



NOTE

Wildlife Science

Genetic variation of mitochondrial DNA in *Phalacrocorax carbo* in Japan

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ABSTRACT. A Japanese resident bird, *Phalacrocorax carbo hanedae* (Japanese name: Kawa-u), was threatened with extinction due to deterioration of its habitat in the 1970s, but the population has since recovered thanks to environmental protection measures. This study analyzed the genetic diversity of 18 Kawa-u individuals living in the basins of the Abe and Warashina rivers in Shizuoka Prefecture, Japan. We obtained seven haplotypes of mitochondrial D-loop sequences and compared them with 49 European *P. carbo* D-loop haplotypes. We identified four new haplotypes but no clear genetic evidence distinguishing the Kawa-u as a distinct subspecies of *P. carbo*. Our results suggest the need for further surveillance of the *P. carbo* genetic lineage, regardless of the geographical distribution.

KEY WORDS: D-loop, European *Phalacrocorax carbo*, mitochondrial DNA, *Phalacrocorax carbo hanedae*

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The great cormorant (*Phalacrocorax carbo*) is morphologically classified into six subspecies: *P. c. carbo* Linnaeus as the nominotypical subspecies; *P. c. sinensis* Blumenbach; *P. c. hanedae* Staunton; *P. c. maroccanus* Hartert; *P. c. lucidus* Licht; and *P. c. novaehollandiae* Stephens [4, 12, 20]. *Phalacrocorax c. hanedae* (Japanese name: Kawa-u) is a Japanese resident with a limited distribution in Japan and its surroundings, Sakhalin, Korea and Taiwan [19]. The body mass of the Kawa-u is the smallest among the *P. carbo* subspecies, and the body length is 80–90 cm [1]. Kawa-u were numerous in Japan before the 1920s, but population numbers decreased precipitously to 3,000 or fewer in the 1970s due to overhunting and environmental deterioration [6]. During the 1980s, improvements in river water quality due to the promulgation of pollution controls and direct protection of cormorants both contributed to range expansion and an increase in the number and size of Kawa-u colonies and roosts in Japan [9]. The estimated number had increased to 100,000 by 2013 [10], but this increase has in turn caused damage to inland freshwater fisheries [9, 13, 17]. According to a report by the National Federation of Inland water Fisheries Cooperatives, the estimated total financial damage attributable to Kawa-u exceeds 10 billion yen (90 million dollars) per annum in Japan [18].

Proper management of wildlife populations requires as much information as possible about those species, including their genetic characteristics. The genetic diversity of the Kawa-u seems vulnerable due to the bottleneck effect associated with its near-extinction in the 1970s. Populations may be reduced drastically again due to low genetic diversity following even minor perturbations to the environment. This study focused on the genetic diversity of the mitochondrial D-loop sequence in Kawa-u inhabiting the Abe and Warashina river basins in the central part of Shizuoka Prefecture, Japan. The obtained D-loop haplotypes were compared with those of European *P. carbo*, as research groups have shown genetic diversity of the European birds [8, 16, 21]

Eighteen bird samples (sample numbers: A1–A14 and A16–A19) morphologically characterized as *P. c. hanedae* were captured under legal control in the basins of the Abe and Warashina rivers in central Shizuoka Prefecture (Fig. 1). Approximately 1 g of kidney (from all samples except A3) or liver (A3) tissue was washed twice with TES-Buffer (10 mM Tris, 1 mM EDTA, 50 mM NaCl, 1% SDS, pH 7.4), minced with scissors, re-suspended in 400 μ l of TES-Buffer, and treated with proteinase K (final concentration: 200 μ g/ml) for 3 hr at 50°C. After this incubation, samples were subjected to phenol-chloroform-isoamyl alcohol DNA extraction, and RNase treatment (5 mg/ml RNase A, 1,000 unit/ml RNase T1) was performed for 1 hr at 37°C. The phenol-chloroform-isoamyl alcohol method was repeated, and the resultant product was dissolved with 100 μ l of TE-Na buffer. Amplification of the D-loop region of mitochondrial DNA, which is located on the 5'-side of the first control region, by PCR was

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Fig. 1. Map of the basins of the Abe and Warashina rivers. Red circles indicate the locations of roosts or colonies of Kawa-u identified by the Shizuoka Prefecture survey (2009). Maps were provided by the Geospatial Information Authority of Japan.

performed using previously reported methods [21, 22]. Sequence analysis was carried out at Operon Biotechnology (Tokyo, Japan). Sequence data were aligned and analyzed using GENETYX version 12 (Genetyx, Tokyo, Japan) and MEGA X [14]. A haplotype network was constructed using PopART [15]. The D-loop sequence of 402 nucleotides, which corresponded to 15,572 to 15,973 of the complete mitochondrial genome of *P. carbo* (GenBank accession number: NC_027267.1), of 18 Kawa-u individuals were deposited in the DNA Data Bank of Japan (Mishima, Japan; accession numbers: LC431061–LC431078). These sequences were classified into seven haplotypes (Kha1–Kha7); Kha1 (A1, A3, A7, A8, A12 and A18); Kha2, (A2 and A4); Kha3, (A5, A9 and A10); Kha4, (A6); Kha5, (A11, A16 and A17); Kha6, (A13 and A14); Kha7, (A19) (Table 1). We compared Kawa-u haplotypes with 49 D-loop haplotypes of European *P. carbo* already registered in GenBank (ha01–ha49; accession numbers: AF101172–AF101193, AF101172–AF101193, AY684305–AY684327 and AY676597–AY676601 [2, 16, 21]). Based on comparisons with the nucleotide sequence of haplotype 1 (ha01, GenBank accession number: AF101172), 30 substitution positions were identified throughout the 402-bp sequence (98.507–99.751% identity). The Kawa-u haplotypes, Kha1, Kha3 and Kha4, were the same as the European *P. carbo* haplotypes, ha04, ha11 and ha15, respectively (see Table 1). Substitutions in the Kawa-u D-loop sequence were found at eight nucleotide positions (positions 15575, 15755, 15781, 15837, 15847, 15879, 15920 and 15928), with 2–4 substitutions in each Kawa-u sequence (99.005–99.502% identity). The C→T substitution at position 209 was common among Kawa-u haplotypes, but was also often observed in other haplotypes (Table 1). The new substitutions were C→T at position 3 and A→G at position 265. Furthermore, four Kawa-u haplotypes (Kha2 and Kha5–Kha7) were classified as new types of *P. carbo* D-loop sequences. Although we examined only 18 Kawa-u individuals obtained from a small area of the Abe and Warashina river basins, given that there are seven Kawa-u haplotypes in this relatively small area, the reduction in the Kawa-u population in the 1970s is considered unlikely to have led to low genetic diversity.

Phylogenetic trees were constructed using the neighbor-joining (NJ) method or the maximum likelihood (MLH) method, using the nucleotide sequence of *P. capillatus* (GenBank accession number: AY676597) as an outgroup. Phylogenetic analysis by the NJ method showed that 53 haplotypes were largely classified into five groups (Fig. 2A). Group A was the first branch linked to the *P. capillatus* sequence used as the outgroup and consisted of nine haplotypes, including three Kawa-u haplotypes (Kha3–Kha5). Kha3 and Kha4 coincided with European *P. carbo* haplotypes ha11 and ha15, respectively, whereas Kha5 formed a new branch. Group B consisted of 14 haplotypes including ha01 and the Kawa-u haplotypes Kha1 and Kha7. The reference sequence in this study, ha01, belonged to this group. Only three European *P. carbo* haplotypes were included in Group C. Group D consisted of 12 haplotypes. Kha2 and Kha6 were located at the end of the branch of this group. Group E contained the largest number of haplotypes, but no Kawa-u haplotypes were assigned to this group. From the above results, seven of the 18 Kawa-u (38.9%) were included in each of Groups A and B, and the remaining four individuals (22.2%) belonged to Group D. In the phylogenetic analysis using the MLH method, bootstrap probability was lower than the results of the NJ method, and fewer branches showed probabilities exceeding 50% (Fig. 2B). *Phalacrocorax carbo* haplotypes were also divided into five groups (I–V) according to the MLH method. The distribution of haplotypes in the phylogenetic trees differed between the two methods. A small genetic distance between both European *P. carbo* subspecies has been identified from mitochondrial DNA, and these haplotypes could be classified into groups N (mainly inhabiting

Table 1. Type of nucleotide substitution in D-loop haplotypes of *Phalachocorax carbo*

| Haplotype | Position ^{a)} | | | | | | | | | | | | | | | | | | | | Number of substitution | | | | | | | | |
|-----------------|------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|------------------------|-------|-------|-------|-------|-------|-------|-------|-------|
| | 15575 | 15748 | 15749 | 15755 | 15772 | 15773 | 15777 | 15781 | 15815 | 15827 | 15828 | 15830 | 15834 | 15837 | 15838 | 15847 | 15866 | 15870 | 15877 | 15886 | | 15894 | 15895 | 15898 | 15920 | 15921 | 15928 | 15929 | 15934 |
| European | C | A | C | T | C | A | T | C | T | A | A | C | A | A | A | A | A | T | A | A | C | T | C | T | T | C | A | T | |
| <i>P. carbo</i> | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| ha01 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | 0 | |
| ha02 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 3 |
| ha03 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 3 |
| ha04 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 2 |
| ha05 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 2 |
| ha06 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 3 |
| ha07 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 3 |
| ha08 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 3 |
| ha09 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 5 |
| ha10 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 4 |
| ha11 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 3 |
| ha12 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 3 |
| ha13 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 3 |
| ha14 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 4 |
| ha15 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 4 |
| ha16 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 6 |
| ha17 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 4 |
| ha18 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 6 |
| ha19 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 5 |
| ha20 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 3 |
| ha21 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 2 |
| ha22 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 1 |
| ha23 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 5 |
| ha24 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 4 |
| ha25 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 5 |
| ha26 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 4 |
| ha27 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 4 |
| ha28 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 3 |
| ha29 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 4 |
| ha30 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 4 |
| ha31 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 3 |
| ha32 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 1 |
| ha33 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 2 |
| ha34 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 3 |
| ha35 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 4 |
| ha36 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 4 |
| ha37 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 5 |
| ha38 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 4 |
| ha39 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 5 |
| ha40 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 5 |
| ha41 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 6 |
| ha42 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 5 |
| ha43 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 5 |
| ha44 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 6 |
| ha45 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 5 |
| ha46 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 2 |
| ha47 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 4 |
| ha48 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 2 |
| ha49 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 2 |
| Kha1 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 2 |
| Kha2 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 4 |
| Kha3 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 3 |
| Kha4 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 4 |
| Kha5 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 4 |
| Kha6 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 3 |
| Kha7 | | | | | | | T | | T | | | | | | | | | | | | | | | | | | | | 3 |

a) Nucleotide positions are cited as the complete mitochondrial genome of *P. carbo* (Genbank accession number: NC_027267.1).

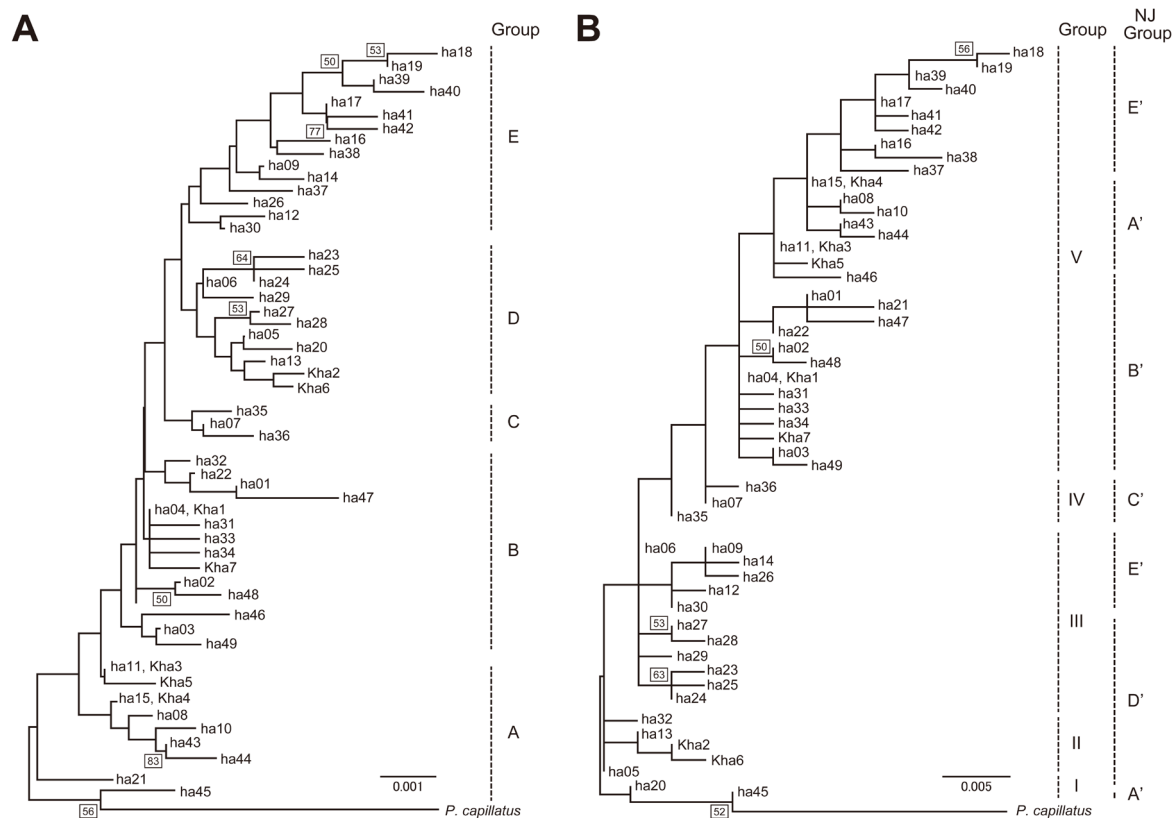


Fig. 2. Phylogenetic trees of 53 D-loop haplotypes of *Phalacrocorax carbo*. A) The neighbor-joining (NJ) method. B) The maximum likelihood (MLH) method. Numbers in squares indicate bootstrap rates after 1,000 trials. Bootstrap values higher than 50 are indicated. Haplotype codes are the same as those in Table 1. “ha” and “Kha” indicate European haplotype and Kawa-u haplotype, respectively. The D-loop sequence of *P. capillatus* (GenBank accession number: AY676597) was used as an outgroup. Phylogenetic analyses by both methods showed that the 53 haplotypes could largely be classified into five groups (NJ: A–E, MLH: I–V). Groups A’–E’ (B) corresponds approximately to groups A–E (A).

Norway), S (*P. c. sinensis*), C (*P. c. carbo*), and U (a fourth type) [16]. From the shape of the NJ phylogenetic tree, we speculated that our groups of A, B, C, and (D + E) corresponded to the previous described groups of N, S, U, and C, respectively. Three of the seven Kawa-u haplotypes (42.9%) were classified into Group A (N), with the other two haplotypes (28.6%) classified into Groups B (S) and D (C), respectively. Unfortunately, we could not obtain any of the D-loop sequences published by Butkauskas *et al.* [2], who reported finding eight additional D-loop haplotypes of the European *P. carbo*. Consequently, the possibility that some of the Kawa-u-specific haplotypes obtained in this study are concordant with these additional European haplotypes cannot be excluded. The haplotype network appears to show a connection between the Kawa-u haplotypes in Groups A and B (Fig. 3). Consequently, Kawa-u cannot be discriminated genetically from subspecies of European *P. carbo*, but they can be discriminated as a subspecies of *P. carbo* based on morphological characters. This inconsistency between the morphological and genetic classifications suggests that there may be some ambiguity regarding the subspecific classification of Kawa-u. Interestingly, one morphological characteristic of Kawa-u from the Japanese archipelago is that it is smaller than any of the other *P. carbo* subspecies, following Foster’s rule [3, 5]. Although we could not clearly distinguish any geographical specificity of Kawa-u haplotypes, unlike the Norway *P. carbo* population [16], most Kawa-u haplotypes belonged to Group N. Recently, the family Phalacrocoracidae has been revised into seven genera of approximately 40 taxa based on results of mitochondrial and nuclear DNA sequencing [11]. Furthermore, duplication of the control region has been reported in the mitochondrial genomes of *P. carbo* and its relatives [7, 22]. The present findings suggest the need for further investigations into the genetic relationships between Kawa-u and other *P. carbo* subspecies. In addition, it is possible that Kawa-u from surrounding areas of Japan moved to Japan, increasing the genetic diversity of the Kawa-u population inland. However, to the best of our knowledge, no information exists on Kawa-u populations in these surrounding areas. Ecological and genetic analyses of Kawa-u populations would be required in order to test this hypothesis and more comprehensively elucidate the genetic variation of Kawa-u in both Japan and the surrounding regions. Such studies would be useful to better understand the ecology and population dynamics of this species in Japan.

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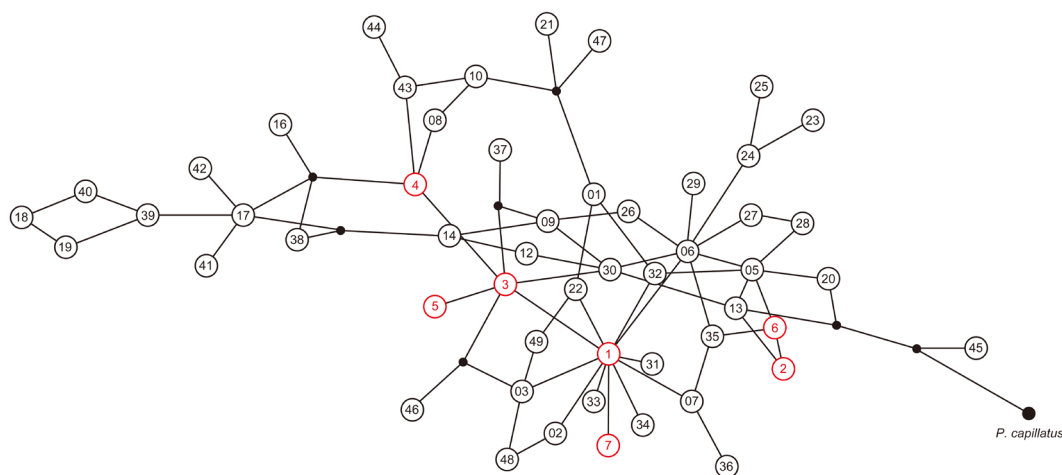


Fig. 3. Haplotype network for Kawa-u and European *Phalacrocorax carbo* subspecies mitochondrial D-loop sequences. Red and black numbers indicate the Kawa-u and European *P. carbo* haplotypes, respectively. Closed circles correspond to unobserved but potential haplotypes.

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