Sex Identification of Owls (Family Strigidae) Using Oligonucleotide Microarrays

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Molecular sexing of the diversified avian family Strigidae is difficult. Sex identification using the intron length difference between W and Z chromosomal CHD1 genes, as visualized by agarose gel electrophoreses, often produces ambiguous results. Here we describe a simple method for sexing a variety of Strigidae species using oligonucleotide microarrays, on which several sex-specific probes operated complementarily or in concert. The sex of 8 owl species was identified clearly on the microarrays through sequence recognition. This sequence-directed method can be easily applied to a wider range of Strigidae species.

DNA microarrays are miniaturized microsystems based on the ability of DNA to spontaneously find and bind to its complementary sequence by hybridization. Labeled DNA molecules in a sample are analyzed using DNA probes tethered at distinct sites on a solid support (Cuzin 2001; Vernet 2002). There are 2 main types of DNA microarrays: cDNA microarrays and oligonucleotide microarrays. The probe size of the former is usually between 500 and 2000 bp and that of the later is around 25 bp (Kim and Watkinson 2002). DNA microarrays have been referred to as “fishing expeditions” (Lockhart and Winzeler 2000; Maughan et al. 2001) that allow widespread application: gene expression, genotyping, infectious, and genetic disease diagnoses, etc (Petrik 2000; Heller 2002).

Strigidae are sexually monomorphic, making sex identification difficult via their appearance. Sex identification is especially important for their breeding and conservation as most of them are endangered species.

The intron length difference of the CHD1 (chromohelicase-DNA-binding protein 1) gene between the W and Z sexual chromosomes has made sexing in many nonratite birds possible (Griffiths et al. 1998; Jensen et al. 2003). Because female birds are ZW, whereas males are ZZ, polymerase chain reaction (PCR) products from males show a single band, whereas those from females show 2 bands on a running gel (Russello and Amato 2001). The CHD1 gene has several introns (Griffiths and Korn 1997). Two introns have been utilized extensively in different bird species, for example, the upstream intron flanked by primers 2550F/2718R (Fridolfsson and Ellegren 1999), and the downstream intron flanked by primers 1237L/1272H (Kahn et al. 1998) or P2/P8 (Griffiths et al. 1998). However, there are still a few species that cannot be identified because of genetic diversity. Some efforts were made, such as using restriction enzymes (Bermudez-Humaran et al. 2002) and designing new primers (Ito et al. 2003). The genetic diversity of Strigidae has also made sexing results doubtful. The length polymorphism of the 1237L/1272H-amplified products of some species was poor, making identification difficult on agarose gels (Kahn et al. 1998). Although the 2550F/2718R-amplified products carried better length polymorphism (Fridolfsson and Ellegren 1999), the sex of some Strigidae species could not be determined. None of these methods have been found generally applicable to all Strigidae members thus far.

This study describes sexing a variety of Strigidae species using oligonucleotide microarrays. The sex of 8 Strigidae species was distinctly identified on microarrays, compared with the ambiguous results obtained on running gel. The complementary binding on microarrays between the bird DNA and the sex-specific probes made the sexing results more reliable. The sex of a wider range of Strigidae species was successfully determined using this sequence-directed approach.
Materials and Methods

Owl samples for sex identification included muscle and blood. All the birds used in this study were of known sex, as determined by autopsy and behavior. Muscle tissues were obtained from carcasses during necropsy. Blood was collected during health checkups by veterinarians. Genomic DNA was extracted by incubating 0.1 g of muscle or 100 μl of blood with 20 μl of proteinase K (Amresco, Solon, OH) and 500 μl of digestion buffer (10 mM Tris–HCL, 2 mM ethylenediaminetetraacetic acid, 10 mM NaCl, 1% sodium dodecyl sulfate, 10 mg/ml dithiothreitol; Amresco) at 56 °C overnight, followed by phenol/chloroform isolation 3 times. The aqueous phase was precipitated in 100% ethanol at 4 °C overnight, followed by phenol/chloroform isolation 3 times. The aqueous phase was precipitated in 100% ethanol at 4 °C overnight, followed by phenol/chloroform isolation 3 times.

DNA was extracted by incubating 0.1 g of muscle or 100 μl of digestion buffer (10 mM Tris–HCL, 2 mM ethylenediaminetetraacetic acid, 10 mM NaCl, 1% sodium dodecyl sulfate, 10 mg/ml dithiothreitol; Amresco) at 56 °C overnight, followed by phenol/chloroform isolation 3 times. The aqueous phase was precipitated in 100% ethanol at 4 °C overnight, followed by phenol/chloroform isolation 3 times.

Amplification was carried out in a reaction volume of 25 μl containing 5 μl of each primer (1 μM), 0.5 μl of Taq DNA polymerase (2.5 U/μl), 2.5 μl of 10× PCR buffer (500 mM KCl, 100 mM Tris–HCL pH 8.3, 15 mM MgCl₂, 1% Triton X-100, 1600 μg/ml bovine serum albumin and 2 mM each deoxynucleoside triphosphate, pH 8.2–8.4), and 2 μl of template DNA. The thermal profile for amplification was 94 °C for 11 min, 30× (94 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s), 72 °C for 5 min. PCR products were separated in 3% agarose gels (Gibco, Grand Island, NY), run in 0.5× tris-acetate-ethylenediaminetetraacetic acid buffer with 0.5 μg/ml of ethidium bromide (Gibco) at 120 V for 6 h, and the corresponding W and Z bands amplified using primers 1237L/1272H in Tawny fish Owls (Ketupa flavipes) and Elegant Owls (Otus elegans) on gels were cut, eluted, and sequenced using an automated sequencer (ABI 3100, Applied Biosystems, Foster, CA). The PCR products from Eurasian-eagle Owls (Bubo bubo) were separated in 3% agarose gels at 80 V for 6 h, and the corresponding W and Z bands amplified using primers 1237L and 1272H were cut, eluted, and sequenced. These sequences were aligned further for probe designing.

A tail composed of 19 T bases was added to each 5’ end of the oligonucleotide probe, including the positive control probe (an oligonucleotide from capsid protein VP1 of human enterovirus 71 gene, 5’-ATGAAGCATGTCAGG-GCTTGGATACCTCG-3’). Ten μM of each probe was then spotted to each specific position on the microarray polymer substrate using an automatic spotting machine (DR. Easy spotter, Miao-Li, Taiwan) and immobilized using a UV Crosslinker (Vilber Lourmat BLX-254, ECC, Marne, France) with 1.2 J for 5 min.

The hybridization reaction between each DNA template and probes was carried out with DR. Chip DIY Kit (DR. Chip Biotech, Miao-Li, Taiwan). The procedures followed the manual and are briefly described below. The PCR product was denatured at 95 °C for 10 min and cooled in an ice bath for 2 min. To the microarray chamber was added 200 μl of hybridization buffer (containing the 5’ end-biotinylated oligonucleotide complementary to the sequence of positive control probe) and 15 μl of denatured PCR product, incubated at 50 °C with vibration for 50 min, and washed twice with wash buffer. The blocking reaction was then performed by mixing 0.2 μl of Strept-AP (Streptavidin conjugate alkaline phosphatase) and 200 μl of blocking reagent at room temperature for 30 min and washing twice with wash buffer. The colorimetric reaction was then implemented by adding 4 μl of nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate indolyl phosphate and 196 μl of detection buffer in the chamber, developing in the dark at room temperature for 5 min, and washing twice with distilled water. The hybridization result was indicated as the developed pattern on the microarray, which was read directly with the naked eyes.

Results

The sexing results based on intronic length polymorphism are shown in Figure 1. Fragments amplified using primers 1237L/1272H were located between 200 and 300 bp, and those amplified using primers 2550F/2718R were distributed between 600 and 1100 bp. This method was successful in 2 species of Strigidae, the Eurasian-scops Owl (Otus scops) and Elegant Owl, using either of these 2 primer pairs. However, the sex of the Brown-wood Owl (Strix
leptogrammica), Tawny-fish Owl, and Collared-scops Owl (Otus bakkamoena) was not identified using primers 1237L/1272H. Furthermore, the sex of the Eurasian-eagle Owl, Mountain-scops Owl (Otus spilocephalus), and Short-eared Owl (Asio flammeus) was not identified using either primers 1237L/1272H or 2550F/2718R in our sexing procedures. 

DNA samples obtained from Eurasian-eagle Owls, Tawny-fish Owls, and Elegant Owls were sequenced, aligned, and analyzed further (Figures 2 and 3). Five nucleotide probes were designed in order to detect sex-specific fragments of diversified Strigidae species. Probe W was employed to recognize female individuals because the W chromosome exists only in females. In addition, Probe ZW was utilized to detect both females and males, which could be taken as a positive control. When both Probe W and Probe ZW dots emerged on a microarray after hybridization, it indicated that the tested individual was a female. If only Probe ZW dots presented, it indicated that the specimen was a male. Several sex-specific probes operated complementarily or performed as multiple confirmation of the sex, making the obtained result more reliable.

The sexing results of 8 Strigidae species using oligonucleotide microarrays are shown in Figure 4. All females exhibited at least one of the 3 Probe W dots as well as the ZW dots. All males displayed only the Probe ZW dots. These findings indicate that the sex of Strigidae can be identified easily and reliably using oligonucleotide microarrays. The sex of 8 Strigidae species in this study was clearly identified through hybridization between DNA templates and probes on oligonucleotide microarrays. The hybridization signals on microarrays were easily read with the naked eye, requiring no laser scanning or imaging systems. The entire microarray manipulation time, including the data readout, was less than 2 h. This was nearly equal to the time needed for gel loading, electrophoresis, ethidium bromide staining, and visualizing of intron lengths under UV light.

The characteristic of sequence-directed hybridization on microarrays, not the length difference on gels, allowed sex identification with CHD1 introns to be more accurate by using a sequence-directed approach, which can be applicable to a wider range of Strigidae species.

Discussion

The CHD1 gene has been used for sex identification in numerous avian species. It exists on the W and also on the Z chromosome. Sex identification with CHD1 must distinguish between these 2 duplicates. A given CHD1 intron, whose length differs between the W and Z chromosomes, could be utilized for sex identification. There are several introns on the CHD1 gene. The 1237L/1272H-amplified intron, whose length polymorphism between the W and Z chromosomes is generally inadequate in Strigidae compared with other avian families, often leads to misjudging a female (ZW) as a male (ZZ). Although the sex of some Strigidae species could be determined through high-resolution electrophoresis, for example, Tawny-fish Owl and Collared-scops Owl, this method is time consuming and cannot be applied to all species (data not shown). Some researchers suggested separating the PCR products through polyacrylamide gels (Kahn et al. 1998) or single-strand conformation polymorphism gels (Cortes et al. 1999). However, both procedures were harder to perform than agarose gels. Although the 2550F/2718R-amplified intron has better length polymorphism between the W and Z chromosomes, this intronic polymorphism does not exist in all Strigidae species. The sex of some species, moreover, cannot be identified on agarose gel, regardless of which primer pairs are employed (Figure 1).

Two primer pairs flanking 2 respective CHD1 introns were employed in this study. Sex identification became more efficient using multiplex PCR. The PCR products of 3 Strigidae species were further sequenced and analyzed. Five probes were designed to generally detect CHD1W and CHD1Z of various Strigidae species. A more accurate method for sex identification is achieved using this sequence-directed approach, which can be applicable to a wider range of Strigidae species.
Figure 3. Sequence alignment of amplified DNA using primers 2550F/2718R. The position of primers and the sequences of the probes are indicated. Ta and El represent the Tawny-fish Owl and Elegant Owl, whereas W and Z symbolize the W and Z chromosome, respectively. Other illustrations are the same as Figure 2.
determination for the species whose \textit{CHD1W} and \textit{CHD1Z} introns are equal or very close in length. The detection sensitivity of the oligonucleotide microarray was also higher than that for agarose gel, making fragments invisible on the gel, visible on the array (Figures 1 and 4, 2250F/2718R-amplified product of Short-eared Owl). Sex identification of a wider Strigidae species range was thus achieved using oligonucleotide microarrays. This approach has also successfully expanded its application to other avian families. The sex of various avian families, including Strigidae, could be determined on 1 oligonucleotide microarray.

Probes designed from only a few species were capable of determining the sex of other species on microarrays. This was probably due to the sequence homology among various species. Some efforts are still needed for those species whose corresponding sequences are much diverged from the general. We recommend that sequencing genes from those species be used to design additional probes. The microarray can efficiently expand its efficacy by recruiting new probes, which would make this device more practicable.

**Supplementary Material**

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

**Figure 4.** Sex identification of 8 Strigidae species using oligonucleotide microarrays. All samples came from known-sex birds. (A) Microarray map. Each dot indicates the spotted position of each probe. P: Positive control, 1: Probe ZW1, 2: Probe ZW2, 3: Probe W1, 4: Probe W2, 5: Probe W3. (B) The sexing results on the microarrays. M: Male, F: Female. 1: Brown-wood Owl, 2: Tawny-fish Owl, 3: Collared-scops Owl, 4: Eurasian-scops Owl, 5: Elegant owl, 6: Eurasian-eagle Owl, 7: Mountain-scops Owl, 8: Short-eared Owl, 9: Negative control.

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**References**


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