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Short communication

Development and Characterization, and Application of Ten Polymorphic Microsatellite Markers in the Crested Ibis *Nipponia nippon* from South Korea

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ABSTRACT

The Asian crested ibis *Nipponia nippon* is one of the world's most endangered species. Except for the Sanxii population from China, it is known that all of the crested ibis populations from East Asia have been extinguished. In these days, most of them are being inbred as captive populations in China, South Korea, and Japan, which caused their low expected genetic diversity. Microsatellite markers are well known as a suitable DNA marker for exploring genetic diversity among captive populations of a variety of endangered species. In the present study, ten microsatellite markers were developed for the captive populations of the South Korean crested ibis, which were employed to examine the level of genetic diversity with the two founders from Sanxii, China and the 70 descendants of them. As a result, the mean number of gene diversity, observed heterozygosity, and expected heterozygosity of the captive population were 0.70, 0.84, and 0.70 respectively. It revealed that the captive population of South Korea is as genetically more stable than we expected. In addition, the principal coordinates analysis and genetic structure analyses showed that the captive population of *N. nippon* can be divided into the two different genetic groups. The developed microsatellite markers here could be helpful for crested ibis conservation in East Asian countries such as China and Japan as well as South Korea.

Keywords: *Nipponia nippon*, Asian crested ibis, endangered species, microsatellite marker, genetic diversity, population genetics, South Korea

INTRODUCTION

The Asian crested ibis *Nipponia nippon* (Pelecaniformes, Threskiornithidae) is an endangered species all over the world, which is published as Endangered on IUCN Red List (IUCN, 2018). The birds were distributed mainly in East Asia encompassing Korean peninsula, China, and Japan (Yamashina, 1975). The population size of *N. nippon* was drastically decreased due to habitat loss caused by detrimental human activities (Li et al., 2009; Kim et al., 2012). Thereby, it was known that the *N. nippon* population has been almost extinguished around from the late of 1970s to the early of 1980s (Yamashina, 1975; Yamashina and Nakanishi, 1983). In 1981, the seven crested ibis individuals were found in the Sanxii region from China, with which its conservation endeavor of the Chinese government was started to be established. Since then, the number of *N. nippon* individuals has been continuously increased in China. In addition, a pair of Chinese *N.*

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nippon individuals including a male and a female were donated to South Korea in October 2008 and two male *N. nippon* individuals were introduced in December 2013. As a result of the conservation efforts of the Ministry of Environment, the South Korean government and the Upo wetland management center, Changnyeong-gun, the population size of *N. nippon* has been dramatically increased up to more than 359 individuals in South Korea as of July 2018 (Kim, 2019). Prevention of both the loss of genetic diversity and the increase of inbreeding is important issues for conservation in the captive population of endangered species because it causes the reduction of population sizes and the increase of extinction risk (Lande, 1988; Taniguchi et al., 2013; Urano et al., 2013; Tsubono et al., 2014). With the pedigree information for relatedness and kinship of founders, molecular genetic data such as mitochondrial DNA or microsatellite markers are necessary to examine the genetic diversities of captive populations (Urano et al., 2013; Tsubono et al., 2014; Kim et al., 2019). Out of various molecular markers, it is known that microsatellite markers are one of the most suitable DNA markers for the purpose of exploring genetic diversity and structure among captive populations of endangered species (Zhang and Hewitt, 2003; Urano et al., 2013). Until now, only a few researches (Ji et al., 2004; He et al., 2006; Urano et al., 2013; Tsubono et al., 2014) have been conducted and reported both the development and application of useful microsatellite markers for the *N. nippon* populations in China and Japan.

In South Korea, Kim et al. (2018) first analyzed the genetic diversity of the South Korean *N. nippon* using 8 microsatellite markers that had developed in China (He et al., 2006), the results of which were compared with those of the Chinese and Japanese crested ibis that had previously been researched by He et al. (2006) and Urano et al. (2013). In this study, we developed and characterized ten novel microsatellite markers for exploring population genetic diversity and structure of South Korean *N. nippon*. The newly developed microsatellite loci were employed to examine the level of genetic diversity in the captive populations of South Korean crested ibis in order to provide genetic evidence for the stability and sustainability of the captive population in South Korea.

Total genomic DNA was extracted from a blood sample of one out of the two founders using DNeasy Blood & Tissue Kit (Qiagen, USA). An Illumina paired-end shotgun library was prepared by shearing 200 ng of DNA using a Covaris S220 (Covaris, Woburn, MA, USA) and following the standard protocol of TruSeqnano DNA Library Kit (Illumina, San Diego, CA, USA). Illumina sequencing was conducted on the Next-Seq 500 platform with 150 bp paired-end reads, resulting in a total of 70,396,357 reads. Read assembly was analyzed using CLC Genomics Workbench software ver. 7 (CLC Bio, Aarhus, Denmark), and Msatcommander program (Faircloth, 2008) was used to extract reads containing microsatellites with di-, tri-, tetra-, penta- and hexanucleotide. In total, there were found 58,900 microsatellite loci, which consisted of 32,405 dinucleotide, 15,750 trinucleotide, 7,017 tetranucleotide, 3,325 pentanucleotide, and 293 hexanucleotide microsatellites. Among the di- and trinucleotide microsatellites, we selected 10 microsatellite loci that could be possible to designing PCR primers and be amplified stably. PCR primers for the 10 microsatellite markers were designed by the Primer 3 program (Untergasser et al., 2012), and then tested for amplification using standard PCR condition. PCR was performed by an ABI 2720 Thermo cycler (Applied Biosystems), and the resultant PCR products were sequenced using ABI 3730x1 (Applied Biosystems). The polymorphism of the selected microsatellite markers was tested using 70 *N. nippon* individuals from the Upo wetland management center, Changnyeong-gun, Gyeongsangdnam-do, South Korea (35°32′46.61″N, 128°24′49.96″E). Allele size calling and genotypic analyses were performed using a GeneMapper Software ver. 4.1 (Applied Biosystems). Microsatellite loci were characterized by estimating the Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium in GENEPOP ver. 4.2 (Raymond and Rousset, 1995). The degree of their polymorphism was estimated using Polymorphic Information Content (PIC) (Botstein et al., 1980) by PowerMarker ver. 3.25 (Liu and Muse, 2005). Expected and observed heterozygosities were also calculated in FSTAT ver. 2.9.3.2 (Goudet, 1995) and Arlequin ver. 3.5 (Excoffier and Lischer, 2010). To explore the genetic structure in the *N. nippon* captive population from South Korea, the principal coordinates analysis (PCoA) and Bayesian clustering analyses were performed with the ten developed markers using the programs of GenAlex ver. 6.5 (Peakall and Smouse, 2012) and STRUCTURE ver. 2.3 separately (Pritchard et al., 2000).

RESULTS AND DISCUSSION

The 10 polymorphic microsatellite markers were developed and characterized for the captive *N. nippon* population from South Korea (Tables 1, 2). According to Botstein et al. (1980), if a PIC value per locus is more than 0.5, the locus is considered a suitable microsatellite marker. As shown in Table 2, the PIC values of the microsatellite markers developed here ranged from 0.547 to 0.729, which implies that the loci are suitable for the use as microsatellite markers. The markers did not show linkage disequilibrium. The developed markers were employed for examining the genetic diversity of the captive South Korean population, with the two founders and the 70 descendants. Some of them were failed in amplifying the marker reigions. It may be due to relatively high degree

Table 1. Characterization of 10 microsatellite markers for captive *Nipponia nippon* in South Korea

Locus	Primer sequence (5′-3′)	GenBank accession No.	*Ta* (°C)	Repeat motif	Expected size (bp)	Allele size (bp)
NIPO-KR5212	CCTCAGCCTAATCCATTATT GTAAGCTACGAAGGAATGTA	MN099152	52	$(AT)_{20}$	260	251, 253, 255, 257, 261, 263
NIPO-KR8395	CAGTTCACATGCAGGATATA GGAAGCAGTTAATTCTTACG	MN099153	52	$(GA)_{19}$	231	210, 236, 238, 240, 242, 246
NIPO-KR10411	AATTCTTCTCTCAGTGTCAG CTGCCTATCCACATTATCAA	MN099154	52	$(TA)_{20}$	273	269, 271, 273, 275, 283
NIPO-KR12706	GCCTGAGATGTTACTACTTC GTAACCAACATTCTCACAAC	MN099155	52	$(TA)_{19}$	231	230, 232, 234, 266, 268
NIPO-KR13569	GGTTGAACACTCTTCAGTA CTCTGCTCCTTCTCTCAT	MN099156	52	$(AT)_{19}$	151	142, 146, 148, 150, 152
NIPO-KR19306	CTTCATTAACTGCTGTGTAC CACCACATGAGCTATTGATA	MN099157	52	$(AT)_{20}$	285	272, 278, 280, 282, 284, 286, 288
NIPO-KR23090	GACTGTGTATGCTCCATC CTCTGTATGTGCTTGATAGA	MN099158	52	$(AT)_{20}$	194	191, 193, 195
NIPO-KR26951	ATGCTATCCACATCTGAAC CATGCATATTCTGAGAAGTG	MN099159	52	$(AC)_{19}$	298	292, 294, 298, 300, 302, 304
NIPO-KR37402	GTACTGTTGATTAGGCTAGT GTACAGACGTTAGAAGAGAA	MN099160	52	$(GAA)_{12}$	276	334, 337, 340, 343, 346, 358, 361
NIPO-KR79219	ATGATCTCACCTTCTACTCA CTTCCATTGATGTGAATTCC	MN099161	52	$(GT)_{19}$	234	226, 228, 230, 232, 234

Table 2. The results of the genetic diversity analyses performed with the 70 descendants out of the captive population of the crested ibis *Nipponia nippon* in South Korea using the 10 microsatellite markers developed in the present study

Microsatellite marker	*N*	*Na*	*Ne*	GD	PIC	H_O	H_E	F_{IS}	HWE
NIPO-KR5212	68	6.000	2.756	0.651	0.617	0.456	0.637	0.284	***
NIPO-KR8395	68	9.000	3.565	0.714	0.676	0.838	0.720	−0.165	***
NIPO-KR10411	70	5.000	3.215	0.688	0.631	1.000	0.689	−0.451	***
NIPO-KR12706	70	7.000	3.057	0.669	0.611	1.000	0.673	−0.486	***
NIPO-KR13569	68	6.000	3.146	0.682	0.628	0.779	0.682	−0.143	***
NIPO-KR19306	70	7.000	4.095	0.756	0.717	0.871	0.756	−0.153	**
NIPO-KR23090	60	3.000	2.675	0.624	0.547	0.983	0.626	−0.571	***
NIPO-KR26951	68	6.000	3.621	0.720	0.679	0.794	0.724	−0.097	***
NIPO-KR37402	66	8.000	4.290	0.764	0.729	0.773	0.767	−0.008	***
NIPO-KR79219	70	5.000	3.700	0.728	0.682	0.900	0.730	−0.233	***
Mean	68	6.200	3.412	0.700	0.652	0.840	0.700	−0.202	

N, number of samples; *Na*, number of alleles; *Ne*, number of effective alleles; GD, gene diversity; PIC, polymorphic information content; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient ($F_{IS}=1-(H_O/H_E)$); HWE, chi-square tests for Hardy-Weinberg Equilibrium (***$p<0.001$, **$p<0.01$)

of sequence variation on their priming sites, inferred from the positioning on the PCoA analysis apart from others. As a result, the mean numbers of alleles (*Na*), genetic diversity (GD), observed heterozygosity (H_O), and expected heterozygosity (H_E) of the captive population were 6.200, 0.700, 0.840, and 0.700 in order. All HWE of the ten microsatellite markers are deviated with statistical significance ($p<0.01$). Except for one marker of NIPO-KR5212, inbreeding coefficients (F_{IS}) for all the remaining microsatellite markers were negative, implying little inbreeding occurrence. It revealed that the captive population of South Korea is as genetically more stable than we expected. In addition, with the PCoA and STRUCTURE analyses, we explored the genetic structure of the *N. nippon* captive population from South Korea. The results coincidently showed that they may be divided into the two different genetic lineages (Figs. 1, 2). It is expected that the

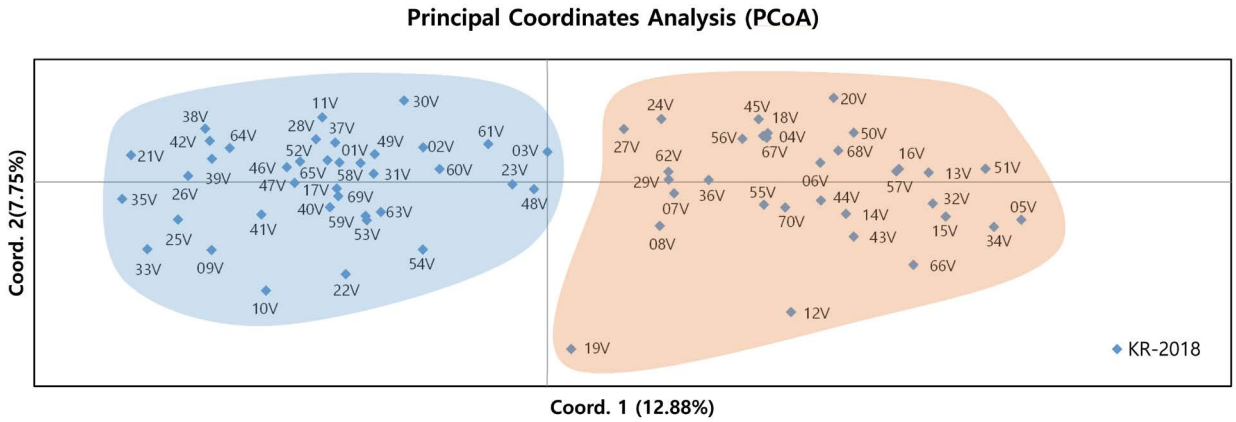

Fig. 1. PCoA analysis of 70 individuals of *Nipponia nippon* based on genotyping result using 10 microsatellite markers in South Korea. The PCoA analysis inferred from the 10 microsatellite markers of 70 individuals of *Nipponia nippon* in South Korea resulted in two different genetic lineages, indicating the possible existence of two different genetic groups.

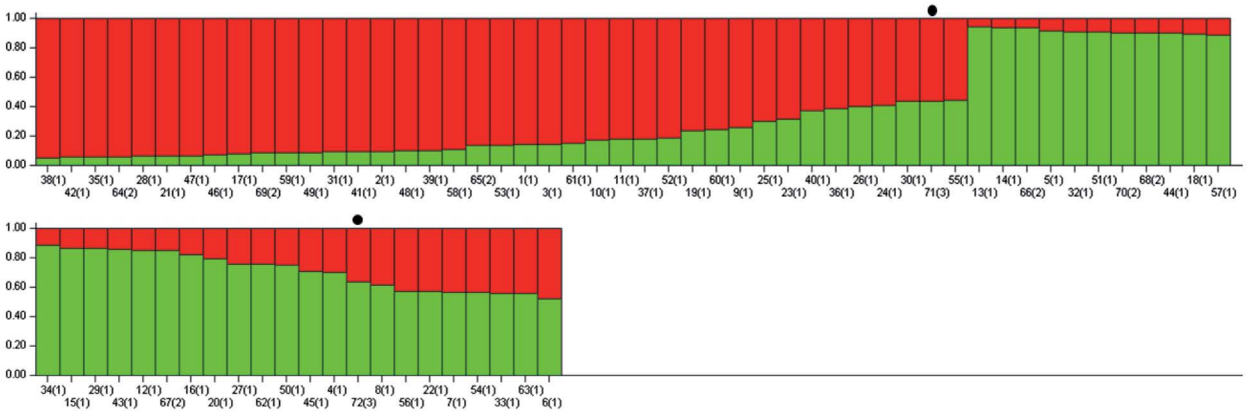

Fig. 2. Genetic structure of the 2 founders and 70 descendant of *Nipponia nippon* in South Korea based on the newly developed 10 microsatellite markers. Each individual is represented by a single vertical line divided into K=2 colors (red and green) and the length of the colored segment indicates the individual's estimated proportion to specific clusters. Two bars with black circles indicate the two founders. It showed the two different genetic lineages indicating the possible existence of two different groups of *Nipponia nippon* in South Korea.

10 developed microsatellite markers developed here could be helpful for crested ibis conservation not only in South Korea but in other East Asian countries including China and Japan.

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CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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REFERENCES

Botstein D, White RL, Skolnick M, Davis RW, 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. American Journal Human Genetics, 32:314-331.

Excoffier L, Lischer HEL, 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources, 10: 564-567. https://doi.org/10.1111/j.1755-0998.2010.02847.x

Faircloth BC, 2008. Msatcommander: detection of microsatellite repeat arrays and automated, locus-specific primer design. Molecular Ecology Resources, 8:92-94. https://doi.org/10.1111/j.1471-8286.2007.01884.x

Goudet J, 1995. FSTAT (version 1.2): a computer program to calculate F-statistics. Journal of Heredity, 86:485-486. https://doi.org/10.1093/oxfordjournals.jhered.a111627

He LP, Wan QH, Fang SG, Xi YM, 2006. Development of novel microsatellite loci and assessment of genetic diversity in the endangered crested ibis, *Nipponia nippon*. Conservation Genetics, 7:157-160. https://doi.org/10.1007/s10592-005-6790-0

IUCN, 2018. The IUCN Red List of Threatened Species. Version 2018-1 [Internet]. Accessed 26 Feb 2020, <http://www.iucnredlist.org>.

Ji YJ, Liu YD, Ding CQ, Zhang DX, 2004. Eight polymorphic microsatellite loci for the critically endangered crested ibis, *Nipponia nippon* (Ciconiiformes: Threskiornithidae). Molecular Ecology Notes, 4:615-617. https://doi.org/10.1111/j.1471-8286.2004.00754.x

Kim DH, Kim YS, Seo JH, Kim SJ, Kong HS, 2018. Genetic analysis of endangered species Crested Ibis (*Nipponia nippon*) microsatellite markers. Korean Journal of Ornithology, 25:77-81. https://doi.org/10.30980/KJO.2018.12.25.2.77

Kim G, Jeong KC, Choi EH, Ryu SH, Lim YJ, Jun J, Lee Y-S, Hwang UW. 2019. The complete mitochondrial genome of an Asian crested ibis *Nipponia nippon* (Pelecaniformes, Threskiornithidae) from South Korea. Mitochondral DNA Part B, 4:3707-3708. https://doi.org/10.1080/23802359.2019.1680321

Kim KA, Cha JS, Park SY, Kim KM, Park HC, 2012. The improved polymerase chain reaction method applied for sex identification of crested ibis, *Nipponia nippon*. Advances in Life Sciences, 2:82-84. https://doi.org/10.5923/j.als.20120204.01

Kim SJ, 2019. Reintroduction of crested ibis in South Korea. In: The 3rd International Symposium for Asian Crested Ibis in China, Japan and Korea: 2019 May 23; Changnyeong, Korea. p. 126.

Lande R, 1988. Genetics and demography in biological conservation. Science, 241:1455-1460. https://doi.org/10.1126/science.3420403

Li X, Tian H, Li D, 2009. Why the crested ibis declined in the middle twentieth century. Biodiversity and Conservation, 18:2165-2172. https://doi.org/10.1007/s10531-009-9580-z

Liu K, Muse SV, 2005. PowerMarker: an integrated analysis environment for genetic marker analysis. Bioinformatics, 21:2128-2129. https://doi.org/10.1093/bioinformatics/bti282

Peakall R, Smouse PE, 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics, 28:2537-2539. https://doi.org/10.1093/bioinformatics/bts460

Pritchard JK, Stephens M, Donnelly P, 2000. Inference of population structure using multilocus genotype data. Genetics, 155:945-959.

Raymond M, Rousset F, 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. Journal of Heredity, 86:248-249. https://doi.org/10.1093/oxfordjournals.jhered.a111573

Taniguchi Y, Matsuda H, Yamada T, Sugiyama T, Homma K, Kaneko Y, Yamagishi S, Iwaisaki H, 2013. Genome-wide SNP and STR discovery in the Japanese crested ibis and genetic diversity among founders of the Japanese population. PLoS ONE, 8:e72781. https://doi.org/10.1371/journal.pone.0072781

Tsubono K, Taniguchi Y, Matsuda H, Yamada T, Sugiyama T, Homma K, Kaneko Y, Yamagishi S, Iwaisaki H, 2014. Identification of novel genetic markers and evaluation of genetic structure in a population of Japanese crested ibis. Animal Science Journal, 85:356-364. https://doi.org/10.1111/asj.12155

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG, 2012. Primer3: new capabilities and interfaces. Nucleic Acids Research, 40:e115. https://doi.org/10.1093/nar/gks596

Urano K, Tsubono K, Taniguchi Y, Matsuda H, Yamada T, Sugiyama T, Homma K, Kaneko Y, Yamagishi S, Iwaisaki H, 2013. Genetic diversity and structure in the Sado captive population of the Japanese crested ibis. Zoological Science, 30:432-438. https://doi.org/10.2108/zsj.30.432

Yamashina Y, 1975. The feeding of Japanese crested ibis. In: Endangered birds (Ed., Templ SA). University of Wisconsin Press, Madison, WI, pp. 161-164.

Yamashina Y, Nakanishi G, 1983. *Nipponia nippon*. Newton Books, Tokyo, pp. 1-301

Zhang DX, Hewitt GM, 2003. Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. Molecular Ecology, 12:563-584. https://doi.org/10.1046/j.1365-294X.2003.01773.x

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