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Mitochondrial DNA variations in Japanese farmed emu populations

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ABSTRACT. The emu (*Dromaius novaehollandiae*) is a new poultry. In this study, we investigated the haplotype composition of mitochondrial DNA among emu populations farmed in Japan. We sequenced the D-loop region in 109 individuals, and detected four substitution sites and three haplotypes (Hap-a, -b, and -c). Hap-a was the most frequently observed haplotype in the Japanese populations. Although Hap-c was a rare haplotype in not only Japanese but also Australian populations, it was detected with high frequency in the Japanese farmed population. The AMOVA indicated that 9% of total variance was "among population". The F_{ST} value was 0.087 and genetic differentiation was significant ($P<0.01$). These results may contribute to conserving the genetic resources available for the Japanese emu industry.

KEY WORDS: emu, genetic structure, mitochondrial DNA

The emu (*Dromaius novaehollandiae*) is ratite bird native to Australia, and is a new poultry that produces meat, eggs and oil. Among these products, the subcutaneous fat, which is rich in unsaturated fatty acids, shows anti-inflammatory effects, and thus, the oil of the emu is one of the most important materials produced by the emu [1, 11]. Over the past few decades, emus have been farmed in Australia, America, and China, and other countries. Recently, emu farming has begun in Japan [14]. Although the emu has a high potential as a novel poultry, research regarding genetic improvements of these birds is in its infancy due to their recent domestication [15].

To effectively improve the genetic ability of productive traits in the emu, it is desirable to maintain and extend genetic diversity in farmed populations. Thus, pedigree information is essential for selective breeding, with the avoidance of inbreeding. However, it is difficult to record pedigree information of the emu due to their reproductive behavior. The emu exhibits monogamy and polygyny, and brooding is carried out by male individuals [4, 9, 11, 13]. Furthermore, the rate of egg laying is influenced by the chemistry between the breeding pair [15]. Therefore, random mating is the most major system for effective reproduction, which means that pedigree information cannot be recorded and genetic diversity in the Japanese farms is difficult to estimate.

Mitochondrial DNA (mtDNA) is one of the most popular markers for estimation of genetic diversity of animal populations. Although Okubo *et al*. (2015) [8] reported that only two haplotypes were found on a Japanese farm, these data were obtained by analysis in individuals hatched at a single year in only one farm. To understand genetic variation of the Japanese emu population, analyzing individuals derived from multiple farms in Japan is required. In this study, we investigated mtDNA haplotypes in three Japanese farm populations based on D-loop polymorphisms and revealed that Japanese emu populations were composed of at least three haplotypes and that many individuals harbored a rare haplotype in a Japanese zoo.

The feather pulps of emus (n=109) were collected from three populations. The Okhotsk Emu Farm (OEF; Abashiri, Hokkaido, Japan), the largest emu population in Japan with more than 1,400 individuals, was established approximately 20 years ago, with emus introduced from farms in the United States, Australia, and Japan. The Japan Eco System, Co., Ltd. (JES; Chikushino, Fukuoka, Japan) Farm was established in 2013 and fostered the second largest population, with more than 300 individuals that originate from the OEF and farms in Australia, Mongolia, and other countries. The Tohoku Safari Park (TSP; Nihonmatu, Fukushima, Japan) has only 19 individuals of unrecorded origin with the purpose of exhibition. Since there are no pedigree information, we obtained specimens by random sampling from OEF and JES. All procedures involving animals met the guidelines described in "The Proper Conduct of Animal Experiments", proposed by the Science Council of Japan, and were approved by the Ethical Care and Use of Animals Committee at the Tokyo University of Agriculture (approval number: 270049). Genomic DNAs were isolated from feather

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Table 1. The detected haplotypes in the D-loop region and its frequency among Japanese populations

Haplotype	Nucleotide position				Haplotype frequency			
Reference (NC_002784)	15792 C	15809 C	15810 C	16114 G	Total n=109	OEF n=32	TSP n=19	JES n=58
a	*	*	T	*	0.79	0.84	0.68	0.79
b	T	*	T	A	0.14	0.13	0.00	0.21
c	T	T	*	A	0.06	0.03	0.32	0.00

Asterisks indicate the identical nucleotide to reference sequence. OEF, TSP, and JES indicate Okhotsk Emu Farm, Tohoku Safari Park, and Japan Eco System, respectively.

pulps using Isogenome (Nippon Gene, Tokyo, Japan), according to the manufacturer's protocol.

We amplified large DNA fragments by long and accurate PCR (LA-PCR) to avoid amplification of nuclear copies of mitochondrial pseudogenes (numts). A 6,410-bp fragment that included a D-loop region was amplified using a primer set previously designed by Okubo *et al.* (2015) (emu_H14335_LA, emu_L4034_LA) with KOD FX (TOYOBO, Osaka, Japan) [8]. After the 1st PCR, 1 μl of its product was used for the 2nd PCR. 2nd PCR was carried out to amplify a 707-bp fragment of D-loop region using a primer set previously designed by Okubo *et al.* (2015) (emu_L15709, emu_H16416) [8] with KOD FX (TOYOBO). The 2nd PCR product was purified using the FastGene Gel/PCR Extraction Kit (Nippon Gene). DNA sequencing was performed by the ABI 3730XL DNA analyzer (Thermo Fisher Scientific).

D-loop sequences were aligned using MEGA 6 software [12], and nucleotide diversity (π) and haplotype diversity (h) were calculated using DNaSP [6]. The haplotype network was also constructed by DNaSP and was drawn by the FigTree version 1.2.2 viewing program. Analysis of molecular variance (AMOVA) [2] and Nei's genetic distance (Da) [7] were calculated using GenAlex version 6.5 [10].

We determined a 348-bp DNA sequence of the D-loop region in 109 individuals, and found 4 substitution sites, and 3 haplotypes: Hap-a, -b and -c, were detected (Table 1). The frequencies of Hap-a, Hap-b and Hap-c were 0.79, 0.14 and 0.06, respectively across all tested individuals. Many of OEF, TSP, and JES individuals harbored Hap-a, and that frequencies were 0.84, 0.68, and 0.79, respectively. Therefore, we suggested that Hap-a was a major haplotype in Japanese emu populations. Meanwhile, Hap-b was found in OEP (0.13) and JES (0.21), but not TSP. Although Hap-c was rarely found in OEF (0.03), its frequency was relatively higher in TSP (0.32). The haplotype network tree also showed that large proportions of Japanese farmed emu possessed Hap-a, and that many TSP individuals harbored Hap-c despite exhibiting a rare haplotype (Fig. 1). In the previous study, 10 haplotypes (A–J) were detected on complete sequence of the D-loop region (1,094-bp) in Australian emu populations that included extinct species [5, 14]. Hap-a and Hap-b detected in this study were identical to hapA–D and hapG–J, respectively, and Hap-b was frequently detected in the extinct species, *Dromaius ater*. Meanwhile, Hap-c that was detected in OEP and TSP, was not found in previous studies, indicating that Hap-c was a rare haplotype in the emu. Therefore, TSP harboring Hap-c with higher frequency may be an important genetic resource in Japan.

To estimate genetic diversity of Japanese emu population, general genetic parameters were calculated based on D-loop polymorphisms. Average number of nucleotide differences (k) showed 0.669, 1.825,

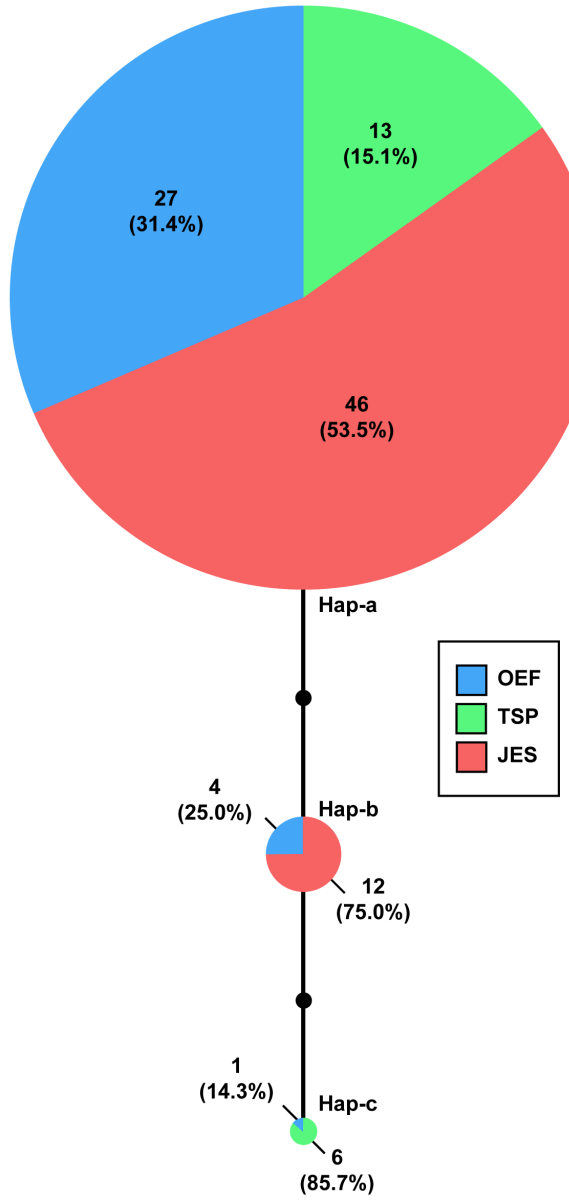

Fig. 1. The haplotype network tree of Japanese farmed emu populations. The pie graph on the interlinking branches means the population structure among haplotypes, and black circles on branches represent missing haplotypes. Pie size correspond to the haplotype frequencies. Blue, green and red colors indicate individuals derived from Okhotsk Emu Farm (OEF), Tohoku Safari Park (TSP), and Japan Eco System (JES), respectively.

Table 2. Genetic parameters of Japanese farmed populations based on D-loop polymorphism

Population	N	No. of haplotypes	No. of mutations	k	$h \pm$ SD	$\pi \pm$ SD
OEF	32	3	4	0.669	0.280 ± 0.095	0.0019 ± 0.0007
TSP	19	2	4	1.825	0.456 ± 0.085	0.0052 ± 0.0010
JES	58	2	2	0.744	0.372 ± 0.064	0.0021 ± 0.0004

OEF, TSP, and JES indicate Okhotsk Emu Farm, Tohoku Safari Park, and Japan Eco System, respectively. k, h, and π indicate average number of nucleotide differences, gene/ haplotype diversity, and nucleotide diversity, respectively.

Table 3. Analysis of molecular variance (AMOVA) of mtDNA D-loop regions analyzed in Japanese emu populations

Source	df	Sum of squares	% of variance	Fixation index (P)
Among population	2	3.564	9%	F_{ST}: 0.087 (0.0002)
Within population	106	45.831	91%	
Total	108	49.395	100%	

Table 4. Pairwise population matrix of F_{ST} (*upper*) and Nei's genetic distance (*lower*)

	OEP	TSP	JES
OEP	-	0.119[a)]	−0.008
TSP	0.060	-	0.151[a)]
JES	0.002	0.064	-

OEF, TSP, and JES indicate Okhotsk Emu Farm, Tohoku Safari Park, and Japan Eco System, respectively. a) $P<0.01$.

and 0.744 in OEF, TSP, and TSP, respectively (Table 2). Although OEF and JES indicated similar degree of values, the highest value of k was observed in TSP. Haplotype diversity (h) was 0.280 ± 0.095, 0.456 ± 0.085, and 0.372 ± 0.064 in OEF, TSP, and JES, respectively. Nucleotide diversity (π) of OEF, TSP, and JES indicated 0.0019 ± 0.0007, 0.0052 ± 0.0010, and 0.0021 ± 0.0004, respectively. Therefore, we revealed that TSP maintained the highest maternal genetic diversity among tested Japanese populations in this study. Although the largest number of haplotypes were observed, the lowest h and π values were shown in OEF. Thus, we confirmed that the maternal genetic diversity of OEF was the lowest among tested Japanese farms, despite it being the largest population size.

To evaluate genetic differentiation among Japanese emu populations, we conducted an AMOVA based on D-loop polymorphisms. The AMOVA exhibited that 9% and 91% of total variance was "among the population" and "within the populations", respectively (Table 3). The F_{ST} was 0.087 among populations ($P<0.01$), indicating that a very weak degree of genetic differentiation was found among Japanese emu populations. To investigate genetic relationships among populations, we calculated genetic distance based on Nei's method (Da). Da showed relatively lower values, which ranged from 0.002 to 0.064 (Table 4). The lowest Da was found between OEP and JES (Da=0.002), and the highest values were shown between OEF/JES and TSP (Da=0.060 and 0.064, respectively). Pairwise F_{ST} also showed a lower value between OEF and JES (0.002), and relatively high values were indicated between TSP and OEF/JES (0.119/0.151) with significant population differentiation. Although relatively higher Da and F_{ST} were observed between TSP and others, the degree of genetic differentiation was very low in Japanese farmed populations. Therefore, the genetic differentiation of Japanese emu populations has not yet been progressed, which is potentially caused by the short history of the Japanese emu industry.

In conclusion, we demonstrated the mtDNA variation among Japanese farmed emu populations based on D-loop polymorphisms and found a rare haplotype in many individuals derived from TSP. Our results may contribute to extending the genetic diversity of Japanese emu populations by the exchange of individuals among farms. The mtDNA haplotype diversity was low in not only Japanese emu, but also Australian populations [4]. Meanwhile, microsatellite analysis indicated high heterozygosity in Australian wild and farmed emu populations [3]. Therefore, we speculated that genetic analysis using microsatellite markers is essential to understanding genetic structures in Japanese emu populations.

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