Two Distinct mtDNA Lineages among Captive African Penguins in Japan

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ABSTRACT. The African penguin (*Spheniscus demersus*) is one of the world's most endangered seabirds. In Japan, although the number of African penguins in captivity continues to increase, genetic data have not been collected for either wild or captive populations. To reveal genetic diversity and characterization in captive African penguins, we analyzed the nucleotide sequences of mitochondrial DNA (mtDNA) from a sample of 236 African penguins. Analysis of 433 bp of the control region and 1,140 bp of cytochrome b sequences revealed the existence of two mtDNA clades. Control region haplotypes were much more divergent (d=3.39%) between the two clades than within each clade. The divergence of these clades may reflect differences at the subspecies or geographical population level in African penguins. These findings suggest that at least two distinct maternal lineages exist in the wild populations of the African penguin.
KEY WORDS: African penguin, control region, genetic diversity, mitochondrial DNA.

The African penguin (*Spheniscus demersus*), which is endemic to southern Africa, inhabits 31 islands and four mainland sites in Namibia and South Africa, ranging from southern Angola in the north to Nelson Mandela Bay in the east [4]. Wild African penguin populations markedly decreased in the 20th century. Approximately 56,000 pairs estimated in 2001 have declined to 21,000 pairs in 2009 [5]. This population decline is thought to be primarily due to food scarcity, resulting from overfishing, overexploitation and environmental fluctuations, such as increased sea surface temperature linked to climate change. In addition, oil pollution has become a major factor in African penguin mortality. Consequently, the African penguin is listed in Appendix II of the Convention on International Trade in Endangered Species and classified as Endangered by the 2010 International Union for Conservation of Nature.

While wild African penguin populations continue to decrease, properly maintained captive populations are increasing year on year. According to the 2011 Japanese regional studbook for the African penguin, their original introduction to Japan was in 1935, and from 1973 to 2011, 156 additional founders were introduced: 92 from South Africa, 30 from overseas zoos and aquariums and the remaining 34 from unknown locations. The time and number of introductions varied. The captive African penguin populations in Japan contain 485 individuals, comprising an estimated 87 different founder lineages. In order to avoid close inbreeding and to maintain genetic diversity, the Japanese Association of Zoos and Aquariums (JAZA) keeps studbooks which it uses to promote longer-term breeding plans. However, no genetic data on the foundering population of African penguins introduced to Japan are described in the studbook, and the genetic relationship of captive African penguins in Japan is unclear.

Genetic data have not been collected on either wild or captive African penguins in Japan so far. In this study, we examined the genetic diversity of the captive Japanese populations of African penguins by analyzing the control region and cytochrome b sequences of mitochondrial DNA (mtDNA), since mtDNA sequences have higher rates of nucleotide substitution than nuclear DNA sequences [2]. Analysis of mtDNA sequences can reveal genetic relationships among closely related species. This molecular information may prove useful to JAZA for future management and implementation of breeding programs.

Blood or feather samples from African penguins (*n*=236) were collected from 20 Japanese zoos and aquariums. Blood samples were also collected from captive populations of Humboldt penguins (*Spheniscus humboldti*) (*n*=20) and Magellanic penguins (*Spheniscus magellanicus*) (*n*=2). Genomic DNA was extracted using Dr. GenTLE™ (Ta-KaRa Bio, Otsu, Japan) or Get pureDNA Kit-Cell, Tissue (Dojindo Molecular Technologies, Kumamoto, Japan). Two mtDNA fragments of 653 bp (control region) and 1,140 bp (cytochrome b) were amplified by polymerase chain reaction (PCR). The control region fragments and the entire cytochrome b gene were amplified using the primer pair L-tRNA^Glu(5′-CCTGCTTGGCTTTTYTCCAAGACC) and H-Dbox (5′-CTGACCGAGGAACCAGAGGCGC) [13] and the primer pair BCL1 (5′-AGGCCTACCTAG-GATCCTTCGCCCT) and BCH1 (GTCTTTGGTTAAT-TACAAGACCAATGTTT) [8], respectively. The H-Dbox, BCL1 and BCH1 primers were also used as sequencing primers. Amplification reactions were carried out in a 50 μl solution containing 10–100 *n*g genomic DNA, 1 × buffer (20 mM Tris-HCl, pH8.0, 100 mM KCL and 2 mM MgCl$_2$), 200

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μM each of deoxyribonucleotides, 1.25 units of *Ex Taq*® HS DNA polymerase (TaKaRa Bio) and 0.2 μM of each primer. Reaction mixtures were incubated in a PCR thermal cycler (TaKaRa PCR Thermal Cycler Dice™; TaKaRa Bio) with initial denaturation of 3 min at 95°C with a typical profile of 32 cycles, each consisting of 30 sec at 95°C, 30 sec at 60°C (for the control region) or 55°C (for cytochrome b) and 30 sec (control region) or 1 min (cytochrome b) at 72°C with final extension of 5 min at 72°C. PCR products were checked by electrophoresis on 1.5% agarose gel with TAE buffer, and the gel was stained with ethidium bromide. PCR products were purified by exonuclease I (Wako, Osaka, Japan) and shrimp alkaline phosphatase (TaKaRa Bio) and were sequenced directly using an ABI PRISM 3130 Genetic Analyzer and BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, CA, U.S.A.). The sequences of 433 nucleotides of the control region and 1,140 nucleotides of cytochrome b were determined. Phylogenetic analyses were conducted using MEGA version 5 [16]. Multiple sequence alignment was performed by using Clustal W [17]. Genetic distances between haplotypes were obtained using the Tamura-Nei model [15]. Phylogenetic trees were constructed by the neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) methods. The reliability of tree topology was assessed by 1,000 bootstrap replications [7]. Lastly, Neighbor-Net analysis [3] was performed to construct a phylogenetic network using SplitsTree4 software [9] (http://www.splitstree.org/).

DNA sequences of the mitochondrial control region of 236 African penguin individuals were determined. Multiple sequence alignments of the 433 bp constituting the partial control region showed 39 polymorphic sites, generating a total of 30 different haplotypes (Table 1). All substitutions were transitions. Our nucleotide sequencing data are available under the accession numbers AB775475-775504 from the DDBJ/EMBL/GenBank databases. The available 45 of the total 64 South African founder populations were also examined, resulting in 27 haplotypes. This indicated that the founder populations were derived from 27 maternal ancestors. These maternal genetic results are consistent with traceable records of captive breeding detailed in the studbook. We also investigated the Humboldt penguin and Magellanic penguin, which belong to *Spheniscus*, the same genus as the African penguin. Eight different haplotypes were obtained from these species (accession numbers AB775505-775512).

NJ phylogenetic tree analysis using mitochondrial DNA control region sequences revealed that the captive African penguin populations kept in a total of 20 Japanese zoos and aquariums clustered into two clades (A and B) as supported by high bootstrap values (Fig. 1). Clades A and B contained 26 and 4 haplotypes, respectively. The 27 haplotypes from South Africa described above belonged to both clades A and B, suggesting 2 maternal lineages derived from South Africa. Five substitutions at nucleotide numbers 110, 166, 217, 225 and 285 in the control region were characteristic of clade B (Table 1). The mean genetic distance of the control region sequences between the 2 clades was 3.39%, and the genetic distances within clades A and B were 0.93% and 1.18%, re-

spectively. Both MP and ML analyses also showed a similar topology with the captive African penguins arranged in 2 different clades (data not shown). A network analysis further supported the division of the maternal lineages of the captive African penguins into these 2 different clades (Fig. 2).

The complete 1,140 bp sequences (accession numbers AB776002-776009) in the cytochrome b gene were obtained from 54 captive African penguins in Japan. There were 8 haplotypes defined by 11 variable sites. Nine nucleotide substitutions were synonymous, and the remaining 2 substitutions were non-synonymous. The same two clades as those of the control region were separated by 1 non-synonymous substitution (data not shown).

The mtDNA diversities of rockhopper penguin (*Eudyptes* spp.) and blue penguin (*Eudyptula minor*) samples from wild populations have been previously reported. The rockhopper penguin, which had been considered a single species with two subspecies [6], was suggested to be two distinct species based on the average genetic distance (6.1%) of the control region sequences between its northern and southern clades [10]. The blue penguin, a single species comprising 6 morphologically determined subspecies [11], can be divided into an Australian-Otago clade and a New Zealand clade from analyses of 3 mtDNA sequences [1]. The control region sequences between these two clades have been found to differ by 11.8%. Moreover, examination of the control region sequences of individuals collected from 7 southern Australian *E. m. novaehollandiae* colonies [11] showed no geographic clustering of closely related genetic variations among colonies [12]. The average genetic distance among colonies of this subspecies was 1.0%, based on our calculations made from sequence data for EU043384-043403. Considering these molecular data found in studies of the rockhopper penguin and blue penguin, the divergence of African penguin clades A and B (*d*=3.39%) seen in the present study may reflect either a difference in geographical populations or the existence of undefined subspecies of African penguins, although it must be noted that our data focused on captive-bred individuals.

This is the first report of molecular data obtained by mtDNA analyses of captive African penguins. Here, we demonstrated the existence of two divergent clades of captive African penguins with moderate genetic distance (*d*=3.39%). Although we currently have no descriptive information on the founders of the captive African penguin population and genetic data from wild African penguin populations must also be examined, our data imply that captive African penguins in Japan are derived from two distinct maternal lines. We are presently analyzing nuclear DNA markers, including microsatellite diversity and repetitive DNA sequences, to elucidate further the genetic characterization of African penguin populations.

In other endangered species, such as the Oriental white stork (*Ciconia boyciana*), mtDNA control region analysis has been utilized to develop an effective breeding plan in Japan [18, 19]. Likewise, careful genetic management is needed to maintain genetic variability in the African penguin and to use captive breeding projects to save this endangered species from extinction.

Table 1. Haplotypes and variable sites of the mitochondrial control region found in Japanese captive populations of African penguins (*Spheniscus demersus*)

Clade	Haplotype	13	31	30	101	107	118	110	117	121	122	134	135	139	141	161	165	166	170	177	184	298	217	220	225	237	239	243	275	285	286	288	292	293	294	395	304	305	306	329	No. of individuals
Clade A	type1	A	G	T	T	T	T	A	A	A	G	A	T	C	T	G	A	A	G	G	C	G	G	G	G	A	T	A	G	A	T	G	G	G	T	T	A	A	T	T	22
	type2	G	.	.	.	.	.	.	G	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	3
	type3	G	.	.	.	C	.	.	G	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	9
	type4	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	20
	type5	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	C	28
	type6	.	.	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	7
	type8	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C	25
	type9	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	27
	type10	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	9
	type11	.	.	.	.	.	.	.	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	3
	type12	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	3
	type13	G	.	.	.	.	.	.	G	G	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	8
	type14	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	18
	type15	G	.	.	.	.	.	.	G	.	.	G	C	.	C	.	.	.	.	.	.	.	.	.	C	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C	8
	type17	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C	3
	type18	G	.	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	1
	type19	G	.	.	.	C	.	.	G	.	A	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
	type20	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	.	.	.	C	4
	type21	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	C	.	.	.	.	C	2
	type22	G	.	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	C	.	1
	type23	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	C	1
	type25	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	1
	type26	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	A	.	.	.	.	.	.	C	3
	type27	G	.	.	.	.	.	.	G	G	.	.	.	.	T	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	11
	type29	G	.	.	.	.	.	.	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	2
	type30	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	3
Clade B	type7	.	A	C	.	C	.	G	G	.	.	.	.	.	A	.	G	.	R	.	.	A	.	A	.	.	.	A	G	.	A	.	.	.	.	.	.	G	.	C	7
	type16	.	.	.	C	C	.	G	G	.	.	.	.	.	A	.	G	.	.	.	A	.	A	G	.	G	A	G	C	A	.	.	.	.	.	.	.	.	.	C	1
	type24	.	.	.	C	C	.	G	G	.	.	.	.	.	A	.	G	.	.	.	A	.	A	G	.	G	.	G	.	A	.	.	.	.	.	.	.	.	.	C	1
	type28	.	A	C	.	C	.	G	G	.	.	.	.	.	A	.	G	.	.	.	A	.	A	.	.	.	A	G	.	A	.	.	.	.	.	.	.	G	.	C	4
																																								total	236

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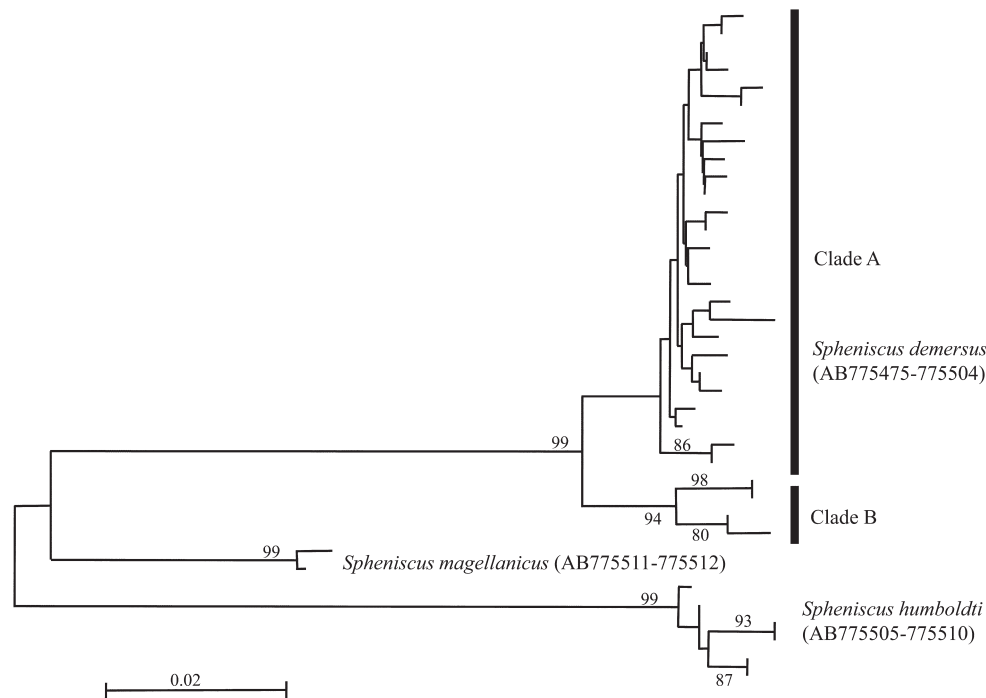8. Hitosugi, S., Tsuda, K., Okabayashi, H. and Tanabe, Y. 2007.

Fig. 1. Neighbor-joining tree of mitochondrial DNA control region haplotypes of African penguins. The tree was constructed as described by Saitou and Nei [14] and was rooted using Humboldt penguins (*Spheniscus humboldti*) as an outgroup. Phylogenetic analyses were conducted with MEGA5. Only bootstrap values greater than 70% are shown.
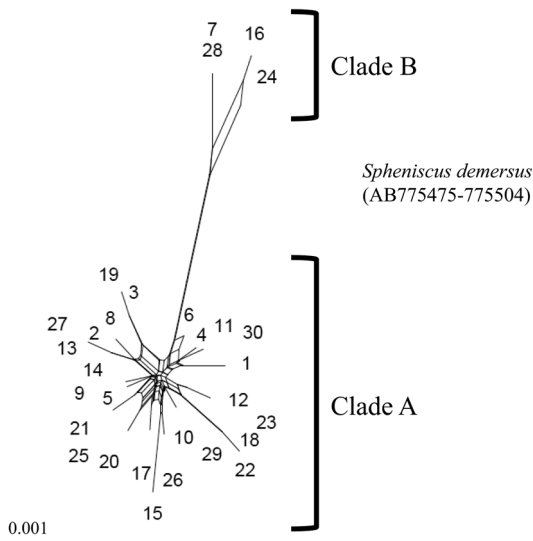

Fig. 2. Neighbor-Net diagram of captive African penguin populations. A network of relationships among the 30 haplotypes detected in 236 individuals of the captive African penguin was generated by Neighbor-Net analysis of polymorphisms of the control region. Digits represent the haplotype number.

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