Sexing Eurasian Oystercatchers *Haematopus ostralegus* from breast feathers collected when ringing

HAZEL K. WATSON, REBECCA J. MOGG, JOANNA M. BOND & SARAH E.A. LE V. DIT DURELL*

*Centre for Ecology and Hydrology (CEH) Dorset, Winfrith Technology Centre, Dorchester, Dorset DT2 8ZD, UK. sld@ceh.ac.uk (*Corresponding author)*

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We describe the methods used to identify the sex of Eurasian Oystercatchers by analysis of DNA extracted from the quills of breast feathers collected when ringing. DNA was successfully extracted and amplified from feathers which had been stored in a domestic freezer for nearly 4 years. DNA analysis has allowed us to check the reliability of sexing oystercatchers by bill measurements, and to identify the sex of those birds we had been unable to sex by bill morphology.

INTRODUCTION

Long-term research into Eurasian Oystercatchers *Haematopus ostralegus* has revealed important differences between the sexes in feeding specialisation (Durell *et al.* 1993), resulting in differential distribution of the sexes on a local and a national scale (Durell & Goss-Custard 1996; Durell & Atkinson 2004). So far, this research has relied upon bill measurements to identify oystercatcher sexes (Durell *et al.* 1993). This method has certain drawbacks. First of all, there will always be differences between observers in taking measurements and a certain degree of observer error. Secondly, birds with intermediate discriminant scores cannot be assigned a sex by this method. Finally, even when birds with intermediate score are excluded, there still remains a 10% probability of birds being assigned the wrong sex (Durell *et al.* 1993).

DNA analysis is a more reliable method for identifying the sex of birds which are monomorphic in their plumage. Most species of bird can be sexed by DNA analysis using a test based on two conserved CHD (chromo-helicase-DNA-binding) genes located on the avian sex chromosomes (Griffiths *et al.* 1998). Unlike mammals, female birds are the heterogametic sex and have a Z and W chromosome, containing the CHD-Z and CHD-W genes respectively. Male birds are homozygous and therefore have two copies of the Z chromosome and only the CHD-Z gene. Sex specific DNA markers take advantage of size differences in the CHD-Z and CHD-W genes, and post PCR (polymerase chain reaction) amplification a single band is observed in males and two bands in females (Griffiths *et al.* 1998). Although this method is known to work in 27 different bird species, its utility in sexing oystercatchers has not, until now, been tested.

In order to carry out genetic testing, a biological sample must be obtained. The collection of blood or tissue samples is not always feasible when the birds are small, or sensitive to excessive human handling. In addition, the collection of blood or tissue samples often requires additional field equipment and specialist training. In contrast, it is usually possible to collect feather samples from birds during routine ringing operations, thus minimising handling time and stress to the birds. However, many researchers do not realise that feathers can now be used for routine genetic testing. In this paper we describe the methods used to sex oystercatchers by DNA analysis, using DNA extracted from breast feathers collected when ringing.

METHODS

Sample material

Three or four breast feathers were collected from 80 oystercatchers caught by cannon-netting at Dawlish Warren, Devon, in November 2000. These feathers were put in individually labelled polythene bags and kept in a domestic freezer until being processed.

Test feathers from birds of known sex were needed to confirm that DNA analysis worked satisfactorily with this species and to provide a standard with which to compare our results. These feathers were obtained from four corpses (two male, two female) which had been found on the Exe estuary between 1996 and 2000 and kept stored in a freezer. These corpses were sexed by gonad analysis.

DNA extraction

Genomic DNA was isolated from 3 feathers plucked from the mid breast of each bird. A small section from the proximal end of the quill was removed and cut into smaller pieces about 1–2 mm in length. These pieces were then placed into 1.2 ml racked microtubes arranged in a 96 well format. One tungsten carbide bead and 50µl of buffer (0.05M Tris, 0.01M EDTA, 0.1 M NaCl), 50µg/ml Proteinase K and 50µg/ml RNase were added to each microtube. The samples were disrupted using a MM300 mixer mill (Qiagen) and then centrifuged at 5000 rpm for 2 minutes. The samples were then incubated overnight at 37°C in 320µl of digestion Chelex

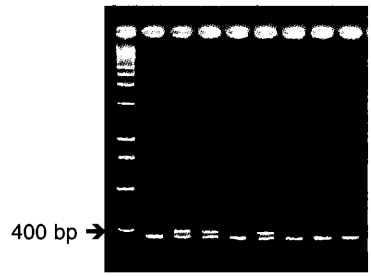

Fig. 1. Identification of oystercatcher sex using DNA extracted from feathers. Samples were separated on a 3% agarose gel. The first lane is a DNA size marker, with oystercatcher samples in lane 2 onwards. Individuals with two bands are females and those with a single band are males.

solution (5% Chelex, 0.05M Tris, 0.01M EDTA, 0.1M NaCl and 1% Triton) with continuous rotation. After overnight incubation the samples were centrifuged at 5000 rpm for 2 minutes and 200µl of the supernatant removed and transferred to a fresh microtube containing 260µl of storage Chelex solution (5% Chelex, 0.01M Tris and 0.001M EDTA). One microlitre of each extract was used as a template in the PCR reaction.

DNA amplification

PCR reactions were set up using the Qiagen Multiplex PCR Kit. This kit contains a master mix specially designed for multiplex PCR applications and contains pre-optimized concentrations of HotStarTaq DNA Polymerase, $MgCl_2$, and dNTPs. The sex identification test employs the P8 (5'-CTCCCAAGGATGAGRAAYTG-3') and P2 (5'-TCTGCATCGCTAAATCCTTT-3') primers (Griffiths *et al.* 1998). PCR amplification was carried out in a total volume of 10µl and contained 1µl of template DNA, 5µl of the Qiagen master mix, and 10 pmoles/µl of primers P2 and P8 respectively. After an initial 15 minute hot start at 94°C, PCR amplification was carried out for 35 cycles consisting of 40 seconds at 94°C, 1 minute 30 seconds at 55°C and 1 minute 30 seconds at 72°C, followed by a final extension of 10 minutes at 72°C. The PCR products (10µl) were electrophoresed on a 3% agarose gel at 80V for 4.5 hours and stained with ethidium bromide.

RESULTS

Using the birds of known sex we determined that males amplified a single band of approximately 380 bp. In contrast female birds amplified two bands 380 bp and 400 bp in size (Fig. 1). Out of 80 oystercatchers, 75 could be confidently assigned a sex following DNA analysis (Table 1). Occasionally, an individual would amplify only the W band. In these cases the PCR was repeated until the Z band was amplified, or the bird was not assigned a sex. Five birds could not be sexed due to failure of the PCR amplification.

When the results of the DNA analysis were compared with the sex assigned to each bird by morphology, it emerged that 9 birds (11%) had been assigned the wrong sex based on bill measurement. DNA analysis also allowed 10 birds (12.5%) to be sexed that could not be assigned a sex by morphology alone.

DISCUSSION

This is not the first time that DNA has been successfully extracted from feathers for genetic studies (Morin *et al.* 1994, Mundy *et al.* 1997), or the first time that DNA from feathers has been used to determine bird sex (Bello *et al.* 2001). However, this is the first time these methods have been tested on the oystercatcher. Additionally, we thought it would be useful for other shorebird researchers to know the techniques that we used. Many shorebirds are sexually monomorphic, particularly during the winter months, and unambiguous sexing is not always possible. Many shorebirds also have sex-related differences in diet and habitat use (Durell 2000) and it would be useful to investigate such differences using unambiguous sexing data.

Our data has shown that the universal primers designed by Griffiths *et al.* (1998) work in oystercatchers and we believe that this molecular sexing method will also work on other shorebirds. The methods used here are relatively easy to learn and can be carried out with basic and inexpensive laboratory equipment. Our study has also demonstrated the value of using feathers, versus blood and tissue samples, as a source of DNA for genetic studies. Collecting breast feathers when ringing birds is relatively non-invasive and rapid, reducing handling time and stress to the bird. Feathers are also very easy to collect and store. Contamination of the feathers in the field, for example with human DNA, is not a problem because this sexing method will only amplify bird DNA. In most cases, only 3 or 4 breast feathers were needed from each bird. Although very little DNA is present in the feathers, this is mitigated by the efficiency of the PCR process.

Previous work has shown that occasionally, when the DNA is at low concentrations, only one band out of the two present is amplified (Taberlet *et al.* 1996, Segelbacher 2002).

Table 1. Comparison of outcomes of oystercatcher sexing methods on a sample of 80 oystercatchers caught at Dawlish Warren, November 2000.

Sex by DNA analysis	Sex by morphology			
	Male	Female	Unknown	Totals
Male	41	5	5	51
Female	4	15	5	24
Unknown	1	2	2	5
Totals	46	22	12	80

When only the W band was amplified in our samples we repeated the PCR to verify the presence of the Z band. However, if only the Z band amplifies in a female the individual may be wrongly sexed as a male. Using DNA extracted from feathers the frequency of this type of error is reported to be around 1.1% (Segelbacher 2002). When carrying out molecular sexing, non-amplification of an allele will only be a problem when a female only amplifies the male specific Z band, therefore the actual error rate will be around 0.55%. It has been noted in Red Knot *Calidris canutus* that the Z allele varies in size (A. Baker pers. comm.), but since our single banded females usually amplified both alleles in later PCRs we believe this explanation is not as likely as the one presented above.

The failure rate during this study was only 6.25%, which is well within the expected failure rate for DNA testing using samples with low DNA content (Bello *et al.* 2001). The failure of this method in some individuals was probably because the quantity of DNA present in the feather quills was too low. However, it is worth noting that in subsequent oystercatcher sexing work all samples amplified successfully, although these feathers had been stored for a much shorter time (a few days rather than nearly 4 years) and by this time we had greater familiarity with the DNA extraction and amplification techniques.

DNA analysis has allowed us to check the reliability of sexing oystercatchers by bill measurements, and to identify the sex of those individuals we had been unable to sex by bill morphology. Understandably, as sexing by bill morphology was done with a 90% probability of being correct, 11% of birds caught in November 2000 had been wrongly assigned a sex (Table 1). Reassuringly, there did not appear to be a bias in the sex of birds that were wrongly assigned a sex by bill morphology, or in those that had not been assigned a sex at all. However in future, given the simplicity of the molecular sexing method, we will prefer to use DNA analysis to sex the birds we colour ring on the Exe estuary.

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