The percentage of polymorphic sites in XZSP (10.88%) is higher than that in CXSP (3.11%). Of all 21 polymorphic loci, six loci exist in both CXSP and XZSP. Furthermore, four polymorphic loci with the lowest dominant frequency (0.0303) were all existed in XZSP.

The loss of population genetic variation is related to the number of founders (Neveu et al., 1998; Rave et al., 1994). The genetic similarity in CXSP (0.9948± 0.0029 S.E.) were higher than that in XZSP (0.9894± 0.0055 S.E.). The CXSP was established with only four wild adults as foundation stocks by local villagers (Webb and Vernon, 1992; only three adults were documented by Wang, 1998, and far smaller than XZSP.

4.2. Within-subpopulation variation

Low genetic variation often is the basis for a number of traits, such as high disease susceptibility and low reproductive success, which result in "species vulnerability" (O'Brien et al., 1985; O'Brien, 1994; Wildt et al., 1987). The fertility and viability of offspring in XZSP from 1993 to 1998 was analyzed by the authors (Wu et al., 1999), but the clutch size was not compared in that paper. The results showed that the mean fertilization rate in the parent generation (P) and the first filial generation (F_1) was 75.66±2.29 S.E.% and 69.24±5.12 S.E.%, respectively, with significant difference ($P<0.01$), and the mean rate of offspring malformed in P and F_1 was 1.85±0.95% and 0.30±0.20%, respectively, with significant difference ($P<0.01$). But the rate of hatching was not significantly different between them. Many factors affect breeding performance of captive animal, such as limited food, environment pollution, and inbreeding (Leonard and Gerber, 1988; Ford and Seigel, 1989; Madsen et al., 1996). Pesticides could also have played a role in the reproductive failure observed in American alligators (Guillette et al., 1996, 1999; Semenza et al., 1997). Lemly (1997) documented a close parallel between metalloid selenium concentrations, incidence of teratogenic deformities in fishes, and magnitude of reproductive failure. Wang and Xie (1997) did not detect high concentration of metal and metalloid selement

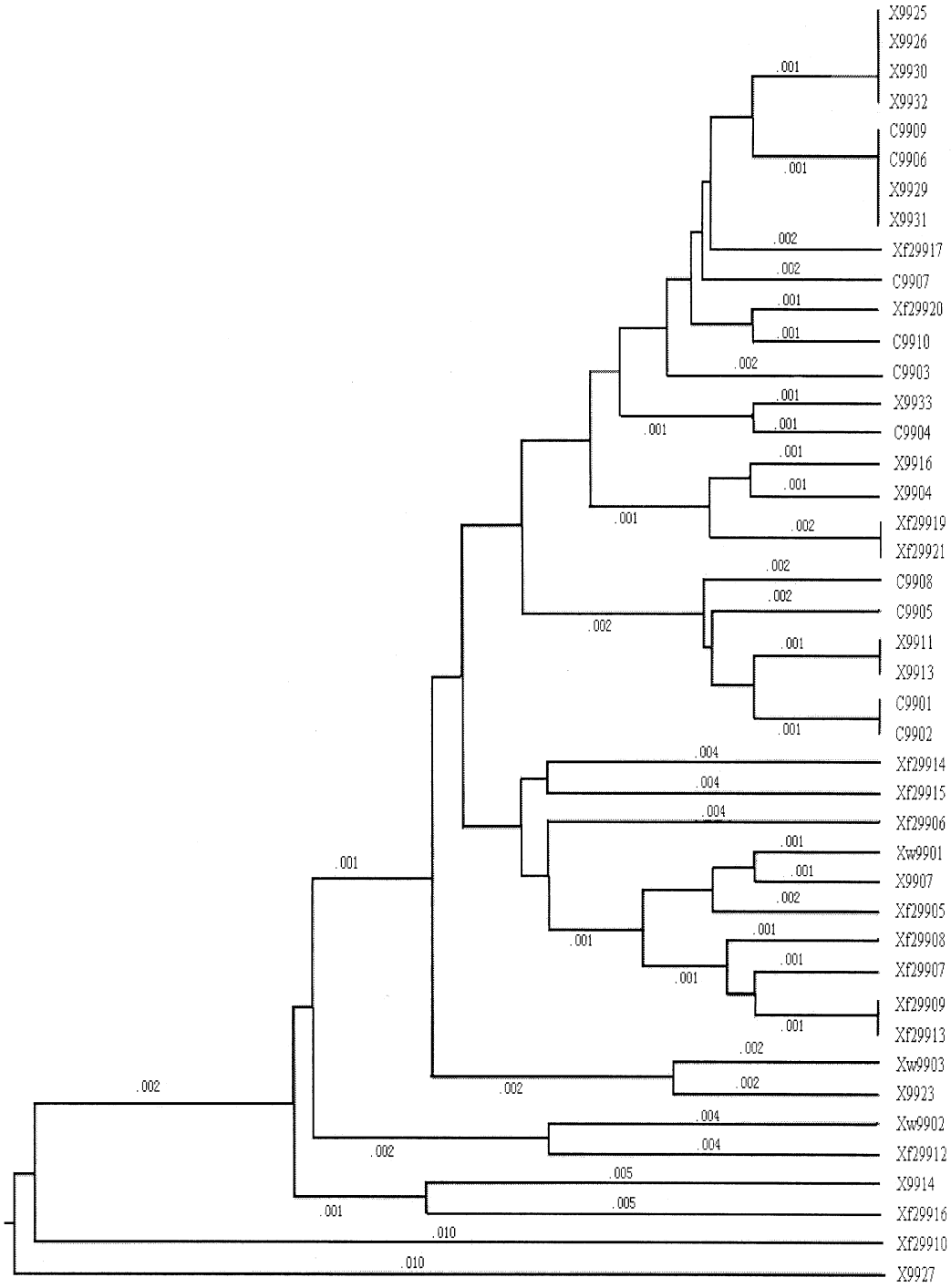

Fig. 2. The dendrogram among 43 individuals constructed by UPGMA.

contaminants in the meat of Chinese alligators. Pesticides were forbidden in ARCCAR. Inbreeding depression is a real phenomenon, especially in captivity. For example, O'Brien et al., (1985) reported that inbreeding caused higher inviability of offspring in mammals (e.g. cheetahs, *Acinonyx jubatus*). Madsen et al. (1996) suggested that the lower reproductive output and viability of snakes (*Vipera berus*) in the isolated population resulted from inbreeding depression. Genetic factors may be one of the reasons for deformities in Chinese alligators, although we can not define which factors affected the reproduction of Chinese alligator.

A control population is very important in conservation genetics, especially when measuring the genetic variation of an endangered population (O'Brien et al., 1985; Sherwin et al., 1991). There are very high genetic

similarities among individuals in XZSP (0.9894 ± 0.0055 S.E.). But we have insufficient information to confirm the loss of genetic variation in captive populations. The number of Chinese alligators is less than 200 in the wild (Thorbjarnarson and Wang, 1999), and it is very difficult to obtain enough wild samples to serve as a control series. In this paper, the three wild samples is insufficient for the analysis of population. CXSP can be considered as a control to XZSP. Owing to the lack of historical records in the rate of malformed offsprings in CXSP, we could not evaluate the relationship between the number of malformed offsprings and genetic variation.

This paper is an account of a preliminary research on genetic structure in population of Chinese alligator. The RAPD data will be useful in determining ESU status of the two alligator subpopulations, but it is important to also analyse mtDNA and microsatellite (Wenink et al., 1994). So, the conclusions here are tentative until mtDNA and microsatellite analyses are performed, and the advice for managers should be given accordingly.

Acknowledgements

The authors greatly appreciate two referees for critical review of early version of this paper. We thank Professor Chen Bihui for his good opinion. This study was sponsored by Jiangsu "333" project programme, Anhui Natural Sciences fund (No. 99042419) and Anhui Special Fund For Training Discipline Leaders.

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