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Development of novel microsatellite loci and assessment of genetic diversity in the endangered Crested Ibis, *Nipponia Nippon*

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The Crested Ibis *Nipponia nippon* is a globally threatened species and has been ranked as ''Endangered'' in the 2004 IUCN List category (http://www.redlist.org). This species historically nested in the Russian Far East, Korea, Japan and China (Yamashina 1975), but habitat loss, increased human population, environmental pollution, etc. have led the wild populations to extinction during the last century. The wild population in China was also thought to be extinct after 1960 but four adults and three nestlings were rediscovered in Yangxian, Shaanxi Province in 1981 (Liu 1981). Since then, great conservation efforts have been taken to restore this small population both in wild and in captivity.

A population viability analysis has been once conducted for the 40 wild ibises of 1998 (Li and Li 1998), arguing the extinction probability of this rare bird was 19.7% in 100 years. Considering that the number of wild ibis individuals has expanded quickly from 40 in 1998 to 210 in 2003 (Xi 2003), clearly there is a requirement to examine the current level of genetic diversity for this species. Although a previous study based on mitochondiral DNA indicated low genetic diversity existed in Crested Ibis (Zhang et al. 2004), the intrinsic drawback of mitochondrial DNA, i.e. a maternal mode of transmission, probably made the results of Zhang et al. well different from genetic variation of nuclear DNA (Wan et al. 2004).

Here, we reported the isolation of eleven novel microsatellite loci from *Nipponia nippon*. These new loci were used to determine the level of genetic diversity in the wild and captive populations in order to provide genetic evidence for designing effective conservation strategies for Crestd Ibis.

Thirty-six samples including blood, muscle or feather pulp were collected from 9 wild ibis and 27 captive ones. Genomic DNA was extracted using a conventional protocol (Sambrook et al. 1989).

The enrichment protocol of Hamilton et al. (1999), with the following specifications and modifications, was used to isolate microsatellite loci. A 200–1000 bp Sau3AI-restricted size fraction was isolated from total genomic DNA, then ligated to linkers SAU-L-A (5′ GCG GTA CCC GGG AAG CTT GG 3′) and SAU-L-B (5′ GAT CCC AAG CTT CCC GGG TAC CGC 3′). The ligated fragments were hybridized to biotinylated $(GT)_{12}$, $(GA)_{12}$, $(AAC)_8$ and $(AAAC)_7$ oligonucleotide probes and the enriched fraction was recovered using streptavidin-coated magnetic beads (Roche). The eluted fragments were amplified using SAU-L-A and SAU-L-B primers, cloned into pGEM-T vector (TaKaRa, Shanghai, China) and transformed into JM109 Competent Cells (TaKaRa, Shanghai, China). Positive colonies were sequenced on automated ABI3700 DNA sequencer. PrimerSelect software (DNASTar Inc. 1996) was used to design primers for loci that contained (GT) > 9, (GA) > 9, (AAC) > 8 or (AAAC) > 6 uninterrupted repeats and had flanking sequence suitable for primer design. A 5′-M13 tail (5′-CAC GAC GTT GTA AAA CGA C) was added to one primer (forward or reverse)

of each primer pair to allow fluorescent labeling during amplification reactions. Primer pairs that gave consistent specific products were further tested for polymorphism.

PCR amplifications were performed in a 10-μl volume using a PTC-220 gradient cycler (MJ Research). For all primers, we used 1 μl of template DNA (20–50 ng/μl), 0.5 units Taq DNA polymerase (TaKaRa, Shanghai, China), 1 μl of 10PCR buffer (TaKaRa, Shanghai, China), 1 μl of 25 mM $MgCl_2$, 0.75 μl of 20 mM $dNTP_S$, 0.1 μl bovine serum albumin (BSA), 0.1 μl of each 10 μM primer and 1 μl of 1 μM IRD labeled M13 primer (LI-COR). The amplification conditions were as follows: denaturation, 94 °C for 3 min; 35 cycles of 30 s at 94 °C, 30 s at the optimized annealing temperature (Table 1), 30 s at 72 °C; and 72 °C for 3 min.

The PCR products were loaded on LI-COR 4200 automated DNA Sequencer. $SAGA^{GT}$ version 3.2 software (LI-COR) was used to size the alleles accurately. Genetic polymorphism was estimated as the number of alleles per locus (A), observed heterozygosity (H_O) and expected heterozygosity (H_E), with the aid of software GENEPOP, version 3.3 (Raymond and Rousset 1995). The GENEPOP program also was used to test linkage disequilibrium and Hardy Weinberg equilibrium (HWE).

Genomic DNA from a blood sample was selected for microsatellite isolation. Approximately 2000 clones were screened and a total of 120 recombinants that potentially contained microsatellite sequence were obtained. Forty-three positive clones were chosen randomly and subject to DNA sequencing. The sequencing results showed that 28 out of 43 clones contained eight repeats or more and had suitable flanking sequences. Eighteen sets of primers were designed from the 28 candidate microsatellite sequences. All of 18 primer pairs succeeded in PCR amplification but only 11 primer dyads were polymorphic and consistently yielded their specific PCR products for each sample. The characteristics of these 11 microsatellite loci were listed in Table 1.

The 11 loci were all polymorphic in both populations, with 2–5 alleles per locus (Table 1). A total number of 28 alleles were identified from this bird but all alleles were shared between two populations. The average expected heterozygosity was 0.506 in wild population and 0.486 in captive one,

Table 1. Characteristics of 11 polymorphic microsatellite loci in Crested Ibis *Nipponia Nippon*

Locus	Primer sequences (5′–3′)	Repeat motif	Ta (°C)	Size range	N_A(n = 36)
Nn01	F: TCAGATAACATTTGTGGGATTG R: AGGGCTAGGTTTGGCATT	$(CA)_{11}$	58	194–206	5
Nn03	F: GATCAACGAACAACCCAA R: CATACGGAAGAAACCTCA	$(CA)_{13}$	57	297–303	2
Nn04	F: GTTTCTTCTGTGCCATCC R: ATGCCTTGCATTATTGCTT	$(CA)_2TA(CA)_{11}C(CA)_2$	57	211–213	2
Nn12	F: TTTCTTCCTCCTGTCAGCTCTTG R: GTGCTCTGCACCCTTCACCTTC	$(GT)_{19}$	57	239–243	3
Nn16	F: CCAGCCAGTGGGAGTGAAATGC R: TGGGATCGTGCTTGGGATCGTG	$(GT)_{12}$	58	237–241	2
Nn17	F: CTGGATGTAGGCTTGCTTGGTG R: AAGGGGCTGGTTAGTGATAGGG	$(GT)_{11}$	59	285–289	2
Nn18	F: TAAAACGAGCCAGACAGTCGCA R: GCCCTGAAGCAGTGGTAGGAAG	$(GT)_{20}$	58	182–196	2
Nn21	F: CCAGCCTCCTATCCTAATCTAATCG R: GAGCCAATCTGTTCCAGTCTCCTT	$(GA)_{12}$	57	175–185	2
Nn23	F: GGTCGCATACAATCCTCA R: AGCCCTTCTTCTGTATGGTGTCCT	$(GTT)_{12}$	57	233–242	2
Nn25	F: TCCAGCTACTCACTTCTTTCGG R: ATAGATACCCAGGGCATTCAGG	$(AAAC)_8$	57	166–178	4
Nn26	F: GCACCCAAGACAAAGAGGCAATG R: CCTGTTCCAGGGTTTGACCACC	$(AAAC)_9$	57	159–167	2

Primer sequences, repeat unit structure, PCR annealing temperature (Ta), number of alleles (N_A) are listed for each locus.

Table 2. Observed (H_O) and expected (H_E) heterozygosity of 11 microsatellite loci

	Nn01	Nn03	Nn04	Nn12	Nn16	Nn17	Nn18	Nn21	Nn23	Nn25	Nn26	Mean over loci
Wild												
n	9	9	9	9	9	9	9	9	9	9	9	9
A	5	2	2	3	2	2	2	2	2	4	2	2.56
H_E	0.791	0.266	0.471	0.601*	0.425	0.523	0.523	0.523	0.503	0.725	0.209	0.506
H_O	0.444	0.222	0.444	0	0.556	0.222	0.667	0.222	0.778	0.778	0.222	0.414
Captive												
n	27	27	27	27	27	27	27	27	27	27	27	27
A	5	2	2	3	2	2	2	2	2	4	2	2.56
H_E	0.702	0.307	0.409	0.668*	0.352	0.475	0.507*	0.484	0.509*	0.700*	0.230	0.486
H_O	0.704	0.222	0.333	0.370	0.370	0.444	0.778	0.630	0.963	0.926	0.259	0.545

*Significant deviation from Hardy Weinberg equilibrium ($P < 0.001$).

respectively (Table 2). Compared with other species with population bottlenecks (Bouzat et al. 1998; Tarr et al. 1998), the genetic diversity of Crested Ibis was relatively high.

Significant linkage disequilibrium ($P < 0.05$) was attained for five pairs of loci, one pair (Nn17-Nn18) in wild population and 4 dyads (Nn03–Nn04, Nn03–Nn12, Nn03–Nn17 and Nn03–Nn26) in captive one. The HWE test revealed that 5 loci significantly deviated from HWE expectations (Table 2). The locus Nn12 showed lower observed heterozygosity than expected heterozygosity in both populations, i.e. heterozygosity deficiency, providing evidence of inbreeding (Genlous and Björn 2003). The loci Nn18, Nn23 and Nn25 presented larger observed heterozygosity than expected one in captive population, indicative of heterozygosity excess. This excess might result from the known serve bottleneck (Cornuet and Luikart 1997).

Eleven polymorphic microsatellite loci were isolated in the present study, the number of alleles per locus ranging from 2 to 5. In a recent study, Ji et al. (2004) developed eight polymorphic loci for the same species, showing 2–3 alleles for each locus. The loci reported here were more polymorphic than those of Ji et al. and therefore more useful.

Currently, the captive ibis population has reached a relatively large size, containing 240 individuals (Xi 2003), thus suggesting a possibility of reintroducing captive-bred ibis into the wild population. However, if significant genetic differences exist between the donor and receipt populations, merging the gene pools of the two populations may result in an overall loss of genetic diversity (Wan et al. 2004). The present study found that the heterozygosities for the captive and wild individuals were not significantly different ($P > 0.05$), indicating similar genetic diversity of the two populations. Therefore, it is feasible to reintroduce captive crested ibis to the wild from a genetic (microsatellite) perspective. Worthy of pointing out, releasing captive crested ibis into the wild has been discussed and tested in a previous study (Xi and Fujihara 2001). Consequently, our markers would be useful for the genetic tracking of released birds in the future.

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