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Gender Identification in Birds using Microsatellite DNA Fingerprint Analysis

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Gender determinations can be performed in sexually monomorphic birds through surgical examination. However, this procedure is not practical when dealing with expensive or endangered species because it is invasive and places the individuals being tested at risk. Alternatively, sex identification can sometimes be made through karyotype analysis (bird sex chromosomes are ZZ and ZW, with females being the heterogametic sex). However, karyotyping can be unreliable due to difficulties in obtaining avian chromosome spreads, lack of distinguishable sex chromosomes, or both (Prus and Schmutz 1987).

The inability to identify gender in a reliable and noninvasive manner in some birds represents an impediment to captive-propagation programs, whether such programs are designed for commercial or restoration purposes. Of value would be the development of DNA-based gender tests for birds. Because the red-blood cells of birds contain nuclei, only a small volume of blood (0.01–0.1 cc) is required to provide sufficient DNA for multiple tests (Longmire et al. 1988). Such volumes of blood can be drawn easily and safely by venipuncture, or collected from a simple nail-clipping procedure. In addition, many endangered avian species are already the subjects of DNA fingerprinting studies to determine levels of genetic diversity and relatedness within natural and captive populations (Brock and White 1991, Longmire et al. 1991, 1992, Geyer et al. 1993, Maltbie 1992). Thus, it would be helpful to identify DNA-sequence probes that enable sex identification in the same analyses that generate highly polymorphic patterns achieved in DNA fingerprinting.

"Minisatellite" and especially "microsatellite" sequences found ubiquitously in the genomes of higher organisms, and commonly used in DNA fingerprint studies, might be used to achieve this goal. Minisatellites consisting of short (approximately 40-base-pair) tandem repeats have been shown to reveal gender-specific patterns in a limited number of species. In birds, the M13 minisatellite detected female-specific restriction patterns in Mauritius Kestrels (*Falco punctatus*) and Peregrine Falcons (*F. peregrinus*; Longmire et al. 1991), and the minisatellite probe 33.15 revealed W-chromosome-specific fragments in Stripe-backed Wrens (*Campylorhynchus nuchalis*; Rabenold et al. 1991).

Microsatellites are very simple (up to six nucleotide) tandem repeats, and have previously been found to be sex-linked in a wider variety of organisms. Kashi et al. (1990) reported that the dinucleotide repeat $(TG)_n$ revealed sex-specific restriction patterns in cattle. The tetranucleotide repeat $(GACA)_4$ was found to provide sex identification in certain reptiles, and the trinucleotide repeat $(TCC)_5$ was shown to be sex-linked in pigeons and chickens (Epplen et al. 1991). The observation that highly polymorphic simple repeats can reveal sex-specific patterns in a range of animals led us to explore the possibility of developing a set of bird sexing probes based on microsatellite repeats.

We investigated the use of three commercially available dinucleotide repeats ($[CT]_n$, $[GT]_n$, and $[CG]_n$), and the trinucleotide repeat $(TCC)_n$ for the ability to detect sex-specific sequences in nine avian species representing eight genera and six orders (Table 1). Blood was collected into lysis buffer, and DNA was isolated as described by Longmire et al. (1991). A 10-μg quantity of each DNA was digested with a 10-fold unit excess of restriction enzyme using buffer conditions recommended by the supplier (New England Bio Labs, Beverly, Massachusetts). Restricted DNAs were electrophoresed in 20 × 25 cm 0.8% agarose gels at 35 volts for approximately 30 h and, subsequently, transferred onto positively charged nylon membranes. Resulting blots were washed for 60 min at 60°C in 0.1× SSC, 0.1% SDS, and then prehybridized for 45 min at 42°C in 6× SSC, 35–40% formamide,

TABLE 1. Sex identification in birds using various microsatellite probes. Numbers indicate restriction enzymes successfully used to reveal high-molecular-weight, female-specific, microsatellite fragments (1 = *Hae*III; 2 = *Hin*fI; 3 = *Pst*I). Zero (0) indicates female-specific microsatellite fragments not detected. Dash indicates that test not done.

Species	Sample size by sex	Microsatellite probe			
		$(CT)_n$	$(GT)_n$	$(CG)_n$	$(TCC)_n$
Canada Goose	14 F, 5 M	2	0	0[a]	2
California Condor	6 F, 3 M	1,2	0	0[a]	0
Peregrine Flacon	9 F, 16 M	1	1	0[a]	0[a]
Greater Prairie-Chicken	9 F, 6 M	1,2	0	0[a]	1,2
Attwater's Prairie-Chicken	2 F, 14 M	1	0	0[a]	1
Wild Turkey	7 F, 4 M	1,3	0	0[a]	—
Whooping Crane	5 F, 5 M	0	0	0[a]	0[a]
Chattering Lory	2 F, 2 M	0	—	0[a]	0
House Sparrow	5 F, 5 M	0	0	0[a]	0

[a] Reduced hybridization signal intensity observed.

0.005 M EDTA (pH 8.0), and 0.25% w/v powdered milk (Vassart et al. 1987). Hybridization was in the same solution containing approximately 10^6 cpm per ml probe. The $(GT)_n$, $(CT)_n$, and $(CG)_n$ oligonucleotides were purchased from Pharmacia LKB (Piscataway, New Jersey). Trinucleotide $(TCC)_n$ with an n of 30 was synthesized using a Beckman System 1 DNA synthesizer. All probes except $(TCC)_n$ were labeled with [^{32}P]dCTP by nick translation (Rigby et al. 1977). The trinucleotide repeat $(TCC)_n$ was labeled with [^{32}P]dCTP using the primer extension method described by Feinberg and Vogelstein (1983). Posthybridization washes were as follows: two times for 15 min at 22°C in 2× SSC, 0.1% SDS; and twice for 15 min at 50°C in 1× SSC, 0.1% SDS. Washed blots were autoradiographed at −70°C in cassettes containing intensifying screens. Gender was confirmed by either cloacal inversion (geese), karyotyping (condors, cranes, lorys), or phenotypic examination (falcons, prairie-chickens, turkeys, sparrows).

Results included the observation of prominent, high-molecular-weight (approximately 50 kb) female-specific restriction fragments in six of the nine species that were tested using at least one combination of enzyme and probe (Table 1, Fig. 1). These sex-specific fragments must originate from clusters of microsatellite sequence uniquely localized on the sex (W) chromosome. Dinucleotide repeat $(CT)_n$ revealed sex-specific fragments in: *Hae*III-digested Peregrine Falcon DNA; California Condor (*Gymnogyps californianus*) DNA digested with *Hae*III or *Hin*fI; Canada Goose (*Branta canadensis*) DNA digested with *Hin*fI; Wild Turkey (*Meleagris gallopavo*) DNA digested with *Hae*III or *Pst*I; Attwater's Prairie-Chicken (*Tympanuchus cupido attwateri*) DNA digested with *Hae*III; and Greater Prairie-Chicken (*Tympanuchus cupido*) DNA digested with *Hin*fI or *Hae*III. Dinucleotide repeat $(GT)_n$ revealed female-specific *Hae*III restriction fragments in the Peregrine Falcon. Trinucleotide repeat $(TCC)_n$ hybridized to female-specific *Hin*fI restriction fragments in the Canada Goose and Attwater's Prairie-Chicken DNA after digestion with *Hae*III, and in Greater Prairie-Chicken DNA following digestion with *Hae*III or *Hin*fI. In the above cases, sex determination by DNA analysis matched that based on morphometric examination. All of the microsatellite probes hybridized to DNAs sufficiently to allow overnight autoradiographic exposures, except $(TCC)_n$, which showed reduced hybridization to DNA from the Peregrine Falcon and Whooping Crane (*Grus americana*), and $(CG)_n$, which produced little or no detectable hybridization to any DNA sample. In addition, each of the microsatellite probes, except $(CG)_n$, revealed highly polymorphic (DNA fingerprint) patterns in all species that were tested.

Thus, variable results were obtained depending upon which microsatellite probes were used with the different species. Certain probes revealed sex-specific patterns in some species but not in others. For example, $(TCC)_n$ detected sex-specific patterns in the Canada Goose, Attwater's Prairie-Chicken, and Greater Prairie-Chicken, but not in the Peregrine Falcon or California Condor. However, only the Peregrine Falcon displayed sex-specific patterns following hybridization to $(GT)_n$. Only $(CG)_n$ was noninformative in all species. The observation that $(CG)_n$ did not hybridize to these avian DNAs is not surprising given that CG is the rarest dinucleotide within vertebrate genomes and may not exist as a moderate copy-number repeat (Stallings 1992, Sved and Bird 1990).

The small set of microsatellite probes tested in this study allowed unambiguous gender testing in the Peregrine Falcon, California Condor, Wild Turkey, Canada Goose, Greater Prairie-Chicken, and Attwater's Prairie-Chicken. Sex-linked fragments were not observed in the House Sparrow (*Passer domesticus*), Chattering Lory (*Lorius garrulus*), and Whooping Crane. Although sex-specific patterns were not visualized in some species in our study, the number of oligomer probes that were used was not exhaustive. Taking into account circular permutation of repeat sequences, as well as strand complementarity, there are 6 dinucle-

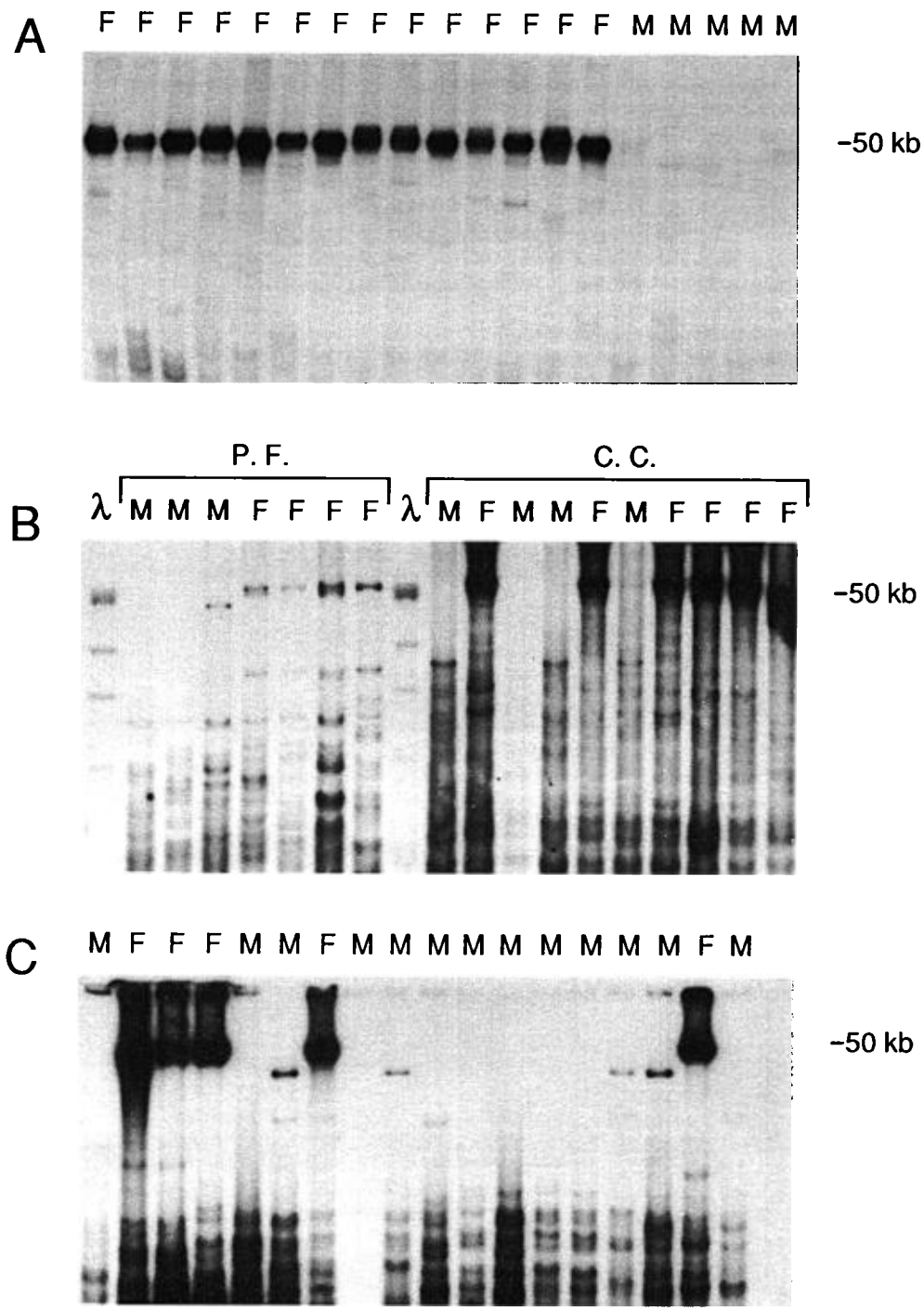

Fig. 1. Representative blots showing sex-specific DNA hybridization patterns. For each DNA, 10 μg digested with excess restriction enzyme and electrophoresed within 0.8% agarose gels. Resulting blots were hybridized to microsatellite probes. (A) *Hin*fI digested Canada Goose DNAs hybridized to $(CT)_n$. (B) Peregrine Falcon (P.F.) and California Condor (C.C.) DNAs digested with *Hae*III and hybridized to $(CT)_n$. (C) *Hae*III digested Peregrine Falcon DNAs hybridized to $(GT)_n$. In each panel, male designated by M, and female designated by F. Size standards (λ) included undigested bacteriophage lambda DNA, *Hin*dIII digested bacteriophage lambda DNA, and *Hae*III digested $\phi \times 174$ DNA. Female-specific fragments indicated in each panel by dashes at approximately 50 kb.

otide, 12 trinucleotide, 39 tetranucleotide, 109 pentanucleotide, and 366 possible hexanucleotide repeat probes. Further experimentation would be required to test for additional gender-determining probes. However, our results indicate that a more comprehensive survey of microsatellites may provide a bank of probes that could be used to identify gender in birds in a more universal manner.

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