

# Isolation and development of a molecular sex marker for *Bassiana duperreyi*, a lizard with XX/XY sex chromosomes and temperature-induced sex reversal

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**Abstract** Sex determination in the endemic Australian lizard *Bassiana duperreyi* (Scincidae) is influenced by sex chromosomes and incubation temperature, challenging the traditional dichotomy in reptilian sex determination. Analysis of those interactions requires sex chromosome markers to identify temperature-induced sex reversal. Here, we report the isolation of Y chromosome DNA sequence from *B. duperreyi* using amplified fragment length polymorphism PCR, the conversion of that sequence to a single-locus assay, and its combination with a single-copy nuclear gene (*C-mos*) to form a duplex PCR test for chromosomal sex. The accuracy of the assay was tested on an independent panel of individuals with known phenotypic sex. When used on offspring from field nests, our test identified the likely occurrence of a low rate of natural sex reversal in this species. This work represents the first report of Y chromosome sequence from a reptile and one of the few reptile sex tests.

**Keywords** GSD · TSD · AFLP · Reptile · Y chromosome

## Introduction

In most vertebrates, sex is determined by the segregation and inheritance of genes borne on sex chromosomes (genotypic sex determination, or GSD). Mammals have male heterogamety, where males are heterozygous with respect to a pair of differentiated sex chromosome homologues (the X and Y) and females are homozygous (two X chromosomes). Birds have an opposite system of female heterogamety, so females are designated as ZW and males as ZZ. Almost all mammals share a homologous pair of X and Y chromosomes (Graves 2006), and similarly, the same Z and W pair is likely to be homologous in all species of birds (Stiglec et al. 2007). It is currently believed that the avian and mammalian sex chromosomes evolved independently from different pairs of ancestral vertebrate autosomes (Nanda et al. 2000; Graves and Shetty 2001; Ezaz et al. 2006).

In contrast to the conservatism of mammals and birds, other classes of vertebrates exhibit remarkable diversity both in their fundamental mechanisms of sex determination and in their sex chromosomes. Both male and female heterogametic GSD are found amongst reptiles, amphibians and fish, but the system of heterogamety, and even the sex chromosome pair, varies amongst closely related taxa (Olmo 1986; Solari 1994). Moreover, many reptiles and some fish exhibit temperature-dependent sex determination (TSD), in which incubation temperature during embryonic development is the dominant influence on the outcome of sexual differentiation (Bull and Vogt 1979; Bull 1980; Conover and Kynard 1981; Valenzuela and Lance 2004). For fish at least, it is accepted that in some species both temperature and sex chromosomal genes interact to determine sex (Conover and Kynard 1981; Devlin and Nagahama 2002; Conover 2004). In reptiles, however, sex determination has

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been traditionally viewed as either genotypic or temperature-dependent, and so systems of interacting influences were considered non-existent (Bull 1980; Janzen and Paukstis 1991; Valenzuela et al. 2003). Recently, this view of reptilian sex determination has been challenged both theoretically (Sarre et al. 2004) and empirically (Shine et al. 2002; Quinn et al. 2007; Radder et al. 2008).

In species where sex chromosomes are present, but developmental temperature also affects sex determination, some individuals develop with a sex phenotype discordant to their genotypic (chromosomal) sex. Temperature-induced sex reversal is sometimes inferred from significantly skewed sex ratios at temperature extremes (e.g. Shine et al. 2002), but alternative explanations, such as differential mortality of the sexes (e.g. Burger and Zappalorti 1988), are possible. Explicitly demonstrating sex reversal in GSD species, and ascertaining which individuals are sex reversed, requires the unambiguous identification of chromosomal sex. In some cases, this may be achieved by cytogenetic methods (e.g. metaphase chromosome staining or banding, or comparative genomic hybridisation). Such approaches are often laborious and technically challenging where cell cultures are required, or simply unfeasible if the level of chromosome differentiation is too subtle for the resolution of the technique.

DNA sex markers provide an alternative means to identify chromosomal sex, but require the isolation of sequences unique to the heterogametic (Y/W) chromosome. Polymorphic X- or Z-linked markers can also be applied, but are less powerful than Y- or W-linked markers because sex is certain for heterozygotes only. For species with little genomic information available, sex markers are detected by molecular genetic approaches which screen the genome for sequences found only in the heterogametic sex. The size of the sex-specific fraction of the genome largely determines the difficulty of finding markers. Commonly applied methods include randomly amplified polymorphic DNA (RAPD) analysis (Welsh and McClelland 1990; Williams et al. 1990) and amplified fragment length polymorphism (AFLP) analysis (Vos et al. 1995). AFLP in particular has been successfully applied to detect sex markers in a diverse range of organisms, including plants (e.g. Reamon-Büttner et al. 1998; Lebel-Hardenack et al. 2002; Peil et al. 2003), birds (Griffiths and Orr 1999), fish (e.g. Griffiths et al. 2000; Ezaz et al. 2004; Felip et al. 2005), reptiles (Quinn et al. 2007), and amphibians (A. E. Quinn, unpublished data). Sex markers obtained using RAPD or AFLP approaches can be subsequently converted into more reliable single-locus PCR tests for the Y or W chromosome sequence that also include the co-amplification of positive control products of higher molecular weight (in both sexes) to eliminate the possibility of incorrect diagnosis of sex due to amplification failure (Griffiths 2000).

The three-lined skink *Bassiana duperreyi* is a montane scincid lizard endemic to southeastern Australia. Chromosome banding has revealed highly differentiated X and Y sex chromosomes (Donnellan 1985). Cyclical incubation temperatures ( $16.0 \pm 7.5^\circ\text{C}$ ), designed to mimic the conditions experienced in the coolest nests at the highest elevations of its range, cause sex reversal of some genotypically female (XX) embryos (Shine et al. 2002; Radder et al. 2008). This lizard is one of only two reptiles (to date) for which temperature-induced reversal of chromosomal sex has been clearly demonstrated through the application of DNA sex markers (Radder et al. 2008; see also Quinn et al. 2007). In this article, we report the isolation of the Y chromosome AFLP marker, and the development of the single-locus PCR sex test, used to demonstrate sex reversal in *B. duperreyi*.

## Materials and methods

### Animals

Adult female *B. duperreyi* were collected from the Brindabella Range ( $148^\circ50'E$ ,  $35^\circ21'S$ ) of southeastern Australia, one week before laying, and allowed to lay in captivity at the University of Sydney (see Radder et al. 2007). Eggs were incubated in moistened vermiculite (water potential  $-200$  kPa). Animals used for AFLP sex marker screening were incubated at a diel cycle of  $20 \pm 7.5^\circ\text{C}$ , and animals used for subsequent PCR analyses were incubated at a diel cycle of  $22 \pm 7.5^\circ\text{C}$  (see Radder et al. 2007). These thermal regimes simulate the conditions experienced in natural nests at low elevations, and produce no significant bias in sex ratio in laboratory incubation experiments (Shine et al. 2002; Radder et al. 2008).

Phenotypic sex of the hatchlings was assessed by hemipene eversion (Harlow 1996; Shine et al. 2002), and verified by histological examination of gonads at 8–10 weeks post-hatching ( $n = 7$ ) (Radder et al. 2007). Tail-tips (10 mm) of hatchlings were removed with a sterile blade and stored in 90% ethanol (at  $-20^\circ\text{C}$ ) prior to DNA extraction and analysis.

### DNA extraction

Tail-tip tissue (ca 5 mm) was macerated, added to 400  $\mu\text{l}$  tissue extraction buffer (40 mM Tris, 20 mM EDTA, 100 mM NaCl, pH 7.2) containing 20  $\mu\text{l}$  proteinase K (10 mg/ml) and 20  $\mu\text{l}$  sodium dodecyl sulphate solution (10% w/v), and incubated overnight at  $55^\circ\text{C}$ . Genomic DNA was purified from the digested tissue by one of two methods. For AFLP analysis, DNA was purified using standard phenol–chloroform procedures (Sambrook and

Russell 2001). For other PCR analysis, DNA was purified using a modified ‘salting-out’ protocol (Miller et al. 1988). Briefly, 150  $\mu$ l of  $\text{NH}_4$  acetate was added to the digested tissue, which was then chilled at  $-80^\circ\text{C}$  for 30 min, before pelleting the cellular debris by centrifugation and transferring the supernatant to a new tube. 1 ml ice-cold 100% ethanol was added to the supernatant, which was again chilled at  $-80^\circ\text{C}$  for 30 min, to precipitate the DNA, followed by centrifugation to pellet the DNA. In both the phenol-chloroform procedure and the salting-out procedure, the DNA pellet was exposed to two consecutive washes of 600  $\mu$ l 70% ethanol followed by centrifugation, then all traces of ethanol were removed and the pellet allowed to dry at RT, followed by resuspension of the purified DNA in TE buffer (10 mM Tris, 0.1 mM EDTA disodium, pH 7.5).

A duplicate DNA extraction was performed for some animals, using an alternative extraction method. A small amount of tail-tip tissue (2–3 mm) was macerated and added to 300  $\mu$ l of a 10% (w/v) solution of Chelex<sup>®</sup> 100 beads (Biorad), along with 10  $\mu$ l of proteinase K (10 mg/ml), and incubated overnight at  $55^\circ\text{C}$ . After digestion, the Chelex-extracted DNA samples were incubated at  $99^\circ\text{C}$  for 5 min, and then allowed to cool to RT. After any period of storage at  $4^\circ\text{C}$ , this ‘boiling’ step was repeated immediately prior to PCR amplification.

#### AFLP analysis

AFLP analyses were performed using the AFLP Analysis System I kit (Invitrogen) or according to the original protocol (Vos et al. 1995). All AFLP-PCRs were performed in a 20  $\mu$ l volume using 1.5U BioTaq<sup>™</sup> Red polymerase (Bio-line). Selective *EcoRI* +3 and +4 primers were labelled with WellRed fluorophores (Sigma), and the selective PCR products were separated by capillary electrophoresis on a CEQ8000 capillary sequencer (Beckman Coulter), and analysed using the associated Genetic Analysis System software. Appropriate negative controls were included for all stages of the AFLP analyses.

#### Sex-linked AFLP marker screening by bulk segregant analysis

Preselective AFLP products for 12 animals were combined into four monosex pools, comprising three males (two pools) or three females (two pools). The pools formed the templates for selective amplification reactions. Forty-four selective primer combinations were tested on the four pools (Table 1). AFLP profiles for the two male pools versus the two female pools were compared visually to detect candidate sex-linked markers, designated as fragments amplifying in one or both of the pools for a single sex only. Selective primer combinations generating candidate sex

**Table 1** Selective AFLP primer combinations screened in the search for a sex-linked marker in *B. dupeireyi*

<i>MseI</i> primer selective nucleotides	<i>EcoRI</i> primer selective nucleotides					
	AAC	AACT	AAG	ACG	ACGG	AGG
CAA	●	●	●	●	●	●
CAC	●	●	●	●	●	●
CAG				●	●	
CAT	●	●	●	●	●	●
CTA	●	●	●	●	●	●
CTC	●	●	●	●	*	●
CTG	●	●	●	●	●	●
CTT	●	●	●	●	●	●

Asterisk denotes selective primer combination which amplified sex-linked AFLP marker Bd199/207Y

markers were tested further by repeating the selective amplification but this time on the preselective products of the 12 animals as separate templates and then on an independent sample set comprising an additional six males and six females (increasing the total sample size to 24).

#### Cloning and sequencing of sex-linked AFLP marker

To facilitate the isolation and cloning of AFLP fragments of interest, the final annealing temperature of the selective PCR was increased from  $56$  to  $60^\circ\text{C}$ , further promoting specific amplification of those fragments. This optimised selective PCR was performed on three males with an increased reaction volume of 60  $\mu$ l, and the PCRs were purified using a High Pure PCR product purification kit (Roche). The purified AFLP products were cloned into a pGEM<sup>®</sup>-T Easy Vector (Promega) and transformed into chemically competent JM109 *E. coli* cells (Stratagene), according to the manufacturers’ instructions. Recombinant clones were identified by blue–white selection and plasmid DNA was isolated from 5 ml overnight cultures in LB medium (including 50  $\mu$ g/ml ampicillin) using the mini-prep procedure described in Sambrook and Russell (2001). Recombinant clones containing the AFLP fragment of interest were verified by PCR amplification with universal M13 forward and reverse primers and were sequenced using the Beckman Coulter Quick Sequencing kit. Sequencing reactions were run on a CEQ8000 capillary sequencer (Beckman Coulter) and sequences were analysed with the accompanying Genetic Analysis System software.

#### Conversion of AFLP marker into single-locus PCR sex assay

PCR primers were designed to amplify the entire genomic sequence of a putative AFLP sex marker (primers BdY-F1/

**Table 2** Primer information for the Y chromosome and *C-mos* primers used in the two variants of the PCR sexing assay

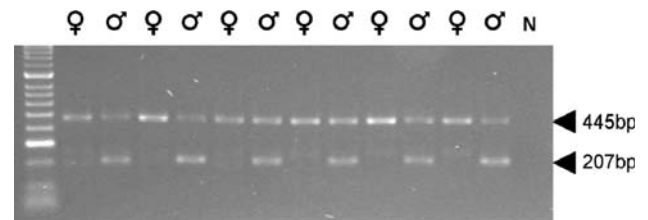
Primer	Sequence (5'-3')	Product
BdY-F1	GAATTCACGGGATGTTGCG	92 bp Y chromosome marker (with BdY-R1) 185 bp Y chromosome marker (with BdY-R2)
BdY-R1	CCATGCGTAACCACCACG	92 bp Y chromosome marker
BdY-R2	TTAACTCCCATGGGCATCAAC	185 bp Y chromosome marker
ScCmosF1	CAGAACTRAATGTGGCACGC	356 bp fragment of the single-copy nuclear gene <i>C-mos</i>
G74 <sup>a</sup>	TGAGCATCCAAAGTCTCCAATC	

<sup>a</sup> From Saint et al. (1998)

BdY-R2), or a nested fragment approximately half the length of the marker (primers BdY-F1/BdY-R1) (Table 2). To provide a positive control for PCR amplification, PCR primers were designed to amplify a fragment of the single-copy nuclear gene *C-mos* (from both sexes) that was larger than the putative sex marker sequence. To do so, a reptile-specific reverse primer (G74; Saint et al. 1998) was paired with a forward primer (ScCmosF1) designed to anneal to lygosomine skink *C-mos* sequences retrieved from Genbank (<http://www.ncbi.nlm.nih.gov/Genbank>).

The primers were combined into two duplex PCRs and optimised to the following conditions. PCR #1 co-amplified the entire AFLP-derived genomic fragment and the *C-mos* fragment, with the following final reaction conditions: 1.5 mM MgCl<sub>2</sub>, 200 μM each dNTP, 5 pmol of primers BdY-F1 and BdY-R2, 10 pmol of primers ScCmosF1 and G74, 0.5U of BioTaq™ Red DNA polymerase (Bioline) and 2 μl of the accompanying 10× PCR buffer were added to 20–50 ng of genomic DNA template in a reaction volume of 20 μl, and thermocycled (94°C for 2 min, then 40 cycles of 94°C for 30 s, 64.5°C for 30 s, and 72°C for 30 s, followed by 72°C for 5 min). PCR #2 co-amplified the shorter, nested section of the AFLP-derived fragment and the *C-mos* fragment, with the following final reaction conditions: 1.5 mM MgCl<sub>2</sub>, 200 μM each dNTP, 5 pmol of primers BdY-F1, BdY-R1, ScCmosF1 and G74, 0.5U of BioTaq™ Red DNA polymerase (Bioline) and 2 μl of the accompanying 10× PCR buffer were added to 20–50 ng of genomic DNA template in a reaction volume of 20 μl, and thermocycled in a touchdown PCR (94°C for 2 min, then 10 cycles of 94°C for 30 s, 66°C for 30 s decreasing by 0.5°C per cycle, and 72°C for 1 min, then 30 cycles of 94°C for 30 s, 61°C for 30 s, and 72°C for 1 min, followed by 72°C for 5 min).

The optimised duplex PCRs were tested on an independent sample set of animals for which sex reversal was not expected. PCR #1 was tested on genomic DNA extracted from 54 animals by the ‘salting-out’ technique (32 males, 22 females). PCR #2 was tested on 27 of those 54 animals (19 males, 8 females), using genomic DNA extracted by the salting-out method, or Chelex-extracted genomic DNA, as template.



**Fig. 1** Agarose gel showing selective AFLP products for six female and six male *B. duperreyi*. Products were amplified with the primer combination *EcoRI*-ACGG/*MseI*-CTC. Only two strong products are visible, a monomorphic 445 bp marker, and a 207 bp marker in males only (Bd207Y), representing Y chromosome sequence. *N* negative control reaction, lane 1 shows molecular weight marker

## Results

### Isolation of Y chromosome AFLP marker

Selective AFLP primer screening by bulk segregant analysis and subsequent amplification from individual templates revealed a single male-linked marker. The combination *EcoRI*-ACGG/*MseI*-CTC amplified a 207 bp AFLP fragment (designated Bd207Y) in five of six males, but in none of six females in the initial sample set. Although most of the selective primer combinations generated 20–50 intense AFLP peaks, this particular primer combination was atypical in that it produced only two intense peaks: a 445 bp fragment in both sexes, and the 207 bp fragment in males only. A strong AFLP product of 199 bp amplified in the male lacking Bd207Y (with equal fluorescence intensity to the Bd207Y marker in the other males), so it appeared likely this band represented a homologous fragment with a deletion in the sequence (see below). Testing this primer combination on another six of each sex (Fig. 1) expanded the sample to 24 individuals and revealed that Bd207Y amplified in 11 of 12 males (the 199 bp marker amplified in the 12th male), but in none of 12 females.

### Sequence of Y chromosome AFLP marker

The Bd207Y marker (two males) and the 199 bp marker were cloned and sequenced. The low complexity of the

**BdY-F1** →  
**GAATTC**ACGGGGATGTTGCGCATGGGGTGGATGTTTTGCATGGGGGATGTTGTACATGGGGGTTAG

ATGCGCATGCGTGGTGGTTACGCATGGGGGATATTGCGCATGGGGTTTTTTGTGCATGGGGATTG  
 CACCACCAATGCGTACC ← **BdY-R1**

TGCGCCTGGGGCTGTTGCGCTGTTGCGCATGGGGTGGATGCCCATGGGAGTTAA  
 CAACTACGGGTACCCTCAATT ← **BdY-R2**

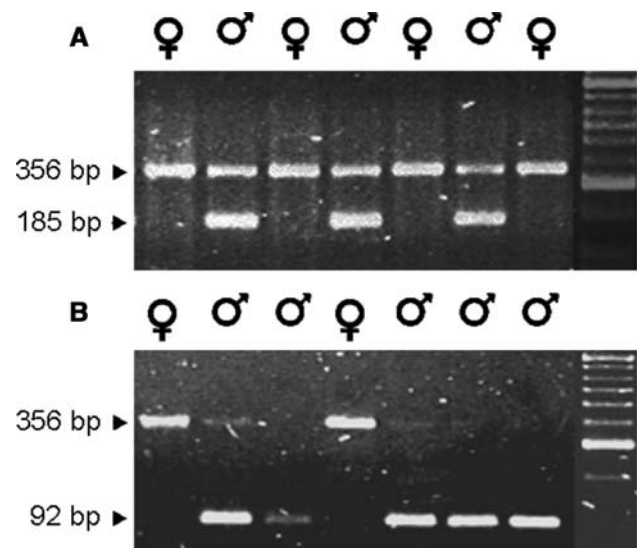
**Fig. 2** Genomic sequence (185 bp) of the AFLP marker Bd207Y (5′–3′). Boxes indicate the sequences and annealing sites for the forward and two alternative reverse primers used to amplify a Y chromosome marker (92 or 185 bp) in the PCR sex assay. Nucleotides in *bold* denote

the *EcoRI* and *MseI* restriction sites for the AFLP marker. Underlined nucleotides denote the 8 bp that were absent from a single male which amplified a 199 bp AFLP marker instead of the 207 bp marker

AFLP profile (only two intense amplification products) made cloning of the markers straightforward; PCR amplification indicated that all recombinant clones included an insert size of either ~200 or ~450 bp. The sequence of the 207 bp fragment was identical for the two males, and the sequence of the 199 bp fragment from the third male was identical to the 207 bp fragment, but with an 8 bp deletion, indicating that it represented the same Y chromosome locus. After accounting for the AFLP adaptor sequences, the Bd207Y marker represented 185 bp of genomic sequence from the Y chromosome (Accession no. EU259191; Fig. 2). Sequence analyses (blastn, blastx, ExPASy) of the 185 bp sequence detected no significant similarity to available database sequences.

#### PCR sex assay testing

PCR #1 (Fig. 3a) amplified a 185 bp fragment in 30 of 32 males (94%), but in only one of 22 females (4%), further confirming the AFLP-derived marker as Y chromosomal sequence (Table 3). Thus, 3 of 54 (5.6%) individuals showed discordance between their sex phenotype and their genotype according to PCR #1. When combined with the original 24 animals genotyped by AFLP, the overall level of discordance was 3 of 78 animals (3.8%). PCR #2 (Fig. 3b) amplified a 92 bp fragment in 17 of 19 males and one of eight females, which included the three animals (two males, one female) shown to be discordant by PCR #1 (Table 3). The genotype results were therefore identical for the 27 animals tested with both variants of the PCR sex test. Agarose gel electrophoresis indicated that the co-amplifying *C-mos* positive control product, amplified from both sexes, was around 350 bp (Fig. 3), which approximates the consensus size of 356 bp for this conserved section of the *C-mos* sequence for other species within the same scincid subfamily (Lygosominae: Accession numbers AF039462 to AF039466). The *C-mos* fragment failed to co-amplify, or co-amplified only very weakly, from Chelex-extracted DNA templates when the male-linked product amplified,



**Fig. 3** Agarose gels showing identification of chromosomal sex for *B. duperreyi* by single-locus PCR assays. **a** Duplex PCR amplification of 356 bp *C-mos* fragment (males and females) and 185 bp Y chromosome fragment (males only) from genomic DNA extracted by salting-out method. **b** Duplex PCR amplification of 356 bp *C-mos* fragment (females only) and 92 bp Y chromosome fragment (males only) from genomic DNA extracted by Chelex method. The Y chromosome fragment is amplified preferentially over the positive-control *C-mos* fragment for the Chelex-extracted DNA although occasionally both fragments are amplified in males. Lane on right-hand side shows molecular weight marker

but this occurred only very occasionally for DNA templates extracted by the salting-out method (Fig. 3).

#### Discussion

We succeeded in isolating a male-linked DNA sex marker for the lizard *B. duperreyi*, by screening amplified fragment length polymorphism (AFLP) markers amplified from pooled monosex templates (i.e. bulk segregant analysis). The heterogametic sex in this species is male (Donnellan 1985), so the isolation of a male sex marker (rather than a

**Table 3** Sex genotypes obtained for 78 *B. duperreyi* sexed by hemipene eversion

	Phenotype	<i>n</i>	Male-linked marker	Discordance
AFLP	Male	12	12	
	Female	12	0	
PCR #1 (*PCR #2)	Male	32 (19)*	30 (17)*	2 (6.3%)
	Female	22 (8)*	1 (1)*	1 (4.5%)
Totals	Male	44	42	2 (4.5%)
	Female	34	1	1 (2.9%)
Combined total for both sexes		78	43	3 (3.8%)

Putative chromosomal sex was established by amplification (or non-amplification) of the Y chromosome AFLP marker Bd209Y, or by amplification (or non-amplification) of a Y chromosome sequence in the duplex PCR sex assay

\* PCR #2 was applied to 27 of the 54 animals genotyped using PCR #1, giving equivalent genotype results

female sex marker) was expected. The lack of significant homology to available sequences indicated by BLAST search implies the sex marker represents novel Y chromosome sequence. The AFLP marker was subsequently converted into a single-locus PCR assay to diagnose chromosomal sex. Two variants of this test, one amplifying a 185 bp Y chromosome marker, and the other a nested 92 bp Y chromosome marker, produced equivalent genotype results. PCR genotyping was effective using two different methods of DNA extraction, including Chelex resin-based extraction. This provides a means of performing rapid and reliable DNA sex identification for *B. duperreyi*, as an alternative to the more expensive, time-consuming, and technically demanding approach of performing AFLP analysis on DNA extracted using commercial kits or by phenol–chloroform methods. The duplex PCR assay favoured amplification of the Y chromosome marker over the co-amplification of the larger *C-mos* positive control fragment. Indeed, for Chelex-extracted DNA templates, the Y chromosome marker out-competed amplification of the *C-mos* product to the extent that the positive control product failed to (visibly) amplify for most XY individuals. *C-mos* still amplified strongly from XX animals, thus serving its purpose as a positive control to prevent misdiagnosis of chromosomal sex in the event of complete PCR amplification failure (Griffiths 2000).

The small proportion of individuals discordant between sex phenotype and genotype observed for the PCR sex assay has four plausible explanations, each of which highlights a specific issue arising in sex marker development. First, it is possible that an error was made in phenotypic sexing of these animals. Given sufficient practice, hemipene eversion is a proven and reliable technique for sexing

skinks (Harlow 1996), and this phenotypic sexing method has been shown to be 100% congruent with gonadal histology for *B. duperreyi* (Radder et al. 2007). So we consider this to be an unlikely explanation. Second, the two “XX” males which failed to amplify the male sex marker could be explained by mutation in the primer sites (null amplification). Mutation rates for non-recombining regions of heterogametic sex chromosomes are typically much higher than for pseudoautosomal regions, so primer sites for Y chromosome markers may be particularly susceptible to point mutations, insertions or deletions. Whilst we cannot rule out this possibility definitively for the forward primer, the two variants of our PCR sex test employed distinct reverse primers (BdY-R1 and BdY-R2) and both failed to amplify the male sex marker in the discordant males. This greatly reduces the likelihood that mutations in the reverse primer site caused null amplification. Even if mutations in the forward primer site do cause occasional false identification of XX males, such mutations cannot account for amplification of the the Y chromosome marker in the single discordant female.

A third possible explanation is that all three discordant animals could be the result of meiotic recombination between the X and Y chromosomes at some point in the patrilineal ancestry of these animals, since a recombination event could have exchanged one (or both) of the primer sites on the Y chromosome with homologous sequence on the X chromosome. This would require the marker to be located within a pseudoautosomal region of the sex chromosomes. The Y chromosome of *B. duperreyi* is much smaller than the X chromosome, and C-banding of metaphase chromosomes indicates that the Y chromosome is largely heterochromatic (Donnellan 1985; T. Ezaz and A. E. Quinn, unpublished data), implying it is highly differentiated from the X chromosome. It seems unlikely then that the Y chromosome marker was isolated from a pseudoautosomal region, but it is certainly not impossible. For example, an AFLP-derived sequence isolated from the highly heterochromatic W chromosome of the bearded dragon lizard *Pogona vitticeps* (Agamidae) (Quinn et al. 2007) appears to be located in a homologous region of the Z and W sex chromosomes still undergoing occasional recombination (A. E. Quinn, unpublished data).

The final, and in our view, most likely explanation for the discordance is that the PCR genotype was a true indication of the chromosomal sex of the three animals in question, and they were therefore sex reversed. The eggs of these hatchlings were incubated under a thermal regime that produces sex ratios that do not depart significantly from 1:1 (Shine et al. 2002; Radder et al. 2007; 2008), but this implies only that this temperature regime does not over-ride chromosomal sex determination in the large majority of embryos. The degree of thermosensitivity in

sex determination may vary in *B. duperreyi*, such that a small proportion of individuals are sex reversed under these incubation conditions. There is some evidence that it is not only sex chromosomes and incubation temperature that can influence sex in this lizard because eggs that produce females are significantly larger than eggs that produce males, irrespective of incubation temperature (Shine et al. 2002). The PCR sex assay we have developed will enable investigation of the possibility that egg size can induce sex reversal in *B. duperreyi*. Chromosomal sex identification with this PCR assay has already shown that application of the steroid sex hormone  $17\beta$ -oestradiol to egg shells can induce male-to-female reversal in XY embryos, suggesting that yolk hormone levels may be able to naturally influence sex determination (Radder et al. 2008). The lability in sex determination clearly evident in this lizard supports the possibility that the discordant animals were rare instances of sex reversal under ‘control’ incubation conditions.

Reports of sex markers, and indeed sex chromosome sequences, are scarce for reptiles. Sequence from the W chromosome of the largest extant species of lizard, the Komodo dragon (*Varanus komodoensis*, Varanidae), was identified by RAPD analysis and converted into a PCR sex test (Halverson and Spelman 2002), which also identified sex in the Australian varanid *V. rosenbergi* (W. Smith, personal communication). More recently, a W chromosome AFLP marker sequence was isolated (Quinn et al. 2007) and converted into a single-locus PCR sex assay for the Australian agamid lizard *Pogona vitticeps* (Agamidae) and shown to diagnose chromosomal sex for other species within the *Pogona* genus (A. E. Quinn, unpublished data). The only other sex-linked marker isolated for a skink is an X chromosome microsatellite locus (Tr4.11) in another Australian species, the shingleback lizard *Tiliqua rugosa* (Cooper et al. 1997), and this locus was subsequently shown to also identify heterozygotes as females in the related Cunningham’s skink (*Egernia cunninghami*) (Stow et al. 2001). To the best of our knowledge, the present study is the first report of a Y chromosome sequence for a reptile.

In contrast to mammals and birds, molecular sex markers developed for reptiles, amphibians and fish are unlikely to have broad taxonomic applicability because of the evolutionary lability of sex chromosomes and sex-determining mechanisms within these vertebrate groups. Genes and non-coding sequences within the sex-specific fraction of the heterogametic chromosome, the target of searches for sex markers, are presumably lost (or exchanged) every time that a sex chromosome pair is replaced by another, and this may also occur whenever a chromosomal mechanism of sex determination is replaced by environmental sex determination. For example, the PCR test for W chromosome sequence for the agamid lizard *P. vitticeps* is ineffective as a diagnostic test for sex beyond the genus (A. E. Quinn,

unpublished data), and a PCR test developed for a Y chromosome AFLP marker in the three-spined stickleback fish (*Gasterosteus aculeatus*, Gasterosteidae) is ineffective for congeneric species (Griffiths et al. 2000). For most reptiles, unless markers happen to be available for related species with highly conserved sex chromosomes, sex markers will need to be developed de novo for species of interest. An important exception may be snakes; conserved sex chromosome sequences may be universally present in this reptile group because the Z and W chromosome pair is conserved (Matsubara et al. 2006), albeit with considerable variation in the degree of W chromosome degeneration between families (Ohno 1967). This study, in conjunction with the recent isolation of W chromosome sequence from an agamid lizard (Quinn et al. 2007), shows that AFLP is a highly effective approach for detecting sex markers in reptiles, provided there is some differentiation of the sex chromosomes.

The Y chromosome sequence isolated from *B. duperreyi* awaits testing of its homology and sex-linkage in populations covering the geographical distribution of this species in southeastern Australia, and also in related species of skinks. Notably, the heteromorphic sex chromosome pair in *B. duperreyi* (pair 7) is also the heteromorphic sex chromosome pair for the two other species in the genus, and also in a further 28 species spanning nine other genera of skinks within the subfamily Lygosominae (Hardy 1979; Donnellan 1985, 1991; Hutchinson and Donnellan 1992). Given this apparent conservation of the sex chromosome pair, the PCR sexing test developed for *B. duperreyi* could prove to be useful for a number of species.

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