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TECHNICAL NOTE

A critique of avian *CHD*-based molecular sexing protocols illustrated by a Z-chromosome polymorphism detected in auklets

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Abstract

The sexes of non-ratite birds can be determined routinely by PCR amplification of the *CHD-Z* and *CHD-W* genes. *CHD*-based molecular sexing of four species of auklets revealed the presence of a polymorphism in the Z chromosome. No deviation from a 1:1 sex ratio was observed among the chicks, though the analyses were of limited power. Polymorphism in the *CHD-Z* gene has not been reported previously in any bird, but if undetected it could lead to the incorrect assignment of sex. We discuss the potential difficulties caused by a polymorphism such as that identified in auklets and the merits of alternative *CHD*-based sexing protocols and primers.

Keywords: *Aethia*, *Alcidae*, auklet, *CHD* genes, *Laridae*, molecular sex determination

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In many birds the sexes are morphologically indistinguishable, even as adults. Sex determination using molecular methods has therefore proved to be a valuable tool in wildlife conservation and for studies of behaviour and sex allocation (e.g. Griffiths & Tiwari 1995; Double & Olsen 1997; Komdeur *et al*. 1997; Lens *et al*. 1998; Sheldon 1998).

Several straightforward molecular protocols are now available for sexing birds based on the simultaneous polymerase chain reaction (PCR) amplification of the sex-linked *CHD-W* and *CHD-Z* genes (Griffiths & Tiwari 1995; see Lessells & Mateman 1996). The most recent methods exploit the difference in length between introns in the *CHD-Z* and *CHD-W* genes (Griffiths *et al*. 1998; Kahn *et al*. 1998; Fridolfsson & Ellegren 1999). We discuss the merits of two such protocols, the Griffiths *et al*. (1998; P2/P8) vs. the Fridolfsson & Ellegren (1999; 2550F/2718R) primers for the molecular sexing of four auklet species.

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Auklets are small seabirds of the North Pacific and Bering Sea. They produce one chick per breeding season and, although socially monogamous (Gaston & Jones 1998), have been recorded having extra-pair copulations (Hunter & Jones 1999). All but the crested auklet (*Aethia cristatella*) are sexually monomorphic. The auklets were sexed to identify sex differences in ornament expression, determine the sex ratio and as part of a study into sperm competition and paternity protection.

Blood was taken from adult auklets whilst brooding and from chicks when they were 30–35 days old. DNA was extracted from blood following Bruford *et al*. (1998). Each 10-μL reaction contained about 50 ng of genomic DNA, 1 μM of each primer and 0.25 units *Taq* DNA polymerase (Thermoprime^{Plus}, Advanced Biotechnologies) in the manufacturer's buffer [final concentrations 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl pH 9.0, 0.01% (w/v) Tween], including 2.0 mM $MgCl_2$ and 0.2 mM of each dNTP. PCR amplification was performed in a Hybaid Touchdown thermal cycler. The reaction profile used with the P2/P8 primers was 94 °C for 2 min, then 40 cycles of 94 °C for 15 s, 50 °C for 20 s, 72 °C for 25 s, followed by 72 °C for 1 min, and for the 2550F/2718R primers was 10 cycles of 94 °C for 30 s, 60–51 °C for 30 s (reducing by 1 °C each cycle), 72 °C for 30 s, followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s,

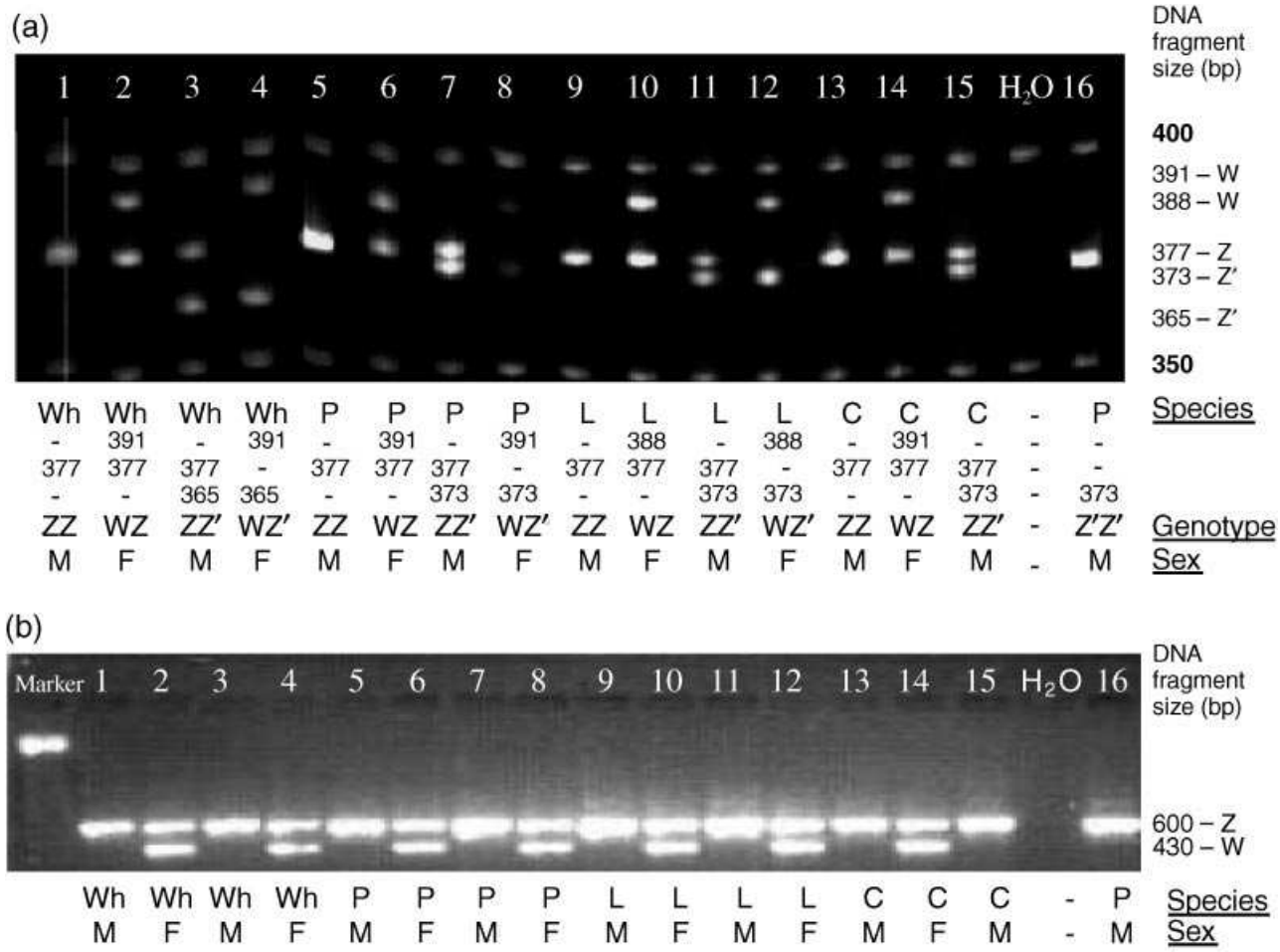

Fig. 1 Sex typing in four species of auklet: whiskered (Wh), parakeet (P), least (L) and crested (C). (a) ABI 377 sequencer gel image using the Griffiths *et al*. (1998) P2/P8 primers; (b) 2% agarose gel image using the Fridolfsson & Ellegren (1999) 2550F/2718R primers.

72 °C for 30 s. The PCR products were visualized either on 2% agarose gels stained with ethidium bromide, or on 5–6% polyacrylamide gels stained with silver (Promega) or analysed on an ABI 377 DNA sequencer.

We sexed a total of 768 auklets [351 whiskered auklets (*Aethia pygmaea*), 228 least auklets (*A. pusilla*), 151 crested auklets (*A. cristatella*) and 38 parakeet auklets (*Cyclorrhynchus psittacula*)] using the P2/P8 primers. Adult crested auklets are sexually dimorphic so we were able to use the adult birds of known sex as controls. For the other three sexually monomorphic species, the majority of the birds sexed were known pairs of breeding partners, so that a male and female were expected in each pair. The auklets could not be sexed on agarose gels due to the small size difference between the amplified products. When visualized on acrylamide gels, four or five different band patterns were observed, depending on the auklet species involved (Fig. 1). We concluded that the two different-sized fragments observed in males when using the P2/P8 primers were the result of a polymorphism in the Z chromosome. All inferred sexes agreed with those expected ($n = 92$ adult crested auklets; 102, 72 and 12 pairs of whiskered, least and parakeet auklets, respectively).

The size difference in the different PCR products obtained from the Z chromosome (Z and Z′) in whiskered auklets was similar (12 bp) to that observed in females (ZW; 14 bp) (Fig. 1). Therefore the actual sizes of the PCR products and not just the patterns (one vs. two bands) had to be carefully checked to ensure the correct assignment of sex. The frequency of genotypes containing the smaller, Z′, allele product ranged from 3.4% in whiskered auklets to 50.0% in parakeet auklets (Table 1). The frequencies of the three male genotypes were in each case consistent with Hardy–Weinberg expectations ($P > 0.05$). There was no difference in the frequency of the two alternative alleles in the two sexes in any of the species. The sex ratio of the adult birds was biased towards 1:1 because virtually all the adult birds sexed were breeding pairs. There was no detectable deviation from a 1:1 sex ratio in the chicks ($P > 0.05$), though the sample sizes were small (Table 1).

Table 1 Frequencies of alternative sex chromosome genotypes detected using the P2 and P8 sexing primers (Griffiths *et al*. 1998) in four species of auklet

Species	Number of birds sexed (chicks)	Genotype frequency					Chromosome frequency	
		Males			Females			
		ZZ	Z Z′	Z′ Z′	ZW	Z′W	Z	Z′
Whiskered auklet	351	161	5	0	178	7	0.977	0.023
(*Aethia pygmaea*)	(134)	(0.459)	(0.014)		(0.507)	(0.020)		
Least auklet	228	103	10	0	112	3	0.962	0.038
(*Aethia pusilla*)	(71)	(0.452)	(0.044)		(0.491)	(0.013)		
Crested auklet	151	78	2	0	71	0	0.991	0.009
(*Aethia cristatella*)	(59)	(0.517)	(0.013)		(0.470)			
Parakeet auklet	38	10	9	1	9	9	0.655	0.345
(*Cyclorrhynchus psittacula*)	(13)	(0.263)	(0.237)	(0.026)	(0.237)	(0.237)		

The intron amplified by the P2/P8 primers is the probable location of the detected length polymorphism, but this has not been confirmed. The 2550F/2718R primers amplify a different intron. We therefore also typed individuals that displayed the full range of different allelic patterns observed with the P2/P8 primers from all four species (totalling 16) using the 2550F/2718R primers, along with 32 random whiskered auklets. The 2550F/2718R primers amplified only two alternative PCR fragments, differing in size by 230 bp (which could be easily resolved on 2% agarose gels). All assigned sexes were consistent between the alternative primers (Fig. 1).

This polymorphism is not a unique example. In the Indian peafowl (*Pavo cristatus*), the P2/P8 primers again amplified four alternative band patterns that could only be resolved on 6% polyacrylamide gels, whereas the 2550F/2718R primers amplified the expected two-band pattern which could also be separated on 2% agarose gels (unpublished data). A second Z allele was also observed in six out of 804 great tit (*Parus major*) individuals (T. Treharne, personal communication) and in five out of 10 Eurasian treecreepers (*Certhia familiaris*) (S.C. Griffith, personal communication) when sexed with the P2/P8 primers.

In most non-passerine species tested (16 out of 18) and many passerine species (eight out of 24) we have found it necessary to use acrylamide gels to resolve the P2/P8 PCR products (unpublished data). The primers designed by Kahn *et al*. (1998; 1237 L/1272H) detect length variation over a region within that amplified by the P2/P8 primers (i.e. including the same intron), and are therefore likely to be subject to the same limitations. The larger size difference between the products amplified by the 2550F/2718R primers should normally make it feasible to use agarose gels as an alternative. However, the 2550F/2718R primers have been tested in far fewer species than P2/P8 and the amplified region is larger, so could be prone to even more polymorphism. The large difference between the sizes of the Z and W products obtained using 2550F/2718R makes it less likely that any polymorphism will lead to scoring error.

One advantage of the P2/P8 primers is that they have been shown to amplify the target regions in a large number of non-ratites and, despite scoring difficulties in some species, sex could be allocated in all cases. The latter include the Eurasian blackbird (*Turdus merula*) (unpublished data) and three other passerines (Fridolfsson & Ellegren 1999), in which the 2550F/2718R primers amplified a single identical product in both sexes.

The P2/P8 primers have the possible disadvantage that the PCR product from the Z allele is smaller than that from the W, and so it may be amplified preferentially, as observed in the Adélie penguin, *Pygoscelis adeliae* (unpublished data). This can lead to the females being mistyped as males, especially when the amplification is weak. In contrast, the design of the 2550F/2718R primers is such that the amplified W fragment is the smaller one, so avoiding this potential problem.

We have highlighted the potential difficulties in *CHD*-based molecular sexing caused by the presence of polymorphism such as that identified in auklets. For the confident assignment of sex in non-ratite birds we conclude that both the Griffiths *et al*. (1998; P2/P8) and Fridolfsson & Ellegren (1999; 2550F/2718R) sexing primers should initially be tested in order to identify the primers best suited to the study species.

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