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

SRI SULANDARI*, MOCH SAMSUL ARIFIN ZEIN, EVY AYU ARIDA, AMIR HAMIDY

*Research Center for Biology, The Indonesian Institute of Sciences (LIPI), Cibinong Science Center,*
*Jalan Raya Jakarta Bogor, Km. 46, Cibinong 16911, Indonesia*

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

*Corresponding author. Phone: +62-21-8765056,
Fax: +62-21-8765068, E-mail: ssulanda@yahoo.co.id

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

(http://www.sdnhm.org/fieldguide/herps/glossary.
html). Laver *et al*. (2012) used SVL-defined as the
straight line distance measured between the tip of
the snout and the cloaca to the nearest millimeter-as
the measure of growth for each individual. In our
study, measurements were made using a flexible
fiberglass tailor's tape.

**Sampling Procedure.** Blood was collected
from the caudal vein, using a 25Gx⅝" or a 23Gx1"
disposable needle, depending on the size of the
specimen. The needle was inserted laterally, at
about 10-30 cm, depending on the size of the animal
(Figure 2). Approximately 100 microliters (µL)
of blood were collected and preserved at ambient
temperature in 2 milliliter (mL) tubes containing a
lysis buffer made of 0.1 M Tris buffer, 0.1 M EDTA,
0.2 M NaCl and 1% sodium dodecyl sulfate, pH
8.0. Blood samples were transported to the genetic
laboratory of Zoology at the LIPI Research Center
for Biology, and stored at -20 ºC.

**DNA Extraction and DNA Quality Assessment.**
DNA was extracted from whole blood samples using
standard phenol/chloroform procedures (Sambrook
*et al*. 2001) based on disruption of red and white cell
membranes, removal of protein contaminants with
organic solvents, and isolation of high molecular
weight nucleic acids in the presence of salt and
ethanol. A Phase Lock Gel™ light (Eppendorf) was
used for a more efficient separation of organic and
aqueous phases. DNA was stored at -20 ºC and then
deposited at the DNA Bank for Indonesian Fauna,
in the genetic laboratory of Zoology at the LIPI
Research Center for Biology.

DNA quality was assessed by visualizing nucleic
acids in agarose gel under ultraviolet light following
electrophoretic reaction. Electrophoresis is a process
by which nucleic acids move along an electric field.
The DNA are run through an agarose gel, a complex
network of polymeric molecules whose average
pore size depends on the buffer composition and
the type and concentration of agarose used. The gel
is placed in the electrophoretic apparatus, which
consists mainly of a buffer reservoir in the form a
square tank with a positive and negative electrodes
placed at the opposite sites of the tank. Agarose
gels are usually run in an electric field of constant
strength and direction under 100 V of power. At
neutral pH values, the DNA is negatively charged
so that fragments loaded into a sample well at the
cathode end of a gel move through the gel towards
the anode end.



Figure 1. From left to right: 13 mm long passive integrated transpoder (PIT), insertion of PIT, checking for PIT presence with
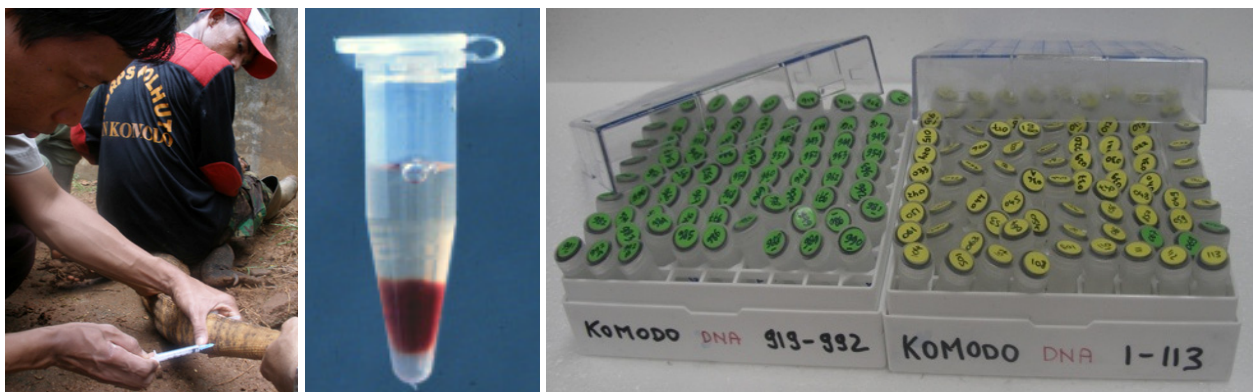       dedicated reader.



Figure 2.  From left to right: Collection of blood sample; blood sample (approximately 100 uL) in 1.5 mL collection tube, and DNA
       sample.

**PCR Amplification of Sex-Specific DNA Fragments.** Species-specific nuclear DNA markers, recently developed by Halverson and Spelman (2002) were employed for sex determination of 81 Komodo dragons in captivity. In PCR, oligonucleotide probes, or primers, anneal to a template of DNA from a single individual. A polymerase that does not denature at high temperatures, called Taq polymerase, catalyzes the replication of that DNA. Changing the temperature controls how the reaction proceeds: the primers anneal, the DNA is replicated, and then the two strands are denatured to make them available for more replication. Through a repeated cycling of temperatures, the template is amplified to make millions of copies. Female Komodo dragons are heterogametic, with one Z chromosome and one W chromosome (Olmo 1986). DNA samples are therefore characterized by alleles of different size, detected in the gel as two distinct bands. Males are homogametic (they have two Z chromosomes) and have alleles of the same size, so that PCR products will run at the same speed during electrophoresis. The two alleles will overlap each other in the gel, and will be therefore represented by a single band. This is the result of PCR primers annealing to both sex chromosomes but amplifying DNA fragments of different lengths.

One set of forward (Ksex 1for) and reverse PCR primers (Ksex3rev) were used (Figure 3) and PCR conditions were optimized for annealing temperature, magnesium concentration, and polymerase. In this study, PCR amplification produced a fragment of 812 base pairs (bp) for females, in accordance with the length of the cloned sequence (Figure 4). The polymerase chain reaction (PCR) protocol for amplification of Komodo dragon sex-related DNA sequences featured the following conditions: 10 µL total volume reaction containing 1x reaction buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 25 µM of each primer and 0.05 U of Taq DNA polymerase (Fermentas). PCR cycling was performed in the Applied Biosystems GeneAmp PCR system 2700 thermocycler for an initial denaturation step at 95 ºC for 5 min, followed by 35 cycles of 30 s at 95 ºC, annealing for 30 s at 57 ºC and extension for 1 min at 72 ºC, with a final extension step of 10 min at 72 ºC.

**Visualisation of PCR Product for Sex Determination.** PCR products were separated by electrophoresis at 100 V for 30 min using a 2% agarose gel. Because DNA is negatively charged, electrophoresis causes PCR products to move through the matrix of an agarose gel towards a positive electrode. The shorter strands move faster, so when there are several DNA sequences of different lengths amplified during PCR, electrophoresis separates PCR products. The Agarose gel is left staining in a buffer solution containing ethidium bromide, a dye that intercalates in DNA and makes the separated PCR products visible under UV light. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through an agarose matrix. The PCR

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       Ksex 1for  →
Tgtccatgccacgcgggcaaaagcggctcatgactgcagatgtaggggtcagaatgacttaaccttgt
Ttgctttgagccaaaaggggagattgggcttggctcgcgaccaaaatcggctcgcgaccacgtttgta
Aaggtcagaatggattaagtttatgctcatcgaaacttatggagaaatagggcttggctcgcgggaaa
Aatcggatcgtgaccacttttgcagttgtgagactggattgacctcattcgcatcgaacgttatgggg
Aaattgtcatgcctcccgaccaaaatcggctcgcgatcacgtttgtaaaggtcagaatgcatttacct
Aatttgcaacgtaccttatgtggaaattggacttatctctcgacaaagataggtttacgaagacgttt
Gcggcggtcagaatggaatgacctaatttccattgaaccttatcaggaaattatcatgcatcgcgacc
Taaatcagctagcgaccacattttcggggtcataatggatcaagtatattcacatcgaacctgatgg
Ggaaattggacttggctcatgaccaaaatcagcttgcgagcgtgtctgtagtggtcagaaaggattga
Ccttctttctatcgaaccttatggggaaatgttcttgcctcgtgggcaaaatcggctctcgaccacat
Ttgtgaaggtcggaatgaattagcctttatttgcatataacctaatggggaaattggacctgcctcgct
acaaaaataggctcgtgacgaggtttgtaggggtcagaatgaattaagtttattggcatggaca
                                        ← Ksex 3rev
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Figure 3. Sequence from the W chromosome of *Varanus komodoensis*. Ksex1for (forward) and Ksex3rev (reverse) PCR primer sequences are underlined and highlighted in grey.
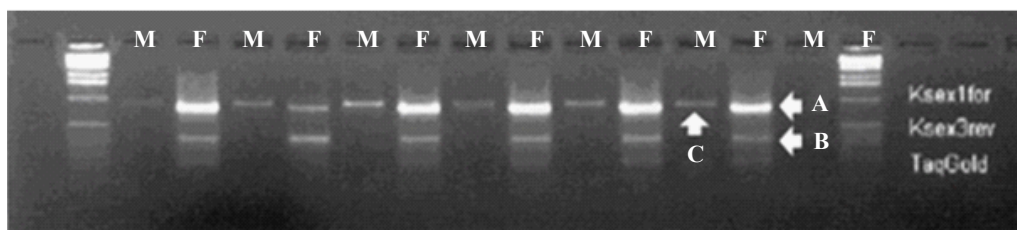


Figure 4. Agarose electrophoresis of PCR amplification products of known sex Komodo Dragon DNA with Ksex 1for and Ksex 3rev. (M: males, F: females).

products appear as bands along the column where they migrated.

The visualization product comparing the male and female pools is shown in Figure 4. Female is indicated by presence of band A. Band B is also restricted to females and, while not predictive of sex, it probably arises from a less homologous binding of primer Ksex 1for. In males, only one band, less intense and about 100 bp longer than female-specific bands, is produced (band C in Figure 4). Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. The longer sequence amplified in males may represent a longer, but similar sequence in the male Z chromosome with a similar annealing sites for Ksex 1for and Ksex 3rev. Alternatively, a mutation event in the male homologous Z chromosome sequence may have resulted in the optimal annealing site for either of the two primers to be located about 100 bp outside the original target sequence.

## RESULTS

**Snout-Vent Length (SVL).** Measurement of Snout-Vent Length (SVL) is presented in Tables 1,

Table 1. Summary of sex determination results for Komodo dragons at Ragunan zoo. Animal identification (ID) is reported along with the name of the enclosure, collection date, passive integrated transponder (PIT) ID, date of arrival at the zoo facilities (or date of birth), life stage, snout-vent length (SVL in cm) and sex as determined by DNA analysis

| ID | Cage | Date | PIT | Arrival | Date of birth | Life stage | SVL | Sex |
|----|------|------|-----|---------|---------------|------------|-----|-----|
| KR1 | Surplus | 28-Mar-06 | 00-01F6-63FD | | 19-Feb-98 | F1 | 87.00 | Female |
| KR2 | Surplus | 28-Mar-06 | 00-01F7-D7B4 | | 10-Mar-98 | F1 | 87.50 | Female |
| KR3 | Surplus | 28-Mar-06 | 00-0648-49AB | 5-May-96 | | F0 | 125.00 | Male |
| KR4 | Penangkaran | 28-Mar-06 | 00-0142-0DDB | | 19-Feb-98 | F1 | 97.70 | Female |
| KR5 | Penangkaran | 28-Mar-06 | 00-01F7-872D | | 15-Apr-98 | F1 | 101.00 | Female |
| KR6 | Penangkaran | 28-Mar-06 | 00-01E5-C3BB | | 19-Feb-98 | F1 | 95.00 | Male |
| KR7 | Penangkaran | 28-Mar-06 | 00-01F7-D567 | | 19-Feb-98 | F1 | 117.00 | Male |
| KR8 | Penangkaran | 28-Mar-06 | 00-01E5-BFEA | | 19-Feb-98 | F1 | 100.00 | Female |
| KR9 | Penangkaran | 28-Mar-06 | 00-01F7-8573 | | 19-Feb-98 | F1 | 102.50 | Female |
| KR10 | Penangkaran | 28-Mar-06 | 00-01F7-8468 | | 19-Feb-98 | F1 | 118.00 | Male |
| KR11 | Penangkaran | 29-Mar-06 | 00-01F6-60E9 | | 19-Feb-98 | F1 | 85.00 | Female |
| KR12 | Penangkaran | 29-Mar-06 | 00-0128-935E | | 19-Feb-98 | F1 | 101.00 | Female |
| KR13 | Penangkaran | 29-Mar-06 | 00-0128-B47A | | 19-Feb-98 | F1 | 96.00 | Female |
| KR14 | Penangkaran | 29-Mar-06 | 00-01E5-701A | | 19-Feb-98 | F1 | 96.00 | Female |
| KR15 | Penangkaran | 29-Mar-06 | 00-0128-9AFA | | 19-Feb-98 | F1 | 98.00 | Female |
| KR16 | Penangkaran | 29-Mar-06 | 00-0127-BC77 | | 10-Mar-98 | F1 | 90.00 | Female |
| KR17 | Penangkaran | 29-Mar-06 | 00-0127-BA1C | | 19-Feb-98 | F1 | 95.00 | Female |
| KR18 | Penangkaran | 29-Mar-06 | 00-01F7-83D1 | | 19-Feb-98 | F1 | 105.00 | Female |
| KR19 | Penangkaran | 29-Mar-06 | 00-01F7-7E59 | | 19-Feb-98 | F1 | 104.50 | Female |
| KR20 | Penangkaran | 29-Mar-06 | 00-01F6-3030 | | 15-Mar-98 | F1 | 117.00 | Male |
| KR21 | Untung 2 | 29-Mar-06 | 00-01F6-55FF | | 10-Mar-98 | F1 | 85.00 | Female |
| KR22 | Suhada 1 | 29-Mar-06 | 00-01E5-7771 | | 10-Mar-98 | F1 | 113.00 | Male |
| KR23 | Suhada 1 | 29-Mar-06 | 00-01E5-808A | | 10-Mar-98 | F1 | 82.00 | Female |
| KR24 | Suhada 2 | 29-Mar-06 | 00-01E5-7FA9 | | 10-Mar-98 | F1 | 87.50 | Female |
| KR25 | Suhada 2 | 29-Mar-06 | 00-0128-B7A5 | | 10-Mar-98 | F1 | 91.00 | Female |
| KR26 | Suhada 2 | 29-Mar-06 | 00-0128-9380 | | 10-Mar-98 | F1 | 101.00 | Male |
| KR27 | Suhada 2 | 29-Mar-06 | 00-0127-C179 | | 10-Mar-98 | F1 | 105.00 | Male |
| KR28 | Suhada 2 | 29-Mar-06 | 00-01F7-8852 | | 10-Mar-98 | F1 | 96.50 | Female |
| KR29 | Suhada 3 | 29-Mar-06 | 00-0127-C315 | | 10-Mar-98 | F1 | 96.00 | Female |
| KR30 | Suhada 3 | 29-Mar-06 | 00-0128-B4B7 | | 10-Mar-98 | F2 | 92.00 | Female |
| KR31 | Suhada 3 | 29-Mar-06 | 00-0648-3B4B | 8-May-96 | | F0 | 105.00 | Male |
| KR32 | Suhada 3 | 29-Mar-06 | 00-01F7-C992 | | 10-Mar-98 | F1 | 104.00 | Male |
| KR33 | Suhada 4 | 29-Mar-06 | 00-0127-D5E5 | | 22-Jun-89 | F1 | 111.00 | Male |
| KR34 | Suhada 5 | 29-Mar-06 | 00-066F-EA3F | | 22-Jun-89 | F1 | 144.00 | Male |
| KR35 | Suhada 4 | 29-Mar-06 | 00-0128-B7D2 | 9-May-96 | | F0 | 118.00 | Female |
| KR36 | Untung 2 | 29-Mar-06 | 00-0142-0D7D | | 15-Apr-98 | F1 | 117.00 | Male |
| KR37 | Untung 2 | 29-Mar-06 | 00-01E5-80EA | | 15-Apr-98 | F1 | 97.50 | Female |
| KR38 | Untung 1 | 29-Mar-06 | 00-012B-667C | 8-May-96 | | F0 | 126.00 | Female |
| KR39 | Untung 1 | 29-Mar-06 | 00-066C-8E3D | 8-May-96 | | F0 | 140.00 | Male |
| KR40 | Untung 1 | 29-Mar-06 | 00-0670-00D2 | 8-May-96 | | F0 | 117.00 | Female |
| KR41 | Untung 1 | 29-Mar-06 | 00-0128-9F28 | 9-May-96 | | F0 | 125.00 | Female |
| KR42 | Untung 3 | 29-Mar-06 | 00-0127-C5A5 | 9-May-96 | | F0 | 121.00 | Female |
| KR43 | Nursery | 29-Mar-06 | 00-066C-F2FB | | 6-Jun-05 | F2 | 30.00 | Female |
| KR44 | Untung 3 | 03-Apr-06 | 00-0127-E61F | 9-May-96 | | F0 | 105.00 | Female |

2, and 3. The average size was 115.1 cm for males and 97.2 cm for females in Ragunan zoo; 101.4 cm for males and 79.1 cm for females in Surabaya zoo; 91 cm for males and 86 cm for females in Gembira loka zoo.

**Sex of Komodo Dragon in Zoo Captivity.** A series of preliminary PCR amplifications were conducted on some samples of known sex, to test for the best protocol for all our DNA samples of Komodo dragons. An example of these results is reported in Figure 5. For males, PCR always resulted in the amplification of the male-specific longer fragment and would sometimes produce the

812 bp fragment specific for females. The first one corresponded to the original annealing site described for the female-specific sequence and the second one was located about 100 bp outside the original target sequence. The first annealing site appears to be not fully complementary to the primer sequence because it does not always result in a successful amplification and, when it does, it often provides PCR products of lighter intensity.

The pattern of sex identification showed in Figure 5 was used subsequently to determine sex of 81 Komodo dragons from Ragunan zoo, Surabaya zoo, and Gembira loka zoo. The sex of

Table 2. Summary of sex determination results for Komodo dragons at Surabaya zoo. Identification (ID) is reported along with the name of the enclosure, collection date, passive integrated transponder (PIT) ID, date of arrival at the zoo facilities (or date of birth), life stage, snout-vent length (SVL in cm) and sex as determined by DNA analysis

| ID | Cage | Date | PIT | Arrival | Date of birth | Life stage | SVL | Sex |
|----|------|------|-----|---------|---------------|-----------|-----|-----|
| KS1 | Depan parker | 12-Apr-06 | 00-01D3-D079 | | 27-Apr-02 | F2 | 86.00 | Male |
| KS2 | Depan parker | 12-Apr-06 | 00-01D2-BEFF | | 1-Apr-03 | F2 | 67.00 | Female |
| KS3 | Depan parker | 12-Apr-06 | 00-01C4-914A | | 4-Apr-03 | F2 | 68.00 | Male |
| KS4 | Depan parker | 12-Apr-06 | 00-012A-01FE | | 3-Apr-03 | F2 | 72.00 | Female |
| KS5 | Depan parker | 12-Apr-06 | 00-0143-616B | | 7-Apr-03 | F2 | 67.00 | Female |
| KS6 | Depan parker | 12-Apr-06 | 00-012E-9FBC | | 7-Apr-03 | F2 | 64.00 | Female |
| KS7 | Depan parker | 12-Apr-06 | 00-0143-70BD | | 2-Apr-03 | F2 | 59.00 | Female |
| KS8 | Depan parker | 12-Apr-06 | 00-01D3-1AF0 | | 3-Apr-03 | F2 | 60.00 | Female |
| KS9 | Depan parker | 12-Apr-06 | 00-0125-1983 | | 25-Jun-02 | F2 | 83.00 | Male |
| KS10 | Depan parker | 12-Apr-06 | 00-0135-0BCD | | 8-Apr-03 | F2 | 64.00 | Female |
| KS11 | Depan parker | 12-Apr-06 | 00-0143-6514 | | 14-Apr-03 | F2 | 72.00 | Female |
| KS12 | Depan parker | 12-Apr-06 | 00-01D2-BE63 | | 12-Apr-03 | F2 | 62.00 | Female |
| KS13 | Depan parker | 12-Apr-06 | 00-013C-4FA9 | | 25-Jun-02 | F2 | 79.00 | Male |
| KS14 | Depan parker | 12-Apr-06 | 00-0143-4886 | | 15-Apr-03 | F2 | 66.00 | Female |
| KS15 | Depan parker | 12-Apr-06 | 00-01D2-BCD8 | | 29-Apr-02 | F2 | 83.00 | Male |
| KS16 | D-Onta T | 12-Apr-06 | 00-01D3-D8FF | | | F1 | 121.00 | Female |
| KS17 | Depan WC | 12-Apr-06 | 00-01D2-BE18 | 16-Jun-92 | | F1 | 113.00 | Female |
| KS18 | Depan WC | 12-Apr-06 | 00-0135-106B | ~1999 | | F0 | 123.00 | Male |
| KS19 | Depan Zebra | 12-Apr-06 | 00-01C4-973D | | 17-Jun-97 | F1 | 99.00 | Female |
| KS20 | Depan Zebra | 12-Apr-06 | 00-012A-0973 | ~1999 | | F0 | 128.00 | Male |
| KS21 | Depan Zebra | 12-Apr-06 | 00-01D2-BE74 | | 28-Apr-98 | F1 | 125.00 | Male |
| KS22 | Depan Zebra | 12-Apr-06 | 00-0143-5BB9 | ~1999 | | F0 | 120.00 | Male |
| KS23 | Depan Zebra | 12-Apr-06 | 00-01D3-D02D | ~1999 | | F0 | 119.00 | Male |
| KS24 | Depan Zebra | 12-Apr-06 | 00-0143-7122 | | | F1 | 94.00 | Female |
| KS25 | Depan Zebra | 12-Apr-06 | 00-01C4-98E4 | 1-Apr-96 | 19-Jul-95 | F1 | 89.00 | Female |
| KS26 | Depan Zebra | 12-Apr-06 | 00-013C-4F3E | ~1999 | | F0 | 97.00 | Female |

Table 3. Summary of sex determination results for Komodo dragons at Gembira Loka zoo. Identification (ID) is reported along with the name of the enclosure, collection date, passive integrated transponder (PIT) ID, date of arrival at the zoo facilities (or date of birth), life stage, snout-vent length (SVL in cm) and sex as determined by DNA analysis

| ID | Cage | Date | PIT | Arrival | Date of birth | Life stage | SVL | Sex |
|----|------|------|-----|---------|---------------|-----------|-----|-----|
| KY1 | Karantina | 3-May-06 | 116233544A | | | F2 | 86.00 | Male |
| KY2 | Karantina | 3-May-06 | 116244117A | | | F2 | 76.00 | Male |
| KY3 | Karantina | 3-May-06 | 114672246A | | | F2 | 60.00 | Male |
| KY4 | Kandang 4 | 3-May-06 | 115752652A | | | F2 | 87.00 | Male |
| KY5 | Kandang 4 | 3-May-06 | 116152164A | | | F2 | 70.00 | Male |
| KY6 | Kandang 4 | 3-May-06 | 116312321A | | | F2 | 72.00 | Female |
| KY7 | Kandang 4 | 3-May-06 | 116335752A | | | F2 | 76.00 | Male |
| KY8 | Kandang 5 | 3-May-06 | 114673364A | | | F1 | 119.00 | Male |
| KY9 | Kandang 5 | 3-May-06 | 114655792A | | | F1 | 100.00 | Female |
| KY10 | | 3-May-06 | 00-0634-AA5E | | | F0 | 132.00 | Male |
| KY11 | | 3-May-06 | 00-0648-4858 | | | F0 | 113.00 | Male |

all 81 individual Komodo dragons were determined successfully, by looking at the difference in length of the female-specific 812 bp fragment and the homologous, longer fragment amplified for males. Gel electrophoresis results for a total of 81 Komodo
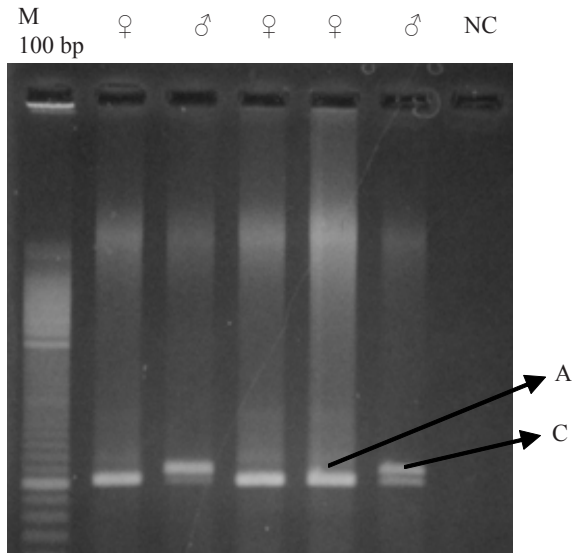


Figure 5. An example of the pattern of PCR amplification products sex-related DNA fragments. (M 100 bp = 100 bp DNA marker,  ♀: Female, ♂: Male, NC: negative control).

dragons are reported for Ragunan zoo (Figure 6), Surabaya zoo (Figure 7), and Gembira loka zoo (Figure 8). First column of the Figures 6A-D, 7A-B, and 8 show a fragment size reference ladder (100 bp ladder). Tables 1, 2, and 3 show the animal identification number (ID) along with the name of the enclosure, collection date, passive integrated transponder (PIT) ID, date of arrival at the zoo facilities (or date of birth there), life stage, snout-vent length (SVL in cm) and sex as determined by DNA analysis.

**Ragunan Zoo.** Jakarta's zoo (Ragunan zoo) is situated in the suburb of Ragunan in the southern part of Jakarta and it has a captive breeding program for endangered species including Komodo dragons. In this study, the sex of 44 Komodo dragons kept in Ragunan zoo was determined (100%). A summary of sex determination results are reported in Table 1. The analysis identified 30 females and 14 males (Figure 6). The position of bands, for females and males was between 800-900 bp. PCR amplification produced the 812 bp fragment specific to females, and the male-specific longer fragment.

**Surabaya Zoo.** Surabaya Zoo is located in Indonesia's East Java province, where an artificial
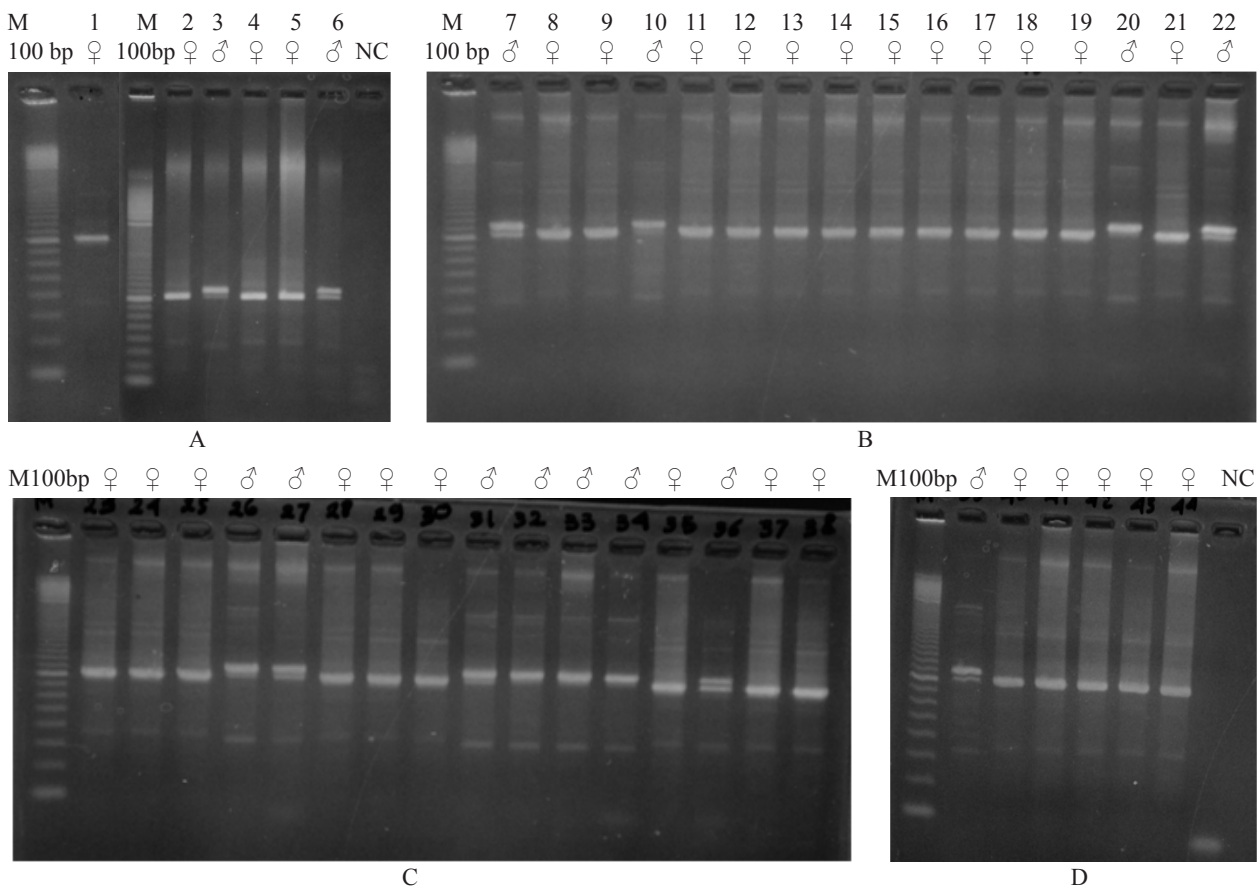


Figure 6A-D. Agarose electrophoresis of PCR sex-related DNA amplification products for Komodo dragons from Ragunan zoo, in Jakarta. M 100 bp = 100 bp DNA marker,  ♀: Female, ♂: Male, NC: negative control.
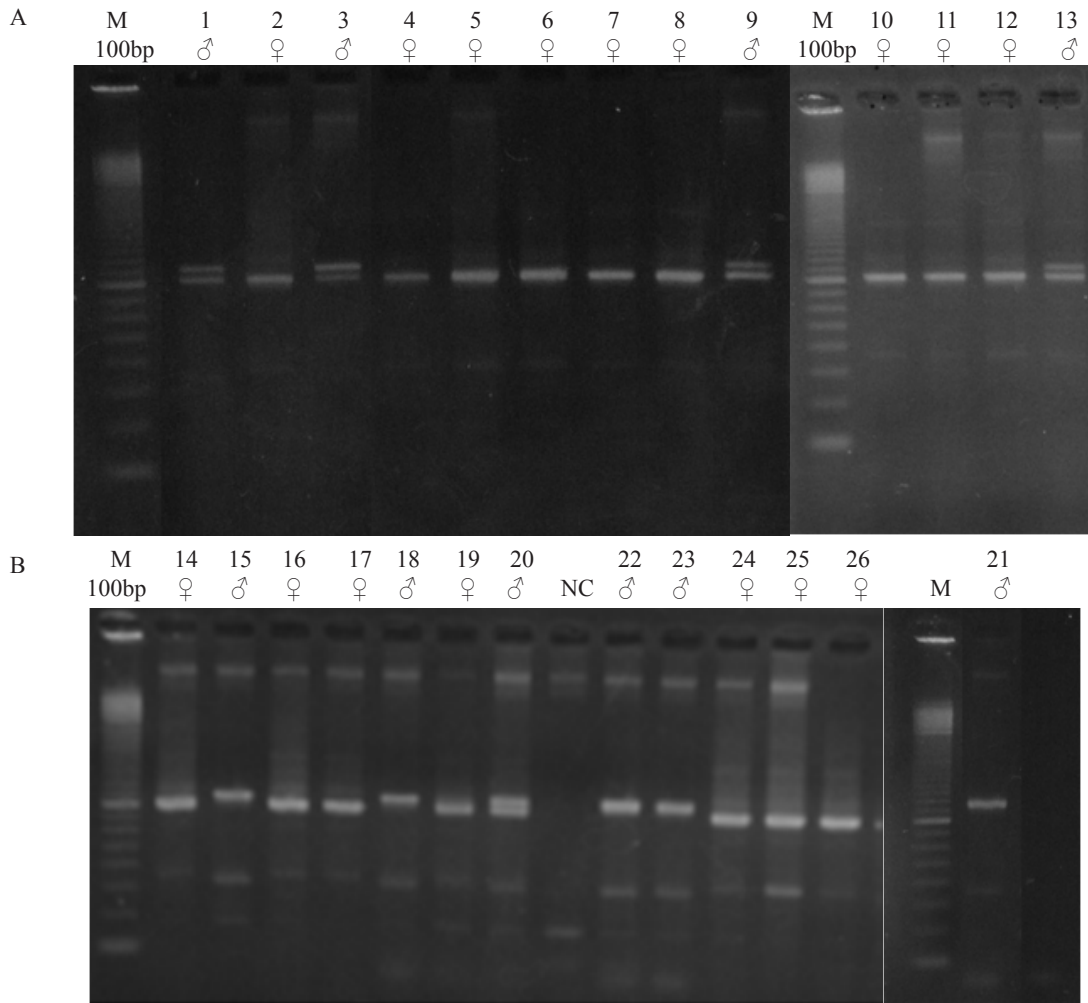
Figure 7A-B. Agarose electrophoresis of PCR sex-related DNA amplification products for Komodo dragons from Surabaya zoo, in Surabaya. M 100 bp: 100 bp DNA marker, ♀: Female, ♂: Male, NC: negative control.
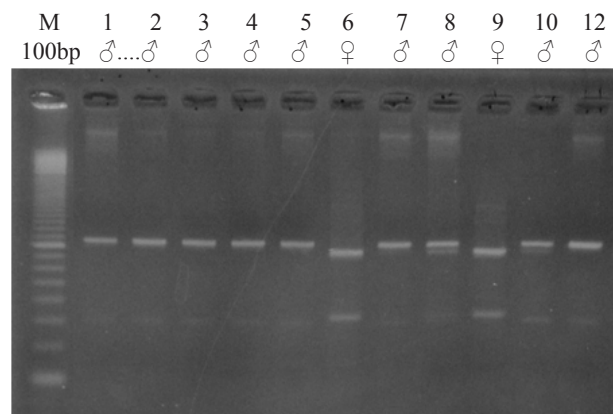


Figure 8. Agarose electrophoresis of PCR sex-related DNA amplification products for Komodo dragons from Gembira Loka zoo, in Yogyakarta. M 100 bp: 100 bp DNA marker, ♀: Female, ♂: Male.

breeding program is underway for Komodo dragons. The sex of 26 komodo dragons kept in Surabaya zoo was determined (100%). Summary of sex determination results are reported in Table 2. Analysis identified 16 females and 10 males (Figure 7). The position of bands for females and males was between 800-900 bp. PCR amplification produced the 812 bp fragment specific to females, and the male-specific longer fragment.

**Gembira Loka Zoo.** The Gembira loka zoo has a large collection of dragons distributed in several cages. The sex of 11 komodo dragons kept in Gembira loka determined (100%). Summary of sex determination results are reported in Table 3. Our results identifed 2 females and 9 males (Figure 8). The position of bands for females and males was between 800-900 bp. PCR amplification produced the 812 bp fragment specific to females, and the male-specific longer fragment.

## DISCUSSION

For animal marking, scientists have often relied upon external tags (e.g. ear and leg bands, dart tags, painted marks, scale clipping), which can also facilitate tracking of single individuals. Such tags are relatively cost-effective, but their external nature makes them susceptible to being lost, scratched,

rendered illegible upon recapture, and also subject to human error while being read (Gibbons & Andrews 2004). Branding, which has been used on such animals as elephants seals, often causes pain and has thus been viewed as inhumane (Galimbert & Sanvito 2000). PIT tags, by contrast, are placed under the skin and so are not as susceptible to damage or loss. They are read by computer scanners, avoiding human error; and they are safe for the animal as they rarely result in infection. Upon the death of the animal, tags can be removed and reused (Neubaum *et al.* 2005; Semmens *et al.* 2007).

The use of internal PIT tags began in the mid-1980s with scientists measuring fish movement, but has since been expanded for use in studying the movement of mammals, amphibians, reptiles, birds and invertebrates (Gibbon & Andrews 2004). PIT tags are also used by veterinarians for identifying household pets and for tracking livestock and zoo animals. Essentially, PIT tags act as a lifetime barcode for an individual animal, analogous to a social security number, and provided they can be scanned, they are as reliable as a fingerprint (Gibbon & Andrew 2004). In this study, a total of 81 Komodo dragons (from Ragunan zoo, Surabaya zoo, and Gembira loka zoo) were tagged as shown in Figure 2 using a micro-sized passive integrated transponder (PIT). Each transponder contains a 10 digit number which uniquely identifies the tagged animal (presented in Tables 1, 2, and 3), and which can be read using an appropriate scanner. After tagging, blood samples were taken individually for DNA sex determination.

**Snout-vent Length (SVL) is a Standard Measurement of Body Length.** Body length measurements were obtained from 81 Komodo dragons at Indonesian Zoos (Gembira loka, Surabaya, and Ragunan). According to Laver *et al.* (2012), Komodo dragons grow until they reach breeding age (about 7 years) after which the growth trajectories diverge, with females tending towards a smaller maximum size than males. Male Komodo dragons continue to grow until reach 160 cm in SVL size (a total length of 3 meters). On the other hand, females stop growing about 120 cm in SVL size (a total length of 2.2 to 2.5 meters). They also concluded that male Komodo dragons keep growing for competing (fighting) with other males stop growing and divert energy for nesting purposes.

Sex determination in Komodo dragons has proved difficult under field conditions ["*for reptile biologists*"]. According to Morris *et al.* 1998, measurements of circulating testosterone and two-dimensional ultrasound imaging together appear useful in the noninvasive determination of gender in juvenile *V. komodoensis* at just over two years of age. Ultrasound procedures have been developed for the Komodo dragon to visualize the internal gonads to determine the sex of the animal. However, this procedure requires an anaesthesia, which can cause unnecessary stress for the animal.

DNA-based sex determination is ideal for sexing animals–especially those that are young and morphologically indistinguishable–since it only requires a small sample such as a drop of blood or a single hair, thereby minimizing animal trauma (Vali & Doosti 2011). Recently, the molecular genetics laboratory of zoology at the Research Center for Biology-LIPI was able to use DNA markers to determine the sex of Komodo dragons. DNA markers linked to the heterogametic sex chromosome was found to provide an easy diagnostic tool for identifying gender.

This study aimed at identifying the gender of captive Komodo dragons by amplifying hetero chromosome sequences of DNA obtained from blood samples collected in the three largest Zoos in Indonesia. In the ideal sex determination test, the final product is an agarose gel showing one band for the homogametic sex and two for the heterogametic sex. In Komodo dragons, females are heterogametic (they have one Z chromosome and one W chromosome), and DNA samples are therefore characterized by alleles of different size, detected in the gel as two distinct bands. Males are homogametic (they have two Z chromosomes) and have alleles of the same size, so that PCR products will run at the same speed during electrophoresis. The two alleles will overlap each other in the gel, and will be therefore represented by a single band. This is achieved through the use of primers that anneal to both sex chromosomes but amplify PCR products of different lengths. Komodo dragons are like birds, males are ZZ and females are ZW. As with the Y chromosome, the W chromosome is a minor chromosome with few functions beyond sex determination. When breeding, a female Komodo dragon can pass on either a Z or W chromosome to her offspring, while a male Komodo dragons can only pass on a Z chromosome.

Interestingly, Watts *et al.* (2006) presented evidence that the Komodo dragon can also produce parthenogenetically (virgin birth). Chapman *et al.* (2007) similarly reported facultative parthenogenesis in hammer-head sharks (*Sphyrna tiburo*). Parthenogenesis is a form of asexual reproduction in which growth and development of embryos occur without fertilization. In both

cases, the parthenogenetic events result in progeny homozygous for all loci, suggesting automictic parthenogenesis with terminal fusion and no recombination (Lenk *et al*. 2005). Watts *et al*. (2006) used genetic fingerprinting to identify parthenogenetic offspring produced by two female Komodo dragons (*Varanus komodoensis*) that had been kept at separate institutions and isolated from males; one of these females subsequently produced additional offspring sexually.

A female Komodo dragon kept alone will have spontaneous genome duplication of an egg. The female Komodo dragon, however, uses the ZW sex determination system, so the egg could either have a Z chromosome and duplication to be a ZZ male, or it could have a W chromosome and duplication to become an unviable WW embryo. In practice, therefore, this means that female Komodo dragons that revert to parthenogenesis will always generate ZZ males. Philip (2007) stated that a single female and her male progeny could start a population, which would have both lowered genetic variation and genetic load. Parthenogenesis in captive populations could also result in unexpectedly high inbreeding, loss of genetic variation and changes in founder contribution.

Ideally a sex-specific chromosome sequence discovered in the Komodo dragon would have utility for other monitor lizards. Unfortunately, the ancient lineage of reptiles has resulted in a lack of conservation of modes of sex determination. It is conjectured that reptilian sex determination has evolved many times. According to Halverson and Spelmann (2002) reptiles exhibit different modes of sex determination including both temperature-dependent determination and chromosomal sex determination. In regards to chromosomal determination some genera have XY sex chromosome, some genera have ZW sex chromosome, and some genera have no recognizable sex chromosome. It is indicated that some reptile families show all three variations. Therefore, it is unlikely that a DNA marker found in one species or genus will be sufficiently conserved to be broadly applicable to other reptiles.

The sex of Komodo dragons from Surabaya zoo, Ragunan zoo and Gembira loka zoo was evaluated in this study by using a protocol developed by our researchers. This was necessary because PCR reactions we attempted, using the settings and original conditions described in Halverson and Spelmann (2002), gave inconsistent results. Our researchers tested different protocols, varying the annealing temperatures, number of cycles, and concentrations of reagents, in order to identify the best protocol for sex determination using our sample set. This specially developed procedure resulted in the amplification of band A in females and band C in males (Figure 5). However, for females, band B was not always visible in this study and, sometimes, a similar, but lighter band was also amplified in males. The sexing results produced by Halverson and Spelmann (2002) showed two clear bands in females, i.e. band A and B, and one band in males. In this study, PCR always resulted in the amplification of the male-specific longer fragment (Figures 6, 7, 8) and the 812 bp fragment specific for females. Thus length of fragment was indicative of sex, rather than the number of bands.

In this study, DNA technology has proved to be a viable technique for sex determination of Komodo dragons, and useful for captive breeding, an important aspect of species conservation.

## ACKNOWLEDGEMENT

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