Reintroduction (2011) 1: 77–80

SHORT COMMUNICATION

New haplotypes in the mitochondrial control region of Oriental White Storks, *Ciconia boyciana*

* **Yoshihiro Yamamoto**[1]

Abstract Previous studies have shown 17 haplotypes in the mitochondrial D-loop region of Oriental White Stork, *Ciconia boyciana*, in Japan (Yamamoto et al. 2000; Murata et al. 2004). We found 7 additional haplotypes while analyzing 11 samples; 2 mounted skin specimens, 5 blood samples and 4 fallen feathers. Altogether, 24 haplotypes have been identified in the Japanese population from live captive individuals and wild white storks, and stuffed specimens. The haplotype diversity (h) and the nucleotide diversity (π) of Japanese captive and re-introduced Oriental White Storks in 2010 were 0.8319 ± 0.0113 and 0.00357 ± 0.00196, respectively. The population size of captive white storks in Japan has increased in recent years, however, the haplotype distribution among breeding centers should be improved. I suggest that new founders should be introduced from Russian or Chinese wild population.

Key words Mitochondria, Haplotype, D-loop, Oriental White Stork, *Ciconia boyciana*

The re-introduction of Oriental White Storks in the Toyooka-city began on April 24 in 2005. The last wild Oriental White Stork had been captured 35 years ago for breeding. The re-introduction was possible because of the recovery of some suitable habitats in Toyooka, and because of the increased number of captive storks following successful breeding in Hyogo Park of the Oriental White Stork. In breeding programs, 2 points should be considered to prevent a decline in the genetic diversity of captive stork populations. The first is that founders belonging to the same mitochondrial haplotype should not form breeding pairs because they might be relatives. The second is that the pedigree status of the offspring should be monitored to avoid inbreeding.

The entire nucleotide sequences of the mitochondrial genome of the Oriental White Stork has been determined (Accession number AB026193), and haplotypes in the control region without repeated sequences (1,203 bp) have been analyzed (Yamamoto et al. 2000). Nine haplotypes were found in captive Oriental Storks (Yamamoto et al. 2000), while an additional 8 have been reported from captive Oriental White Storks and stuffed samples (Murata et al. 2004). Therefore, 17 Oriental White Stork haplotypes were known previously. In this study, I analyzed 2 mounted skin specimens (SCB601 & SCB602), 5 blood samples (Stud number 19, 21, 208, 818 and 819), and 4 feather roots, and I was able to identify 7 novel haplotypes. The 4 feather samples (FCB412, FCB414, FCB415 and FCB603) were derived from wild Oriental White Storks during the wintering season in Japan.

Whole cellular DNA was extracted from blood by the standard method with protease K and phenol (Sambrook et al. 1989). DNA extraction from feather roots was performed with the DNeasy Blood & Tissue Kit (QIAGEN). The PCR conditions and sequencing methods were the same as described previously (Yamamoto et al. 2000; Murata et al. 2004). Three PCR primer sets (STM3 & STM4; STM5 & STM6; STM7 & STM8) were used to cover 1,248 bp which corresponds to the control region (D-loop) excluding the repeated sequences. Combined nucleotide sequences of 1,210 bp without overlaps and primer sequences were treated as haplotype sequences (Yamamoto et al. 2000), and were analyzed further. All determined sequences were compared and analyzed using the GENETYX software (GENETYX Co., Tokyo, Japan).

Seven new haplotypes (18–24) were found after a comparison of 11 nucleotide sequences, and they have been listed with their nucleotide differences in Table 1. The previously reported haplotypes (1–17) are also shown in Table 1. Nucleotide polymorphisms are present at 34 sites, and 24 haplotypes have been determined. Haplotypes 18, 19, and 20 were found in captive storks. Haplotype 21 contains 3 unique polymorphic sites and a deletion of 3

[1] Department of Genetics, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo, 663-8501 Japan
* E-mail: yosyam@leto.eonet.ne.jp

Table 1. Nucleotide differences and haplotypes of the Oriental white storks in the control region (D-loop) of the mitochondrial genome.[a)]

TYPE	146	181	189	203	222	237	289	292	307	312	318	319	334	336	342	348	351	352	363	364	371	393	396	417	446	463	505	546	756	935	995	1000	1142	1145	sample[b)]	N[c)]
1	G	T	A	G	C	A	−	C	T	A	G	T	G	G	T	G	G	T	A	T	A	A	G	G	G	A	G	G	T	A	T	C	T	T	(A)	31
2	A	−	−	−	−	−	−	T	−	−	−	−	−	−	−	−	−	−	−	−	−	−	A	−	C	−	−	−	−	−	−	−	−	−	(A)	43
3	−	A	−	A	−	−	−	−	−	−	−	−	−	−	−	−	A	−	G	C	G	−	−	−	C	G	−	−	−	−	−	−	−	−	(A)	16
4	−	C	−	A	−	G	−	−	−	−	−	−	−	−	C	−	−	−	−	G	−	G	−	−	C	−	−	−	C	−	−	−	−	−	(A)	18
5	−	−	−	−	−	−	−	T	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	C	−	−	−	−	−	−	−	−	−	(A)	30
6	−	−	−	−	−	−	−	T	−	−	−	−	−	−	C	−	−	−	−	−	−	−	−	−	C	−	−	−	−	−	−	−	−	−	(A)	69
7	−	−	−	−	−	−	−	−	−	−	−	−	−	−	C	−	−	−	−	−	−	−	−	−	C	−	−	−	−	−	−	−	−	−	(A)	
8	−	A	−	A	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	G	−	−	−	C	−	−	A	−	−	−	−	−	−	(A)	
9	A	−	−	A	−	−	−	T	C	−	A	−	−	−	−	−	−	−	−	−	−	−	−	−	C	−	−	−	−	−	−	−	−	−	(A)	
10	−	A	−	A	−	−	−	−	−	−	−	−	−	−	C	−	−	−	−	−	G	−	−	−	C	−	−	A	−	−	−	−	−	−	(B)	24
11	A	−	−	A	T	−	−	−	−	G	A	−	A	−	−	−	−	−	−	−	−	−	−	A	C	−	−	−	−	−	−	−	−	C	(B)	
12	A	−	−	A	−	−	−	−	−	G	A	−	A	−	−	−	−	−	−	−	−	−	−	A	C	−	−	−	−	−	−	−	−	−	(B)	
13	A	−	−	A	−	−	−	−	−	G	A	−	A	−	−	−	−	−	−	−	−	−	−	A	C	−	−	−	−	−	−	−	−	C	(B)	
14	A	−	−	A	−	−	−	T	C	G	A	−	A	−	−	−	−	−	−	−	−	−	−	−	C	−	−	−	−	−	−	−	C	−	(B), SCB601	
15	−	C	−	A	−	G	−	−	−	−	−	−	−	−	C	−	−	−	−	G	−	G	−	−	C	−	−	−	C	−	−	T	−	−	(B), FCB412, FCB415	
16	−	C	−	A	−	G	−	−	−	−	−	−	−	−	C	−	−	−	−	G	−	G	−	−	C	−	−	−	C	−	−	−	C	−	(B)	
17	−	−	−	A	−	G	−	−	−	−	−	−	−	−	C	−	−	−	−	−	−	G	−	−	C	−	−	−	−	−	−	−	C	−	(B)	
18	−	−	−	−	−	−	−	−	−	−	−	−	−	−	C	−	−	−	−	−	−	−	−	−	C	−	A	−	−	−	−	−	−	−	19	2
19	−	A	−	A	−	−	−	−	−	−	−	−	−	−	−	−	A	−	G	−	G	−	−	−	C	G	−	−	−	−	−	−	−	−	21	
20	−	C	−	−	−	−	−	−	−	−	−	−	−	A	−	−	−	−	−	−	−	−	−	−	C	−	−	−	−	−	−	−	−	−	208	2
21	−	A	−	−	−	−	G	−	−	−	−	C	−	−	C	A	−	G	−	C	G	−	−	−	C	−	−	A	−	−	−	−	−	−	818, 819	2
22	−	A	−	−	T	−	−	−	−	−	−	−	−	−	−	−	−	−	−	C	G	−	−	−	C	−	−	A	−	−	−	−	−	−	FCB414	
23	−	C	G	A	−	−	−	−	−	−	−	−	−	−	C	−	−	−	−	G	−	−	−	−	C	−	−	−	C	C	C	T	−	−	FCB603	
24	−	−	−	−	−	−	−	T	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	SCB602	

[a)] Polymorphic sites found within a 1,210 bp region of the mitochondrial genome of Oriental white storks are displayed (Accession number AB026193).

[b)] Samples analyzed in this paper are indicated by their individual numbers. Previously reported haplotypes are indicated by (A) or (B). (A) Yamamoto et al. 2000. (B) Murata et al. 2004.

[c)] The numbers of live captive and wild Oriental white storks belonging to each haplotype in Japan. The haplotype diversity (h) and nucleotide diversity (π) were estimated using Arlequin (Schneider et al. 2000).

bp between 354 and 356. It is interesting that haplotype 21 was only found in specimens 818 and 819, which were captured from the same nest in Khabarovsk and were transferred to Japan in 2003. These individuals had been transferred in order to increase the nucleotide diversity within the captive Japanese population of Oriental White Storks. It is conceivable that these individuals also contain nucleotide differences in nuclear DNA relative to other captive birds, making them suitable for the purpose of introduction.

Haplotype analysis of 4 wild Oriental White Stork feathers revealed 2 new haplotypes; 22 and 23. Haplotype 22 was found in feather FCB414, which was obtained in the Chiba Prefecture in 2005, while haplotype 23 was found in FCB603, which was collected at Okinoshima in the Shimane Prefecture in 2006. Two additional feathers, FCB412 (collected at the Kameoka, Kyoto Prefecture in 2004) and FCB415 (collected in the Chiba Prefecture in 2005), were found to belong to haplotype 15. This haplotype was previously reported in 3 mounted specimens, SS13, SS14 and SS15, stored in the Izushi Village near the Toyooka city (Murata et al. 2004). It is remarkable that Oriental White Storks with the same haplotype 15 as individuals that visited in the past are still coming to Japan for wintering. Haplotype 24 was found in a mounted specimen with no available information stored at Museum of Nature and Human Activities, Hyogo.

The genetic structure of Chinese populations of Oriental White Storks in new breeding areas was recently described by Zan et al. (2008). New breeding colonies were found in wintering areas and at some stopover sites. Zan et al. (2008) reported that 37 haplotypes were found in 66 individuals from the southern and northern breeding areas, and from zoo colonies. It has also been noted that the Chinese population has greater genetic diversity than the Japanese population, with a haplotype diversity (h) of 0.953 ± 0.013 and a nucleotide diversity (π) of 0.013 ± 0.007. However, since previously reported haplotypes and haplotype frequencies within the Japanese population (Yamamoto et al. 2000; Murata et al. 2004) were determined from captive and mounted specimens, it may not be appropriate to compare these values directly.

Regional and International Studbooks for the Oriental White Stork have been published every year by the Japanese Association of Zoological Gardens and Aquariums, and it has been recorded that 183 storks were in captivity in Japanese zoos in 2010 (Regional studbook for the Oriental White Stork 2010). Mitochondrial haplotypes of almost all captive individuals had been identified and recorded, including those of the re-introduced (wild) white storks. The individual numbers of 10 haplotypes in captive and wild Oriental White Storks in Japan are listed in Table 1. The genetic diversity of the Japanese population in 2010 was estimated to be 0.8319 ± 0.0113 for haplotype diversity (h) and 0.00357 ± 0.00196 for nucleotide diversity (π) using Arlequin 2.000 (Schneider et al. 2000). The low nucleotide diversity is considered to be attributable to the small number of haplotypes, which is present within the captive population. Although the genetic diversity of the Japanese population in 2010 was relatively lower than that of the Chinese population (Zan et al. 2008), it was higher than that reported for threatened or near-threatened avian populations such as the Red-crowned crane population of East Asia population (Hasegawa et al. 1999; h is 0.78) or the Resplendent Quetzal (Solorzano et al. 2004; h is 0.8180).

A minimum-spanning network among 24 haplotypes of the Oriental White Stork was estimated using the TCS1.21 software (Clement et al. 2000), as shown in Fig. 1. These haplotypes were obtained from a mixed population containing captive storks, mounted specimens and fallen feathers of wild birds. Therefore, the circle size is fixed because it is not meaningful to compare the number of individuals or samples belong to each haplotype. Fifteen haplotypes were found within the captive Japanese population, while 4 and 9 haplotypes were reported from wild and mounted specimens, respectively. In addition, 5 hap-

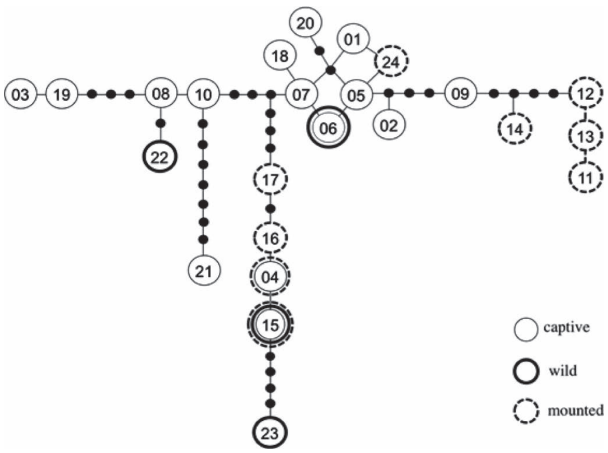

Fig. 1. Haplotype network of the Japanese population of Oriental white storks.

lotypes, 7, 8, 9, 15 and 19 have been recently lost by death within the captive Japanese population. Therefore, individuals belong to these haplotypes are absent in Table 1. The rest 10 haplotypes contained 183 individuals in captivity in 2010 (Regional studbook for the Oriental White Stork 2010). However, the distribution of haplotypes of the captive population is biased toward the Japanese zoo and Hyogo Park of the Oriental White Stork. For instance, each one individual belong to haplotype 3 and 4 is reared in Hyogo Park of the Oriental White Stork, while 15 for haplotype 3 and 17 for haplotype 4 in other Japanese zoo. Exchanges of individuals between breeding centers and introduction of new founders from the Russian and/or Chinese wild populations are required to increase the genetic diversity of the Japanese captive population of Oriental White Storks. The re-introduction program of Oriental White Storks is now successfully in progress. Additional studies will be required to assess the genetic diversity of captive and wild populations by using nuclear DNA in addition to mitochondrial DNA.

Acknowledgement

I am grateful to M. Sato and Y. Mitsuhashi from Hyogo Park of the Oriental White Stork for assistance with sampling of the Oriental White Stork populations. I would also like to thank S. Satake from NPO Sicchi Net for his support during the sampling.

References

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Molecular Ecology, 9: 1657–1660.

Hasegawa O, Takada S, Yoshida MC, Abe S (1999) Variations of mitochondrial control region sequences in three crane species, the Red-crowned Crane *Grus japonensis*, the Common Crane *G. grus* and the Hooded Crane *G. monacha*. Zoo Science, 16: 685–692.

Murata K, Satou M, Matsushima K, Satake S, Yamamoto Y (2004) Retrospective estimation of genetic diversity of an extinct Oriental White Stork (*Ciconia boyciana*) population in Japan using mounted specimens and implications for reintroduction programs. Conservation Genetics, 5: 553–560.

Tama Zoological Park (2000) Regional Studbook for the Oriental White Stork, *Ciconia boyciana* (2000) Tama Zoological Park, Hino.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning—a Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 458–459.

Schneider S, Roessli D, Excoffier L (2000) Arlequin: A software for population genetics data analysis. Ver. 2.000. Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva.

Solórzano S, Baker AJ, Oyama K (2004) Conservation priorities for resplendent quetzals based on analysis of mitochondrial DNA control region sequences. Condor, 106: 449–456.

Yamamoto Y, Murata K, Matsuda H, Hosoda T, Tamura K, Furuyama J (2000) Determination of the complete nucleotide sequence and haplotypes in the D-loop region of the mitochondrial genome in the Oriental White Stork, *Ciconia boyciana*. Genes and Genetic Systems, 75: 25–32.

Zan S, Zhou L, Jiang H, Zhang B, Wu Z, Hou Y (2008) Genetic structure of the Oriental White Stork (*Ciconia boyciana*): implications for a breeding colony in a non-breeding area. Integrative Zoology, 3: 235–244.

(Accepted: 29 September 2011)

ニホンコウノトリにおける新しいミトコンドリアのハプロタイプ

*** 山本義弘**[1]

[1] 兵庫医科大学遺伝学講座
663-8501　兵庫県西宮市武庫川町1-1

* E-mail: yosyam@leto.eonet.ne.jp

ニホンコウノトリ（*Ciconia boyciana*）の遺伝的多様性については，ミトコンドリアのD-loop領域のハプロタイプの解析により17の遺伝系統が見いだされていた．今回の分析では，11の解析用試料，すなわち2体の剥製，5体の血液試料，及び4本の羽根を調べることにより，新たに7つのハプロタイプを見いだした．従って日本のコウノトリのハプロタイプは24となった．しかしながら，遺伝的多様性の解析ではハプロタイプ多様度（0.8319±0.0113）は他種集団と比較しても低くはなかったが，ヌクレオチド多様度（0.00357±0.00196）が低い値を示した．総飼育個体数は増加しているので，飼育施設間でのハプロタイプの偏りを解消することに加えて，ロシアや中国からの新しい血統の導入が望まれる．

キーワード　ミトコンドリア，ハプロタイプ，D-ループ，コウノトリ，*Ciconia boyciana*