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Chromosome-specific Intron Size Differences in the Avian CHD Gene Provide an Efficient Method for Sex Identification in Birds

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Adults and particularly offspring of many avian species are monomorphic, making determination of sex difficult. Probes that hybridize to the female-specific *W* chromosome have led to molecular solutions to this problem for some species (e.g. Griffiths and Holland 1990, Quinn et al. 1990, Rabenold et al. 1991, Dvorák et al. 1992, Longmire et al. 1993, Ogawa et al. 1997), but their use requires considerable expertise, and often their taxonomic range is limited. More recently, PCR-based approaches that are technically simpler and that have broad taxonomic utility have been developed (Ogawa et al. 1997).

The discovery of a highly conserved gene (CHD) that is linked to the *W* and a non-*W* chromosome in most birds allowed Griffiths et al. (1996) to develop a PCR-based approach that can be used in a wide variety of avian taxa. The technique involves two steps: DNA is amplified from the CHD genes to produce a 110-bp PCR product and is subsequently digested with a restriction endonuclease that digests either the *W* or non-*W* specific amplification. In practice, with some gel systems the cleaved bands are small enough that it is easiest to use the presence or absence of the heaviest band as diagnostic. However, the cleaved fragments yielded by the digest tend to fall into the size range of "primer dimers" generated in PCR reactions, which can lead to confusion in interpretation.

Ellegren (1996) developed an assay for Collared Flycatchers (*Ficedula albicollis*) within the CHD gene that used primers flanking an intron to amplify both the Z and *W* chromosomal genes. He found that differences in intron size between those two chromosomes allowed a direct detection of the two chromosomes without the need for a restriction digest. The size difference between the *W* and *Z* introns was slight, necessitating the use of acrylamide gel electrophoresis to obtain clear resolution. It has been indicated that this primer pair is not effective for sex identification among divergent avian taxa (Ellegren and Sheldon 1997).

We have taken a similar one-step approach to that used by Ellegren (1996) except that we designed primers that are highly conserved (and hence amplify CHD sequences from a wide variety of taxa) and that flank an intron that varies considerably in size between the sex chromosomes of many avian species. The intron reported here intervenes between the CHD helicase and DNA-binding regions (Fig. 1). The information from this intronic region provides a simple and efficient way to screen birds for sex determination. The most significant distinction regarding the work reported here is that this intron and primer pair provide a simple procedure for sex identification almost universally across avian taxa. Throughout this paper, we make the assumption that non-*W* chromosomal product comes from the Z chromosome, following the work by Griffiths and Korn (1997), Griffiths et al. (1996), and Ellegren (1996). This has not been formally assessed in many of the taxa included in this work. However, for purposes of determining sex, this has no negative effect on the assay that we describe.

Methods.—DNA was extracted by a variety of methods. Samples from Snow Goose (*Anser caerulescens*), Tundra Swan (*Cygnus columbianus*), Canvasback (*Aythya valisineria*), and Ostrich (*Struthio camelus*) were prepared from blood following the "small volume" approach of Quinn and White (1987). Sage Grouse (*Centrocercus urophasianus*) DNA was extracted from muscle following Kahn et al. (unpubl. data). DNA from whole blood was extracted from Moun-

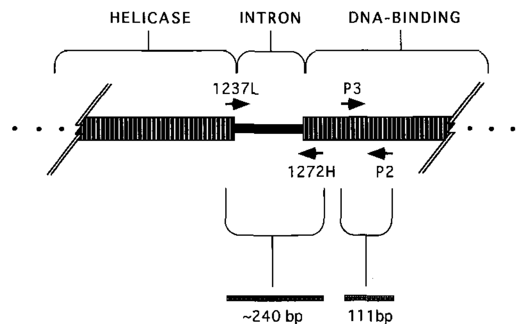

FIG. 1. Schematic representation of the region in the CHD gene where primer sets 1237L/1272H (this manuscript) and P3/P2 (Griffiths et. al. 1996) bind with respect to the location of the intron reported in the text. The amplification by the primer pair 1237L/1272H produces one band from males (from the two Z-chromosomes) and two from females (from the W- and the Z-chromosome); the region being amplified is composed of nucleotide bases almost entirely from an intron. The size difference between *W* and *Z* copies of the 1237L/1272H product varies from species to species, as does the overall size of the amplification products (as shown in Fig. 2). These results are attributable to sex-chromosome-specific and species-specific differences in intron size.

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TABLE 1. Amplification products by sex from a variety of avian taxa.

Species	Source	No. tested[a]		No. bands generated[a]		Sexed by
		Male	Female	Male	Female	
Struthioniformes						
Ostrich	Blood	4 (4)	4 (4)	1 (1)	1 (1)	Dissection
Casuariiformes						
Emu	Blood	3 (3)	1 (1)	1 (1)	1 (1)	Dissection
Anseriformes						
Tundra Swan	Heart	3	2	1	2	Dissection
Snow Goose	Blood	5	5	1	2	DQSG10/cloacal exam
Mallard	Blood	1	1	1	2	Plumage
Canvasback	Serum	1	1	1	2	Plumage
Redhead	Blood	1	1	1	2	Plumage
Falconiformes						
Red-tailed Hawk	Blood	2 (1)	2 (1)	1 (1)	1 (2)	Necropsy/behavior
Galliformes						
Sage Grouse	Muscle	5	5	1	2	Morphology/plumage
Gruiformes						
Pukeko[b]	Blood	1	1	1	1	Behavior
Charadriiformes						
Mountain Plover	Blood	3	3	1	2	Behavior
Spotted Sandpiper	Blood	5	4	1	2	Morphology
Common Tern[b]	Blood	1	1	1	1	Behavior
Caspian Tern[b]	Blood	1	1	1	1	Behavior
Black Skimmer[b]	Blood	1	1	1	2	Morphology
Strigiformes						
Great Horned Owl	Blood	3 (3)	3 (3)	1 (1)	1 (2)	Necropsy/behavior
Passeriformes						
Florida Scrub-Jay[b]	Blood	1	1	1	2	Behavior

[a] Brackets denote number of samples tested and number of bands resolved on acrylamide gel. Otherwise, agarose gel alone was used. Very faint bands observed in a few amplifications are not included.
[b] James S. Quinn (pers. comm.).

tain Plover (*Charadrius montanus*) and Emu (*Dromaius novaehollandiae*) using the Chelex-based method described by Walsh et al. (1991), except that we used 10% Chelex (vs. 5%), incubation at 65°C (vs. 56°C), and a 15-min boiling step (vs. 8 min). Several preextracted DNA samples were provided by other researchers (Table 1; see Acknowledgments).

Published chicken (Funahashi et al. 1993) and mouse (Delmas et al. 1993) CHD sequences and newly derived Sage Grouse and Snow Goose sequences (N. Kahn and T. Quinn unpubl. data) from the region surrounding the junction of the helicase and DNA-binding domains were aligned. Comparison of these cDNAs with our genomic sequence revealed that we found an intervening intron. Using this alignment, we found two highly conserved regions that flank the intervening intron and that are common to both the *Z*-linked and the *W*-linked CHD genes, and we synthesized the corresponding primers (Genosys; 1237L [5'-GAGAAACTGTGCAAAACAG-3'] and 1272H [5'-TCCAGAATATCTTCTGCTCC-3']). The relative locations of primers 1237L and 1272H on the CHD gene are shown in Figure 1. PCR amplifications were performed in a Perkin-Elmer DNA thermal cycler, using approximately 30 ng of genomic DNA as template in a 25-μL volume. In addition, each reaction included primers at 1 μM each, 250 μM each dNTP, and 0.125 U *Taq* DNA polymerase in a 1× *Taq* buffer (67 mM Tris-HCl pH 8.0, 6.7 mM $MgSO_4$, 16.6 mM AmSO4, 10 mM β-mercaptoethanol). Another PCR buffer system also works well (J.S. Quinn pers. comm.). Each reaction was overlaid with two drops of light mineral oil, preheated at 94°C for 2 min, and amplified for 30 cycles with the following thermal profile: denaturation, 94°C for 30 s; annealing, 56°C for 1 min; and extension, 72°C for 2 min. A 10-min extension at 72°C followed the last thermal cycle. A 5-μL sample of each PCR product was electrophoresed through a 2% Nusieve gel (FMC) in 1× TBE with 0.5 μg/mL ethidium bromide and visualized under UV light. Typically, electrophoresis was at 5 V/cm for 1.25 h.

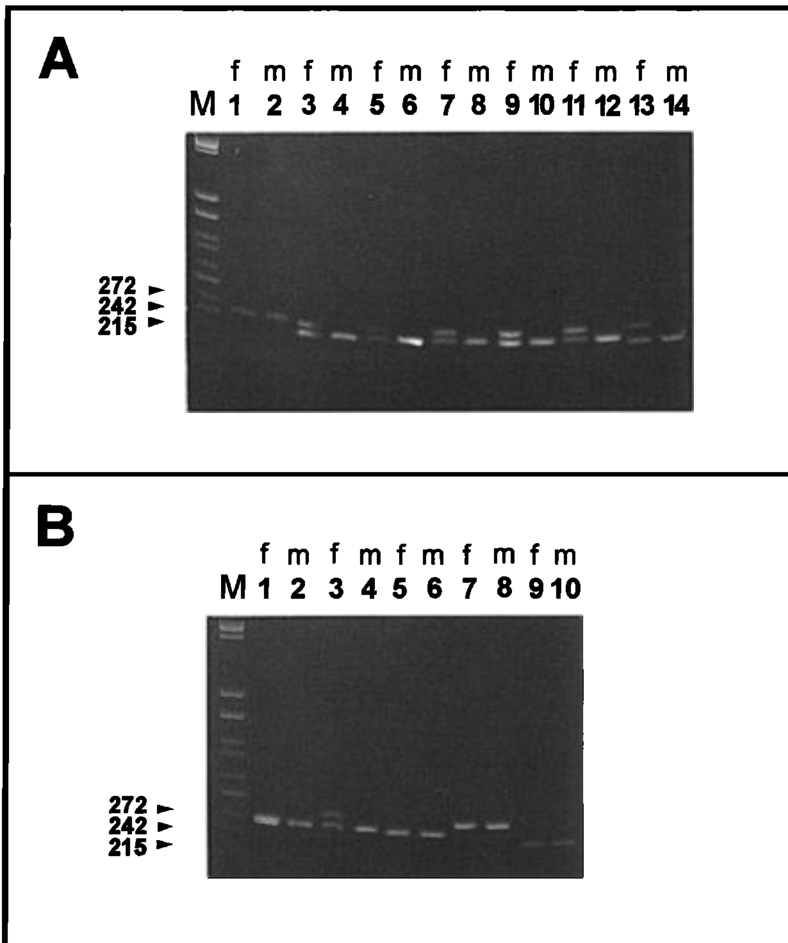

FIG. 2. PCR amplifications from a variety of avian taxa, electrophoresed on 2% Nusieve agarose in 1× TBE at 5V/cm. Individuals alternate between female and male from the marker on the left. (A) Lanes 1 and 2, Ostrich; 3 and 4, Snow Goose; 5 and 6, Tundra Swan; 7 and 8, Canvasback; 9 and 10, Mallard (*Anas platyrhynchos*); 11 and 12, Redhead (*Aythya americana*); 13 and 14, Sage Grouse. (B) Lanes 1 and 2, Mountain Plover; 3 and 4, Spotted Sandpiper (*Actitis macularia*); 5 and 6, Great Horned Owl; 7 and 8, Red-tailed Hawk; 9 and 10, Emu. Numbers to the left of marker indicate relevant marker band sizes in bp. Among waterfowl, the band found only in females appears to be approximately 20 bp larger than the band that is common to both males and females.

Some samples, appearing as single bands in both male and female samples on agarose gels, were subsequently electrophoresed through a 5% nondenaturing polyacrylamide gel, stained with 0.5 μg/mL ethidium bromide, and visualized under UV light following Sambrook et al. (1989).

Results and Discussion.—Amplifications from 1237L and 1272H appeared robust and specific, producing clear amplification products of one or two sizes for all species tested (Fig. 2). In most species, males had one band and females had two, presumably reflecting differing intron sizes of the W versus the Z chromosomes (Table 1). Included in this category are members of both of the major avian infraclasses (Neoaves and Eoaves) of Sibley et al. (1988). In many cases, differential resolution of bands, and hence sex determination, was done easily using standard agarose gel electrophoretic conditions (Fig. 2). Typically, the first attempt at amplification was successful and specific for samples extracted by our lab and by other labs. Amplifications were equally robust for the variety of different extraction methods outlined above.

Because this methodology takes advantage of intron-specific differences, the sizes of the amplification products differ over a range of 210 to 285 bp for

each species. Presumably, this intron is poorly conserved and will change in length independently in a given lineage, as well as diverge differentially on the *W* and *Z* chromosomes. This is reflected by the observation that size differences between the *W*- and *Z*-specific copies of this amplification vary among species (Fig. 2). Thus, it is not surprising to find some species in which the size difference is very small, as we found in Great Horned Owl (*Bubo virginianus*) and Red-tailed Hawk (*Buteo jamaicensis*). Curiously, in cases where bands of different sizes were clearly discernible (Fig. 2), the *W* copy was always larger, suggesting some preference for deletion on the *Z* or insertion on the *W*.

In some species, size differences could not be resolved on standard agarose gels. In species where the size difference between the *W* and *Z* copies of the intron are very small (Great Horned Owl, Red-tailed Hawk, and presumably Common Tern [*Sterna hirundo*], Caspian Tern [*S. caspia*], and Pukeko [*Porphyrio porphyrio*]), higher-resolution gels should allow sex identification from the 1237L and 1272H PCR product. In two of the cases that we tested further (Great Horned Owl, Red-tailed Hawk), differences between the sexes were discerned easily using nondenaturing polyacrylamide gel electrophoresis (not shown). If a species has *W* and *Z* copies of this intron that are the same size, the DNA base-composition of the *W*- and *Z*-chromosome versions still would probably be different (if the *W* and *Z* chromosomes are evolutionarily isolated) and therefore could be separated by use of a single-strand conformation polymorphism gel.

The only exception to the ability of 1237L and 1272H to identify sex appears to be for ratites. The Ostrich has been indicated to have just one copy of the CHD gene on a non-*W* chromosome, or two copies that are identical (Ellegren 1996); this may also be the case for other ratites. Our results show that amplifications by 1237L and 1272H in Ostrich and Emu produce only single bands for both males and females, as resolved on agarose and on 5% nondenaturing polyacrylamide gels. DNA sequencing of this intron from both male and female Ostriches (N. Kahn and T. Quinn unpubl. data) consistently reveals only one sequence.

We recommend that laboratories using this sexing method incorporate the primers directly into whatever PCR system they are already using, using their standard buffer and their usual nucleotide concentrations, etc. The ease and reliability of this test in conjunction with rapid DNA extraction methods should greatly simplify the sexing of many bird species.

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