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New primers for molecular sex identification of waders

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Determining the sex of individuals through molecular methods is an increasingly important research tool, particularly for studies of sexually monomorphic species. However, currently available primer sets to molecularly sex birds are known to cause difficulties in sexing Red Knots *Calidris canutus* and several other waders, due to amplification failure of, and polymorphisms on, the CHD-Z gene. To counter these problems, we developed a new primer set, 2602F/2669R, and show that these primers can be used to reliably sex Red Knots and eight other wader species in four genera. We expect these primers to be useful in a wide range of wader and possibly non-wader species.

Keywords

molecular sexing
Red Knot
primers 2602F & 2669R
CHD gene
shorebirds

INTRODUCTION

The ability to determine the sex of individuals is a key methodological step in both evolutionary ecology and conservation research. However, in many bird species the sexes overlap in morphological traits, or are morphologically indistinguishable (e.g. Price & Birch 1996, Griffiths *et al.* 1998, Piersma & van der Velde 2009). Consequently, phenotypic traits have only limited usefulness to reliably sex individuals in birds (e.g. Baker *et al.* 1999). This is especially true for waders, in which less than half of the species can be sexed using morphology (dos Remedios *et al.* 2010). Fortunately, molecular DNA techniques have been developed to tackle this problem (e.g. Griffiths *et al.* 1998, Fridolfsson & Ellegren 1999). Using these relatively quick and inexpensive methods, individual birds can now be sexed using DNA extracted from a small drop of blood or a single feather. See Box 1 and dos Remedios *et al.* (2010) for more detailed information on PCR-based molecular sexing in birds.

There are, however, some potential problems with molecular sexing using the method of Griffiths *et al.* (1998). Both allelic drop-out of the W-allele of the Chromo-Helicase-DNA binding protein gene (CHD), and polymorphism on the CHD-Z locus might lead to false assignments of the male and female sex, respectively (Dawson *et al.* 2001,

Lee *et al.* 2002, Dawson *et al.* 2016; see also Box 1 for pitfalls in molecular sexing). These and other factors have also been attributed to causing difficulties in sexing waders (Schroeder *et al.* 2008, Casey *et al.* 2009, Trimbos *et al.* 2013). In addition, when using the Griffiths *et al.* (1998) method in waders, the difference in PCR product size between CHD-Z and CHD-W is observed to be small for several species (Black-tailed Godwit *Limosa limosa*: Schroeder *et al.* 2008; Upland Sandpiper *Bartramia longicauda*: Casey *et al.* 2009; Snowy Plover *Charadrius nivosus*: Conway *et al.* 2004; Kentish Plover *Charadrius alexandrinus*: Küpper *et al.* 2009; Eurasian Golden-Plover *Pluvialis apricaria*: Sheffield Molecular Genetics Facility bird sex-typing database: <http://www.shelf.ac.uk/nbaf-s/databases/birdsexing>). As it requires toxic polyacrylamide gels, expensive DNA sequencers, or additional restriction enzyme digestion steps to separate such similar sized Z- and W-alleles, this method is less attractive to use.

For waders, therefore, the method of Fridolfsson & Ellegren (1999) appears to be a good alternative to molecularly sex individuals. In this method, the PCR product from CHD-W is smaller than from CHD-Z, making it less sensitive to W-allele dropout, and polymorphisms on the CHD-Z locus have not been reported. Moreover, there is a relative large size difference in PCR product between CHD-W

and CHD-Z, allowing simple analysis on 2% agarose gels. With the Fridolfsson & Ellegren method, however, we also encountered problems sexing a wader species, the Red Knot *Calidris canutus*. Using this method in knots, only the W-allele reliably amplifies, giving a single band on agarose gels for females and no bands for males (O. Haddrath unpubl. data; Fig. 2 top right). The Z-allele amplified only in a very small subset of individuals. This is an undesirable situation, as samples that technically failed could not be distinguished from males. To prevent such misidentification of sex, dos Remedios *et al.* (2010) recommend to use two different sexing markers.

Since Red Knots are a main study subject of our respective research groups, we decided to develop a new primer set based on the same gene region described in Fridolfsson

& Ellegren (1999). In doing so, we aimed to keep the advantageous traits of the Fridolfsson & Ellegren methodology, whilst solving the problem of non-amplification of the Z-allele in Red Knots. In this paper we describe the development of these new primers to molecularly sex Red Knots and their application in other wader species.

METHODS

DNA extraction

DNA was extracted from tissue or whole blood stored in 95% alcohol by standard phenol-chloroform extraction methods or the ammonium acetate method described in Richardson *et al.* (2001).

Box 1. PCR-based molecular sexing in birds

Molecular sexing of birds is most commonly based on PCR amplification of an intron (non-coding DNA region) in the Chromo-Helicase-DNA binding protein (CHD) gene located on the sex chromosomes (denoted by Z and W in birds). The CHD gene has several introns, and some molecular sexing methods differ in the specific intron targeted in PCR (e.g. Griffiths *et al.* 1998, Kahn *et al.* 1998 vs. Fridolfsson & Ellegren 1999, this study). For all these methods, the length of the intron differs between the gene copies on the Z- (CHD-Z) and W-chromosomes (CHD-W). For schematic representation see Fig. 1. PCR amplification of this intron in males (having two identical sex chromosomes: ZZ) will result in two PCR products of the same size, whereas in females (having two different sex chromosomes: ZW) two PCR products of different size will be produced. Visualisation of the PCR products after electrophoresis on an agarose gel will typically show a single band in males and two bands in females. There are two main pitfalls with PCR-based molecular sexing. One is called W-allele dropout, meaning that the CHD-W copy of the intron is not amplified. This can be due to mutation(s) in the primer binding site on CHD-W or preferential amplification of CHD-Z over CHD-W. In such cases, only the Z-allele will be amplified in these females, making them indistinguishable from males. A second pitfall is variation in intron length of CHD-Z within a species (known as polymorphism). In species with polymorphism on CHD-Z, some males will have two CHD-Z introns of differing lengths. PCR-based molecular sexing of these males will result in two PCR products of different sizes. PCR products of such males will thus appear as two bands when separated by agarose gel electrophoresis, and consequently may be falsely scored as females (see Lee *et al.* 2002). Nowadays, automated sequencers are often used to separate PCR products for molecular sexing, and then the above mentioned pitfalls may be less of a problem.

Fig. 1. Schematic representation of part of the CHD gene on avian W- and Z-chromosomes. Grey boxes are exons (gene coding regions), lines are introns (non-coding regions) and arrows indicate priming sites (forward or reverse direction) in PCR-based molecular sexing. Here, as for the primers in our study (2602F and 2669R), the CHD-Z intron is larger than that of CHD-W, but depending on the species, specific intron amplified, and method used, this can be the other way around.

PCR and electrophoresis

PCR reactions were carried out in 10 µl volumes, containing 0.1–0.2U Taq polymerase (Roche Diagnostics or Life Technologies), 1x Buffer (10mM TrisHCl, pH 8.3), 2.5–3.0 mM MgCl₂, 0.2 mM dNTPs, 0.5 µM of each primer, and 1–2 µl DNA template. For the PCR program of primers 2550F/2718R see Fridolfsson & Ellegren (1999). This PCR program was also used for the new primer set 2602F/2669R. Additionally, for primers 2602F/2669R the following PCR program was also used: initial denaturation for 1 min at 94°C was followed by 35 cycles of 30 sec at 94°C, 60 sec at 60–62°C, 60 sec at 72°C, with a final extension for 2 min at 72°C. PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining and UV-transillumination. Different PCR programs used and ranges in variables mentioned above are differences in protocols between labs and species analysed.

Primer development and design

To obtain sequence data from which to design new primers, the Z- and W-allele were amplified using primers 2550F/2718R (Fridolfsson & Ellegren 1999) in different bird species: a passerine, Rifleman *Acanthisitta chloris*; a gull species, the Silver gull *Chroicocephalus novaehollandiae*; and two wader species, Dunlin *Calidris alpina* and Red Knot (RK1). The Silver Gull and Rifleman were both males and consequently only the Z-allele was recovered. The Dunlin and Red Knot were both females, but only the W-allele was recovered as the Z-allele did not amplify in these individuals using these primers. As the Z-allele was observed to amplify in a small proportion of Red Knot samples, one Z-allele was also amplified and recovered in Red Knot (RK2). All PCR products were retrieved from a 2% agarose gel and purified using a QIAquick PCR Purification Kit (QIAGEN) before sequencing them on an

ABI PRISM 3100 Genetic Analyzer, using BigDye Terminator (Applied Biosystems) sequencing in both forward and reverse direction using primers 2550F/2718R. Sequences were aligned using MAFFT software package (Katoh *et al.* 2005) and new primers 2602F/2669R were designed using FastPCR software (Kalendar *et al.* 2009) (Fig. 3; primer 2602F: 5'CAGATGGTGAGGATGCTGGAC3' and 2669R: 5'CCCTTTTATTGATCCATCAAGYCTCTRAAGAG3').

Primer testing

The new primer set 2602F/2669R was tested for accurate sexing on 13 samples of Red Knots of known sex. Subsequently, it was tested in eight other wader species: Great Knot *Calidris tenuirostris*, Sanderling *C. alba*, Semipalmated Sandpiper *C. pusilla*, Black-tailed Godwit, Bar-tailed Godwit *Limosa lapponica*, Ruddy Turnstone *Arenaria interpres*, Eurasian Golden-Plover, and Black-bellied (Grey) Plover *Pluvialis squatarola* (Table 1). The sex of the tested samples was known through (a combination of) dissection, observed behaviour (mates in a breeding pair), and/or molecular sexing with P2/P8 (Griffiths *et al.* 1998) or 2550F/2718R (Fridolfsson & Ellegren 1999).

RESULTS & DISCUSSION

The new primer set 2602F/2669R worked well in Red Knots. It produced the expected bands of the theoretically predicted sizes: two (568 and 340 bp) in females and one (568 bp) in males (see Fig. 2 top left for a subset of tested individuals). The most probable cause for the failure of the Fridolfsson & Ellegren (1999) primers 2550F/2718R to consistently amplify the Z-allele in this species is thought to be a mutation in the priming site of 2550F, since the combination of primers 2550F and 2669R also failed to amplify the Z-allele, whereas the combination

Table 1. Assessment of the 2606F/2669R primers for sex-typing waders. *n* = sample size of known-sex males (♂) and females (♀). *T_a* = annealing temperature in PCR program (TD = touchdown annealing temperature as described in Fridolfsson & Ellegren 1999). Successful = number of correctly sexed males (♂) and females (♀).

Species	<i>n</i> ♂/♀	<i>T_a</i> (°C)	Successful ♂/♀
Red Knot <i>Calidris canutus</i>	5/8	TD	5/8
Great Knot <i>Calidris tenuirostris</i>	5/5	62	5/5
Sanderling <i>Calidris alba</i>	5/5	62	5/5
Semipalmated Sandpiper <i>Calidris pusilla</i>	9/8	TD	9/8
Black-tailed Godwit <i>Limosa limosa</i>	5/5	62	5/5 ¹
Bar-tailed Godwit <i>Limosa lapponica</i>	5/5	62	5/5
Ruddy Turnstone <i>Arenaria interpres</i>	3/3	TD	3/3
Eurasian Golden-Plover <i>Pluvialis apricaria</i>	4/4	60	4/4
Black-bellied Plover <i>Pluvialis squatarola</i>	7/3	TD	7/3

¹CHD-Z did not amplify in some females.

Fig. 2. Agarose gel (runtime 30 min at 120V) with PCR products amplified in three male (♂) and three female (♀) Red Knots with four different primer combinations 2602F/2669R (top left), 2550F/2718R (top right), 2602F/2718R (bottom left) and 2550F/2669R (bottom right). Note that the primer combinations 2550F/2718R and 2550F/2669R failed to amplify the Z-allele in both males and females. Hence, only the W-allele is visible on the right side of the gel. Control = negative control. For further explanation see *Results & Discussion*.

2602F and 2718R did amplify the Z-allele (Fig. 2). The latter combination of primers could therefore also be used to sex Red Knot, but we continued with 2602F/2669R as these produced a slightly shorter PCR product, which might be advantageous when processing samples of lower DNA quality.

The new primers also worked well for other wader species (Table 1). All but one species showed two bands in females and one in males. Black-tailed Godwits provided an exception. For one of the five tested females, the Z-allele was not amplified (but a larger-sized, probably non-specific, band was amplified) and only the W-allele was visible on agarose gels, whereas for most females both the Z- and W-allele were amplified (see Fig. 4 for a subset of tested individuals). It seems that there are two different Z-alleles in Black-tailed Godwits, one (Z) that can be amplified, and one (Z*) that cannot be amplified with the primers 2602F/2669R (i.e. no Z-allele band visible on the gel). Subsequent analyses of more Black-tailed Godwits showed that this variation observed with primers 2602F/2669R correlated well with the Z-allele length polymorphism observed with the Griffiths *et al.* (1998) P2/P8 primers in this species (Fig. 4; Schroeder *et al.* 2008, M. van der Velde unpubl. data). This suggests that Black-tailed Godwits have linked polymorphisms on the CHD-Z gene.

Fig. 3. Aligned DNA sequences using MAFFT v7.036b software of the CHD-Z and/or CHD-W allele for Riffliman *Acanthisitta chloris*, Dunlin *Calidris alpina*, Silver Gull *Chroicocephalus novaehollandiae*, and two Red Knots *Calidris canutus* designated RK1 and RK2. Primer sequences of 2602F and 2669R are highlighted grey above the aligned sequence. a, c, g, t = nucleotides, – = gap, * = monomorphic position.

In conclusion, as all wader species (even the aberrant Black-tailed Godwits) tested in this study could be reliably sexed, the primer set 2602F/2669R is a useful tool in shorebird studies. The primers have been used to sex hundreds of individuals in ongoing studies on knot species, godwit species and Sanderling. As far as we know there have been no reports of 2602F/2669R failing to sex a wader species. The new primer set might also be applicable in research on a much broader range of bird species, as it

Fig. 4. Agarose gel with PCR products amplified in two male (M) and three female (F) Black-tailed Godwits with primers 2602F/2669R. Note that in female F* the Z-allele was not amplified, but a larger-sized band was amplified. This band might be an aberrant Z-allele, but more likely a non-specific amplification, which to a lesser extent also occurs in the other individuals (hardly visible on the picture). The non-specific amplification might be increased in female F* because primers may fail to anneal to the target locus on the Z-chromosome in this individual. NC = negative control. For further explanation see *Results & Discussion*.

has also been used successfully in another non-passerine species (Swift *Apus apus*; M. van der Velde unpubl. data), as well as being a match for the passerine species sequenced here (Rifleman *Acanthisitta chloris*).

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