

SHORT COMMUNICATION

External features and molecular sexing of the anomalous Pintail, *Anas acuta*, found at Hyo-ko Waterfowl Park, Niigata Prefecture, Japan

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Akira CHIBA^{1,#}, Koichi MURATA², Shigeki MIZUNO³, Ryuhei HONMA⁴ and Fumio SUGIMORI⁵

¹ Department of Biology, Nippon Dental University School of Dentistry at Niigata, Niigata 951–8580, Japan

² Department of Wildlife Science, College of Bioresource Sciences, Nihon University, Fujisawa 1866, Kanagawa 252–8510, Japan

³ Department of Agricultural and Biological Chemistry, College of Bioresource Sciences, Nihon University, Fujisawa 1866, Kanagawa 252–8510, Japan

⁴ Niigata Prefecture Wild Bird Conservation Group, Shiunji Town, Niigata 957–0231, Japan

⁵ Research Center, Yamashina Institute of Ornithology, Abiko, Chiba 270–1145, Japan

Phenotypic variation of the plumage including albinism and melanism is widely known among birds. On the other hand, morpho-chromatic variation of the plumage caused by hybridization has also been found in nature. Such an example is occasionally found in the Anatidae (Kuroda 1939), and possible hybrids of the Pintail *Anas acuta* with other species, e.g., Mallard (*A. platyrhynchos*), Teal (*A. crecca*), Baikal Teal (*A. formosa*), Gadwall (*A. strepera*), Wigeon (*A. penelope*), Shoveler (*A. clypeata*) and Pochard (*Aythya ferina*), were documented previously (Kuroda 1939). In fact, a presumptive hybrid between the Pintail and Baikal Teal was observed also in Hyo-ko Waterfowl Park, located in a suburb of Niigata City, Niigata Pref., Japan (Chiba, unpubl. data). During the course of bird-banding study in this Park, we incidentally found 2 unusual male Pintails, which were morphologically different from any known hybrids. The present study, therefore, was conducted to clarify the characters of these anomalous Pintails on the basis of macroscopic examination and molecular sexing.

MATERIALS AND METHODS

1) Birds

Thirteen individuals of Pintail were used in this study (Table 1). They were captured (by permission of the Ministries of the Environment and Culture, Japan) at Hyo-ko Waterfowl Park (37°50'N, 139°14'E) in the suburb of Niigata City, Niigata Prefecture, Japan, during 2 winter seasons, from January 2000 to March 2001. The birds were caught humanely by hand-made net or by hand, marked with a metal ring, and kept in plastic cages (80 cm×50 cm×25 cm) for a while. Then, their external features were macroscopically examined and recorded in photographs, and measurement of the body was made. Sex and age were checked mainly based on their plumage and cloacal structure. The contour feathers as a source of DNA, 5 to 7 feathers per bird, were plucked from the mid breast region by using sterilized forceps, put into clean plastic bottles, and stored in a refrigerator at –10°C before extracting the DNA. After examination, the birds were released back to the wild.

2) Isolation of genomic DNA

Genomic DNA was isolated from 3 contour feathers plucked from the mid breast of each bird. The method of DNA extraction followed Walsh et al., (1991), Murata & Masuda (1996) and Murata et al. (1998), with a slight modification. Briefly, a length of calamus about 3 mm was removed from the proximal end with clean scissors and cut into smaller pieces, which were incubated in 200 µL of 5%(w/v) Chelex^R (Bio-Rad) at 56°C overnight, and then boiled in a water-bath for 8 min. After centrifugation at 12,000 rpm for 5 min, the supernatant was used as template DNA for PCR.

2) Isolation of genomic DNA

3) PCR for gender determination

Primer sets used in this study for amplifying the Z/W chromosome-specific DNA sequences of Pintail were those used for sexing domestic duck (*A.*

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#Corresponding author: E-mail: chiba@ngt.ndu.ac.jp

platyrhynchos var. *domestica*) as reported by Itoh et al. (2001). The sequences of sexing primer for amplifying the W chromosome-specific DNA (~190 bps) were 5'-ACAGTTTGTCTGTCTCCGGGGAA-3' (AWS03) and 5'-AGCTGGAYTTCAGWSCATCTTCT-3' (USP3), and those of internal control primer for amplifying the Z/W chromosome-common DNA (~250 bps) were 5'-CTCTGTCTGGAAGGACTT-3' (INT-R) and 5'-ATAGAAACAATGTGGGAC-3' (INT-F). Detailed information about these primers and related sequences was given elsewhere (Itoh et al. 1997, 2001; Ogawa et al. 1997).

PCR was carried out in a 25- μ L mixture containing a 0.2 mM concentration of each dNTP, 50 pmoles of primers AWS03, USP3, INT-R and INT-F, 3 μ L of DNA extract, 0.25 units of *Taq* polymerase (Amersham), and 2.5 μ L of 10 \times PCR buffer. The PCR conditions used for the thermal cycler (PE Applied

Biosystems, 9700) were as follow: initial DNA denaturation at 95°C for 3 min followed by 35 cycles of 95°C, 80 sec for denaturation; 59°C, 90 sec for annealing; 72°C, 60 sec for extension, and lastly 72°C, 9 min for final elongation. PCR products (8 μ L) were electrophoresed on a 2% agarose gel (A-6013, SIGMA) in 0.5 \times TBE (44.5 mM Tris-borate, 44.5 mM Boric acid, 0.5 mM EDTA) buffer at 100V for 35 min, stained with ethidium bromide, and visualized under a UV transilluminator.

RESULTS

Externally, 2 birds in question, Sp-1 (ring number, 10A-80409) and Sp-2 (10A-75898), were characterized by nuptial plumage of the male type (Fig. 1A). Measurement values of the wing, tail and body weight of these birds approximately corresponded to



Fig. 1. External features (A) and external aspect of the disclosed vent (B) of an anomalous Pintail (Sp-2). Corresponding part of an adult male with normal plumage (C) and that of an adult female with normal plumage (D) are also shown. dl, dorsal lip of cloaca; p, phallus (artificially everted and erected); vl, ventral lip of cloaca. Scale bar, 1 cm

Table 1. Measurements of anomalous males (SP-1 and SP-2) of the Pintail, *Anas acuta*, and of control (normal plumage) birds for comparison

	Wing*	Tail*	Bill*	Tarsus*	Body Weight**
Sp-1 (10A-80409)	259.0	148.0	46.5	40.8	810.0
Sp-2 (10A-75898)	259.0	173.0	47.3	42.5	790.0
Control					
Normal males (N=5)	260.8±5.4	145.4±38.5	50.6±2.4	41.8±2.0	836.0±50.0
Normal females (N=5)	224.6±10.6	109.8±11.2	47.7±2.0	38.9±2.3	722.0±65.5
“Buff” mutant female	260.0	110.0	45.3	39.0	670.0

*, mm; **, g; Numerals are presented as the average±SD

those of control males rather than to those of control females (Table 1). However, their plumage was unclear in comparison with normal male plumage; i.e., their heads were light brown in color, the breast and upper belly not brilliantly white, but finely striped, the back and sides roughly striped, the black spot on the scapulars indistinct, and the dark undertail coverts and creamy caudal belly unclearly demarcated (Fig. 1A). As so far studied, no morphological evidence of hybridization with other species has been detected in the present birds. Interestingly, neither phallus (Fig. 1C) nor its equivalent in the cloacal region (Fig. 1B), which macroscopically appeared just the same as that seen in the female duck (Fig. 1D).

The W chromosome-specific DNA fragments (~190 bp) were PCR-amplified from feather extracts of 2 unusual males, Sp-1 and Sp-2, one “Buff” mutant female, and normal-plumage females. In contrast, only Z/W chromosome-common DNA fragment (~250 bp) was amplified from normal-plumage males (Fig. 2). No DNA fragment was amplified from negative control (distilled water). Thus, the results indicated that the 2 atypical males in question have DNA sequences common to the sequences of Z and W chromosomes.

DISCUSSION

The present study provided new data available for characterization of unusual individuals of Pintail. The molecular sexing data suggested two possibilities, i.e., (1) that the anomalous birds studied are genetically female (Z/W) irrespective of their male-type plumage and (2) that they represent the individuals of sex chromosomal aberrations, e.g., ZZW. Apart from these possibilities, one may presume that the birds in question are immature (under-yearling) males. However, the third possibility can be excluded, for they

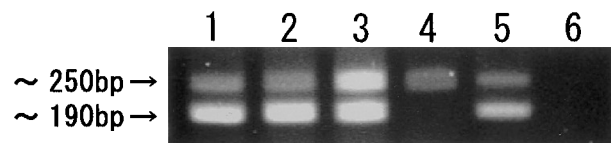


Fig. 2. Electrophoretic pattern of DNA for gender determination of Pintail. The W chromosome-specific DNA fragments (~190 bp) were amplified from plucked contour feathers of anomalous Pintails (lanes 1 and 2), “Buff” mutant female (lane 3) and normal-plumage female (lane 5), while only Z/W chromosome-common DNA fragment (~250 bp) was amplified from normal-plumage male feathers (lane 4). No sex-specific DNA band was observed in the negative control using distilled water (DW, lane 6).

had no phallus in the cloaca, although no anatomical evidence on the gonads was obtained. In favor of this view, a study made long ago showed that immature Mallard males killed between late July and early November had a macroscopically distinct phallus (Höhn 1960). Furthermore, recovery data of the marking showed that at least one of them, Sp-2, was a 2+-year-old adult bird.

If the anomalous birds studied are genetic females, we have to explain why did the genetic females have a male character for their plumage? Previous studies cited in a review paper (Witschi 1961) may be helpful for discussing this point. In the duck, Wolff and Wolff (1949) and Wolff (1950) showed that the development of accessory sex organs, syrinx and phallus, depends on the gonads in the prehatching stages: gonadectomy caused various degrees of masculinization in the female embryos, i.e., the enlargement of syrinx and the development of the phallic tubercle. Unfortunately, however, these studies provided no information about the effect of gonadectomy on the plumage. In birds, it is generally known that gonadectomy or deprivation of sex steroids causes the sex-related characters to turn to the phenotype of the

homozygote (genetic male), not heterozygote (genetic female), of the sex chromosomes. The present birds may be a case of this phenomenon. If so, we may speculate that the present birds may have dysfunctional ovary or they may have been physiologically ovariectomized, presumably in an earlier life stage.

Currently, we have no data to exclude the second possibility, i.e., the sex chromosomal aberration. It is also known that the domestic fowls of ZZW-genotype show masculinization in the post-hatching early life stages (Naito 1998). In any case, we need further information about the anomalous Pintail from studies on the gonads, chromosomes, genes, plasma concentration of sex steroids, and so on. Recent progress made by studies on the Z/W sex chromosomes of birds has been reviewed with respect to the mechanisms of sex determination and sex differentiation (Naito 1998; Ellegren 2001; Mizuno 2001).

So far as we surveyed during the recent 3 years, the incidence of the present anomaly in the study area is estimated to be 0.02–0.03%. Exact causal factor(s) of the anomaly found in the Pintail remain unknown, but it seems to be important to examine possible relationship between the anomaly and the global pollution. Future comprehensive studies on sexually anomalous birds, both in the field and laboratory, may contribute to various aspects of avian biology and environmental chemistry.

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