A PCR-based method for sex identification in *Hippopotamus amphibius*

Richard Beckwitt[1]*, Jennifer Shea[1], Deirdre Osborne[1], Stephen Krueger[2] & William Barklow[1]

[1]*Department of Biology, Framingham State College, Framingham, MA 01701-9171, U.S.A.*

[2]*The Toledo Zoo, Toledo, OH, U.S.A.*

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Identification of the sex of aquatic mammals such as the hippopotamus (*Hippopotamus amphibius*) is difficult in the field. We have developed a PCR-based method for sex identification in the hippopotamus. This method amplifies a short fragment of the ZFX and ZFY genes from the X and Y chromosomes. The PCR products are then digested with the restriction endonuclease *Hae*III. The method was verified using tissue samples taken from six captive animals in zoos. We then collected 54 tissue samples from free-ranging hippos in Kruger National Park, South Africa, using a biopsy dart and cross-bow. The PCR method correctly identified the sex of all individuals for whom the sex was known (either zoo animals, or those exhibiting clear behaviours or morphologies in the field). Field identification of the sex of other individuals was prone to error (6 of 22 misidentified, 27 %).

Key words: hippopotamus, sex identification, ZFX, ZFY, PCR.

INTRODUCTION

Determining the sex of individuals in the field is often critical for behavioural and ecological studies. But when there is limited sexual dimorphism, and the animals are difficult to observe, estimating sex is subject to error. We found this to be the case for the hippopotamus (*Hippopotamus amphibius*). When hippos are in the water, often only the head and neck are visible. In adult males the neck and jaw muscles are more pronounced than in females, which allows adult males to be identified with some confidence. For females, however, it is very difficult to distinguish adults from sub-adult males, and it is impossible to sex calves when they are in the water. In this note, we describe a method based on collecting skin biopsy samples with a crossbow, and subsequent analysis using PCR, that allows unambiguous sex identification of hippos of any age.

There are several DNA-based methods for identifying the sex of mammals, which make use of X- or Y-chromosome specific markers. Aasen & Medrano (1990) described a PCR assay that clearly identifies the sex of a wide variety of mammals, including humans, cattle, sheep and goats, by amplifying the ZFX or ZFY genes: zinc-finger encoding genes that are found on the X and Y chromosomes, respectively. The ZFX and ZFY genes of most mammals are very similar, but not identical in DNA sequence. As a result, it is often possible to use the same set of PCR primers to amplify a product from each gene, and then use one or more restriction endonucleases to identify sites that are present in one gene and not the other. The resulting restriction fragment length polymorphism (RFLP) can then distinguish between males and females. Amstrup *et al.* (1993) used a similar technique with polar bears, as did Pande & Totey (1998) with water buffalo. Brown *et al.* (1991a, 1994) used a Southern-blot technique, with probes based on the ZFY gene, with beluga and right whales. Pomp *et al.* (1995) used PCR to amplify both the *Sry* gene and the ZFX/ZFY genes of pigs in a single reaction. Berube & Palsboll (1996) used a multiplexed PCR method to amplify ZFX or ZFY specific products from several species of whales. As part of a larger study on the population genetics of the hippopotamus, we have modified the method of Aasen & Medrano (1990), and used it successfully to identify the sex of both zoo and field sampled hippos.

*Author for correspondence. E-mail: rbeckwi@frc.mass.edu

MATERIALS & METHODS

Sampling

Skin biopsy samples were collected from hippos using a crossbow (458 Magnum Treestand, Hunter's Manufacturing Company, Suffield) fitted with an open-faced spinning reel and a specially designed biopsy dart (Krueger *et al.* 1998; modified

from Brown *et al.* 1991b). Six samples came from various zoos in the United States, from individuals of known sex. An additional 54 samples were collected from Kruger National Park, South Africa, in June–July 1997 (CITES permit number ZA9147174). These samples were taken from five herds located along the Olifants and Letaba Rivers. Some herds were in adjacent stretches of river, while others were separated by up to 40 km.

In the field, individual tissue samples (0.3–0.5 g) were cut into 1-mm pieces with a razor blade and placed into 1 ml of tissue preservation buffer (20 % DMSO, 0.25 M EDTA, saturated with NaCl, pH 8.0, Seutin *et al.* 1991). To avoid cross-contamination, each sample was prepared on a new piece of aluminum foil, using new, disposable tools (e.g. razor blades, wooden toothpicks). Samples were held at ambient temperature in the field for up to four weeks, and then stored at 4 °C until used. Usable DNA has been recovered from tissue stored in this fashion for up to three years.

DNA analysis

Total genomic DNA was prepared from 50–100 mg of hippo skin using the 'High Pure Genomic DNA Preparation Kit' (Roche Molecular Biochemicals, Indianapolis, IN). Genomic DNA was eluted with 200 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Amplification of the ZFX and ZFY regions of the hippo genome were performed using primers based on Aasen and Medrano (1990): P1-5EZ , 5'-ATA ATC ACA TGG AGA GCC ACA AGC T-3'; P2-3EZ, 5'-GCA CTT CTT TGG TAT CTG AGA AAG T-3'. Oligonucleotides were synthesized by Gibco Custom Primers (Life Technologies, Rockwell, MD). Since we were not sure of the exact sequences in the hippo genome, we used a 'touch-down' protocol in which the annealing temperature is initially set quite high, and is then lowered (Don *et al.* 1991; Roux 1994). The touch-down procedure is useful when the exact target sequence is not known. In the absence of complete sequence information, it is not possible to calculate the proper annealing temperature exactly. If the annealing temperature is too high, there will be no amplification, and if it is too low, non-target sequences may also be amplified. With a hot-start, the primers will anneal at a higher temperature to the correct template sequence, and these products will be more numerous in the final PCR reaction, even if non-specific amplification occurs at the lower annealing temperatures. The complete protocol was: 95 °C, 5 min; followed by 20 cycles of 95 °C, 1 min, 55 °C, 1 min (lowered by 0.5 °C each cycle); 72 °C, 3 min; 35 cycles of 95 °C, 1 min; 45 °C, 1 min; 72 °C, 3 min; final extension at 72 °C for 5 min. PCR reactions consisted of 1 µl genomic DNA (approximately 1 µg), 1 µl of each primer (0.2 µmolar), 45 µl Platinum PCR supermix (Life Technologies, Rockwell, MD). In this reaction mix, the *Taq* polymerase is complexed to an antibody that is denatured during the first period at 95 °C, providing an automatic 'hot-start.'

PCR products were purified using the 'High Pure PCR Product Purification Kit' (Roche Molecular Biochemicals, Indianapolis, IN). Purified samples were eluted into 40 µl TE buffer. Restriction digests were done using 17 µl of purified PCR product and four units of *Hae*III (Life Technologies, Rockwell, MD).

To confirm high molecular weight genomic DNA, 10 µl was run on a 1 % agarose gel (TAE buffer: 4 mM Tris-acetate, 1 mM EDTA, pH 8.0), using *Hin*d III-digested Lambda DNA (Life Technologies, Rockwell, MD) as a size standard. PCR products and restriction digests were run on 1.5 % agarose gels, using *Hae*III-digested ϕX174 DNA (Life Technologies, Rockwell, MD) as the size standard.

RESULTS AND DISCUSSION

Krueger *et al.* (1998) describe the design and use of the biopsy dart. Field samples came from five herds located on the Olifants and Letaba rivers in Kruger National Park, South Africa. Each herd was observed for one or more days before sampling. Where possible, individuals were identified on the basis of characters such as scars and colouration.

We also attempted to identify the sex of each individual on the basis of morphology and behaviour. Using three crossbows, we were able to collect no more than 5 samples from one herd in one day. Very young individuals were particularly difficult to sample unless they were approached while sleeping on land, since they remained submerged except for brief periods to breathe.

Hippos reacted only briefly to being sampled. The simplest method was to approach quietly to within 20–40 m while the animals were sleeping on land. After the darts were fired, all of the animals in the herd would move quickly into the water and briefly vocalize. Once in the water, they returned to their normal behaviour within five minutes, although they remained sensitive to our presence. There was no obvious difference in the

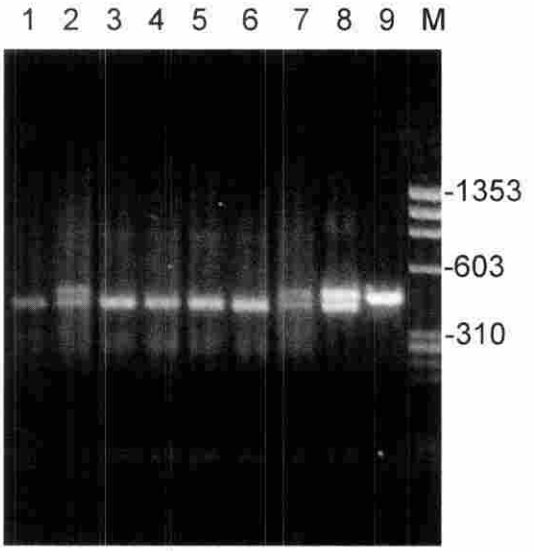

Fig. 1. ZFX and ZFY PCR products digested with restriction enzyme *Hae*III and separated on a 1.5 % agarose gel. Lanes 1, 3, 4, 5 and 6 are female; lanes 2, 7 and 8 are male; lane 9 is undigested. M = markers (ϕX174/HaeIII digest). Numbers along the right are sizes in base-pairs of selected markers.

behaviour of either the target animal or others in the herd, whether the dart hit or missed. Once the animals were in the water, they would not leave while we were present. However, if we remained still for a period of time, they would begin to sleep in the water. This often presented a second opportunity to take a sample, if the animal was facing away from us.

In total, of 253 shots were fired. Out of 74 shots that hit (29 %), only 54 were recovered successfully (samples were lost when the fishing line or dart broke). Of these, 52 had sufficient tissue to be used for PCR amplification.

PCR amplification of the ZFX and ZFY regions of hippopotamus DNA, using the P1-5EZ and P2-3EZ primers, produced a single product of about 450 base pairs. This corresponded well to the products seen in other mammals (Aasen & Medrano 1990). We surveyed several restriction enzymes. Of these, *Hae*III gave the clearest results (Fig. 1). In females, there is a single *Hae*III site in the ZFX product, which results in two fragments of approximately 400 and 50 base pairs (the 50 base pair band was not always visible). When PCR products from males were digested with *Hae*III, three bands were visible: the 400 and 50 base pair bands seen in females, as well as the 450 base pair band seen in undigested samples. We interpret this to mean that there is no *Hae*III site in the ZFY product. This allows an unambiguous interpretation of restriction digest patterns: individuals with both the 450 and 400 base pair bands are males (with both X and Y chromosomes), while individuals with only the 400 base pair band are females (X chromosome only). It also allows an unambiguous rejection of samples in which the digest failed: they would show only the single band at 450 base pairs. We also attampted to use primers based on Amstrup *et al.* (1993), but could not obtain consistent results: PCR reactions often produced multiple bands, and repeated restriction digests of the same samples gave differing banding patterns.

We were able to verify the accuracy of the PCR method for sex identification by testing six specimens collected from zoos. The method provided the correct sex in all cases. We made tentative sex identifications for 22 individuals observed in the field. Of these, the PCR method confirmed 16 (27 % error rate). For five individuals for which it was possible to make a definite field identification based on direct observation of secondary sexual characteristics or mating behaviour, the PCR method was in agreement each time. We did not collect a random sample of individuals in any herd. Since the tissue samples for this report were collected as part of an ongoing study of population genetics and paternity in hippo herds, we focused on sampling the alpha male (identified by behaviour) as well as any adult females with calves. Other individuals were sampled as the occasion arose.

The ability to identify the sex of individuals in the field is necessary to correctly interpret behavioural or ecological observations. As we have shown, determining the sex of hippos based solely on morphology is prone to error, even for observers with considerable experience. Karstad & Hudson (1986), for example, used visual cues to distinguish sex while hippos were in the water, and concluded that adult females comprised 36 % of the herd. They also used the same sexing technique to estimate the number of adult females involved in agonistic encounters. The results of our study suggest their numbers may be in error by as much as 27 %.

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