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AN EVALUATION OF THE USE OF FLOW CYTOMETRY TO IDENTIFY SEX IN THE FLORIDA SCRUB JAY

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Abstract.—Two-parameter flow cytometry was used to measure sex differences in nuclear DNA content in the Florida Scrub Jay (*Aphelocoma c. coerulescens*), a sexually monomorphic, cooperative breeder. Blood samples were obtained from 39 individuals, comprising 14 known males, 14 known females and 11 birds of unknown sex. Although significant among-session variation complicated the analysis, the standard estimate of genome size for known males (3.159 ± 0.019 pg DNA per nucleus) was significantly larger than that of known females (3.095 ± 0.019 pg), and the distributions of genome size for the two sexes did not overlap. Sex of most birds of unknown sex could thereby be assigned unambiguously. The mean value for genome size was 3.129 ± 0.036 pg (SD), and intraspecific variation was 4.63% of the mean (range 3.195–3.045 pg). Analysis of feather pulp samples, complicated by high levels of debris and variability in the fluorescence distributions, was improved by use of electronic bitmap gating. Although flow cytometric techniques will probably not allow identification of sex in all cases, flow cytometry is nonetheless a potentially useful method for determining the sex of live birds.

EVALUACIÓN DEL USO DE LA CITOMETRÍA DE FLUJO PARA DETERMINAR EL SEXO EN INDIVIDUOS DE *APHELOCOMA C. COERULESCENS*

Sinopsis.—Se utilizaron dos parámetros de citometría de flujo, para determinar las diferencias sexuales en el ADN nuclear de individuos de *Aphelocoma c. coerulescens,* el cual es una especie monomórfica. Se tomaron muestras sanguíneas de 39 individuos, que comprendían a 14 machos, 14 hembras y a 11 individuos de sexo desconocido. Aunque hubo variaciones significativas entre las secciones, lo que complicó el análisis, el estimado estandarizado del tamaño del genoma de los machos (3.159 ± 0.019 pg de ADN por nucleo), resultó significativamente mayor que el de las hembras (3.095 ± 0.019 pg) y la distribución del tamaño del genoma entre los sexos, no se solapó. Por ende, el sexo de la mayoría del grupo desconocido, se determinó sin ambigüedades. El valor promedio del tamaño del genoma fue de 3.129 ± 0.036 pg (DE), y la variación intraespecífica resultó ser 4.63% del promedio (alcance 3.195–3.045 pg). El análisis de la pulpa de muestras de plumas (complicado por altos niveles de impurezas y variabilidad en la distribución de las fluorescencias), fue mejorado con la utilización de "bitmat gating." Aunque la citometría de flujo probablemente no permitirá la identificación del sexo en todos los casos, la técnica es de uso potencial para determinar el sexo de aves vivas.

Research in avian biology frequently requires knowledge of the sex of live birds. Identification of sex, however, can present significant problems for ornithologists dealing with sexually-monomorphic species, juveniles and endangered species (Quinn et al. 1990, Tiersch et al. 1991). In response to these problems, Nakamura et al. (1990) described a rapid

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and inexpensive procedure, which is based on the difference in nuclear DNA content resulting from sex chromosome heteromorphism, for identifying the sex of birds by single-parameter flow cytometry.

The utility of the technique remains to be fully evaluated, however, in samples collected under field conditions, in birds other than adults, in passerines, in feather pulp samples and in analyses performed at different times. In this paper, we address these factors and extend the findings of Nakamura et al. (1990) by use of two-parameter flow cytometry and electronic "bit-map" gating, and evaluate the utility of the flow cytometric technique for identifying the sex of adults and nestlings in a wild population of Florida Scrub Jays (*Aphelocoma c. coerulescens*).

This species is particularly appropriate for such an investigation. First, males and females are identical in plumage, and although males tend to be slightly larger than females, reliable sex identification can usually be accomplished only by observation of behavior (Schoech et al. 1991, Woolfenden and Fitzpatrick 1984). Second, because the Florida Scrub Jay has been recently designated as "Threatened" by the U. S. Fish and Wildlife Service, potentially stressful surgical techniques such as laparoscopy, should be avoided if possible. Third, because Florida Scrub Jays are cooperative breeders with female-biased dispersal (Woolfenden and Fitzpatrick 1990) there are theoretical reasons to expect an imbalance in primary sex ratio under some circumstances (e.g., Emlen et al. 1986). Thus, a reliable and efficient means of identifying sex from small quantities of blood or feather pulp would be valuable in this and similar species.

METHODS

Field work was conducted at Archbold Biological Station in Highlands County, Florida, the site of a 21-yr study of the population biology of the Florida Scrub Jay (Woolfenden and Fitzpatrick 1984, 1990). On three occasions between 29 Apr. and 28 May 1990, blood samples were obtained from 39 Florida Scrub Jays, including five birds that were sampled on two different occasions at 2–4 wk intervals. We studied 14 known males, 14 known females and 11 birds of unknown sex, including five nestlings from two different broods and six unknown-sex adults. Sex of known-sex adults was generally determined by examining behavior (e.g., "hiccup vocalization," courtship feeding, development of a brood patch, participation in paired territorial flights; Schoech et al. 1991, Woolfenden and Fitzpatrick 1984). Identifying the sex of nonbreeders is problematical, and these birds comprised the six adults of unknown sex used in this study.

For adults and 11-d-old nestlings, blood samples were obtained by brachial venipuncture. For each bird, 20–80 μl of blood was collected in 100-μl microhematocrit tubes that had been rinsed with ACD anticoagulant solution (Becton-Dickinson Vacutainer #4606) prior to sample collection. In addition to blood, pulp feathers were collected from five molting adult birds. Each feather was placed shaft down into about 0.2 ml of ACD in the bottom of a 2-ml tube. Samples were refrigerated,

shipped in a styrofoam cooler and analyzed as coded samples within 3 d of collection.

Nuclear DNA content was measured with a PROFILE model flow cytometer (Coulter Electronics, Hialeah, Florida) according to the method detailed in Tiersch et al. (1989). Prior to analysis, cryopreserved cells from channel catfish (*Ictalurus punctatus*) and domestic chicken (*Gallus gallus*) were thawed and analyzed as a mixture with fresh human leukocytes to calibrate the flow cytometer. For analysis, fresh blood cells from catfish and scrub jays were suspended in 0.5 ml of lysis-staining buffer containing 0.1% sodium citrate, 0.1% Triton X-100, 25 μl RNase (1 mg/ml) and 25 μg of propidium iodide. The suspension was kept at room temperature and analyzed within 15 min. Cells from the feathers were prepared for analysis by mincing of the feather pulp (with a small pair of scissors) in 0.5 ml of lysis-staining buffer.

Two parameters were analyzed simultaneously for each nucleus. One parameter was fluorescence in the red wavelengths (around 650 nm), which is directly proportional to DNA content and intercalation of the propidium iodide stain. The other parameter was forward angle light scatter (FALS), which provides an estimate of nuclear size. The propidium-iodide-stained nuclei were analyzed at a rate of 200–300 per second, and the fluorescence and light scatter signals were used to generate pulse-height histograms from the analysis of at least 30,000 cells. Fractional mode channel and percent coefficient of variation (% CV) were calculated for the fluorescence peaks by the PARA 1 program (Coulter Electronics). The DNA content per cell was estimated relative to a value of 7.0 pg per leukocyte nucleus for the human male, according to the formula: pg DNA $= (A/B) \times (C/D) \times 7.0$, where B and C are the fractional mode channels of the catfish (reference) cells in the A-B and C-D mixtures; A is the fractional mode channel of the avian sample, and D is the fractional mode channel of cells from a human male (one of us, TRT).

Methods for gating or specifying certain populations of cells or nuclei are found on many flow cytometers. We used bitmap gating, a standard feature of the PROFILE flow cytometer, to improve resolution in analysis of feather pulp. By use of a stylus, a bitmap can be drawn within a two-dimensional data display around any population of cells contained in a mixture of cells and debris. By collecting data only from the specified population, extraneous signals can be eliminated. For feather pulp samples, individual bitmaps were drawn around the populations corresponding to blood cells for each bird studied.

RESULTS

Typical histograms showing the fluorescence distribution and light-scatter profiles of blood cells from catfish and Florida Scrub Jay are depicted in Figure 1. In this system, intensity of fluorescence was directly proportional to the DNA content of nuclei. Thus cells from the jays contained more DNA than did cells from the catfish (used as an internal reference). Forward angle light scatter was greater in Florida Scrub Jays

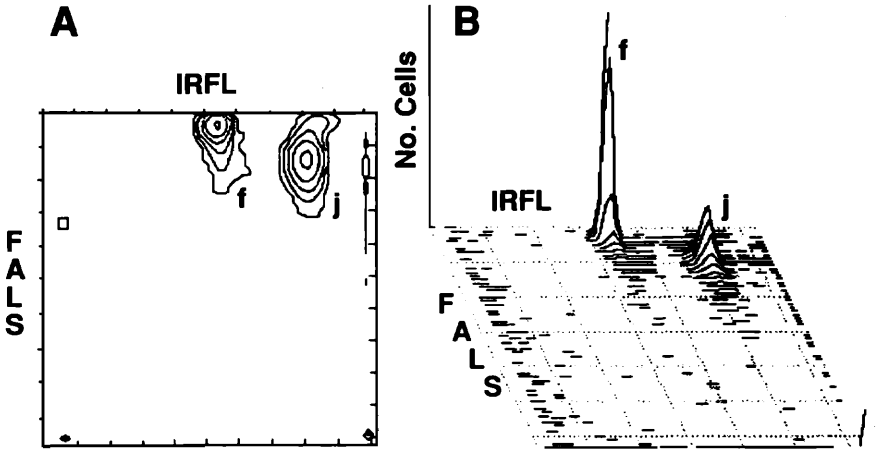

FIGURE 1. Two-dimensional (A) and three-dimensional (B) plots of simultaneous two-parameter analysis by flow cytometry of blood cells from catfish (f) (used as an internal reference) and Florida Scrub Jay (j). IRFL, integrated red fluorescence (linear propidium iodide fluorescence—directly proportional to DNA content of nuclei); FALS, forward angle light scatter (a measure of nuclear size). The FALS axis increases from top to bottom of the graph.

than in catfish, indicating that the jay cells possessed larger nuclei than did the catfish cells. The mean value for diploid genome size of the Florida Scrub Jay was 3.129 ± 0.036 pg (SD); intraspecific variation in genome size was 4.63% (range 3.195–3.045 pg).

Unstandardized values of diploid genome size observed in the three analyses are given in Table 1. Significant differences were found among the mean values of genome size for the three separate sessions (ANOVA $F_{2,41} = 29.50$, $P < 0.0001$). In order to standardize the values for direct comparison among the analyses, a correction factor was employed. The values obtained in Analyses 2 and 3 were standardized in relation to Analysis 1, and thereby to each other, by multiplication with a ratio (the mean male value of Analysis 1 divided the mean male value of either Analysis 2 or 3 respectively). Standardization reduced the variation observed for repeated analysis of particular animals in separate sessions ($n = 5$; see Table 1) from a mean (±SD) of 0.071 ± 0.048 pg (2.2%) to 0.019 ± 0.011 pg (0.61%).

Distributions of the standardized values for birds of known sex and unknown sex are shown in Figure 2. Within each of the three analyses, the genome sizes of all known males were greater than the genome sizes of all known females; this was true for standardized and unstandardized values. Genome size of males was on average 2.1% larger than the genome size of females. The mean for male values (3.159 ± 0.019 pg) and the mean for female values (3.095 ± 0.019 pg) were significantly different ($P < 0.001$; t-test).

TABLE 1. Unstandardized (raw) values of diploid (2C) nuclear DNA content (pg) for 39 Florida Scrub Jays as determined from three separate analyses by flow cytometry. Letters in parentheses indicate five individuals that were sampled twice. All values given in descending order of genome size.

Analysis 1		Analysis 2		Analysis 3	
DNA	Sex	DNA	Sex	DNA	Sex
3.182	Unknown	3.296	Male	3.292	Male (a)
3.177	Male	3.279	Male	3.281	Male
3.172	Male	3.259	Male	3.272	Male (b)
3.163	Male	3.256	Unknown	3.263	Male (c)
3.160	Male	3.255	Unknown	3.248	Female (d)
3.159	Unknown	3.254	Male	3.224	Female
3.149	Unknown	3.253	Male	3.217	Female (e)
3.145	Unknown	3.251	Male (b)	3.216	Female
3.128	Unknown	3.246	Male (a)	3.213	Unknown
3.123	Male (c)	3.242	Unknown	3.169	Female
3.118	Female (e)	3.225	Male		
3.108	Female	3.221	Unknown		
3.093	Female	3.219	Female		
3.091	Female	3.203	Female		
		3.201	Female		
		3.198	Female (d)		
		3.173	Female		
		3.170	Female		
		3.170	Female		
		3.155	Unknown		

There was no overlap in standardized DNA values for known males (range, 3.195–3.123 pg) and known females (3.121–3.054 pg) when calculated to three decimal places (10^{-3} pg). When rounded to two decimal places, however, the values of the lowest male and the two highest females were the same (3.12 pg; Fig. 2). Of the 11 unknown-sex birds, one (a nestling) also had a value of 3.12 pg, and another had a value at the low end of the male range (3.13 pg). The sex of the other nine unknown-sex birds could be assigned unambiguously (seven male, two female).

The DNA values obtained for feather pulp were 5.64 ± 2.61% larger than the DNA values obtained for blood from the same individual. Analysis of feather pulp generated broad, asymmetric fluorescence peaks, and in some cases, multiple peaks or shoulders (Fig. 3). The presence of the multiple peaks complicated analysis and increased the % CV values (4.10 ± 0.23) in relation to the % CV values obtained for blood (1.96 ± 0.15). To correct for this we employed bitmap gating, which allowed collection of data from specific cell populations within the mixture of cell types and debris present in feather pulp (Fig. 3). Use of the bitmap reduced the DNA values for feather pulp to a mean of 2.69 ± 1.26% greater than blood, and reduced the % CV values (2.97 ± 0.57).

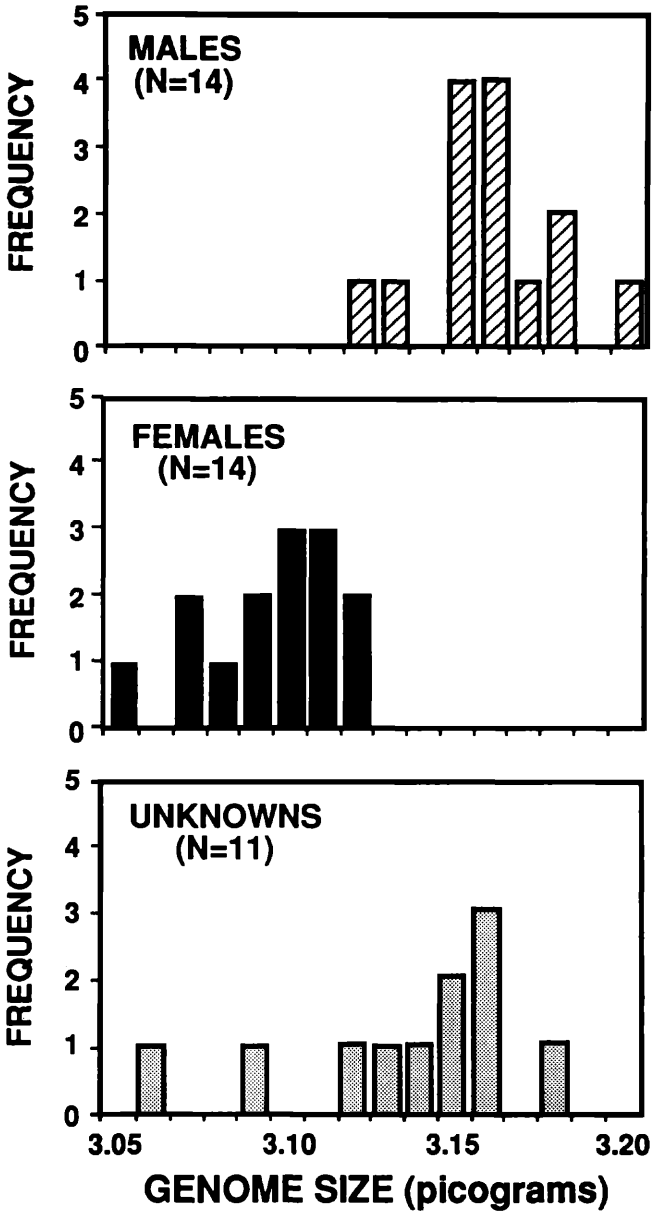

FIGURE 2. Frequency distribution of standardized values of diploid (2C) nuclear DNA content (picograms) as determined by flow cytometry for male, female and unknown-sex Florida Scrub Jays.

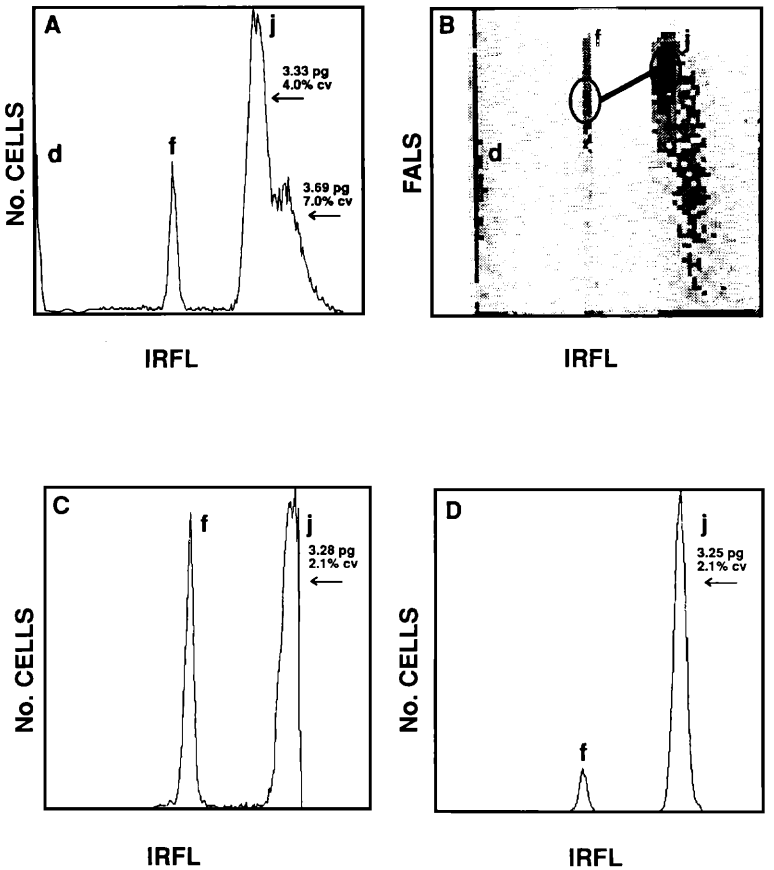

FIGURE 3. Analysis by flow cytometry of DNA content of cells from feather pulp (A); f, catfish; j, Florida Scrub Jay; d, debris. Diploid DNA content (pg) and % CV of fluorescence peaks are indicated. Two-parameter analysis of feather pulp showing area (within dumbell shape) used for data collection in bitmap procedure (B); IRFL, integrated red fluorescence (directly proportional to DNA content of nuclei); FALS, forward angle light scatter (a measure of nuclear size). This procedure allowed collection of data from specific cell populations within a mixture of cell types. Appearance of feather pulp sample following analysis with bitmap procedure (C). Analysis of blood cells from same bird used for analysis of feather pulp (D).

DISCUSSION

Estimates of genome size derived by flow cytometry are sufficiently precise to allow the sex of Florida Scrub Jays to be identified unambiguously in the majority of cases. As shown in Table 1 and Figure 2, values of genome size for known males abutted those of females, but the two distributions did not overlap.

For the 11 unknown-sex birds analyzed, flow cytometric data allowed

sex to be assigned unambiguously in all but two cases. Of the six unknown-sex adults sampled, five were classified as male and one as female. For the five unknown-sex nestlings, assignment of sex was less certain. One of the five nestlings was clearly female and two were clearly male. Estimated genome size for a fourth nestling (corrected value of 3.128 pg) fell at the lower end of the range of male values, whereas estimated genome size of a fifth nestling (corrected value of 3.123 pg) fell at the boundary between the smallest genome size recorded among known males (corrected value of 3.123 pg) and the largest genome size recorded among known females (corrected value of 3.121 pg). We suspect, however, that the latter unclassified nestling was a small-genome male; its nestmate had the second lowest value of genome size recorded (corrected value of 3.059 pg) and was clearly a female. The 0.062 pg difference in genome size between these two siblings corresponds closely to the mean male-female difference in genome size (0.064 pg) and is explained most parsimoniously as being a result of a sex difference.

Studies of intraspecific genome size in birds are limited. In the approximately 100 species of birds for which genome size has been measured, there are only three species in which more than 10 individuals have been studied [domestic chicken, Golden Pheasant (*Chrysolophus pictus*) and Japanese Quail (*Coturnix japonica*); see Tiersch and Wachtel 1991] and most values are derived from analysis of only one or two birds. The variation in Florida Scrub Jays (4.6%) is small compared to values of intraspecific variation observed for other vertebrates (e.g., Gold et al. 1990) and is well below the extreme value of 35% observed in pocket gophers (*Thomomys* spp.) (Sherwood and Patton 1982).

In this study, analysis of feather pulp yielded multiple peaks and high levels of debris. The additional peaks could have been caused by differences in DNA content among various cell types present in feather pulp or by differences in the staining properties of the various cell types. Electronic gating procedures, such as bitmapping, could improve resolution sufficiently to enable identification of sex by analysis of feather pulp. Blood and feather pulp samples were analyzed within 3 d of collection, in this study. Flow cytometric methods would be considerably more useful to ornithologists, however, if samples obtained in the field could be preserved for extended periods in alcohol or by freezing prior to analysis (e.g., Vindeløv et al. 1983).

Our study has also shown that the direct comparison of flow cytometric results collected at different times is potentially problematic. Blood samples were analyzed during three separate sessions, and among-session differences were found in the resulting estimates. Thus samples from known males and females need to be run concurrently with samples from unknown-sex birds, and values of genome size obtained from separate analyses could require standardization to allow comparison.

In spite of this difficulty, flow cytometric analysis of sex is a useful technique that may find an important place in the avian biologist's toolkit. Although flow cytometry will probably not allow for 100% unambiguous

identification of sex in all cases for all species, and thus cannot substitute for cytogenetic and molecular techniques of sex determination (e.g., Quinn et al. 1990, Rabenold et al. 1991), it is a viable option that should be considered by ornithologists requiring rapid and accurate identification of sex in living birds.

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LITERATURE CITED

EMLEN, S. T., J. M. EMLEN, AND S. A. LEVIN. 1986. Sex-ratio selection in species with helpers-at-the-nest. Am. Nat. 127:1–8.

GOLD, J. R., C. J. RAGLAND, AND L. J. SCHLIESING. 1990. Genome size variation and evolution in North American cyprinid fishes. Genet. Sel. Evol. 22:11–29.

NAKAMURA, D., T. R. TIERSCH, M. DOUGLASS, AND R. W. CHANDLER. 1990. Rapid identification of sex in birds by flow cytometry. Cytogenet. Cell Genet. 53:201–205.

QUINN, T. W., F. COOKE, AND B. N. WHITE. 1990. Molecular sexing of geese using a cloned Z chromosomal sequence with homology to the W chromosome. Auk 107:199–202.

RABENOLD, P. P., K. N. RABENOLD, W. H. PIPER, AND D. J. MINCHELLA. 1991. Density-dependent dispersal in social wrens: genetic analysis using novel matriline markers. Anim. Behav. 42:144–146.

SCHOECH, S. J., R. L. MUMME, AND M. C. MOORE. 1991. Reproductive endocrinology and breeding suppression in the cooperatively breeding Florida Scrub Jay (*Aphelocoma c. coerulescens*). Condor 93:354–364.

SHERWOOD, S. W., AND J. L. PATTON. 1982. Genome evolution in pocket gophers (genus *Thomomys*). II. Variation in cellular DNA content. Chromosoma 85:163–179.

TIERSCH, T. R., R. W. CHANDLER, S. S. WACHTEL, AND S. ELIAS. 1989. Reference standards for flow cytometry and application in comparative studies of nuclear DNA content. Cytometry 10:706–710.

———, R. L. MUMME, R. W. CHANDLER, AND D. NAKAMURA. 1991. The use of flow cytometry for rapid identification of sex in birds. Auk 108:206–208.

———, AND S. S. WACHTEL. 1991. On the evolution of genome size of birds. J. Heredity 82:363–368.

VINDELØV, L. L., I. J. CHRISTENSEN, N. KEIDING, M. SPANG-THOMSEN, AND N. I. NISSEN. 1983. Long term storage of samples for flow cytometric DNA analysis. Cytometry 3:317–322.

WOOLFENDEN, G. E., AND J. W. FITZPATRICK. 1984. The Florida Scrub Jay: demography of a cooperative-breeding bird. Princeton University Press, Princeton, New Jersey. 406 pp.

———, AND ———. 1990. Florida Scrub Jays: a synopsis after 18 years of study. Pp. 239–266, *in* P. B. Stacey and W. D. Koenig, eds. Cooperative breeding in birds: long-term studies of ecology and behavior. Cambridge University Press, Cambridge, United Kingdom.

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