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SEX IDENTIFICATION IN THE EGYPTIAN VULTURE BY FLOW CYTOMETRY AND CYTOGENETICS[1]

DELIA CAVALLO, RAFFAELE DE VITA[2] AND PATRIZIA ELEUTERI
Environmental Department, Environmental Epidemiologic Unit, ENEA Casaccia, S.P. 016, Via Anguillarese 301, I-00060 Rome, Italy

ROBERT H. R. BELTERMAN
Biological/Veterinary Department, Royal Rotterdam Zoological and Botanical Gardens, P.O. Box 532, 3000 AM Rotterdam, The Netherlands

GIACOMO DELL'OMO
Laboratorio di Medicina Veterinaria, Istituto Superiore di Sanità, Viale Regina Elena 299, I-00161 Rome, Italy

Abstract. The genome size and sex of the Egyptian Vulture (*Neophron percnopterus*), a sexually monomorphic bird, were identified from blood samples by the use of flow cytometry (FCM). This technique allowed evaluation of the slightly higher amount of DNA content in the homogametic male compared to that of the heterogametic female. A karyotypic analysis also was performed in order to confirm the FCM results. Sex identification by both FCM and cytogenetic analyses was concordant in all cases. The average DNA content was estimated to be 5.6% higher in males than in females.

Key words: genome size, sex identification, Egyptian Vulture, flow cytometry, karyotype.

Many species of birds do not exhibit pronounced sexual dimorphism, and identification of sex is a significant problem for ornithologists, particularly in the case of endangered species (Tiersch and Mumme 1993). We examined the sexually monomorphic Egyptian Vulture (*Neophron percnopterus*), which has been listed as "Endangered" by the European Committee for the Conservation of Nature and Natural Resources (1994). Captive breeding of individuals obtained from rehabilitation centers has been suggested as one of the tools for restocking local populations in France (Terrasse 1990) and Italy (Ceccolini 1995).

Common procedures for sex identification include observation of courtship behavior, analysis by cytogenetic, immunological, biochemical or molecular approaches, cloacal examination, and laparoscopy. Most of these methods of sex identification are time consuming and costly, and laparotomy in particular is invasive. Identification of sex on the basis of the genome can be conducted by a qualitative analysis of the karyotype or by a quantitative evaluation of total DNA content.

In birds, the female is the heterogametic sex and carries the distinguishable sex chromosomes (ZW) with W smaller than Z, and the male is homogametic ZZ (Rothfelds et al. 1963). Whereas the cytogenetic procedures for identifying the mitotic chromosomes have been developed and commonly used for decades (Shields 1982, Belterman and De Boer 1984), flow cytometry (FCM) recently has been used to evaluate the small difference in DNA content between males and females from blood samples (Nakamura et al. 1990) and feather pulp (Tiersch and Mumme 1993). Although several studies investigated the genome of birds of prey (Renzoni and Vegni-Talluri 1966, De Boer and Sinoo 1984), few data are available for the Egyptian Vulture (Ansari and Kaul 1986).

FCM allows a rapid and accurate quantitative estimate of cellular DNA content in thousands of interphase cells (Kent et al. 1988). FCM has been used to investigate the genetic damage (Otto et al. 1981) occurring in natural populations exposed to environmental mutagens (Deaven, 1982, Bickham et al. 1988, McBee and Bickham 1988). In this study, blood samples from captive vultures were analyzed by flow cytometry to discriminate the small DNA content difference between males and females, and the results were compared with those obtained from analysis of karyotypes and with birds of known reproductive history. The aim was to extend and validate the use of FCM as a rapid and noninvasive method of sex identification.

METHODS

SAMPLES

Blood samples from 24 Egyptian Vultures were analyzed by flow cytometry. Cytogenetic analysis was performed on 15 of these birds. Blood samples for both analyses were collected at the same time by wing vein puncture. The remaining nine vultures were sexed on the basis of their reproductive history.

FLOW CYTOMETRIC ANALYSIS

Ten microliters of blood were collected and dropped immediately into 2 ml of phosphate buffer. The cellular suspension was then stored at 4°C. Erythrocytes of

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[2] Corresponding author: e-mail: devita@casaccia.enea.it

male chicken and lymphocytes of male human separated from peripheral blood were used as reference cells.

The technique of Krishan (1975), with modifications (Tiersch et al. 1989, Tiersch and Mumme 1993), was used for DNA staining. Reference cells and blood cells from Egyptian Vultures were suspended in 1 ml of lysis-staining buffer which consisted of 0.1% sodium citrate, 0.1% triton × 100 and 50 μg ml^{-1} propidium iodide. Fifty microliters of RNase (1 mg ml^{-1}) were added just prior to the addition of cells. The stained suspension was then filtered through a 53-μm nylon mesh, kept in the dark at 4°C, and analyzed within 15 min. For each specimen, erythrocytes of male chicken were used as internal reference cells and analyzed simultaneously with erythrocytes from the birds studied. Moreover, a mixture of male chicken erythrocytes and male human lymphocytes, with known DNA content, was analyzed to calibrate the flow cytometer before each stock of measurements. For each specimen, at least six measurements were performed to assess the reproducibility of results. Most measurements were carried out within 48 hr of collection.

The samples were analyzed by a PAS II flow cytometer (Partec, Munster, Germany). Excitation wavelengths around 488 nm of a 100 W mercury arc lamp were selected. Signals were collected and accumulated on a 512-channel memory. The linearity of the amplifier was verified. A total of at least 2×10^4 events was accumulated for each histogram. The histograms were analyzed using the PAS/FLOW software (Partec, Munster, Germany) to evaluate the DNA Index (DI) and the coefficient of variation (CV). The CV of the fluorescence distributions from stained avian erythrocytes and human lymphocyte cells ranged from 1.0–3.0%. These values were obtained by setting $G_{1/0}$ modal value of human lymphocytes peak at channel 100 and the internal standard (chicken erythrocytes) at channel 35. The DNA content was expressed as mass relative to a standard value of 7.0 pg per human male lymphocyte nucleus according to the formula: pg DNA = $7.0(X/S_A)(S_B/H)$, where X is the fractional mode channel of the avian samples under study and S_A is the fractional mode channel of the internal reference cells (chicken erythrocytes) in the X-S_A mixture, S_B is the fractional mode channel of the internal reference cells (chicken erythrocytes) in S_B-H mixtures, and H is the fractional mode channel of human male lymphocytes. Student's *t*-test was used to detect differences in DNA content between males and females.

CYTOGENETIC ANALYSIS

Cytogenetic analysis was conducted on 15 of the 24 birds also examined by FCM. Blood was stored at 4°C and analyzed within 24 hr. Blood cultures were prepared as described in Belterman and De Boer (1984) with the following modifications. From each blood sample, two cultures were made. For each culture, 0.5 ml of whole blood was added to 5.0 ml Iscove's Modified Dulbecco's medium (Gibco) containing 0.1 ml phytohaemagglutinin (Difco) and 0.1 ml penicillin-streptomycin solution (Sigma). Incubation time was 72 hr at 40°C. One hour before harvesting the cells, 0.1 ml of a 0.005% solution of colchicine was added. Cultures were fixed with Carnoy's fixative (methanol:acetic acid = 3:1) and chromosomes were prepared using the air-drying technique of Rothfelds et al. (1963). Chromosome preparations were stained with 4% Giemsa and studied on a Zeiss phase-contrast microscope. Metaphases were photographed and the karyotype reconstructed.

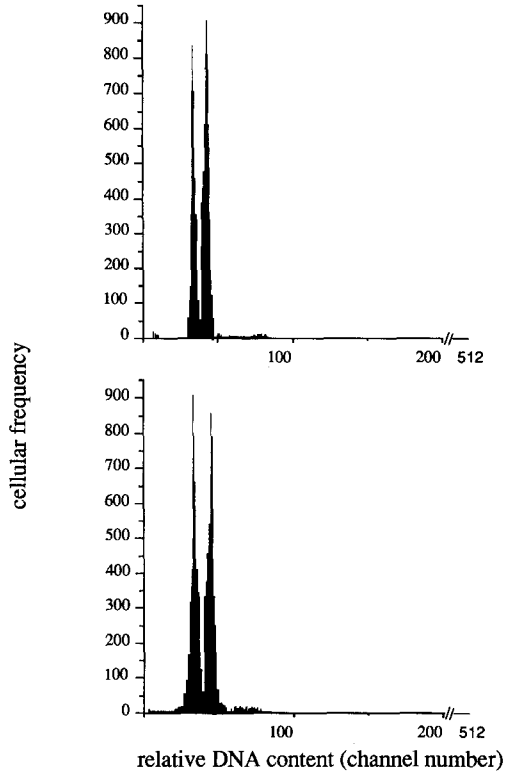

FIGURE 1. Flow cytometric DNA content distributions of domestic chicken internal reference cells (first peak from the left) together with erythrocytes of Egyptian Vulture (second peak). Top: female, bottom: male.

RESULTS

Evaluable FCM histograms were obtained in all cases (Fig. 1). The reproducibility of the flow cytometric measurements demonstrated a good reliability of the method. In fact, the values obtained from the six different measurements of the same specimen did not produce significant differences, each individual CV was ≤ 3.3%.

Cytogenetic analysis was successful in 14 of the 15 samples analyzed, whereas in one case insufficient metaphases were found. In agreement with the karyotype previously described by Ansari and Kaul (1986), a total of 66 chromosomes were present. The analysis of metaphases gave nine female heterogametic (ZW) karyotypes (Fig. 2) and five male homogametic karyotypes (ZZ). These results were confirmed by FCM. Moreover, FCM analysis confirmed the sex of the other nine birds with known reproductive histories. All fe-

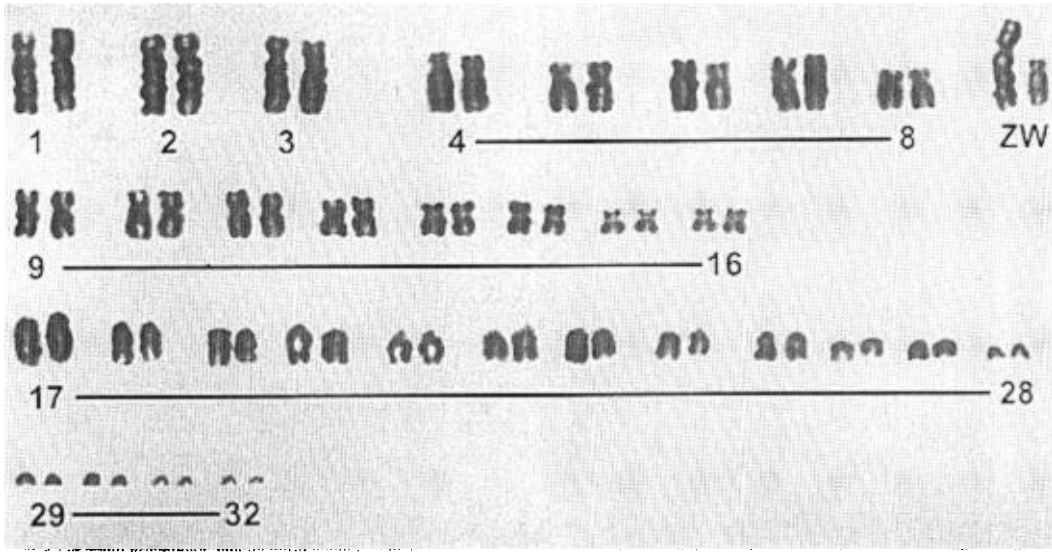

FIGURE 2. Representative karyogram of a female Egyptian Vulture, *Neophron percnopterus*, showing typical macro- and microchromosomes. The Z chromosome is a large submetacentric element, similar to one of the first three pairs of autosomes, whereas the W is a medium-size submetacentric chromosome.

males showed a lower DNA content than males (Fig. 3; *t*-test, $t_{22} = 11.1$, $P < 0.001$). DNA content was 5.6% greater in males than females ($\bar{x} \pm SD = 3.24 \pm 0.03$ for males and 3.06 ± 0.04 for females).

DISCUSSION

We used flow cytometric analysis to discriminate the small DNA content difference between the homogametic (ZZ) male and the heterogametic (ZW) female Egyptian Vulture with the aim to extend and validate a rapid and noninvasive method of sex identification. Moreover, a karyotypic analysis was performed in order to confirm the FCM results. The results obtained in our study by FCM showed in all cases a small but statistically significant difference between the DNA content of the two sexes, with the DNA content of males greater than that of females as expected. The reproducibility of results was not affected by storage of the samples for three weeks after their collection. This suggests that FCM can be applied in field studies where samples can be collected several weeks before analysis, providing that adequate temperature conditions are maintained during storage. The addition of alcohol to the buffer can make the sample suitable for longer storage at ambient temperature.

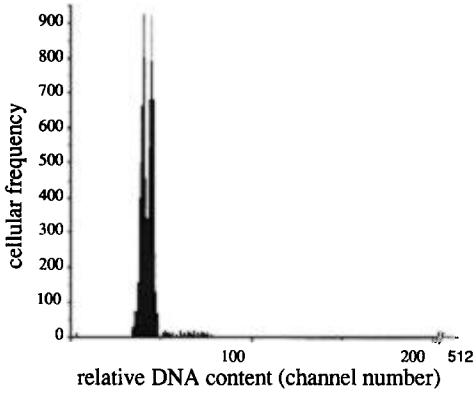

FIGURE 3. Flow cytometric DNA content distribution of a mixture of erythrocytes taken from a female (first peak from the left) and a male (second peak) Egyptian Vulture.

This study also allowed us to better describe the genome of this rare species for which the only data currently available are the karyotype description of Ansari and Kaul (1986). Cytogenetic analysis furnished valuable results in 14 of 15 cases, nine heterogametic females and five homogametic males, and showed, in agreement with the previous description of Ansari and Kaul (1986), a karyotype of 66 chromosomes. The high number of chromosomes, many of which are microchromosomes, made the karyotype reconstruction laborious. Compared to other Falconiformes (Tiersch and Wachtel 1991), genome size of Egyptian Vulture appears to be slightly higher and similar to that of the California Condor (*Gymnogyps californianus*) (Rasch et al. 1985) and the Cinereus Vulture (*Aegypius monachus*) (De Vita et al. 1994), the only data actually available on genome size of vultures. However, no data are available on genome size of vultures evaluated by FCM.

The results obtained in this study, performed on only a few microliters of blood from each animal, confirm the utility of FCM analysis as a rapid, simple, accurate, and statistically reliable technique in identi-

fying the sex of birds on the basis of nuclear DNA content.

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