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Genetic Diversity of Emu Population in a Japanese Farm Based on Microsatellite DNA Analysis

By

Yuichi KOSHIISHI*, Michiko MURATA-OKUBO**, Gaku SHIMOI*, Hiroki HIRAYAMA**, Kousaku SOUMA** and Kenta WADA*†

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Summary：The emu (*Dromaius novaehollandiae*) is predicted to be a new livestock animal for oil, meat and egg production. However, the genetic structure of emu populations in Japanese farms is scarcely known. The aim of this study was to determine the genetic diversity and population structure in the largest emu farm in Japan. We collected feather pulps of emu chicks (N＝131) from 40, 20, 23, and 48 individuals hatched at 2013, 2014, 2015, and 2016, respectively, in the Okhotsk Emu farm in Abashiri, Hokkaido, Japan. Using six microsatellite markers, we investigated the genetic diversity and structure of this farmed emu population. The number of alleles (N_A) were 4.83, 4.17, 4.17, and 7.17, in individuals hatched in 2013, 2014, 2015, and 2016, respectively. Expected and observed heterozygosity (H_E ; H_O, respectively) was 0.466/0.339, 0.426/0.325, 0.433/0.384, and 0.550/0.347, in each year, respectively. A high inbreeding coefficient (F_{IS}) was observed in all tested generations (0.113-0.369). The Structure program and unrooted phylogenetic tree analysis showed that the Abashiri emu population is largely divided into three to five different clades. Our results suggested that the genetic diversity in the Abashiri emu population is low, and that it contains three to five genetic lineages. These data may help guide a more sustainable breeding of emus in Japan.

Key words：emu, genetic diversity, genetic structure, microsatellite markers

Introduction

The emu (*Dromaius novaehollandiae*) is a ratite native to Australia, and a potential new poultry for production of low-fat red meat, eggs, and oil. Currently, emus are farmed in the USA, Europe, and China[1] because emu oil has therapeutic[2-6] and cosmetic[7,8] properties. In the Abashiri farm located in north-eastern Japan, a pair of emus originating from a farm in the USA was first introduced in 1999, and another 20 individuals from farms in Australia and Japan were added shortly thereafter. Currently, the Abashiri emu farm is composed of more than 1,400 individuals, including approximately 200 breeding stock, and contains the largest emu population in Japan.

Emu domestication is a relatively recent event[9]. Despite the emu potential as a new livestock, genetic improvement of its productive traits has hardly reached the advanced state of other livestock. To develop emu farming in Japan, its genetic improvement is one of the most important issues, and genetic diversity is one of the foremost parameters for efficient and sustainable breeding of these animals[10]. In general, genetic diversity of livestock should be managed by selective mating while preventing inbreeding. However, the reproductive system of emus is comparatively complex with monogamy, polygamy, and polyandry, and eggs are brooded by the male[11-13]. Moreover, mating by pairing often fails owing to lack of attraction between male and female. Therefore, the Abashiri emu farm is carrying out breeding by random mating for the effective proliferation of individuals. In this mating system, because parents of hatched chicks are not specifically chosen, genetic diversity is more readily assessed using DNA from hatched chicks in the Abashiri

* Laboratory of Animal Resources and Development
** Laboratory of Livestock Production and Management, Graduate School of Bioindustry, Tokyo University of Agriculture
† Corresponding author（E-mail : k3wada@nodai.ac.jp）

farmed population.

Microsatellite sequences are useful genetic markers to evaluate genetic diversity in animal populations[14]. In Japan, Okubo *et al.* (2015)[15] first reported the degree of genetic diversity in farmed emus hatched in 2013 using six microsatellite markers that were characterised in previous studies[16–18]. However, the genetic diversity of multiple generations has still not been investigated, and that information is crucial for accurate estimation of genetic diversity in this population.

In this study, using microsatellite marker analysis, we investigated the genetic diversity in emus hatched from 2013 to 2016 in the Abashiri farm, and suggested that the genetic diversity of this population is low. In addition, the Abashiri farmed population can be divided into three to five genetic lineages.

Materials and Methods

Sample collection and DNA extraction

The total number of individuals hatched in 2013, 2014, 2015, and 2016 was 83, 317, 525, and 477, respectively. We randomly collected feather pulps of emu chicks (N = 131) from 40, 20, 23, and 48 individuals hatched in 2013, 2014, 2015, and 2016, respectively, at the Okhotsk Emu farm in Abashiri, Hokkaido, Japan. We re-analysed some individuals hatched in 2013 and used by Okubo *et al.* (2015)[15]. These 131 individuals may comprise siblings and consanguinity because they were produced by random mating in large-scale rearing[15]. Genomic DNA was isolated from feather pulps using Isogenome (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions.

Microsatellite genotyping

We used six markers (*emu18*, *emu63*, *Dn02*, *Dn10*, *Dn28*, and *Dn35*) characterised in previous studies[16,18]. PCRs with fluorescently labelled primers (Beckman dye 2–4 ; SCIEX, Brea, CA, USA) were performed using PrimeTaq DNA Polymerase (M&S Techno Systems, Tokyo, Japan) according to the manufacturer's instructions. We used PCR conditions as described in previous studies[16,18]. Alleles were detected and analysed using a CEQ8000 Genetic Analyzer (SCIEX, Brea, CA, USA).

Data analysis

An online tool[19,20] was used to calculate allele number, frequency, and heterozygosity, and to test for Hardy-Weinberg equilibrium (HWE). Inbreeding coefficient (F_{IS}) values were calculated based on expected heterozygosity (H_E) and observed heterozygosity (H_O)[21]. For population cluster analyses, Structure version 2.3.4[22] was used, and the maximum likelihood number of clusters (K) was predicted using Structure Harvester version 0.6.94[23]. Genetic distance (Da)[24] and allele shared distance (ASD)[25] were calculated using Populations version 1.2.32[26], and a phylogenetic tree based on ASD was constructed using Neighbor-joining method[27], and visualized using FigTree version 1.2.2.

Results and Discussion

Genetic diversity of the emu population

We tested the utility of 17 microsatellite markers characterised in previous studies[16,18] in the Abashiri farmed population. Unfortunately, 11 of the 17 tested markers could not be amplified by PCR. Consequently, only six of these markers were used for analysis in the Japanese emu population. To estimate the genetic diversity within the population, we genotyped six microsatellite loci in 131 individuals obtained from 2013 to 2016. The number of alleles (N_A) were 9, 4, 13, 12, 5, and 8 in the *Dn28*, *Dn35*, *emu18*, *emu63*, *Dn02* and *Dn10* loci, respectively (Table 1). The expected and observed heterozygosities (H_E/H_O) were 0.768/0.670, 0.154/0.072, 0.696/0.517, 0.742/0.467, 0.063/0.048, and 0.399/0.318, respectively (average : 0.469/0.349). The F_{IS} values were 0.116, 0.529, 0.257, 0.371, 0.240, and 0.202, respectively (average : 0.286). Four markers (*Dn28*, *Dn35*, *Dn02*, and *Dn10*) showed no significant deviation from the HWE, whereas two markers (*emu18* and *emu63*) deviated significantly from the HWE (Table 1). These results suggest that loss of random mating occurred in this population, or that null alleles were included in the genotypes detected with the *emu18* and *emu63* markers. Unfortunately, we could not exclude *emu18* and *emu63* in this study, because few genetic markers for the emu are available.

We compared the genetic diversity of chicks hatched for four years to estimate the difference between generations. Average N_A and A_E (N_A/A_E) in individuals hatched in 2013, 2014, 2015, and 2016 were 4.83/2.37, 4.17/2.46, 4.17/2.33, and 7.17/2.88, respectively (Table 2). The H_E/H_O were 0.466/0.339, 0.426/0.325, 0.433/0.384, and 0.550/0.347 respectively, and none of the tested generations

Table 1 Characterisation of six microsatellite markers in the Abashiri emu population

Marker	N_A	H_E	H_O	F_{IS}	HWE
Dn28	9	0.758	0.670	0.116	0.052
Dn35	4	0.154	0.072	0.529	0.333
*emu18**	13	0.696	0.517	0.257	0.007
*emu63**	12	0.742	0.467	0.371	0.003
Dn02	5	0.063	0.048	0.240	0.500
Dn10	8	0.399	0.318	0.202	0.750
Average	8.5	0.469	0.349	0.286	0.274

N_A, Number of allele; HWE, Hardy-Weinberg's equilibrium; H_E, Expected heterozygosity; H_O, Observed heterozygosity; F_{IS}, Fixation index. Asterisks indicate a significant deviation from the HWE ($P < 0.05$).

showed a significant deviation from the HWE (Table 2). From 2013 to 2015, large differences of N_A, A_E and heterozygosity were not observed, although individuals hatched in 2016 showed higher N_A and H_E than those of other generations. Although the highest N_A and H_E were observed in individuals hatched in 2016, the difference between H_E and H_O was the highest among all tested generations. High F_{IS} values were observed in hatched individuals of all tested generations (0.272, 0.237, 0.113, and 0.369, respectively), indicating that this farmed emu population shows a tendency of inbreeding (Table 2). Hammond *et al.* (2002) investigated the genetic diversity of Australian and Thailand emu populations of 9–20 individuals per population using five microsatellite markers. The average H_E/H_O of Australian wild populations indicates a high heterozygosity, 0.87/0.87 and 0.80/0.84, and of Australian farmed populations range from 0.82 to 0.89/0.78 to 0.86[17]. In addition, Hammond *et al.* (2002) reported that relatively low H_E/H_O is found in the Thailand farmed population (0.79/0.65). Negative F_{IS} values are observed in wild populations, 0.032–0.077 in farmed populations in Australia, and 0.202 in farmed populations in Thailand. Thus, the heterozygosity of the Abashiri population was lower than both the Australian and Thailand farmed populations, and inbreeding coefficients were comparable to, or even higher than, those of populations farmed in Thailand. These results suggest that the Abashiri farmed emu population has low genetic diversity, at least in chicks hatched for the past four years.

Table 2 Genetic diversity of each generation in the Abashiri emu population

Year	N_A	A_E	HWE	H_E	H_O	F_{IS}
2013	4.83	2.37	0.179	0.466	0.339	0.272
2014	4.17	2.46	0.260	0.426	0.325	0.237
2015	4.17	2.33	0.423	0.433	0.384	0.113
2016	7.17	2.88	0.171	0.550	0.347	0.369

N_A, Number of alleles; A_E, Effective number of alleles; HWE, Hardy-Weinberg's equilibrium; H_E, Expected heterozygosity; H_O, Observed heterozygosity; F_{IS}, Fixation index.

Population structure of the emu population

To estimate the genetic structure of the population, we performed population structure analysis based on six markers with the Structure software. The Structure Harvester analysis indicated that the most likely K value was 3, predicting that this farmed emu population was genetically divided into three clusters (Fig. 1A). Predictably, the Abashiri farmed population separated in clearly divided clusters in $K=3$ (Fig. 1B). Although analysis at $K=3$ did not indicate clear genetic differentiation among hatching years, the genetic structure of individuals hatched in 2016 was slightly different from that of other generations (Fig. 1C). In the case of $K=2$, a more obvious difference was observed between individuals hatched in 2016 and the others, which was supported by the highest Nei's genetic distance (Da ; 0.151–0.161) and

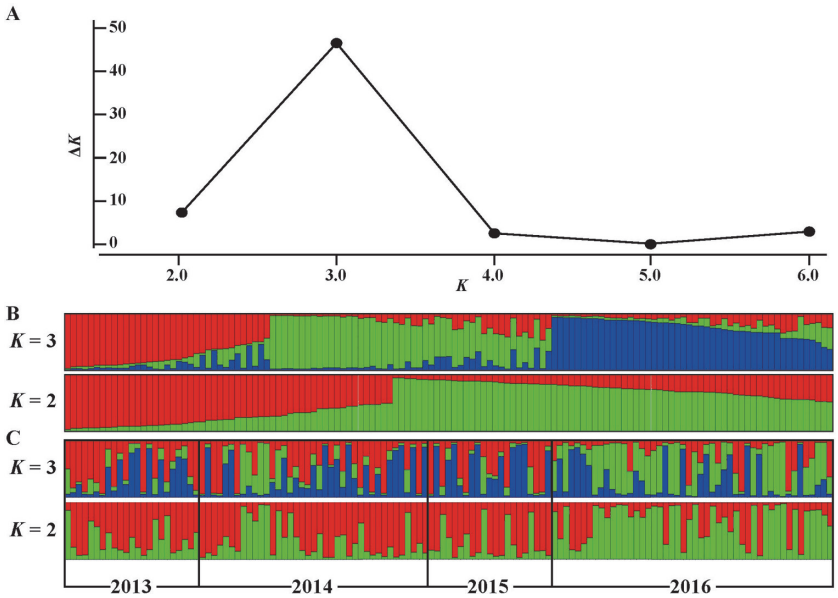

Fig. 1 Genetic structure of Abashiri emus hatched in 2013, 2014, 2015, and 2016. (A) The optimal K value predicted using the Structure harvester software. The maximum likelihood K value was 3 in the Abashiri emu population. (B) Clustering of the Abashiri emu population using Structure with a K value of 3 (*upper*) and 2 (*lower*). (C) Clustering of the Abashiri emu population using Structure with a K value of 3 (*upper*), and 2 (*lower*) in each hatched year.

F_{ST} values (0.0687–0.0768) between the 2016 generation and the others (Table 3).

An unrooted individual phylogenetic tree based on ASD showed that the Abashiri farmed emu population was largely divided into five clades (Fig. 2). The total number of individuals in clade I, II, III, IV, and V were 27, 18, 39, 28, and 13, respectively (Table 4). Clades II, III, IV, and V included individuals from each of the tested years. However, clade I comprised a large number of individuals hatched in 2016 (70.37 %), which corresponded to 40.43% of the individuals hatched in that year. These results suggest that the genetic composition of individuals hatched in 2016 was different from that of other generations. Although the number of tested individuals varied, a clear genetic difference was found between 2016 and other generations, and therefore we conclude that the generations in the Abashiri farmed emu population have different genetic structures. Unfortunately, we could not fully explain the different allelic composition among generations. At present, we predict that the different genetic composition in these populations might be caused by genetic drift with loss of minor alleles, or by unnatural selection of the breeding stock in farmer. Thus, the selective breeding between individuals possessing differ-

Table 3 Genetic distance (Nei's Da ; *upper*) and F_{ST} (*lower*) between populations of Japanese emus hatched in different years.

Year	2013	2014	2015	2016
2013	-	0.060	0.057	0.155
2014	0.000	-	0.037	0.161
2015	0.005	0.005	-	0.151
2016	0.069	0.075	0.077	-

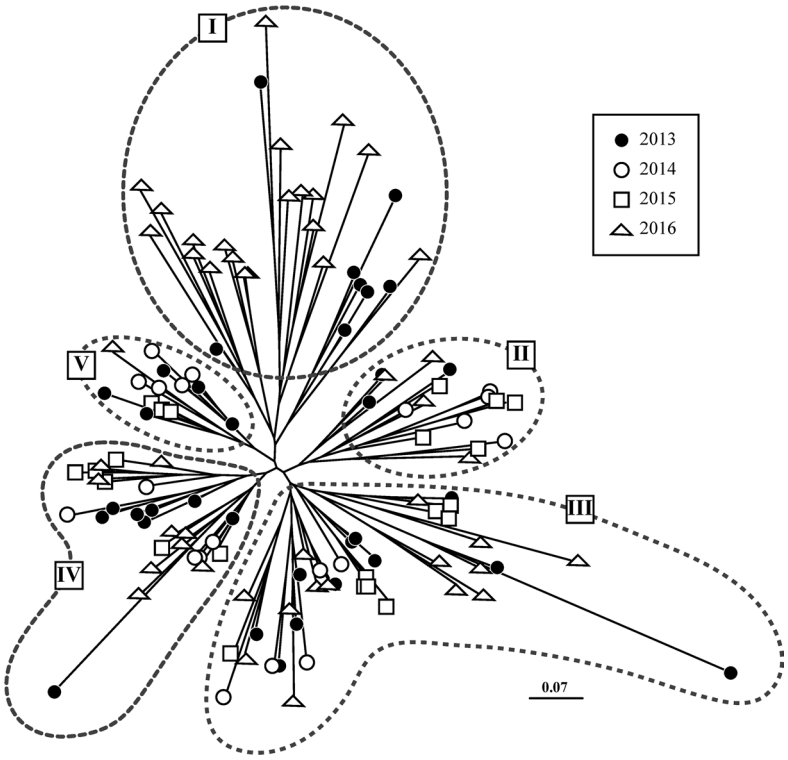

Fig. 2 Unrooted individual phylogenetic tree based on shared allele distance calculated from the genotypes of six microsatellite markers. Black circles, white circles, squares, and triangles indicate individuals hatched in 2013, 2014, 2015, and 2016, respectively. Dashed circles indicate the largely divided clades (I–V) in the Abashiri emu population.

Table 4 Number of individuals and their percentages in each clade

Year	Number of individuals (%)									
Clade	I		II		III		IV		V	
2013	8	(29.63/21.62)	4	(22.22/10.81)	12	(30.77/32.43)	8	(28.57/21.62)	5	(38.46/13.51)
2014	0	(0.00/0.00)	5	(27.78/25.00)	5	(12.82/25.00)	5	(17.86/25.00)	5	(38.46/25.00)
2015	0	(0.00/0.00)	5	(27.78/23.81)	8	(20.51/38.10)	6	(21.43/28.57)	2	(15.38/9.52)
2016	19	(70.37/40.43)	4	(22.22/8.51)	14	(35.90/29.79)	9	(32.14/19.15)	1	(7.69/2.13)

Numbers in parentheses indicate the percentage of individuals per cluster (*left*) and per year (*right*).

ent allelic compositions might be useful for conservation of the genetic diversity of this population.

Genetic information of the emu is very poor, despite the fact that these animals are being bred in various countries. The genetic diversity and structure of farmed emu populations have not been defined except for Australia, Thailand[17], and Chile[16]. Our study revealed that the Abashiri population possesses a low genetic diversity despite being the largest farm in Japan. Therefore, we suggest that conservation and enrichment of the genetic diversity of Japanese emu populations are crucial to sustain and develop emu farming. Further research is needed to investigate the genetic compositions of other emu populations in Japan to characterise the available gene pool.

Moreover, the number of genetic markers available for the emu is very low compared to other livestock species. In this study, only six usable microsatellite markers were confirmed, and thus additional markers should be identified to facilitate reliable research on emu genetic diversity. Genomic analysis of the emu also remains to be performed despite its potentially high economic value. We predict that for future analyses, a larger number of genetic markers will be needed, not only for estimation of genetic diversity but also for pedigree and genomic analyses of the emu.

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マイクロサテライト分析に基づく日本のエミュー飼養集団における遺伝的多様度

輿石雄一*・大久保倫子**・下井　岳**・平山博樹**・相馬幸作**・和田健太**†

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要約：エミュー（*Dromaius novaehollandiae*）は食肉，卵およびオイルを生産する新規動物資源となることが期待されている。しかしながら，エミュー産業の歴史は浅く，その生産形質の遺伝的改良はほとんど進んでいない。我々は，日本で最大規模となる北海道網走市のエミュー牧場の個体群を対象としてマイクロサテライト解析に基づく遺伝的多様度を経年的に調査した。検出されたアレルの数（N_A）は 2013，2014，2015 および 2016 年でそれぞれ 4.83，4.17，4.17 および 7.17 であり，ヘテロ接合率（H_E/H_O）はそれぞれ 0.466/0.339，0.426/0.325，0.433/0.384 および 0.550/0.347 であった。近交係数（F_{IS}）は調査したすべての世代において正の値を示し，2016 年に孵化した個体では 0.369 と最も高い値が観察された。Structure プログラムを用いた解析では，本集団は 3 つのクラスターに分かれ，2016 年に孵化した個体群は明らかに他の世代とは異なる遺伝的構成を示した。またアレル共有率に基づく系統樹は 5 つのクレードを示し，2016 年に孵化した個体の約半数は一つのクレードに属した。本研究は，網走市のエミュー集団は遺伝的多様度が低いこと，遺伝的に 3 − 5 の異なる系統から構成されること，ならびに 2016 年に孵化した個体の遺伝的構成が他の世代とは異なることを確認した。

キーワード：エミュー，遺伝的多様度，遺伝的構造，マイクロサテライトマーカー

* 東京農業大学大学院生物産業学研究科生物産業学専攻
** 東京農業大学生物産業学部北方圏農学科
† Corresponding author（E-mail : k3wada@nodai.ac.jp）