

SHORT COMMUNICATION

## A DNA test to sex most birds

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### Abstract

Birds are difficult to sex. Nestlings rarely show sex-linked morphology and we estimate that adult females appear identical to males in over 50% of the world's bird species. This problem can hinder both evolutionary studies and human-assisted breeding of birds. DNA-based sex identification provides a solution. We describe a test based on two conserved CHD (chromo-helicase-DNA-binding) genes that are located on the avian sex chromosomes of all birds, with the possible exception of the ratites (ostriches, etc.; *Struthioniformes*). The CHD-W gene is located on the W chromosome; therefore it is unique to females. The other gene, CHD-Z, is found on the Z chromosome and therefore occurs in both sexes (female, ZW; male, ZZ). The test employs PCR with a single set of primers. It amplifies homologous sections of both genes and incorporates introns whose lengths usually differ. When examined on a gel there is a single CHD-Z band in males but females have a second, distinctive CHD-W band.

**Keywords:** CHD, CHD-W, CHD-Z, W chromosome, sex identification

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### Introduction

DNA should provide a versatile way to discriminate male and female birds. Unfortunately, the selection of a suitable sex-linked marker has proved difficult. The obvious source is the W sex chromosome as this occurs in the female (ZW) and not the male (ZZ). But, similar to the human Y chromosome, it is small and offers a disproportionate amount of junk DNA (Stefos & Arrighi 1971). Such sequences evolve rapidly, even between closely related species; therefore they provide sex-linked markers of a limited range (Lessells & Mateman 1998). An improved basis for a DNA sexing technique is to use a gene. Because this is a conserved coding region the test will encompass many more species.

The first and only avian W chromosome gene that has been discovered is the chromobox-helicase-DNA-binding gene (CHD-W; Griffiths & Tiwari 1995). This gene is remarkably conserved and it has been shown that a single set of PCR primers can be used to sex birds throughout the class Aves, with the exception of ratites (Griffiths & Tiwari 1996; Griffiths *et al.* 1996). These primers simultaneously amplify homologous parts of CHD-W and the related gene CHD-Z

(CHD-Z was referred to as CHD-NW but is actually Z linked (Griffiths & Korn 1997)). Because CHD-Z occurs in both sexes it should always be amplified and this ensures that the PCR reaction has worked. Unfortunately, the two CHD products were of the same size; therefore Griffiths *et al.* (1996) used a restriction enzyme to selectively cut a fragment from the CHD-Z version before gel electrophoresis. Females, therefore, had two bands and males one.

In this study we describe a new test based on the two CHD genes. It does not require the use of a restriction enzyme to separate the PCR products and is therefore quicker, less expensive and simpler. The test employs two PCR primers which anneal to conserved exonic regions but then amplify across an intron in both CHD-W and CHD-Z. Because these introns are noncoding they are less conserved and their lengths usually differ between the genes. As a result, the PCR products vary in size from the start. Therefore gel electrophoresis immediately reveals one band in the male and two in the female. We then proceeded to test the method successfully on a wide variety of birds.

### Materials and methods

The two CHD genes in domestic chickens are  $\approx 5000$  bp in size (Griffiths & Korn 1997). The P2 and P3 primers

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described by Griffiths & Tiwari (1995) provided a basis from which to search for introns within these genes. Sequencing revealed that the P2 primer was positioned 132 bp downstream of an intron. A sequence alignment of the mouse CHD1 gene (Delmas *et al.* 1993) and the chicken CHD-Z gene (Griffiths & Korn 1997) was used to design a new forward primer (P8) which, in conjunction with the P2 primer, would amplify a region of the CHD genes which included the intron. The forward primer (P14, 5'-ACTTTTCCAATATGGATGAAGA-3') and the reverse primer (P9, 5'-TAAGGTCTGTCTCAGAYTTRTCNAC-3' R = A/G, Y = T/C, N = A/T/C/G) were designed externally to P8 and P2 (P14 > P8 > intron P3 > P2 < P9 where the arrow heads denote their 5' to 3' direction using the PRIMER program (Lincoln *et al.* 1991)). They were used to check the target sequences of the P8 and P2 sexing primers in zebra finch and chicken RT-PCR cDNA libraries or genomic DNA. All necessary precautions and controls were implemented to prevent PCR contamination (Newton & Graham 1994). Sequencing was carried out using 50–100 ng of template DNA by the Molecular Biology Support Unit, University of Glasgow using an ABI model 373A automated sequencer.

The sex identification test employs just the P8 (5'-CTC-CCAAGGATGAGRAAYTG-3') and P2 (5'-TCTGCATC-GCTAAATCCTTT-3') primers and PCR amplification was carried out in a total volume of 10 µL. The final reaction conditions were as follows: 50 mM KCl; 10 mM Tris-HCl pH 9 (25 °C); 1.5 mM MgCl<sub>2</sub>; 0.1% Triton X-100; 200 µM of each dNTP; 100 ng of each primer and 0.15 units of *Taq* polymerase (Promega). Between 50 and 250 ng of genomic DNA was used as template. PCR was performed in a Genius or Progene thermal cycler (Techne). An initial denaturing step at 94 °C for 1 min 30 s was followed by 30 cycles of 48 °C for 45 s, 72 °C for 45 s and 94 °C for 30 s. A final run of 48 °C for 1 min and 72 °C for 5 min completed the program. Most problems associated with poor amplification were solved by a 1–3 °C increase or decrease of the annealing temperature. PCR products were separated by electrophoresis for 45–60 mins at 7–10 V/cm in a 3% agarose gel stained with ethidium bromide. For the purposes of speed or for species in which there was found to be only a small difference in intron size between the two CHD gene PCR products, an 8% denaturing acrylamide gel (silver stained on completion (Promega)) was used (Double & Olsen 1997).

To test the applicability of the P2 and P8 sexing method on other bird species, DNA samples used were taken from six or more ( $N \geq 6$ ,  $P < 0.016$ ) known-sex individuals obtained from a diverse range of avian taxa (see Table 1.). The 28 test species were from 23 families, and include 11 of the 23 avian orders. Several species

were included from the two largest orders, the Ciconiiformes and Passeriformes because they contain 70% of the total bird species.

## Results and Discussion

The P2 and P8 sexing method was successful for 27 of the 28 bird species from across the class Aves on which it was tested (Table 1). Thirteen of these were used to demonstrate the technique and Fig. 1 shows their analysis on a simple 3% agarose gel. In 11 species the males have a single band but the females have two; both are readily discernible. In most cases the female CHD-W gene yields the larger product but in the rock pigeon and the European bee-eater the reverse is true. Product size also differs between species; for example, the two bands in the kestrel are larger than the two in the jackdaw. This variation provides an advantage, as it provides an extra guard against cross-species contamination (also see Newton & Graham 1994). This is important as the human CHD-1 gene can be such a contaminant (R. Griffiths, unpublished observation).

To investigate the reason for the success of the test, sequence data were collected from the chicken and the zebra finch, species which represent the two avian infra-classes (Fig. 2). These were compared to the mouse CHD-1 sequence (Delmas *et al.* 1993) and they showed that the important 3' regions of P2 and P8 match the targets exactly. Due to the taxonomic distance between the two birds and the mouse, this demonstrates the conservation of the CHD genes and provides evidence for the idea that P2 and P8 will amplify the CHD genes in all birds. The sequence data also included information on the intron that falls between the two primers. This showed poor conservation but provided the length differences between CHD-W and CHD-Z that made the sexing test successful.

The ostrich and the owl were not easily sexed by agarose gel analysis (Fig. 1). The former is a ratite which has W and Z chromosomes that are morphologically similar to autosomal chromosomes and show little divergence in either size or banding patterns (Ansari *et al.* 1988; Tagaki *et al.* 1972). If the CHD gene has remained on the sex chromosome in the ostrich, which is probable, then there will be two alleles of a single gene which are unlikely to diverge to form CHD-Z and CHD-W in the near future.

The tawny owl could not be sexed, for a different reason. The PCR did produce CHD-W and CHD-Z bands but the introns were so similar in size that they could not be distinguished on a 3% agarose gel. One solution is to use an 8% denaturing acrylamide gel whose resolution is easily sufficient to discriminate the two products (Fig. 3). Other solutions are the use of single-strand conformation polymorphism (SSCP) analysis (Ellegren 1996) or to differentially cut the PCR products with enzymes such as

Order	Family	Species
Struthioniformes	Struthionidae	ostrich ( <i>Struthio camelus</i> )*
Galliformes	Phasianidae	domestic chicken ( <i>Gallus domesticus</i> )
Anseriformes	Anatidae	mute swan ( <i>Cygnus olor</i> )
Coraciiformes	Alcedinidae	laughing kookaburra ( <i>Dacelo novaeguineae</i> )
	Meropidae	European bee-eater ( <i>Merops apiaster</i> )
Psittaciformes	Psittacidae	Spix's macaw ( <i>Cyanopsitta spixii</i> )
		crimson rosella ( <i>Platycercus elegans</i> )
		glossy black-cockatoo ( <i>Calyptorhynchus lathami</i> )
Apodiformes	Apodidae	swift ( <i>Apus apus</i> )†
Strigiformes	Strigidae	tawny owl ( <i>Strix aluco</i> )†
		morepork ( <i>Ninox novaeseelandiae</i> )†
Columbiformes	Columbidae	rock pigeon ( <i>Columba livia</i> )
Gruiformes	Otididae	houbara bustard ( <i>Chlamydotis undulata</i> )
Ciconiiformes	Burhinidae	bush stone-curlew ( <i>Burhinus grallarius</i> )
	Laridae	lesser black-backed gull ( <i>Larus fuscus</i> )
	Alcidae	black guillemot ( <i>Cepphus grylle</i> )
	Accipitridae	marsh harrier ( <i>Circus aeruginosus</i> )
	Falconidae	kestrel ( <i>Falco tinnunculus</i> )
Passeriformes	Maluridae	superb fairy-wren ( <i>Malurus cyaneus</i> )
	Pardalotidae	white-browed scrubwren ( <i>Sericornis frontalis</i> )
		brown thornbill ( <i>Acanthiza pusilla</i> )
	Sylviidae	african marsh warbler ( <i>Acrocephalus baeticatus</i> )
	Corvidae	white-winged chough ( <i>Corcorax melanorhamphos</i> )
	Callaeatidae	North Island kokako ( <i>Callaeas cinerea</i> )
	Sturnidae	starling ( <i>Sturnus vulgaris</i> )
	Paridae	blue tit ( <i>Parus caeruleus</i> )
	Passeridae	zebra finch ( <i>Taeniopygia guttata</i> )

\*Cannot be sexed.

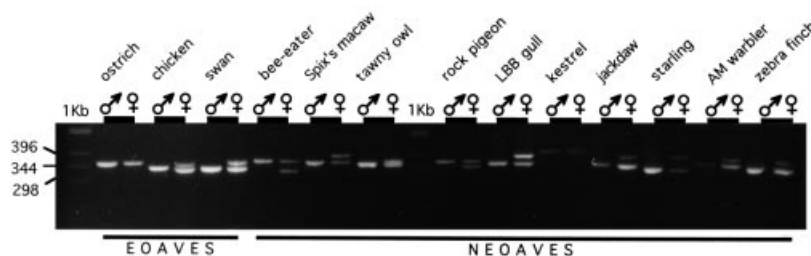
†Sexed using an acrylamide gel.

**Table 1** The P2 and P8 sexing technique will sex 27 of these 28 species that are sampled from across the class Aves. Classification is based on Sibley *et al.* (1988)

*HaeIII* or *MaeII* to allow their separation on an agarose gel (Griffiths *et al.* 1996).

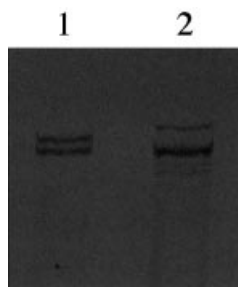
Besides increasing resolution, polyacrylamide gel electrophoresis can also increase the number of samples run per gel as batches can be run sequentially before silver staining. In addition, this helps to overcome the occasional, species-specific problem of primer competi-

tion. The competition occurs because the primers may match one CHD gene slightly less well than the other (see Fig. 2). This results in differential amplification such that one band is less bright than the other on an agarose gel. This fault is usually obvious and can be resolved by lowering the PCR annealing temperature to reduce the primer competition. If this does not solve the problem,



**Fig. 1** DNA sex identification using PCR with a single set of primers P2 and P8. The known sex of each individual is indicated; those birds with two bands are females and those with one band are males. The species name is indicated above the figure, where LBB gull indicates lesser black-backed gull and AM warbler indicates African marsh warbler. 1Kb is a size marker (BRL) providing the base-pair sizes that are indicated to the left of the figure.

Mouse	CHD1	CTCCCGAGAA TGAGAAACTG TGCAAAGCAG N(?) ATAAGTTTCA ATGGAAGTGA AGGGAGGCGG
Chicken	CHD-W	..T..A.....A... N(178) ..C..C..T. ....A... ..AT.C
Chicken	CHD-Z	.....G.....A... N(161) ..C..C..T. ....G.....A..A..C
Zebra Finch	CHD-W	T.G..A..G.....A... N(207) ..C..C..T. ....AA.AC
Zebra Finch	CHD-Z	..T..A.....A... N(171) ..T..C.....C.....A..C
Primer P8		CTCCCAAGGA TGAGRAAYTG
Mouse	CHD1	AGTAGAAGCA GGAGATATTC TGGATCTGAT AGTGATTCAA TCTCGAAAG GAAACGCGCG
Chicken	CHD-W	.....G.....A.....C.....A.....A.....A..A
Chicken	CHD-Z	.....G.....A.....C..C..A.A.....A.....A
Zebra Finch	CHD-W	.....G..G..A.....C..GG.....A.....A.....A..A
Zebra Finch	CHD-Z	.....G..T..A.....C.....C..C.....A.....A.....A..A
Mouse	CHD1	AAGAAACGTG GCGACCCCG CACTATCCCT CGGAGAATA TTAAAGGATT TAGTGATGCG GA
Chicken	CHD-W	..A.....A.....A.....T..C..T..A..C.....A..
Chicken	CHD-Z	..A..G.....AA.....T..A..C..T.....A..A.....A..
Zebra Finch	CHD-W	..A.....AA.....G..A.....T.....A..A.....C.....A..
Zebra Finch	CHD-Z	..A.....AA.....A..A.....T.....A..A.....A.....A..
Primer 2		TTTCCTAA ATCGCTACGT CT



**Fig. 3** The resolution of female P2/P8 PCR products on an 8% denaturing acrylamide gel. The first lane is a tawny owl where the two bands of a similar size have been separated, whilst the second is a chicken where both CHD-Z and CHD-W (upper) can still be identified although the former has 10× the concentration of the latter.

the sensitivity of the acrylamide technique often ensures that both CHD-W and CHD-Z bands remain easy to score (Fig. 3).

To conclude, the P2/P8 sexing test is robust and almost universal. It does not suffer extensively from variation in the concentration of the DNA sample and the single primer pair effectively reduces the chance of contaminating bands. Overall, it is an effective way to distinguish a male from a female bird.

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The sequences, including introns, are deposited in GenBank with Accession nos AF006659–62. We thank Kate Lessells, Simon

**Fig. 2** The figure shows the nucleotide sequence of a region of the CHD genes in the mouse (Delmas *et al.* 1993), chicken and zebra finch. The illustrated sequences include portions of two conserved exons whilst the poorly conserved intron (italic) has been removed but N (nucleotide) and a number indicate the size of this region. The P2 and P8 PCR primers are also shown. In the primer P8 the code R = A/G and Y = T/C. A full point indicates identity with the mouse sequence.

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Richard Griffiths, Kate Orr and Bob Dawson work in a new Molecular Evolution Unit at the University of Glasgow. At the moment we are concentrating on perfecting this DNA sexing technique but we are also using it in a practical sense for ecology. Michael Double, based in the Australian National University, is primarily investigating sexual selection in superb fairy-wrens. In collaborative studies he also uses the sexing technique described in this paper to investigate parental allocation, siblicide, sex allocation and population sex ratios.

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