

TECHNICAL ADVANCES

Heteroduplex molecules cause sexing errors in a standard molecular protocol for avian sexing

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Abstract

Molecular methods are a necessary tool for sexing monomorphic birds. These molecular approaches are usually reliable, but sexing protocols should be evaluated carefully because biochemical interactions may lead to errors. We optimized laboratory protocols for genetic sexing of a monomorphic shorebird, the upland sandpiper (*Bartramia longicauda*), using two independent sets of primers, P2/P8 and 2550F/2718R, to amplify regions of the sex-linked *CHD-Z* and *CHD-W* genes. We discovered polymorphisms in the region of the *CHD-Z* intron amplified by the primers P2/P8 which caused four males to be misidentified as females ($n = 90$ mated pairs). We cloned and sequenced one *CHD-W* allele (370 bp) and three *CHD-Z* alleles in our population: Z° (335 bp), Z' (331 bp) and Z'' (330 bp). Normal ($Z^\circ Z^\circ$) males showed one band in agarose gel analysis and were easily differentiated from females ($Z^\circ W$), which showed two bands. However, males heterozygous for *CHD-Z* alleles ($Z'Z''$) unexpectedly showed two bands in a pattern similar to females. While the Z' and Z'' fragments contained only short deletions, they annealed together during the polymerase chain reaction (PCR) process and formed heteroduplex molecules that were similar in size to the *W* fragment. Errors previously reported for molecular sex-assignment have usually been due to allelic dropout, causing females to be misidentified as males. Here, we report evidence that events in PCRs can lead to the opposite error, with males misidentified as females. We recommend use of multiple primer sets and large samples of known-sex birds for validation when designing protocols for molecular sex analysis.

Keywords: 2550F/2718R, *Bartramia longicauda*, *CHD-Z*, molecular sexing, P2/P8, upland sandpiper.

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Introduction

Reliable methods for determining the sex of birds in laboratory and field studies provide the essential demographic information that is required for many questions in ecology and evolutionary biology. In birds, the sex of neonates can rarely be determined during early development, and more than 50% of bird species are sexually monomorphic in body size and plumage as adults (Jensen *et al.* 2003). Molecular methods for sex identification are a useful tool and have been widely applied to studies of brood sex ratios (Andersson *et al.* 2003; Szekely *et al.* 2004), sex-specific life-history characteristics (Merila *et al.* 1997; Lopes *et al.* 2006; Remisiewicz & Wennerberg 2006), and captive breeding programmes (Griffiths & Tiwari 1995; Jarvi & Banko 2000).

At least three universal primer sets for avian molecular sexing have been developed, the most commonly used being P2/P8 (Griffiths *et al.* 1998), 1237L/1272H (Kahn *et al.* 1998), and 2550F/2718R (Fridolfsson & Ellegren 1999). All three methods are based on the co-amplification of homologous, sex-linked *CHD-Z* and *CHD-W* genes and generally work for all birds, except ratites (Ellegren 1996). Sex in birds is determined genetically by sex chromosomes, where males are the homogametic sex (*ZZ*) and females are the heterogametic sex (*ZW*). The P2/P8 and 1237L/1272H primers amplify an intron within the *CHD* gene, where size differences between the *Z* and *W* homologues can be used to determine the sex of male and female birds (Griffiths *et al.* 1998). The 2550F/2718R primers operate in a similar way, except they amplify a different *CHD* intron. Thus, after electrophoresis, homozygous males (*ZZ*) and heterozygous females (*ZW*) are identified by one or two bands, respectively. In the case of the P2/P8 primers, the homologues of *Z* and

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are sometimes similar sized fragments, and digestion with restriction enzymes specific to one of the homologues may be used to increase separation on a gel (Griffiths *et al.* 1998).

Despite greater assurance of accuracy of sex assignment when compared to morphological approaches, errors are still possible with molecular techniques. To date, molecular sexing errors detected in birds have been primarily due to allelic dropout, where females are mis-sexed as males (Arnold *et al.* 2003; Robertson & Gemmell 2006). A second possible source of error could be due to Z-chromosome polymorphisms. Z-chromosome polymorphisms have been reported with the use of the P2/P8 primers and occur in at least 18 species of birds, encompassing five families and three orders (Dawson *et al.* 2001; Lee *et al.* 2002; Dawson 2005). Sexing errors could occur if Z-polymorphisms were the result of large insertions or deletions, but known Z indels are generally small (4–40 bp, Dawson *et al.* 2001; Lee *et al.* 2002). Nevertheless, Z-polymorphisms might be a concern if mutations generate different alleles, if those alleles interact together during the polymerase chain reaction (PCR) process, and if interactions create heteroduplex molecules, which are double-stranded DNA molecules composed of two different alleles (Nagamine *et al.* 1989). Although never previously reported for birds, heteroduplex molecules could lead to errors in avian sexing by disrupting the normal gel-banding patterns for males and females.

The upland sandpiper (*Bartramia longicauda*) is a sexually monomorphic shorebird that breeds in the grasslands of North America and winters in the pampas of South America (Houston & Bowen 2001). This species is socially monogamous; both sexes take part in incubation and males provide uniparental care during brood-rearing. During a six-year study of upland sandpiper population biology, we used the P2/P8 protocol to determine the sex of adults in our population. Here, we report the first evidence for PCR-based sexing errors in birds due to the formation of heteroduplex DNA molecules in polymorphic males.

Materials and methods

Field methods

We sampled mated pairs of upland sandpipers at Konza Prairie Biological Station, Manhattan, Kansas, from 2001 to 2006. Adult sandpipers were captured at night with the use of spotlights and a long-handled net. We attached radio transmitters (PD-2, Holohil Systems) to each sandpiper, and tracked each radio-marked bird until we located its nest (Mong & Sandercock 2007). We used mist nets to capture the mate of each radio-marked bird at the nest site. At first capture, we banded each sandpiper with a numbered metal band, a unique combination of coloured plastic bands, and collected a 200 µL blood sample from the brachial wing vein. Blood was stored in Queen's Lysis

Buffer in a refrigerator until DNA extractions were completed (Seutin *et al.* 1991).

Molecular sexing

DNA was extracted using the DNeasy Extraction Kit (QIAGEN) and used in PCR with primers P2 and P8 to amplify introns from the *CHD-Z* and *CHD-W* genes, following the protocols of Griffiths *et al.* (1998). Each 20 µL reaction contained 4 µL of genomic DNA, 1.5 µM of each primer, and 1 U of *Taq* DNA Polymerase (Promega) in the manufacturer's buffer, including 1.75 mM MgCl₂ and 0.2 mM of each dNTP. PCR was performed in an Eppendorf Mastercycler ep gradient thermal cycler. In the reaction profile for the P2/P8 primers, an initial denaturing step at 94 °C for 2 min was followed by 45 cycles of 94 °C for 30 s, 48 °C for 45 s and 68 °C for 45 s, then a final step at 68 °C for 10 min. Agarose gels did not provide sufficient resolution to differentiate between the resulting *CHD-Z* and *W* fragments for upland sandpipers. Thus, we used a *CHD-Z* specific restriction enzyme, *HaeIII*, to selectively digest the *CHD-Z* fragments. PCR products were visualized on 3% agarose gels stained with GelStar (Lonza Group). If the P2/P8 primers gave questionable results, we used an independent set of primers to re-evaluate our molecular-based sex determination (2550F/2718R, Fridolfsson & Ellegren 1999). For the second set of primers, each 10 µL reaction contained 2 µL of genomic DNA, 2 µM of each primer, and 0.5 U of *Taq* DNA Polymerase in the manufacturer's buffer, including 1.75 mM MgCl₂ and 0.2 mM of each dNTP. The reaction profile used with the 2550F/2718R primers was 94 °C for 2 min, then 10 cycles of 94 °C for 30 s, 54 °C for 15 s and 72 °C for 30 s, followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s, and finally 72 °C for 5 min.

Cloning and sequencing

We amplified *CHD-Z* and *CHD-W* alleles using the P2/P8 primer pair for six individuals: two males and two females whose sex determination matched using both primer sets, and two unknown individuals who were sexed differently by the two independent primer sets. PCR products were sequenced directly from the first two males. Both females and two unknown individuals produced gels with lanes containing different-sized bands (i.e. heterozygotes). Thus, we isolated the PCR products by running them on a 3% agarose gel and by excising the resulting DNA fragments directly from the gel. The isolated fragments were purified using a QIAquick Gel Extraction Kit (QIAGEN) and cloned using XL-1 Blue Supercompetent Cells and pBluescript II Phagemid Vectors (Stratagene). We sequenced all DNA fragments with an ABI 3730 DNA Analyser, using Applied Biosystems BigDye chemistries for sequencing. Sequences were aligned using the program Sequencher

(Gene Codes) and deposited in GenBank (Accession nos. EU784665–EU784668).

Assessing formation of heteroduplex molecules

To test for the possible formation of heteroduplex DNA molecules, we amplified each *CHD-Z* allele variant from our clone library with a 20 μ L P2/P8 PCR. Using these PCR products, we created synthetic combinations of the *Z* alleles by mixing them in equal proportions of 5 μ L each. Each combination was produced in duplicate for further experimentation. One tube of each combination was left at room temperature as a control, while the second tube was subjected to five PCR temperature cycles to recreate the original P2/P8 thermal kinetics within the experimental samples. We then visualized the resulting products on a 3% high resolution 3:1 (low:high gelling temperature) agarose gel, formulated for separation of small DNA fragments (Sigma-Aldrich).

Results

Gel electrophoresis of P2/P8 products

We determined the sex of 90 mated pairs of upland sandpipers using the P2/P8 primer set, and discovered 86 male–female pairs and four putative female–female pairs. We reanalysed the eight birds in the four female–female pairs with an independent set of primers and found that the 2550F/2718R primers identified four of the putative females as males. Upon closer inspection of our PCR products at the P2/P8 locus, we found three distinct genotypes in our population of upland sandpipers, both before and after digestion with the restriction enzyme, *HaeIII* (Fig. 1). Without digestion with *HaeIII*, there was a thick single band for normal homozygous males ($Z^{\circ}Z^{\circ}$), two similar sized bands for females ($Z^{\circ}W$), and a different double-band pattern with a greater size difference for an individual who had conflicting sex identification (Fig. 1). Digestion with *HaeIII* reduced the size of both fragments in the normal male and the individual of unknown sex, but the bands had similar relative positions on the agarose gel. The digested products for the female were smaller at *Z* but not *W*, as expected (Fig. 1). We initially inferred that the individuals of unknown sex were males heterozygous for the *CHD-Z* intron, with an approximately 70 base pair (bp) insertion in one of the *CHD-Z* alleles, according to mobility of fragments on our agarose gels when compared to 100 bp size standard (Promega).

Cloning and sequencing P2/P8 products

The size of the PCR products based on sequence analysis did not agree with the size expected based on separation of

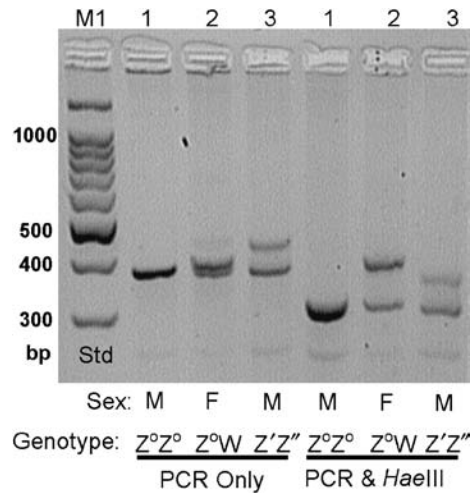


Fig. 1 Comparison of sexing results with P2/P8 primers under standard PCR conditions on high resolution agarose. In the original protocol, *HaeIII* was used with P2/P8 primers to increase differences in *CHD-Z* and *CHD-W* alleles. The difference between misdiagnosed males and females was negligible in low-resolution gels, leading to misidentification of some males as females (not shown). Sample 1 is a normal ($Z^{\circ}Z^{\circ}$) male (M), sample 2 is a normal ($Z^{\circ}W$) female (F), and sample 3 is a male whose sex assignment differed between the two primer sets. The left three lanes include the PCR products before digestion with *HaeIII*, whereas the right three lanes include the same samples after digestion with *HaeIII*. M1, 100 bp size standard (Promega).

the fragments on agarose gel. We cloned and sequenced one *W* allele (370 bp) from the two females we examined. From the normal males and unknown individuals, we cloned and sequenced three different *CHD-Z* alleles: Z° , Z' and Z'' . The Z° allele was 335 bp in length, and was found in the two females and the two males whose sex was determined correctly with both independent sets of primers. The sequences from the two unknown individuals contained both the Z' and Z'' alleles. Unexpectedly, neither fragment contained a large insertion. Instead, the Z' allele contained a 4-bp deletion with respect to Z° , whereas the Z'' allele contained a different 5-bp deletion (Table 1).

Determination of heteroduplex formation

To explain the discrepancy between the results of our gel mobility and sequence analyses, we visualized the three *CHD-Z* alleles independently and in synthetic mixtures on an agarose gel. We exposed mixtures to one of two conditions: room temperature or five temperature cycles of denaturing and re-annealing. When run independently and in synthetic mixtures at room temperature, all three *CHD-Z* alleles appeared to be approximately the same size (Fig. 2a). However, after mixing the alleles and then cycling synthetic mixtures through a PCR thermal regime, we

Table 1 Nucleotide sequence alignment of the three *CHD-Z* alleles. Nucleotide position is indicated relative to the 335 bp *CHD-Z*⁰ PCR product of the primers P2 and P8. Absent bases are represented by dashes. The restriction enzyme *HaeIII* cuts the *CHD-Z*⁰ fragment at restriction sites centred on positions 290 and 308.

Accession no.	Total fragment length	Allele												1	1	1	1	1	1	1	1	1	1	1
			4	5	6	7	8	9	0	1	2	3	...	4	4	4	4	5	5	5	5	5	5	6
EU784665	335	<i>CHD-Z</i> ⁰	G	T	T	T	G	T	T	G	G	G	...	A	A	A	C	T	T	T	A	C	T	T
EU784666	331	<i>CHD-Z</i> '	G	T	T	T	G	T	T	G	G	G	...	A	A	A	-	-	-	-	-	G	T	T
EU784667	330	<i>CHD-Z</i> ''	G	T	T	-	-	-	-	G	G	G	...	A	A	A	C	T	T	T	A	C	T	T

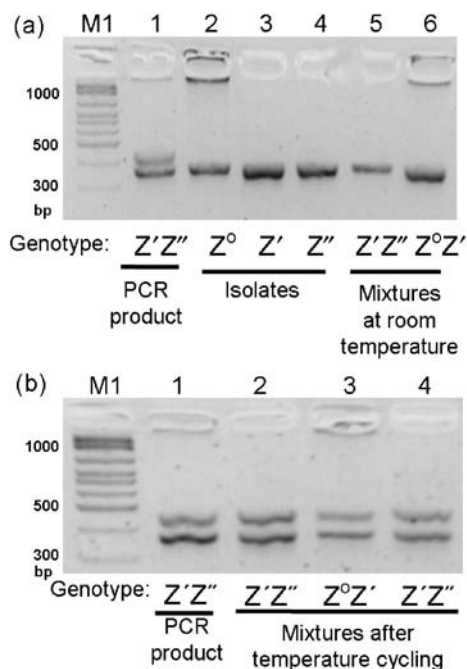


Fig. 2 Formation of heteroduplex molecules in synthetic mixtures of three *CHD-Z* alleles. (a) PCR product of a *Z'Z''* male (lane 1), isolates of the three *CHD-Z* alleles (lanes 2–4), and synthetic mixtures of the alleles after incubating them at room temperature (lanes 5–6); (b) PCR product of a *Z'Z''* male (lane 1), and synthetic mixtures of the alleles after treating them with five cycles of denaturing and annealing (lanes 2–4). M1, 100 bp size standard (Promega).

obtained the same double-band pattern that lead to sexing errors in *Z'Z''* males (Fig. 2b).

Discussion

PCR-based sexing errors are a concern for researchers using molecular methods to sex young or monomorphic species of birds. The most common direction of error has been females misidentified as males, which has been reported in eight species of birds (Robertson & Gemmell 2006). Here, we present the first report of avian molecular sexing errors where males can be misidentified as females

due to the formation of heteroduplex DNA molecules. This phenomenon was identified in upland sandpipers, but our results are potentially relevant to many species of birds due to the universality and widespread use of the P2/P8 primers.

The small differences in fragment size among the three *CHD-Z* alleles (4–5 bp) in upland sandpipers should not have caused sexing errors when compared to a *CHD-W* fragment that was 40 bp larger. However, we have characterized an interaction between different *Z* alleles which annealed to form heteroduplexes. When the hybrid molecules were run on agarose gels, their mobility on the gel appeared to be that of a much larger fragment, presumably because of looping of mismatched areas. Heteroduplex molecules have not been documented previously with primers for molecular sexing, but *Z*-chromosome polymorphisms have been reported in auklets and moorhens with the P2/P8 primers, and in red knots (*Calidris canutus*) with the 1237L/1272H primers (Baker & Piersma 1999; Dawson *et al.* 2001; Lee *et al.* 2002). Given the widespread occurrence of *CHD-Z* polymorphisms in birds (20 species), *Z*-polymorphisms may cause undetected sexing errors in other species. For example, Gunnarsson *et al.* (2006) found two unexplained errors of males mis-sexed as females in a study of black-tailed godwits (*Limosa limosa*), which the authors attributed to possible masculinization of genotypic females (Jacob & Mather 2004). Our results suggest that low-frequency *Z*-chromosome polymorphisms could be an alternative explanation. Errors due to heteroduplex formation would not occur if individuals were analysed through automated fragment analysis, because fragments are denatured before analysis, and small differences in fragment length (< 5 bp) are easily identified. Nevertheless, agarose gel analysis is the most widely used method for molecular sexing because the equipment is widely available and the cost is comparatively low.

The *Z*-chromosome polymorphisms reported here were in low frequency in upland sandpipers, but higher frequencies have been found in other species. In auklet species, 3 to 50% of individuals carried a polymorphic *CHD-Z* allele and 32% of common moorhens (*Gallinula chloropus*) males carried a *Z*-polymorphism (Dawson *et al.* 2001; Lee *et al.*

2002). To ensure continued reliability of molecular sexing methods, we make three recommendations for future studies. First, the low frequency polymorphisms we have reported will be difficult to detect if validation samples of known sex birds are small. We were only able to detect the sexing errors because we were working with mated pairs of sandpipers. To reduce uncertainty in molecular sexing protocols, it will be desirable to have large validation samples of known-sex birds based on morphology, behavioural observations, laparoscopy, or necropsy. Second, we advocate the use of independent primer sets as a first step in optimizing a molecular sexing protocol for untested species of birds. Last, if independent primer sets yield conflicting results, comparison of fragment mobility with and without restriction enzymes, and on agarose gels vs. a capillary sequencing system will be useful in testing for the presence of heteroduplex molecules.

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References

- Andersson M, Wallander J, Oring L *et al.* (2003) Adaptive seasonal trend in brood sex ratio: test in two sister species with contrasting breeding systems. *Journal of Evolutionary Biology*, **16**, 510–515.
- Arnold KE, Orr KJ, Griffiths R (2003) Primary sex ratios in birds: problems with molecular sex identification of undeveloped eggs. *Molecular Ecology*, **12**, 3451–3458.
- Baker AJ, Piersma T (1999) Molecular vs. phenotypic sexing in red knots. *Condor*, **101**, 887–893.
- Dawson DA (2005) SMGF bird sex typing database. Available at: <http://www.shef.ac.uk/misc/groups/molecol/smgfbirdsexing.html>. (Accessed on 28 February 2008).
- Dawson DA, Darby S, Hunter FM *et al.* (2001) A critique of avian CHD-based molecular sexing protocols illustrated by a Z-chromosome polymorphism detected in auklets. *Molecular Ecology Notes*, **1**, 201–204.
- Ellegren H (1996) First gene on the avian W chromosome (CHD) provides a tag for universal sexing of non-ratite birds. *Proceedings of the Royal Society B: Biological Sciences*, **263**, 1635–1641.
- Fridolfsson A-K, Ellegren H (1999) A simple and universal method for molecular sexing of non-ratite birds. *Journal of Avian Biology*, **30**, 116–121.
- Griffiths R, Double MC, Orr K, Dawson RJG (1998) A DNA test to sex most birds. *Molecular Ecology*, **7**, 1071–1075.
- Griffiths R, Tiwari B (1995) Sex of the last wild Spix's macaw. *Nature*, **375**, 454–454.
- Gunnarsson TG, Gill JA, Goodacre SL *et al.* (2006) Sexing of black-tailed godwits *Limosa limosa islandica*: a comparison of behavioural, molecular, biometric and field-based techniques. *Bird Study*, **53**, 193–198.
- Houston CS, Bowen DE Jr (2001) Upland sandpiper (*Bartramia longicauda*) Account 580. In: *The Birds of North America* (eds Poole A, Gill F). The Academy of Natural Sciences, The American Ornithologists' Union, Washington D.C.
- Jacob J, Mather B (2004) Sex reversal in chickens. In: *Factsheet PS-53*. Institute of Food and Agricultural Services, University of Florida, Gainesville, Florida.
- Jarvi SI, Banko PC (2000) Application of a PCR-based approach to identify sex in Hawaiian honeycreepers (*Drepanidinae*). *Pacific Conservation Biology*, **6**, 14–17.
- Jensen T, Pernaesetti FM, Durrant B (2003) Conditions for rapid sex determination in 47 avian species by PCR of genomic DNA from blood, shell-membrane blood vessels, and feathers. *Zoo Biology*, **22**, 561–571.
- Kahn NW, St John J, Quinn TW (1998) Chromosome-specific intron size differences in the avian CHD gene provide an efficient method for sex identification in birds. *Auk*, **115**, 1074–1078.
- Lee PLM, Brain PF, Forman DW, Bradbury RB, Griffiths R (2002) Sex and death: *CHD1-Z* associated with high mortality in moorhens. *Evolution*, **56**, 2548–2553.
- Lopes RJ, Marques JC, Wennerberg L (2006) Migratory connectivity and temporal segregation of Dunlin (*Calidris alpina*) in Portugal: evidence from morphology, ringing recoveries and mtDNA. *Journal of Ornithology*, **147**, 385–394.
- Merila J, Sheldon BC, Ellegren H (1997) Antagonistic natural selection revealed by molecular sex identification of nestling collared flycatchers. *Molecular Ecology*, **6**, 1167–1175.
- Mong TW, Sandercock BK (2007) Optimizing radio retention and minimizing radio impacts in a field study of upland sandpipers. *Journal of Wildlife Management*, **71**, 971–980.
- Nagamine CM, Chan K, Lau YFC (1989) A PCR artifact — generation of heteroduplexes. *American Journal of Human Genetics*, **45**, 337–339.
- Remisiewicz M, Wennerberg L (2006) Differential migration strategies of the wood sandpiper (*Tringa glareola*) — genetic analyses reveal sex differences in morphology and spring migration phenology. *Ornis Fennica*, **83**, 1–10.
- Robertson BC, Gemmell NJ (2006) PCR-based sexing in conservation biology: wrong answers from an accurate methodology? *Conservation Genetics*, **7**, 267–271.
- Seutun G, White BN, Boag PT (1991) Preservation of avian blood and tissue samples for DNA analyses. *Canadian Journal of Zoology*, **69**, 82–90.
- Szekely T, Cuthill IC, Yezerinac S, Griffiths R, Kis J (2004) Brood sex ratio in the Kentish plover. *Behavioral Ecology*, **15**, 58–62.