Molecular Ecology Notes (2004) **4**, 615–617 doi: 10.1111/j.1471-8286.2004.00754.x

PRIMER NOTE

Eight polymorphic microsatellite loci for the critically endangered crested ibis, *Nipponia nippon* (Ciconiiformes: Threskiornithidae)

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Abstract

The Chinese crested ibis, *Nipponia nippon*, is a critically endangered bird. The current population of this species has developed from four wild individuals rediscovered in 1981. Given its conservation status, there is considerable interest in assessing the genetic diversity and individual relatedness in this species. For this purpose, a set of eight polymorphic di- or trinucleotide microsatellite loci was developed for the crested ibis. The expected heterozygosity at these loci ranges from 0.01 to 0.50, with less than four alleles being observed at individual loci, a reflection of the serious population bottleneck experienced by this species.

Keywords: black-headed ibis, cross-species amplification, microsatellite, *Nipponia nippon*, *Threskiornis melanocephalus*

Received 7 May 2004; revision received 23 June 2004; accepted 23 June 2004

The crested ibis *Nipponia nippon* (Ciconiiformes: Threskiornithidae) is a critically endangered bird (Collar *et al*. 1994) that was thought to be extinct in the wild until a colony of four individuals was rediscovered in 1981 in Yangxian of the Shaanxi province of China (Liu 1981). It formerly occurred across Russia, China, the Korea Peninsular and Japan. The population declined rapidly from 19th century due to over-hunting and the degradation of habitats caused by the severe deforestation, drying out of wetland and the abuse of chemical fertilizers and pesticides. The four individuals found in the wild are two pairs of adult birds. Earlier efforts in conservation have been focused on increasing population size. Thus the overall population size has now increased to more than five hundred individuals, largely due to natural habitat restoration and captive-breeding programs implemented over the last 20 years. However, initially not enough attention was paid to breeding management and pedigree records, and there is practically no information on genetic relatedness of the four founding individuals. This will certainly affect further effective management of the crested ibis population and place the population under potential risk of serious inbreeding. A study on genetic diversity, relatedness and kinships of the current ibis populations is clearly needed. Microsatellite DNA should be the most suitable DNA marker so far available for this purpose (Zhang & Hewitt 2003). Here we report on the isolation and characterization of eight polymorphic di- and trinucleotide microsatellite DNA loci in *Nipponia nippon*.

The enrichment method used for isolation of the microsatellite loci has been described in Ji *et al*. (2003). In total, 1145 recombinant clones were screened with biotin-labelled oligonucleotide probes using CDP-Star™ Universal Detection Kit (Sigma), and 229 positive clones were found. Inserts of positive clones were isolated using polymerase chain reaction (PCR) amplification directly from bacterial colonies using M13 universal and reverse sequencing primers (−47 and −48, respectively, New England Biolabs), then sequenced with ABI BigDye™ Terminators Cycle Sequencing Kit (version 2.0) in an ABI PRISM3100 automated sequencer. Sequencing analysis showed that 20% of the recombinant clones contained microsatellite DNA.

Nineteen microsatellite sequences were selected for designing oligonucleotide primers, using OLIGO® 6.31

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Table 1 Characteristics of eight polymorphic and five monomorphic microsatellite loci in the crested ibis *Nipponia nippon* (Nn). Primer sequences, repeat unit structure, expected (H_E) and observed (H_O) heterozygosities, number of alleles in the crested ibis (Nn), PCR annealing temperature (T_a) for Perkin-Elmer GeneAmp 9700 and number of alleles in the black-headed ibis, *Threskiornis melanocephalus* (Tm) are indicated for each locus

Locus	Primer name	Primer Sequence (5′–3′)	Repeat type (Nn)	T_a (°C)	Labeling dye	No. of Alleles (Nn)	No. of Alleles (Tm)	Size range (bp, Nn)	H_E (Nn)	H_O (Nn)
NnAF4	AF4F*	AAAAAAGCTGATAACATCTAGAA	$(TC)_3TG(TC)_7$	57	FAM	2	2	146–148	0.010	0.010
	AF4B	TTTGCATTAGTGAAGAGATAAAG	$AG(TC)^3$							
NnBF7	BF7F*	GAAACTTGTTTTGTTACACACT	$(CTG)_4C(CTG)_2$	59	FAM	3	1	108–118	0.019	0.019
	BF7B	TCCAACTGGTGCCTTACTC								
NnCE11	CE11F*	GAGTAGCAGAAGCGTGAGT	$(CTG)_8$	59	HEX	2	1	188–197	0.502	0.990
	CE11B	GGGTGGCACAAGGGTGTC								
NnCG3	CG3F	TCTTGAGTGGCTGCCTAGT	$(CAT)_4CTT$	59	NED	2	1	252–270	0.019	0.000
	CG3B*	TTTGGATGTTCATCAGGAAAGT	$CTC(CHT)_{14}$							
NnDD9	DD9F*	GAACTGCTTTTGCCCAGGA	$(CAT)_7$	59	HEX	2	2	123–126	0.019	0.000
	DD9B	TAGGGTTGGAATATGTTAAGA								
NnEB12	EB12F	TGTTAGAAGCCAGATCCAAGT	$(CA)_{11}$	55	HEX	2	3	92–99	0.010	0.010
	EB12B*	AATGTTTGCCTCTATAGATTT								
NnHB12	HB12F	AAAGACGGTGGTGACAGGA	$(GT)_8$	59	HEX	2	1	185–187	0.019	0.000
	HB12B*	TCTGCTGTACCTTTTGGGGA								
NnNF5	NF5F	TTTGAAGGCAGGTGGAGAAGT	$(TAGA)_2$	57	NED	3	2	115–130	0.371	0.463
	NF5B*	AGCTGAAAAGAAGATAATTAGGA	$TA(CA)_8$							
NnEA9	EA9F*	CCTTAAACTTCCACTAAATATC	$(CT)_{12}$	53	HEX	1	2	121	ML	ML
	EA9B	CATATCTTGGTGTTGTGTTCT								
NnAD10	AD 10F	TGTTAGGCTGGTGTAAAGGT	$(GT)_2TT(GT)_4$	66	FAM	1	1	87	ML	ML
	AD 10B*	AATGCAACAACAGAAAAGAAGA	$(GTGC)_2$							
NnEH10	EH10F*	CAGAGCTTTACACTAGTTAGA	$(GT)_3ATG(GT)_3$	55	FAM	1	1	127	ML	ML
	EH10B	TTTGCACAGTTTGACTAAAGA	$GC(GT)_5(AT)_2$							
NnGF4	GF4F*	GATACTTCATATATCAATAGCTC	$(TA)_2(GA)_2$	57	NED	1	1	180	ML	ML
	GF4B	TTTCCTCGCTCTTTCACTGAC	$(TA)_2(CA)_8$							
NnLF11	LF11F	TTTCTTCTGGACTTCTTTAGG	$(CA)_8CGC$	59	FAM	1	1	225	ML	ML
	LF11B*	AAAACATTGGAAGTGGTTATAG	$ATA(CA)_4$							

*denotes primers that are labelled with fluorescent dyes. ML, Monomorphic locus. GenBank Accession nos: AJ699404–AJ699416

primer analysis software (National Biosciences Inc.). Oligonucleotides were synthesized by Genecore Biotech (Shanghai). After extensive optimization, we found that 18 pairs of primers gave satisfactory results and each appeared to be a single-copy sequence in the genome. Further analysis with high-resolution Metaphor agarose gel (FMC, Marine, USA) suggested that 13 pairs are likely to be polymorphic in the ibis population. With these 13 loci, one primer of each pair was end-labelled with a fluorescent dye, either 6-FAM, HEX or NED (Table 1). They were further tested by genotyping with 107 crested ibis individuals on an ABI PRISM® 3100 automated sequencer using Pop4 gel matrix with GENESCAN® 400HD (ROX) as the internal size standard, and eight loci are found to be polymorphic. Final PCR conditions employed for these loci are as follows: A 10 μL reaction containing 20–30 ng of template DNA, 0.2 mM of each dNTP, 1.5 × PCR buffer (HuaMei Biotech, Beijing), 1.5 mM Mg^{2+}, 0.6 U of *Taq* DNA polymerase (HuaMei Biotech, Beijing), and 0.3 μM of each primer, was denatured at 94 °C for 4 min, then followed by 36–40 cycles of 20 s at 94 °C , 30 s at the appropriate annealing temperature (Table 1), and 10 s at 72 °C. The reaction was terminated by a final extension of 2 min at 72 °C.

Table 1 shows the characteristics of these 13 microsatellite loci. The expected heterozygosity at the eight polymorphoic loci (calculated using the program GENEPOP, Raymond & Rousset 1995) ranges from 0.01 to 0.50. Only two or three alleles were observed at those polymorphic loci in the sample analysed, reflecting the severe bottleneck effect experienced by this bird.

Since the crested ibis belongs to a monospecific genus, cross species applicability of these microsatellite loci was tested in three siblings of the black-headed ibis, *Threskiornis melanocephalus*. All loci can amplify successfully in the black-headed ibis (Table 1), with five loci being polymorphic in the three sibling individuals tested. PCR

conditions for these loci in the black-headed ibis are identical to those for the crested ibis. Therefore, the microsatellite loci we characterized in the crested ibis should be useful in other ibises as well.

Acknowledgements

This work was supported by NSFC grants (30025008, 30170140) to DXZ and CQD, and the CAS 'Bai Ren Ji Hua' professorship to DXZ. We thank Beijing Zoo and the Crested Ibis Conservation and Observation Station of Shaanxi (Yangxian) for the ibis samples. Individual contribution: YJJ — construction and screening of genomic library, primers design and preparation of the manuscript; LYD — test and optimization of primers, genotyping; DXZ — Supervision and manuscript preparation; CQD — specimen collection.

References

Collar NJ, Crosby MJ, Stattersfield AJ (1994) *Bird to Watch 2: the World List of Threatened Birds*. BirdLife International, Cambridge, UK.

Ji YJ, Zhang DX, Hewitt GM, Kang L, Li DM (2003) Polymorphic microsatellite loci for the cotton bollworm *Helicoverpa armigera* (Lepidoptera: Noctuidae) and some remarks on their isolation. *Molecular Ecology Notes*, **3**, 102–104.

Liu Y (1981) Recovery of Japanese crested ibis in Qin-Ling range. *Acta Zoologica Sinica*, **27**, 273.

Raymond M, Rousset F (1995) GENEPOP Version 1.2: population genetics software for exact test and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Zhang DX, Hewitt GM (2003) Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. *Molecular Ecology*, **12**, 563–584.