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ORIGINAL ARTICLE

Screening and application of microsatellite markers for genetic diversity analysis of Oriental White Stork (*Ciconia boyciana*)

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Abstract The Oriental White Stork (*Ciconia boyciana*) is a large and endangered waterbird in East Asia. Research on conservation genetics of this species is urgently needed. In this study, microsatellite marking technology was used for screening and analysis of genetic diversity of microsatellite markers in Oriental White Storks. A total of 36 pairs of microsatellite primers were used, of which 7 pairs came from *Ciconia ciconia*, 12 pairs from *Nipponia nippon* and 17 pairs from *Ardea herodias*. Microsatellite loci were screened from 23 individuals of the Oriental White Stork and 11 microsatellite loci were found with high polymorphism. The number of population alleles ranged from 3 to 11, averaging 7.09. The average expected heterozygosity (H_e) was 0.7816 and the average polymorphism information content (PIC) 0.7172, suggesting a relatively high genetic diversity in the population.

Keywords microsatellite molecular marker, *Ciconia boyciana*, genetic diversity

Introduction

The Oriental White Stork (*Ciconia boyciana*) is a large and endangered waterbird, extensively found in East Asian countries. As a result of hunting, environmental pollution, habitat fragmentation and other causes, its breeding area is constantly shrinking. In the 1970s, wild breeding populations were extinct in Japan and in both South Korea and North Korea (Collar et al., 2001). In recent years, breeding pairs have been found in succession in the lower and middle Yangtze River floodplain and the Yellow River Delta (Yang et al., 2007; Xue et al., 2010) and their breeding area is expanding southward. In breeding and wintering areas of Russia and China, population protection and habitat restoration are underway, while in the historic breeding areas of South Korea and Japan, reintroduction plans are in progress. Research on conservation genetics of the population is very urgent.

At present, research on conservation genetics of the Oriental White Stork is not thorough enough and is confined to preliminary research using mitochondrial markers (Murata et al., 2004; Zan et al., 2008a, 2008b). Therefore, more approaches to molecular markers are required. Use of microsatellite markers, characterized by extensive distribution, dominant inheritance, high morphism and their capacity in detecting slight variations, is an extremely valuable approach to study the genetic background of this population (Tauta, 1989; Zane et al., 2002). The screening of microsatellite primers which has been applied to the Wood Stork (*Mycteria americana*) can also be used with microsatellite markers in research on genetic diversity of the Oriental White Stork (Zhang et al., 2010). However, only two loci can be screened with this method and one pair shows moderate polymorphism, thus restricting research on the genetic structure of this bird. In this study, the conservatism of the flanking sequence of a microsatellite locus was brought into use and a cross-species amplification method was employed in the screening of microsatellite markers of the Oriental White Stork. These microsatellite markers will be very useful for studies of population genetics and genetic conservation.

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Materials and methods

Sample collection

A total of 23 samples of Oriental White Stork were collected, of which 22 were from wild birds and one from a captive-bred bird (from Hefei Wildlife Park, with parental generation from the Chinese Alligator Nature Reserve of Anhui). Four feather samples were contour feathers of fledglings, three muscle samples were taken from dead birds and sixteen blood samples were taken from wing veins (Table 1). These samples were preserved with dehydrated alcohol (99%) and placed in a refrigerator at a temperature of –20°C.

Extraction of genomic DNA

The feather roots with pulp from the fledglings were cut into pieces after being washed with double distilled water; 0.2 g of muscles or 5 μL of blood were taken. The samples were then placed in a 2 mL centrifuge tube and 500 μL of the following solution was added: 10 mmol·L^{-1} Tris-HCl, 100 mmol·L^{-1} EDTA, 150 mmol·L^{-1} NaCl and 0.8% SDS, followed by the addition of K 40 μg·mL^{-1} proteases. Genomic DNA was extracted using the classical phenol-chloroform extraction method (Sambrook et al., 1989). The products obtained were supplemented with 50 μL of TE (pH 8.0) and cryopreserved at –20°C after electrophoresis with a 1% agarose-Ethidium Bromide (EB) gel.

PCR amplification of microsatellite markers

We synthesized 36 pairs of microsatellite primers, of which 7 pairs came from *Ciconia ciconia* (Shephard et al., 2009), 12 pairs from *Nipponia nippon* (He et al., 2005) and 17 pairs from *Ardea herodias* (McGuire and Noor, 2002). The synthesis of all the primer pairs was entrusted to the Shanghai Bioengineering Co., Ltd.

The gross volume of the PCR was 25 μL. The reaction system used was as follows: 100 ng of a DNA template, 0.4 μmol·L^{-1} per primer, 1.5 mmol·L^{-1} $MgCl_2$, 0.2 mmol·L^{-1} dNTP and 0.8 U Taq enzyme. The PCR proceeded on an Eppendorf thermal cycler. The settings of the reaction cycle were as follows: the products were pre-degenerated at 95°C for 15 min, de-natured at 94°C for 30 s, annealed at 54–58°C for 90 s, extended at 72°C for 60 s and after 40 cycles, the products were extended at 72°C for 10 min. The reaction was terminated at 4°C. The PCR products were detected by 1% agarose-EB gel electrophoresis. In this way, effective SSR primer pairs were selected.

Genotyping

Primers with stable reaction results were synthesized into fluorescence primers marked with FAM, HEX and TAMRA which were then subjected to another PCR. The genotyping of reaction products was carried out by the BGI Life Tech Co., Ltd.

Data analysis

Interpretation of microsatellite data after genotyping was performed using GeneMarker1.85 software and the data were integrated in an Excel table. Format transformation was conducted using the Excel Microsatellite Toolkit 3.1.1 software (http://animalgenomics.ucd.ie/sdepark/ms-

Table 1 Samples for microsatellite analysis of Oriental White Storks

Collection site	Sampling time	Number	Source description
Xuzhou Zoo	2006	5	Tongshan (blood samples)
Jinan Zoo	2006	2	Breeding individuals from the Yellow River Delta N.N.R. in Dongying, Shandong Province (blood samples)
Shenjin Lake National Nature Reserve (N.N.R.)	2006	1	Shengjin Lake (blood samples)
Nanjing Hongshan Forest Zoo	2006	2	Shengjin Lake N.N.R. (blood samples)
Hefei Wildlife Park	2010	2	Tianchang City (1 muscle sample), Hefei City (1 muscle sample)
Yellow River Delta N.N.R.	2010–2011	10	Breeding individuals from the Yellow River Delta N.N.R. (6 blood samples, 4 feather samples)
Yancheng N.N.R.	2010	1	Breeding individuals from Yancheng N.N.R., Jiangsu Province (muscle samples)

toolkit/). The number of alleles (N_a), polymorphism information content (PIC), expected heterozygosity (H_e) and observed heterozygosity (H_o) of each locus were obtained and the mean number of alleles (MNA), average apparent heterozygosity (H_{obs}) and the degree of unbiased genetic diversity (H_{exp}) of each locus were calculated. The probability (p value) of each locus was calculated using GENEPOP version 3.4 (Raymond and Rousset, 2000), in order to check if each locus satisfied the Hardy-Weinberg balance.

Results

Screening of microsatellite loci

The results of PCR amplification of 36 pairs of microsatellite primers on DNA of 23 samples showed that 12 pairs of primers did not have amplification products, of which 5 pairs of primers came from *C. ciconia* and 6 pairs from *A. herodias*. Stable products could be amplified from the remaining 24 pairs of primers. Random sampling was conducted and PCR repeated tests were performed using the 24 pairs of primers. After the typing results were interpreted, 11 pairs of polymorphism microsatellite primers were screened (Table 2). Among these 11 pairs, 5 pairs were from *C. ciconia* and 6 pairs from *A. herodias*.

Polymorphism of microsatellite loci

From 11 microsatellite loci a total of 79 alleles were detected in the Oriental White Stork (Table 3). Of these, a maximum of 11 alleles were detected at locus Ah343, while a minimum of 3 alleles was detected at loci Ah210 and Ah 211. All 11 loci were polymorphic (PIC > 0.5), with the lowest mean polymorphism information of 0.5556. Expected heterozygosity ranged from 0.6376 (Cc07) to 0.8848 (Ah341), while the observed heterozygosity ranged from 0.2000 (Cc02) to 0.9474 (Ah341). At locus Ah341, the expected heterozygosity was 0.8848, the observed heterozygosity 0.9474 and the polymorphism information 0.8467; each of these was a maximum value. Loci with p values larger than 0.05 were Cc01, Cc05, Cc06, Ah208, Ah211 and Ah341, all of which were balanced. The only locus with a p value smaller than 0.05 but larger than 0.01 was the unbalanced Cc07. Loci Cc02, Ah209, Ah210 and

Table 2 Primer information of 11 microsatellite loci

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)
Cc01	F: TTCTTGCATTTGCTCCAGTG R: CACAAACATCAGCAAGGACAG	$(TTCT)_{16}$*	54
Cc02	F: CGCTCGCTGTCTTTATCTCC R: CTGCTGCATGCCAGTTGAT	$(AC)_3 + (AC)_3 + (AC)_{10}$	57
Cc05	F: GGAGGAATTTCAGCAATGGA R: TGGGAAACCAGGAAACTGTC	$(CA)_9$*	54
Cc06	F: CTCGCTGTCTCCTCTGCTCT R: GAACAGCAATATCGCATCTACA	$(TG)_{13}$	53
Cc07	F: GCATGAAAATGCATAGAGCAGA R: CCACCGTTATGATCCTTTGG	$(AAAG)_{10}$	57
Ah208	F: GCTAATAACACCCAGTGTGGACC R: GACCCTGTACATACACTTCTAAAACCC	$(CA)_{10}$	58
Ah209	F: GAAACACATCAGTGCAAGAGCAG R: AGTTAAGGAACAAATGTTTGGAAGGAATG	$(AC)_{16}$	58
Ah210	F: ACGGGAACGTTTCAAAAATTTAAGATGTG R: ACGTTTCTATGGCTCAGAAACTGG	$(CA)_{11}$	58
Ah211	F: GCTCATCAGGAGTTGAATCTGGC R: TCTGTCATTCAGCAATGGACC	$(CA)_{13}$	56
Ah341	F: GGTAATGATTCTGATTTACCACTGAGGG R: ATGTGTTATCATATCTGGTCTTCACAGC	$(AC)_{12}$	58
Ah343	F: CATTGCTTAACTTCTGAAGAAAC R: CTTGACCCAGCATTTGTGAATAAAACTG	$(AC)_{17}$	58

Notes: "*" represents an interruption of this repeating unit by one nucleotide, while "+" in the repeating unit represents the linking of two repeating units by several random nucleotides. T_a means annealing temperature.

Table 3 Genetic indices of 11 microsatellite loci of Oriental White Stork populations

Locus	Allele size range (bp)	N_a	H_e	H_o	PIC	p
Cc01	167–205	8	0.8148	0.9286	0.7588	0.1237
Cc02	146–156	5	0.7789	0.2000	0.6918	0.0003
Cc05	152–212	10	0.8369	0.8462	0.7810	0.6573
Cc06	182–210	8	0.8115	0.7000	0.7648	0.0790
Cc07	201–207	4	0.6376	0.3571	0.5556	0.0103
Ah208	116–158	8	0.8734	0.9412	0.8307	0.0963
Ah209	145–181	10	0.7678	0.8667	0.7062	0.0000
Ah210	122–150	3	0.6690	0.7333	0.5724	0.0002
Ah 211	94–114	3	0.6578	0.8235	0.5629	0.1254
Ah341	178–204	9	0.8848	0.9474	0.8467	0.3442
Ah343	137–183	11	0.8651	0.7143	0.8185	0.0041
All		7.1818	0.7816	0.7325	0.7172	

N_a, the number of alleles, refers to the number of alleles of each locus; H_e, expected heterozygosity; H_o, observation heterozygosity; PIC, polymorphism information content; p, probabilities of Hardy-Weinberg balance tests, representing Hardy-Weinberg balance conditions; $p < 0.05$ indicates a significant deviation from the Hardy-Weinberg balance condition.

Ah343 with p values smaller than 0.01, were extremely unbalanced.

Genetic diversity of the population

From the data analysis of genotyping 23 Oriental White Storks we arrived at the following results about diversity: the mean number of alleles (MNA) of the population was 7.09 ± 3.02; H_{obs} was 0.7326 ± 0.0341; H_{exp} was 0.7816 ± 0.0270; PIC was 0.7172.

Discussion

Analysis of interspecific microsatellites

The flanking sequence of the two ends of the microsatellites suggests relatively excellent conservatism in the case of close genetic relationships between species, making the acquisition of microsatellite loci through cross-species amplification possible. The closer the genetic relationship, the greater the probability to acquire microsatellite loci, else the lower the success rate of cross-species amplification (Primmer et al., 1996, 2005; Barbar et al., 2007). Compared with other methods for screening microsatellite primers, cross-species amplification is charaterized by convenience, speed and stable amplification of targeted DNA and can be developed and applied rapidly. One pair of primers with 35 microsatellite loci was designed by selecting one EST microsatellite sequence and a two-end flanking sequence of Zebra Finch (*Taeniopygia guttata*) homologous with Domestic Chickens (*Gallus gallus domesticus*). Among the 35 loci, 33 are suitable for research on 17 species of passerine birds, while 99% of the microsatellite loci are suitable for research on five species of non-passerine birds. The genotyping results of four birds of each species showed that microsatellite loci registered polymorphism in 24–76% (average 48%) of passerine birds, while the polymorphism was greatly reduced in non-passerine birds, at only 18–26% (average 21%) (Dawson et al., 2010). Thirty-six pairs of microsatellite primers from *N. nippon, A. herodias* and *C. ciconia* were used in the PCR of a DNA template extracted from Oriental White Storks, of which 24 pairs could amplify clear products and 11 pairs showed polymorphism, with a success rate of 30.6%. Of these, five pairs were from *C. ciconia* with a success rate of 71.4% and six pairs from *A. herodias* with a success rate of 35.3%. *N. nippon* did not show any polymorphism. Before the 1990s, the Oriental White Stork was recognized as a subspecies of *C. ciconia,* but it is now an independent species (Kyoko, l991). Throughout our study, we realized that the closer the genetic relationship in the cross-species amplification using microsatellite, the higher the success rate of the acquisition of microsatellite loci.

Analysis of genetic diversity of population

For the Hardy-Weinberg balance to be satisfied, the population must be an ideal population (no immigration and emigration of individuals; random mating). In the analysis

of genetic diversity of the population, the genotype distribution of microsatellite loci is generally checked to see if it complies with the requirements of the Hardy-Weinberg balance. Eleven pairs of microsatellite markers, screened with Oriental White Storks, were used to perform the Hardy-Weinberg balance check on 23 birds. Locus Cc07 deviated significantly from the Hardy-Weinberg balance. Loci Cc02, Ah209, Ah210 and Ah343 also deviated significantly from the Hardy-Weinberg balance. Small populations, inbreeding, mutation of alleles, null alleles and migration were important factors leading to the deviation from the Hardy-Weinberg balance (Hartl and Clark, 1997).

MNA, H_o and H_e were the three major indices for the measurement of genetic diversity of the population and they typified the degree of mutation of the genetic structure. MNA was susceptible to sample size (Maudet et al., 2002). The average MNA was 7.09 ± 3.02, the average H_{exp} 0.7816 ± 0.0270 and the average H_{obs} 0.7326 ± 0.0341, suggesting relatively low genetic consistency, high heterozygosity and profound genetic diversity. These indices indicate that the population, up till now, possessed relatively high evolutionary potential.

Botstein et al. (1980) were of the opinion that when the polymorphism information content (PIC) of the gene locus > 0.5, it is a gene locus with high polymorphism; with PIC between 0.25 and 0.5, the gene locus has moderate polymorphism and when PIC < 0.25, polymorphism is low. The lowest PIC of a gene locus was 0.5556 in our study and all 11 gene loci were highly polymorphic. The PIC of Oriental White Storks was 0.7172, suggesting high genetic diversity.

Although for reasons such as habitat fragmentation and low reproductive rate the Oriental White Stork was believed to be an endangered species, our study shows that this species is still able to maintain a high level of genetic diversity, compared with other endangered or vulnerable birds such as the *Nipponia nippon* (PIC = 0.3230) and the *Otis tarda dybowskii* (PIC = 0.5497) (Tian et al., 2006; He and Fang, 2007). Gene flow in the population might contribute to high genetic diversity in the Oriental White Stork. Some birds of the Chinese population had migrated to Japan and historically, genetic exchanges may have occurred between the Chinese and Japanese populations (Zan et al., 2008a). In addition, progress in protection measures, such as reducing human disturbance and habitat restoration, may have resulted in a slight increase in the population and a gradual expansion of the breeding region may have been able to maintain the stability of the breeding population and its high level of genetic diversity (Marcia et al., 2005).

Eleven pairs of Oriental White Stork microsatellite primers were screened in this study, which provides an effective tool for the analysis of genetic mutation and genetic structure of the Oriental White Stork at the level of nuclear genes. The size of our sample of Oriental White Storks was relatively small in relation to the number of microsatellite markers suitable for testing large samples, which might reduce the accuracy of data analysis for genotyping. The sample size needs to be expanded and more microsatellite markers should be screened in future studies, which will enable a more thorough analysis of genetic diversity of the Oriental White Stork population.

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东方白鹳微卫星遗传标记的筛选及其在种群遗传分析中的应用

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摘要：东方白鹳（*Ciconia boyciana*）是分布于东亚的大型濒危涉禽，亟待深入开展其种群的保护遗传学研究。本研究采用微卫星标记技术筛选东方白鹳的微卫星位点，并进行种群的遗传多样性分析。所用的36对微卫星引物7对来自白鹳（*Ciconia ciconia*），12对来自朱鹮（*Nipponia nippon*），17对来自大蓝鹭（*Ardea herodias*）。从23个东方白鹳个体中筛选获得11个多态性丰富的微卫星位点。群体等位基因数为3–11，平均7.09。平均期望杂合度为0.7816，平均多态信息含量为0.7172，显示出东方白鹳较丰富的种群遗传多样性。

关键词： 微卫星分子标记，东方白鹳（*Ciconia boyciana*），遗传多样性