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Molecular Sex Determination of Captive Komodo Dragons (*Varanus komodoensis*) at Gembira Loka Zoo, Surabaya Zoo, and Ragunan Zoo, Indonesia

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Captive breeding of endangered species is often difficult, and may be hampered by many factors. Sexual monomorphism, in which males and females are not easily distinguishable, is one such factor and is a common problem in captive breeding of many avian and reptile species. Species-specific nuclear DNA markers, recently developed to identify portions of sex chromosomes, were employed in this study for sex determination of Komodo dragons (*Varanus Komodoensis*). Each animal was uniquely tagged using a passive integrated micro-transponder (TROVAN 100A type transponders of 13 mm in length and 2 mm in diameter). The sex of a total of 81 individual Komodo dragons (44 samples from Ragunan zoo, 26 samples from Surabaya zoo, and 11 samples from Gembira Loka zoo) were determined using primers Ksex 1for and Ksex 3rev. A series of preliminary PCR amplifications were conducted using DNA from individuals of known sex. During these preliminary tests, researchers varied the annealing temperatures, number of cycles, and concentrations of reagents, in order to identify the best protocol for sex determination using our sample set. We thus developed our own PCR protocol for this study, which resulted in the amplification of band A in females and band C in males. Results from band B, however, turned out to be non-determinative in our study because, for females, band B was not always visible, and for males sometimes a similar, but lighter band was also amplified, making interpretation difficult. In this study, sex determination was based mainly on the difference in size between the female-specific 812 bp fragment and the homologous, longer fragment amplified for males.

Keywords: captive breeding, zoo, komodo dragon, molecular sexing, PCR

INTRODUCTION

Sex identification provides valuable insights into species breeding strategies, which informs conservation and management programs (Helander *et al*. 2007; Garcia *et al*. 2009; Naim *et al*. 2011) and reproduction programs for threatened species (Ellegren & Sheldon 1997). The most basic and important information in establishing captive breeding programs is a clear understanding of sex ratio; that is, the number of males and females in a population. Sex determination in captivity allows individual identification for mating, and it is useful in order to set breeding procedures on the basis of data on male-to-female ratios available from field studies. However, sex determination is often hampered by the occurrence of sexual monomorphism, in which the male and female are not easily distinguished. Komodo dragons have long been popular zoo attractions, thanks to their size and fearsome reputation. The Komodo dragon *Varanus komodoensis* is the world's largest lizard, up to 3 m in length and over 70 kg in weight. It is restricted to five small islands in Eastern Indonesia (Ciofi & De Boer 2004). It is endemic to southeast Indonesia and protected throughout its range, which includes Komodo National Park and the island of Flores. The species is now listed in CITES Appendix 1, and has been proposed for designation as "endangered" on the IUCN red list (Ciofi & De Boer 2004). Although males tend to grow bulkier and bigger than females, Komodo dragons have no obvious morphological differences between sexes except in the arrangement of a specific part of precloacal scales. So far, however, examination of the precloacal scales to determine sex has been proved to be difficult in the field. The scale pattern is not always clear, and probing the cloaca for presence or absence of inverted hemipenes is troublesome since females have hemiclitoreal sacs at approximately the same position of males hemipenes, and gender can often be confused. Sexing Komodos remains a challenge to researchers; the dragons themselves appear to have little trouble figuring out who is who.

It is relatively easy to restrain Komodo dragon in captivity, so a number of accurate alternative tests can be used to determine sex. Laparoscopy provides direct visualization of gonads by means

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of an endoscope. Radiography of the cloacal region shows the presence or absence of the bones in males associated with the hemipenis. Differences in testosterone concentrations can be assessed in fresh blood samples. Finally, ultrasound can be used to assess whether ovarian follicles are present. Although accurate, these techniques have not given consistent results for young animals of under 10 months of age, and their employment is sometimes hampered by the requirement for quality equipment. It is therefore important to have an accurate and relatively non-expensive technique available for sex determination. Thus far, DNA technology has provided the most consistent results (Halverson & Spelman 2002).

Animal molecular sexing techniques are usually based on polymerase chain reaction (PCR) amplification of deoxyribonucleic acid (DNA) sequences from the Y (mammal) or W (bird) chromosome-linked loci (Putze *et al*. 2007), with concomitant amplification of X or Z, respectively. Alternatively, sexing techniques are based on differences in the PCR yielded fragment of the X-Y homologous gene amelogenin from the X and Y chromosome. Nevertheless, X and Z chromosome-linked loci are not usually used as stand-alone techniques for animal sex determination (Dubiec & Zagalska-Neubauer 2006). Sexing technique methods are usually based either on the presence or absence of PCR amplification products, on the differences in band lengths and/or the resulting banding patterns on gel, and/or on differences in sequences ('qualitative sexing'), which enable researchers to distinguish between X and Y chromosomes (mammals) or W and Z chromosomes (birds) [restriction fragment length polymorphisms (RFLPs, Sacchi *et al*. 2004), amplified fragment length polymorphism (AFLPs, Griffiths & Orr 1999), single strand conformation polymorphism (SSCPs, Ramos *et al*. 2009), microsatellite alleles (Nesje & Roed 2000), and oligonucleotide-microarrays (Kalz *et al*. 2006; Wang *et al*. 2008)].

Sex determination tests using DNA markers have been used in many vertebrate species, including whales (Baker *et al*. 1991), birds and reptiles (Millar *et al*. 1996, 1997; Fleming *et al*. 1996). Reptiles exhibit different modalities of gender determination, including both temperature-dependent determination and chromosomal sex determination. In regards to chromosomal determination, some genera have XY sex chromosomes, some genera have ZW sex chromosomes (such as in Varanus; Olmo 1986), and some genera have no recognizable sex chromosomes. Indeed, some reptile families show all three variations. It is unlikely that a DNA marker found in one genus will be sufficiently conserved that it can be broadly applied in other reptiles. Within a single genus, however, sometimes markers for sexing one species can be applied to other species. However, until recently, no sex determination markers where available for the genus *Varanus*. For Komodo dragons, species-specific DNA markers for sex determination have been developed by Halverson and Spelmann (2002) to identify portions of the heteromorphic female sex chromosomes. These were the first probes for sex determination in varanid lizards.

The goal of this study was to determine the sex of captive Komodo dragons kept at the Zoos in Surabaya (Surabaya zoo), Yogyakarta (Gembira loka zoo), and Jakarta (Ragunan zoo). The sex of individual Komodo dragons in captivity was identified using a pair of PCR primers (Ksex 1for and Ksex 3rev) as decribed by Halverson and Spelmann (2002).

MATERIALS AND METHODS

Animal Marking. A total of 81 Komodo dragons were studied, including 44 Komodo dragons kept in Ragunan zoo (Jakarta), 26 Komodo dragons kept in Surabaya zoo (Surabaya), and 11 Komodo dragons kept in Gembira loka zoo (Yogyakarta). The zoos of Ragunan, Gembira loka, and Surabaya are the three largest Zoos in Indonesia and hold the most easily accessible populations of Komodo dragons.

Each animal was uniquely tagged using a passive integrated micro-transponder. We used TROVAN 100A type transponders of 13 mm in length and 2 mm in diameter. Each transponder easily scanned by an appropriate reader-contains a 10 digit barcode that is uniquely assigned to the tagged animal. Transponders were inserted under the skin of the upper part of the right hind leg of the animal (Figure 1). Most animals, however, already had transponders in the upper portion of one of the front legs, implanted previously by zoo staff. Therefore, animals were checked for the presence of signal from an existing transponder and, if no signal was detected, a new device was inserted in the upper part of the right hind leg of the animal. This technique provides permanent tagging with no injury to the animal being treated.

Measurement of Snout-Vent Length (SVL). After tagging, Snout-Vent Length (SVL, cm) was measured for each animal. This is a standard measurement of body length taken from the tip of the nose (snout) to the anus (vent), excluding the tail