1 **Identification of novel genetic markers and evaluation of genetic structure in the**
2 **Japanese population of the Japanese crested ibis**
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19 Running head: GENETIC MARKER OF JAPANESE CRESTED IBIS

ABSTRACT

Japanese population of the Japanese crested ibis *Nipponia nippon* was founded by 5 individuals gifted from **the People's Republic of China**. **In order to exactly evaluate genetic structure, we first performed development of novel genetic makers using** 89 microsatellite primer pairs of related species for cross-amplification. Of them, only 3 primer pairs were useful for the genetic markers. Additionally, we sequenced allelic PCR products of these 3 markers together with 10 markers identified previously. Most markers showed typical microsatellite repeat units, but 2 markers were not simple microsatellites. Moreover, over half of the markers did not have the same repeat units as those of the original species. These results suggested that development of novel genetic markers in this population by cross-amplification is not efficient, partly because of low genetic diversity. Furthermore, the cluster analysis by STRUCTURE program using 17 markers showed that the 5 founders were divided into 2 clusters. However, the genetic relationships among the founders indicated by the clustering seemed to be questionable, because the analysis relied largely on a small number of triallelic markers**, in spite of addition of the 3 useful markers**. Therefore, more efficient methods for identifying large numbers of **single nucleotide polymorphisms** are desirable.

Key words: *cross-amplification*, *genetic diversity*, *microsatellite*, *Nipponia nippon*

1 **INTRODUCTION**

2 The Japanese crested ibis *Nipponia nippon* was a popular bird in East Asia until
3 the early 20th century, but nowadays, it is considered to be an endangered species due to
4 overhunting or reduction in available habitats (IUCN 2012). In Japan, the Japanese
5 crested ibis in the wild was extinct. After the extinction, 5 individuals (2 individuals in
6 1999, 1 individual in 2000, and 2 individuals in 2007) were introduced from **the**
7 **People's Republic of China**. As a result of the conservation efforts for captive-breeding
8 programs implemented at the Sado Japanese Crested Ibis Conservation Center, Niigata,
9 Japan, the number of the captive individuals at present are about 180 and about 90
10 individuals have been released in the Sado island.

11 In the case of small captive-breeding populations for the conservation of the
12 endangered species, it is important not only to increase the population size, but also to
13 prevent increase in inbreeding coefficient and loss of genetic diversity which cause
14 diminution of future adaptability to changing environment and reduction in the average
15 individual fitness (Lande 1988). Moreover, inbreeding depression results in reduction in
16 population growth rate and increase in extinction risk (Frankham & Ralls 1998).
17 Therefore, the exact knowledge of the current genetic diversity and structure can lead to
18 prevention of genetic deterioration, and be vital to the administration of captive
19 populations and fundamental to the success of metapopulation management strategies,
20 including the reintroduction of captive-bred individuals into the wild.

21 Historically, pedigree information has been utilized to understand the genetic
22 diversity and structure in captive populations (Ballou & Foose 1995). However, Henkel
23 *et al*. (2012) has recently reported that pedigree knowledge alone is not sufficient for the
24 exact understanding of the genetic diversity and structure, under the situation that the
25 founders of the captive population are related to each other. The Japanese population of
26 the Japanese crested ibis is applicable to this situation. Therefore, molecular genetic as

1 well as pedigree information are needed to assess the genetic diversity or infer the
2 structure of the population.
3 Among various molecular markers, microsatellite DNA, which is simple
4 repetitive sequences present widely throughout eukaryote organisms and have high
5 levels of polymorphism (Jarne & Lagoda 1996; Zhang & Hewitt 2003), is one of the
6 most useful DNA markers for the study on the genetic diversity and structure (Zhang &
7 Hewitt 2003). For the Japanese crested ibis, 24 microsatellite markers were developed
8 (Ji *et al*. 2004; He *et al*. 2006), and 9 of these markers were polymorphic in the
9 Japanese population (Urano *et al*. 2013). Microsatellite PCR primer pairs derived from
10 one species are often found to amplify microsatellite loci in related species (Engel *et al*.
11 1996; Primmer *et al*. 1996; Slate *et al*. 1998). Especially, cross-amplifying markers
12 might be worthwhile for bird studies, since the avian genome has been shown to contain
13 fewer microsatellites than organisms of other classifications (Primmer *et al*. 1997). We
14 previously showed the polymorphisms of 10 markers developed for related species (1
15 from the wood stork, 1 from the roseate spoonbill, and 8 from the black-faced
16 spoonbill) in the Japanese population and performed the assessment of genetic diversity
17 and the inference of population structure using a total of 19 markers (Urano *et al*. 2013).
18 Simulation studies showed that increasing the number of loci used improved
19 the inference of the population structure (Evanno *et al*. 2005; Hubisz *et al*. 2009). Thus,
20 in this study, **in order to exactly evaluate genetic structure,** we **first** attempted to
21 identify new genetic markers useful for the Japanese population using microsatellite
22 primers developed in the related species. Subsequently, we used the whole of useful
23 markers identified in the present and previous studies to assess the genetic diversity and
24 infer the genetic structure of the Japanese population.
25
26 **MATERIALS AND METHODS**

1 **Sample**

2

3 We used the 5 founder birds for the Sado captive Japanese crested ibis
4 population, and 138 descendant birds of which 77 individuals were alive in Japan and
5 61 were dead or returned to **the People's Republic of China** by 2011. The descendant
6 birds had hatched from 2001 to 2009. For the founder birds, blood samples were
7 collected from the founders at the Sado Japanese Crested Ibis Conservation Center
8 (Niigata, Japan) and used for genomic DNA preparation. For the descendant birds, the
9 post-hatch eggs stored at the Sado Japanese Crested Ibis Conservation Center were used.
10 The vascularized chorioallantois membrane samples were excised from the eggs and
11 used for genomic DNA preparation, as described by Urano *et al*. (2011). In addition to
12 these samples, we used the liver sample of a dead individual for the examination of
13 PCR amplification. DNA was extracted from these samples using the DNeasy Blood &
14 Tissue Kit (QIAGEN, Hilden, Germany).

15

16 **PCR amplification and length polymorphism detection**

17

18 We used 42, 8, 12, 1, 7, 9, and 10 microsatellite primer pairs developed for the
19 humboldt penguin (*Spheniscus humboldti*) (Ohta A, 2010, unpublished data), the little
20 penguin (*Eudyptula minor*) (Billing *et al*. 2007), the yellow-eyed penguin (*Megadyptes*
21 *antipodes*) (Boessenkool *et al*. 2008), the african penguin (*Spheniscus demersu*) (Akst *et*
22 *al*. 2002**, we used only B3-2 marker for the african penguin**), the white stork
23 (*Ciconia ciconia*) (Shephard *et al*. 2009), the american white pelican (*Pelecanus*
24 *erythrorhynchos*) (Hickman *et al*. 2008), and the great white pelican (*Pelecanus*
25 *onocrotalus*) (De Ponte Machado *et al*. 2009), respectively. **These bird species were**
26 **considered as the related species to the Japanese crested ibis, according to**

1 **Sibley-Ahlquist taxonomy of birds (Sibley & Ahlquist 1990). The information on**
2 **these primer pairs were also obtained from the literatures published up to 2009.** All
3 forward primers included the universal sequence (TGTAAAACGACGGCCAGT) at 5'
4 end. Firstly, we examined PCR amplifications of these primer pairs with DNA of a dead
5 individual. PCR reactions were performed using a 20 μL final volume containing 20 ng
6 genomic DNA, 0.4 μmol/L each of primers, 0.2 mmol/L each of dNTPs, 1 × *Ex taq*
7 buffer (Takara, Shiga, Japan), and 0.5 U *Ex Taq* DNA polymerase（Takara）with the
8 following temperature profile: an initial denaturation step at 94°C for 5 min, and then 35
9 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C
10 for 1 min. PCR products were run on a 2% agarose gel and we selected the primer pairs
11 which showed a single band with a length less than 500 bp. Secondly, to detect length
12 polymorphisms in PCR products from the primer pairs, DNA panel of the 5 founders
13 were tested using 2 rounds of PCR amplification. The first-round amplifications were
14 carried out under the same condition as above. We performed second-round
15 amplifications using the first-round PCR products as the template DNA and universal
16 sequence fluorescently end-labeled with 6-FAM as the forward primer. PCR reactions
17 were performed using a 20 μL final volume containing 1 μL PCR products, 0.4 μmol/L
18 each of primers, 0.2 mmol/L each of dNTPs, 1 × *Ex taq* buffer (Takara) and 0.5 U *Ex*
19 *Taq* DNA polymerase（Takara）with the following temperature profile: an initial
20 denaturation step at 94°C for 5 min, and 30 cycles of denaturing at 94°C for 30 s,
21 annealing at 53°C for 45 s, and extension at 72°C for 45 s, followed by a final extension
22 step at 72°C for 10 min. For determining sizes of the PCR products, a portion of PCR
23 products (1 μL) was combined with 10 μL of formamide loading dye that contained the
24 fluorescent labeled GeneScan-500LIZ size standard (Applied Biosystems, Foster city,
25 CA), and resolved on the 3730xl DNA analyzer (Applied Biosystems). We used
26 GeneMapper software version 4.0 (Applied Biosystems) to score the PCR products. The

1 primer pairs showing polymorphisms among the 5 founders were ascertained the
2 effectiveness as genetic markers by comparing the genotypes of individuals with
3 Mendelian parent-offspring relationships. Subsequently, the markers which were
4 seemed to be effective were genotyped on the descendants.

5

6 **Validation of polymorphic markers as microsatellites**

7

8 We sequenced the polymorphic markers identified in this study and the 10
9 related species markers identified in the previous study (Urano *et al.* 2013) and
10 examined the differences in the sequence among PCR products of different size. PCR
11 amplifications were carried out on the same condition as in the examination of
12 amplification, except for the temperature profile for the previously identified markers,
13 which followed the protocols described by He *et al.* (2006), Sawyer and Benjamin
14 (2006), Tomasulo-Seccomandi *et al.* (2003), and Yeung *et al.* (2009). The PCR products
15 were examined by electrophoresis through a 2% agarose gel to determine the quality
16 and quantity for DNA sequencing. DNA sequencing of PCR products was performed by
17 direct sequencing following excision from the gel. Alternatively, the PCR products were
18 cloned into TA cloning vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and at least 2
19 positive clones were sequenced. Sequencing was carried out by Greiner Japan Co., Ltd.
20 (Tokyo, Japan). Sequence alignments and comparisons were made with GENETYX
21 version 10 (Software Development, Tokyo, Japan). Nucleotide polymorphisms were
22 identified by comparison of sequence traces between the PCR products of different size.

23

24 **Data analysis**

25

26 Based on the whole of useful markers identified in the present and previous

1 studies, basic genetic diversity parameters including allelic diversity (A; Fernández *et al.*
2 2005), effective number of alleles (n_e), observed heterozygosity (H_O), unbiased
3 expected heterozygosity (H_E; Nei 1987), and polymorphic information content (PIC;
4 Boststein *et al.* 1980) of the Japanese population were estimated using EXCEL
5 MICROSATELLITE TOOL KIT version 3.1.1 (Park 2001). The deviation from
6 Hardy-Weinberg equilibrium (HWE) at each locus was tested using a Markov-chain
7 algorithm (Guo & Thompson 1992) in GENEPOP version 4.0 (Raymond & Rousset
8 1995). All pairs of loci were also tested for genotypic linkage disequilibrium using the
9 exact test in GENEPOP version 4.0. The sequential Bonferroni adjustment (Rice 1989)
10 was applied to correct for multiple testing of deviation from HWE and linkage
11 disequilibrium.
12 To investigate the genetic structure of the population, program STRUCTURE
13 version 2.3 (Pritchard *et al.* 2000) was used. Since we found that different runs could
14 produce different likelihood values, 10 runs were carried out for each data set in order to
15 quantify the amount of variation of the likelihood for each K, or the number of clusters.
16 The range of possible Ks we tested was from 1 to 8. Runs with a burn-in period of
17 100,000 followed by 100,000 MCMC iterations were performed, using the model with
18 correlated allele frequencies and assuming admixture (Falush *et al.* 2003). The α, or the
19 Dirichlet parameter for the degree of admixture, was the same for all clusters and
20 allowed to vary between runs. For each value of K, the log-likelihood values of the data
21 defined as ln Pr(X/K) (Pritchard *et al.* 2000) were averaged and standard deviation was
22 calculated. We tried to infer the appropriate K by comparing both the averaged ln
23 Pr(X/K) and calculated ΔK statistic (Evanno *et al.* 2005). This procedure was repeated 3
24 times. Additionally, the same analysis was carried out, using the subsets of markers in
25 order to investigate the influence of the marker sets used on STRUCTURE results.
26

1 **RESULTS**

2 **Identification of novel useful marker**

3

4 Of the 89 primer pairs (Billing *et al*. 2007; Boessenkool *et al*. 2008; Hickman
5 *et al*. 2008; De Ponte Machado *et al*. 2009; Shephard *et al*. 2009; Ohta A, 2010,
6 unpublished data), 25 (13 from the humboldt penguin, 3 from the american white
7 pelican, 5 from the white stork, and 4 from the great white pelican) succeeded in
8 amplifying (out of the other 64 primer pairs, 18 showed multiple bands, but 46 showed
9 no PCR products). Out of the 25 primer pairs, 4 (hSTR1-78, hSTR1-92 and hSTR1-150;
10 from the humboldt penguin, and PEL086; from the great white pelican) had PCR
11 products with different size among the 5 founders (Table 1). Remaining 21 loci
12 amplified as a monomorphic microsatellite product in this study: hSTR1-56,
13 hSTR1-102, hSTR1-109, hSTR1-139, hSTR1-162, hSTR1-233, hSTR1-268,
14 hSTR1-308, hSTR1-312, and hSTR1-344 from the humboldt penguin, PeEr03, PeEr05,
15 and PeEr06 from the american white pelican, Cc01, Cc02, Cc05, Cc06, and Cc07 from
16 the white stork, and PEL149, PEL207, and PEL304 from the great white pelican. We
17 should note that all the 5 founders had the same hetero-genotypes at each of 2 primer
18 pairs (hSTR1-78 and hSTR1-92). To ascertain whether the 4 primer pairs are useful as
19 genetic markers, the PCR products were genotyped using DNA from individuals with
20 Mendelian parent-offspring relationships. As a result, we obtained the genotypes
21 consistent with Mendelian parent-offspring relationships at 3 primer pairs (hSTR1-78,
22 hSTR1-150, and PEL086), but that was not the case at 1 primer pair (hSTR1-92) where
23 all the individuals showed the same hetero-genotypes (data not shown). Thus, the 3
24 polymorphic markers were chosen for large-scale genotyping of the 138 descendants for
25 the **exact** analysis of genetic diversity and structure in this study.

Table 1

26 For the 3 markers together with the 10 related species markers identified in the

1 previous study, we sequenced allelic PCR products of different size. Of the 13 markers,
2 11 had repeat regions and there were differences in the number of repeat unit among
3 each allele, while 2 markers did not seem to be simple microsatellite markers (Table 2,
4 Fig. 1). There were three and one partial differences in sequences between alleles of
5 PM1-17 and PM2-37 loci, respectively, and the variations of single nucleotide were
6 considered to be sequencing errors or single nucleotide polymorphisms. For the 11
7 markers which had simple repeat regions, comparing the sequences of repeat motifs
8 with those of original species, we found that 6 had the same repeat motifs as those of
9 the original species, whereas 5 had different sequences from the original species.

Table 2

Fig. 1

10

11 **Analysis of genetic diversity**

12

13 The genetic diversity parameters were shown in Table 3. The values of the first
14 19 markers were cited from Urano *et al*. (2013), but slightly different values were
15 obtained because we recalculated them excluding some data which were inconsistent
16 with Menderian parent-offspring relationships probably because of typing error. The 3
17 useful markers identified in this study had 7 alleles in total (3 alleles at PEL086 locus
18 and 2 alleles each at hSTR1-78 and hSTR1-150 loci). For the whole of 22 useful
19 markers (3 identified here and 19 identified previously), average A was 2.18 in both the
20 founders and descendants and average n_e, H_E, H_O, and PIC were 1.73, 0.38, 0.40, and
21 0.31, respectively. PEL086 locus had the second highest values for these parameters,
22 and hSTR1-78 and hSTR1-150 loci had moderate values. The 77 descendants which are
23 still alive in Japan inherited all alleles of 22 markers from the founders and the genetic
24 diversity measures similar to those of the 138 descendants were obtained (data not
25 shown). The Nn26, NnNF5, and PM2-20 loci and the overall showed a deviation from
26 the HWE ($P < 0.05$) and the NnNF5 locus significantly deviated from HWE even after

1 sequential Bonferroni correction for multiple testing. The PM2-20 locus may be
2 Z-linked since the locus had Ho lower than expected, and in the original species, or
3 black-faced spoonbill, it was suggested that the marker was Z-linked (Yeung *et al*.
4 2009). In addition, pairwise tests for genotypic disequilibrium revealed that all the 3
5 markers identified in this study (hSTR1-150, hSTR1-78, and PEL086) were in
6 significant linkage disequilibrium with previous markers (Nn01, PM2-28, and Aaju02)
7 after accounting for multiple testing.

Table 3

8

9 **Cluster analysis**

10

11 For the analyses of the composition of genomes among the founders and the
12 descendants using the program STRUCTURE, we used 17 markers except for the
13 PM2-20 locus which seemed to be Z-linked, NnNF5 locus which showed a significant
14 deviation from HWE even after sequential Bonferroni correction, and hSTR1-150,
15 hSTR1-78, and Aaju02 for the 3 pairs of loci which were in significant linkage
16 disequilibrium. In the program STRUCTURE, the true number of clusters (K) is often
17 identified using the maximal value of ln Pr(X/K) (Ciofi *et al*. 2002; Vernesi *et al*. 2003;
18 Hampton *et al*. 2004). The highest value of ln Pr(X/K) was observed at K = 2 for all 3
19 replicates. Furthermore, the ΔK statistic was found to be also obviously maximum at K
20 = 2, giving averaged ΔK values for 3 replicates of 89.28 at K = 2, 2.01 at K = 3, 3.79 at
21 K = 4, 2.66 at K = 5, 2.68 at K = 6, and 5.56 at K = 7. This finding suggested that the
22 Japanese population contains 2 clusters. At K = 2, as shown in Fig. 2, the 2 clusters
23 were distributed with approximately equal proportion in total and with considerable
24 inter-individual variation, suggesting that the founder genomes were inherited equally in
25 the population and with various admixture patterns at individual level. Given that this
26 population was founded by 5 individuals, the result of only 2 clusters was likely to be

Fig. 2

1 consistent with a considerably low level of genetic diversity in the Japanese population.

2 We carried out the cluster analysis with the subsets of the 17 markers. The 3 result obtained by excluding the 4 triallelic markers (Nn01, PM2-21, PM2-37, and 4 PM3-13) from the 17 markers was largely different from that of the 17 markers (Fig. 3), 5 giving the highest value of ln Pr(X/K) at K = 1. However, inclusion of the 4 triallelic 6 markers, irrespective of the markers chosen, resulted in the result similar to that of the 7 17 markers. Even if we used the 4 triallelic markers in combination with the 6 biallelic 8 markers which had low PIC values (Nn17, Nn18, PM2-16, PM2-37 PM3-13, and 9 Wsu13), we obtained the similar result at K= 2 (data not shown). Therefore, it was 10 suggested that the triallelic markers had an essential effect on the clustering result.

11 Fig. 3

12 **Genetic relationships among the founders**

13

14 The analysis by STRUCTURE showed that 5 founders were divided into 2 15 clusters. One cluster contained founder C which was accounted for mostly by dark gray 16 cluster and another cluster contained founder A, B, D, and E that was accounted for 17 mostly by light gray cluster. Furthermore, founder C was also distinct from the other 18 founders at K= 3 to 7. However, the genotype data did not show that there was an 19 apparent difference between the 2 clusters (Table 3). The founder C had a specific allele 20 for Nn01 and specific genotypes for PM221, hSTR1-150, and PEL086, but over 80% of 21 the genotypes of founder C were shared by the other founders. Therefore, the genome of 22 founder C did not seem to be apparently distinct from the others' genomes.

23

24 **DISCUSSION**

25 Because the isolation of microsatellite markers is extensive and 26 time-consuming, it is generally efficient to test the cross-amplification of the primer of

1 the related species (Galbusera *et al*. 2000). In this study, of the 89 microsatellite markers
2 developed for the related species, 25 were successfully amplified, 4 showed PCR
3 products of different size in the 5 founders, and eventually, **only** 3 were **indicated** to be
4 useful as a genetic marker for the Japanese population. In the previous study, 44 out of
5 the 50 markers developed for the related species were successfully amplified and 10
6 showed polymorphisms in the 5 founders (Urano *et al*. 2013). The proportion of the
7 markers which were successfully amplified or showed polymorphisms in this study was
8 extremely low, as compared with that of the previous study, being consistent with the
9 fact that the related species utilized in this study were evolutionally more distant from
10 the Japanese crested ibis than those of the previous study. The species utilized in this
11 study belong to different families from the Japanese crested ibis, while the most of the
12 species used in the previous study belong to the same family as the Japanese crested ibis
13 (Sibley & Ahlquist 1990; Urano *et al*. 2013). It is suggested that the evolutionally more
14 distant the related species used are from the target species, the lower the probability of
15 amplification success or polymorphism detection is (Primmer *et al*. 1996, 2005).
16 Particularly, in bird species, it was reported that in the case where the related species
17 used belonged to a different family **of the same order**, the rate of success in
18 amplification **(33 ± 3%)** or detection of polymorphisms **(28 ± 6%)** was lower than that
19 of the case where they belonged to **a different genus of** the same family **(60 ± 2% or**
20 **43 ± 3%)** (Primmer *et al*. 2005; Barbará *et al*. 2007). We should note that it is also
21 difficult to identify new markers in a population with lower genetic diversity such as the
22 population of the Japanese crested ibis.

23 It is necessary to determine the sequence of alleles in order to validate the
24 genetic markers from the cross-amplification as microsatellite, because it is unclear that
25 the primers amplify the microsatellite sequences in the target species. Thus, we
26 sequenced the alleles of the 3 markers identified in this study and the 10 markers

1 identified previously by the cross-amplification (Urano *et al*. 2013). As a result, we
2 found that while 11 markers have tandem repeat sequences, 5 markers have the repeat
3 sequences different from those of the original species. Also, 2 markers were not simple
4 microsatellite. These results suggested that over half of the markers did not amplify
5 orthologous genome regions to the original species.

6 Using the whole of useful markers identified in the present and previous studies,
7 we calculated genetic diversity parameters in the Japanese population. Although the 3
8 markers were added and one of those was triallelic, values of the parameters were not
9 much different from the previous study by Urano *et al*. (2013) and were similar to those
10 of other species with population bottleneck (Bouzat *et al*. 1998; Tarr *et al*. 1998). These
11 results, together with the result that the maximum number of alleles at single locus was
12 3 in spite of the founding by 5 individuals, support our previous idea of a considerably
13 low level of genetic diversity in the Japanese population (Urano *et al*. 2013).

14 In the result of the cluster analysis by STRUCTURE, the 5 founders were
15 divided into 2 clusters. On the other hand, comparing the actual genotype data, there
16 was not apparent difference between 2 clusters. Furthermore, by excluding the 4
17 triallelic markers, the founders were not divided into 2 clusters. These results suggest
18 that the cluster analysis relied largely on more polymorphic markers and the differences
19 in the genotypes of the triallelic markers made a significant contribution to the
20 STRUCTURE result. Thus, the genetic relationships among the 5 founders indicated by
21 the cluster analysis with STRUCTURE using 17 markers seemed to be questionable,
22 because the analysis relied largely on a small number of triallelic markers**, in spite of**
23 **addition of the 3 useful markers**. Therefore, development of large numbers of **single**
24 **nucleotide polymorphisms** by means of more efficient methods such as those using
25 next generation sequencing technologies and further molecular genetic studies with
26 those markers will be needed for more exact understanding of genetic diversity and

1 structure in the Japanese population of the Japanese crested ibis. Additionally, for the
2 future conservation management strategy, it is important to investigate not only neutral
3 polymorphisms in microsatellite markers but also functional mutations related to fitness
4 in MHC (Major histocompatibility complex) regions (Kohn *et al*. 2006; Agudo *et al*.
5 2011).
6

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26
27

1 **Figure Legends**

2 Fig. 1 Nucleotide sequence alignment of the two PCR products by primers
3 PM1-17 (a) and PM2-37 (b). The upper and lower rows show the sequence of the one
4 and the other PCR product, respectively.

5

6 Fig. 2 Genetic structure of the Japanese population in Japan consisting of the 5
7 founders and 138 descendants based on the STRUCTURE software with 17 markers.
8 Each animal is represented by a single vertical line divided into K = 2 colors, where K
9 is the number of clusters assumed and the length of the colored segment represents the
10 individual's estimated proportion of membership to a particular cluster. The characters A
11 to E indicate the 5 founders. Among the 10 runs carried out for K = 2, only the graphical
12 representation of the highest estimates for Ln Pr(X/K) value is given.

13

14 Fig. 3 STRUCTURE result obtained with 13 markers, removing 4 triallelic
15 markers from the whole 17 markers. Each animal is represented by a single vertical line
16 divided into K = 2 colors and the length of the colored segment represents the
17 individual's estimated proportion of membership to a particular cluster. The characters A
18 to E indicate the 5 founders.

19

Fig.1(a)

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CCTCCTTCTGGTCCCTCCCGAATATTTATTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CCTCCTTCTGGTCCCTCCCGAATATTTATTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCT

TTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTT------------CTTTCTTTCTTTCTTT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||            ||||| ||||||| ||
TTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCCTTCTTTCCTTCTTTCCTTCTTTCCTT

CTTTCCTTCTTTCCTTCTTTCCTTCTTTCCTTCTTTCCTTCCTTCCTTCTTTCCTTCCTTCTTT--------CCTTCTTTCCTTCTTCCC
||||||||||||||||||||| |||||||||||||||||||| |||||||||||| ||||||||||        |||||||||||||||| ||
CTTTCCTTCTTTCCTTCTTTCTTTCTTTCCTTCTTTCCTTCTTTCCTTCTTTCTTTCCTTCTTTCCTTCTTTCCTTCTTTCCTTCTTTCC

TTCTTTCCTTCTTTCCTTCTTTCTTTCCTTCTTTCTTT--------CCTTCTTTCCTTCCTTCTTTCCTTCTTTCTTTTCTTTCTCTGTC
||||||||||||||||||||||||||||||||||||||        ||||||||||||||||||| |||||||||||||||||||||| ||
TTCTTTCCTTCTTTCCTTCTTTCTTTCCTTCTTTCTTTCCTTCTTTCCTTCTTTCCTTCCTTCTTCCCTTCTTTCTTTTCTTTCTCTTTC

TGTCTCTCTTTCTCTCTCTTCCTCTCTTCCCATATTGATTGTAAAATTATGAGTCTACAGCACGAGCAACTCTGCAG
|||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
TGTCTCTCTTTCTCTCTCTTCCTCTCTTCCCATATTTATTGTAAAATTATGAGTCTACAGCACGAGCAACTCTGCAG
```

Fig.1(b)

```
CATACAAGCAATGCCTAACCATACCTCTGAGTTTCATGTATGTAGATAGATTTCCCTTCTCTTAGAAATGGAGGTTAAGTGTTGTCCAAA
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CATACAAGCAATGCCTAACCATACCTCTGAGTTTCATGTATGTAGATAGATTTCCCTTCTCTTAGAAATGGAGGTTAAGTGTTGTCCAAA

TCAATACTGATTCAATTGTACTATTCAAAGATTTCCTCACTGTTATGAATTTAGGGTATTTTTGAAAAAGAAAGAAAGAAGGAAAGAGAG
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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AGAAAGAAAGAAAGAACGAAAGAACGAAAGAAC------------------------------------AAAAGAAAGAAAGAATGAAAG
|||||||||||||||||||||||||||||||||                                    ||||||||||||||||| |||
AGAAAGAAAGAAAGAACGAAAGAACGAAAGAACGAAAGAAAGAATGAAAGAAAGAACGAAAGAAAGAACAAAAGAAAGAAAGAATGGAAG

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||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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GAAAAAGAAAAAGAAAAAGAAAAAAGTTGTGTAGTTTCAGTCTGAGT
|||||||||||||||||||||||| ||||||||||||||||||||||
GAAAAAGAAAAAGAAAAAGAAAAAGGTTGTGTAGTTTCAGTCTGAGT
```

Fig.2

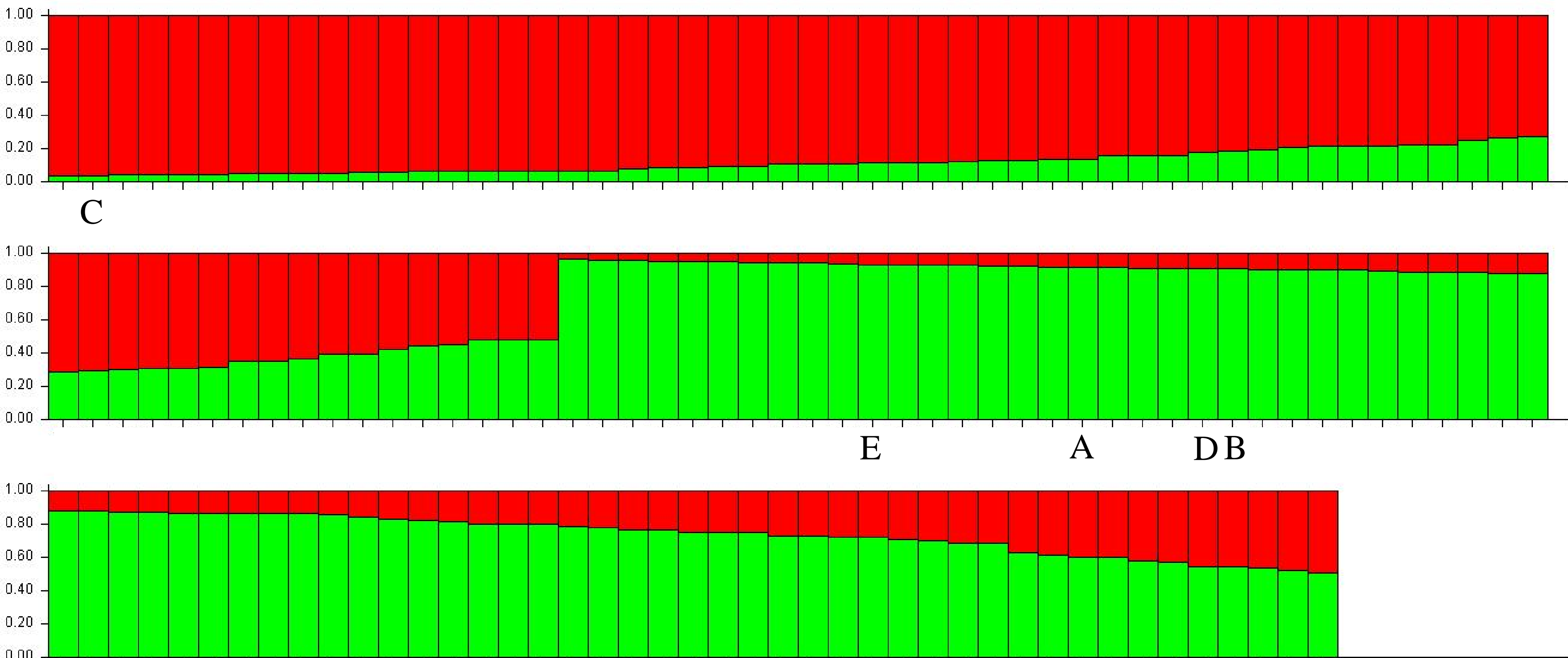

Fig.3

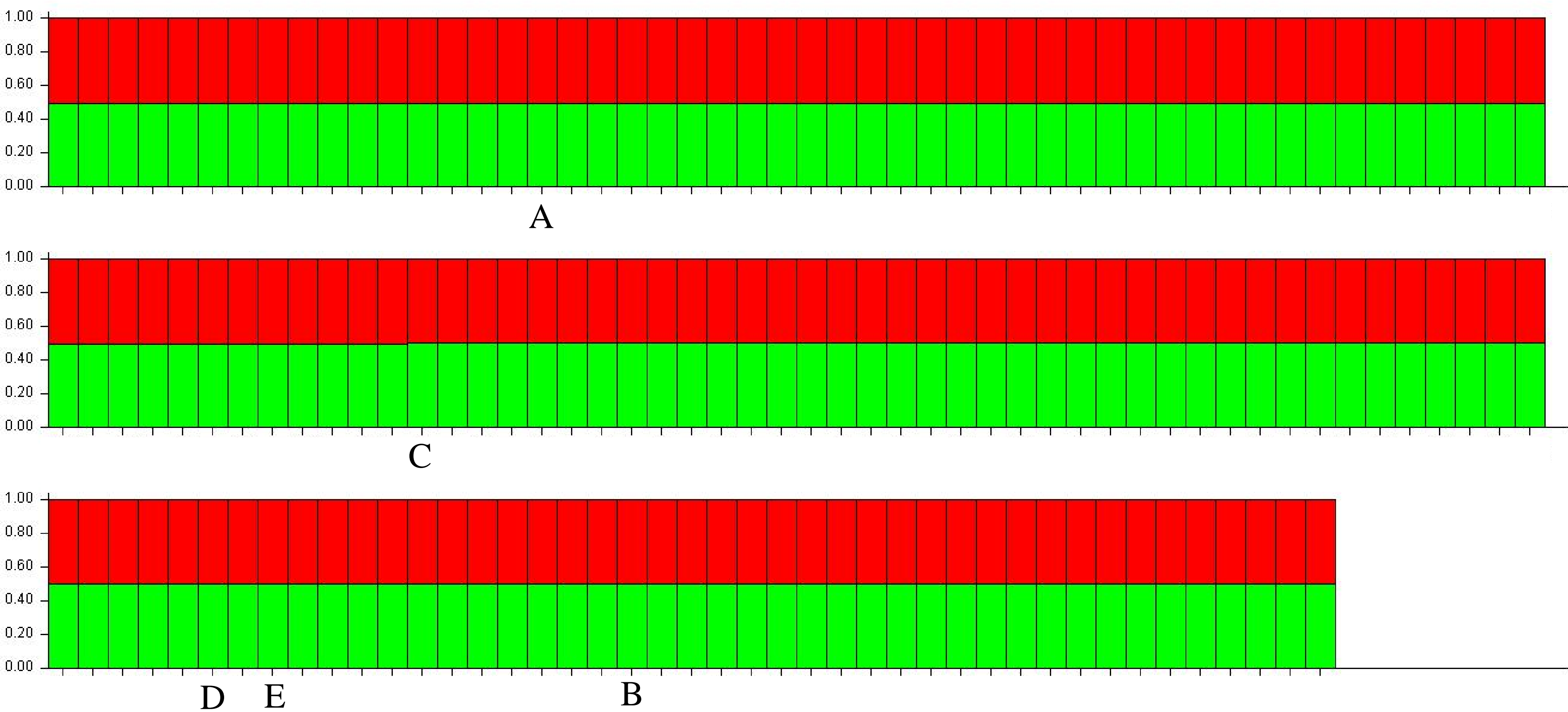

Table 1 The size of PCR products for 5 founders

Locus	Source species	Size of PCR products for each founder† (bp)					Reference
		A	B	C	D	E	
hSTR1-78	Humboldt penguin	267, 271	267, 271	267, 271	267, 271	267, 271	Ohta A, unpublished data
hSTR1-92	Humboldt penguin	111, 115	111, 115	111, 115	111, 115	111, 115	Ohta A, unpublished data
hSTR1-150	Humboldt penguin	320, 325	320, 325	325	320, 325	320, 325	Ohta A, unpublished data
PEL086	Great white pelican	189, 193	185, 193	185, 189	189, 193	189	De Ponte Machado et al. (2009)

†The characters A to E represent founders.

Table 2 The repeat motif and the number of repeat unit of 2 or 3 alleles for each loci

Locus	Repeat motif of original species	Repeat motif[†]	Number of repeat motif[†]
PM1-17	AAAG	-	-
PM2-16	AAAG	$(TTTA)_n$	n=6 or 7
PM2-20	AAAG	$(AAAG)_n$	n=12 or 13
PM2-21	AAAG	$(GGAAGAAA)_n$	n=12, 14 or 17
PM2-28	AAAG	$(AAAG)_n$	n=6 or 7
PM2-29	AAAG	$(AAAG)_n$	n=13 or14
PM2-37	AAAG	-	-
PM3-13	CA	$(CA)_n$	n=5 or 6
Aaju02	$(AACT)_{13}TA(CTTT)_6CT(CTTT)_3$	$(CTTTT)_mCTTTTT(CTTTT)_n$	m=20 and n=2 or m=23 and n=1
Wsu13	AC	$(AC)_n$	n=13 or 14
hSTR1-78	AC	$(CAAA)_n$	n=3 or 4
hSTR1-150	TATCC	$(AGAAC)_n$	n=4 or 5
PEL086	TATC	$(TATC)_n$	n=8, 9 or 10

[†]The characters “m” and “n” represent the number of repeat unit.

Table 3 Allelic diversity (A), genotypes of the 5 individuals, effective number of alleles (n_e), unbiased expected heterozygosity (H_E), observed (direct count) heterozygosity (H_O), polymorphic information content (PIC) and deviation from HWE in the Japanese population (the founders and descendants) of 22 microsatellite markers

Locus	Allelic diversity	Genotype of founder†					n_e	H_E	H_O	PIC	HWE‡
		A	B	C	D	E					
Nn01	3	12	12	**13***	12	**11**	2.01	0.50	0.53	0.45	
Nn04	2	12	**22**	12	12	12	1.90	0.47	0.54	0.36	
Nn12	3	13	23	23	**12**	13	2.98	0.67	0.72	0.59	
Nn17	2	22	22	22	22	**1*2**	1.02	0.02	0.02	0.02	
Nn18	2	22	12	12	22	12	1.43	0.30	0.33	0.26	
Nn21	2	12	**11**	12	**22**	12	1.99	0.50	0.58	0.37	
Nn25	2	12	12	22	22	12	1.87	0.47	0.43	0.36	
Nn26	2	**11**	12	12	12	12	1.63	0.39	0.46	0.31	*
NnNF5	2	12	22	12	22	12	1.57	0.37	0.48	0.30	*, (*)
PM1-17	2	**11**	12	12	12	12	1.91	0.48	0.51	0.36	
PM2-16	2	11	**12**	11	22	22	1.36	0.26	0.28	0.23	
PM2-20	2	11	12	11	12	11	1.25	0.20	0.16	0.18	*
PM2-21	3	13	12	**11**	12	13	2.39	0.58	0.58	0.50	
PM2-28	2	**11**	12	12	12	12	1.69	0.41	0.46	0.32	
PM2-29	2	12	22	12	22	22	1.80	0.45	0.42	0.35	
PM2-37	2	22	22	22	**1*2**	22	1.01	0.01	0.01	0.01	
PM3-13	2	**1*2**	22	22	22	22	1.21	0.17	0.19	0.16	
Aaju02	2	22	12	12	22	12	1.80	0.45	0.40	0.35	
Wsu13	2	11	11	12	12	11	1.31	0.24	0.28	0.21	
hSTR1-78	2	12	12	12	12	12	1.66	0.40	0.44	0.32	
hSTR1-150	2	12	12	**22**	12	12	1.49	0.33	0.33	0.28	
PEL086	3	**13**	23	**12**	23	**22**	2.83	0.65	0.67	0.57	
Mean	2.18						1.73	0.38	0.40	0.31	*

†The number 1, 2, 3 represents each allele, the allele specifically carried by one founder is indicated by asterisk, and the genotype specifically carried is indicated in bold.

‡Significant deviation from Hardy-Weinberg equilibrium is indicated by asterisk ($P < 0.05$), and that after correction using Bonferroni procedure is indicated by asterisk in bracket ($P < 0.05/22=0.002$).