

Z and W sex chromosomes in the cane toad (*Bufo marinus*)

John Abramyan · Tariq Ezaz ·
Jennifer A. Marshall Graves · Peter Koopman

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Abstract The cane toad (*Bufo marinus*) is one of the most notorious animal pests encountered in Australia. Members of the genus *Bufo* historically have been regarded as having genotypic sex determination with male homogamety/female heterogamety. Nevertheless, as with many toads, karyotypic analyses of the cane toad have so far failed to identify heteromorphic sex chromosomes. In this study, we used comparative genomic hybridization, reverse fluorescence staining, C-banding, and morphometric analyses of chromosomes to characterize sex chromosome dimorphism in *B. marinus*. We found that females consistently had a length dimorphism associated with a nucleolus organizer region (NOR) on one of the

chromosome 7 pair. A strong signal over the longer NOR in females, and the absence of a signal in males indicated sex-specific DNA sequences. All females were heterozygous and all males homozygous, indicating a ZZ/ZW sex chromosomal system. Our study confirms the existence of sex chromosomes in this species. The ability to reliably identify genotypic sex of cane toads will be of value in monitoring and control efforts in Australia and abroad.

Keywords Cane toad · Sex chromosomes · Nucleolus organizer region · Chromomycin A3 · CGH · *Bufo marinus*

Abbreviations

DAPI	4',6-diamidino-2-phenylindole
NOR	Nucleolus organizer region
GSD	Genotypic sex determination
CGH	Comparative genomic hybridization
SSC	Standard saline citrate
CMA3	Chromomycin A3
dDNA	Genomic deoxyribonucleic acid
rDNA	Ribosomal deoxyribonucleic acid
dUTP	2'-Deoxyuridine 5'-Triphosphate

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J. Abramyan · P. Koopman (✉)
Institute for Molecular Bioscience,
The University of Queensland,
Brisbane QLD 4072, Australia
e-mail: p.koopman@imb.uq.edu.au

T. Ezaz · J. A. M. Graves
Comparative Genomics Group, Research School
of Biology, Australian National University,
Canberra ACT 0200, Australia

T. Ezaz
Institute for Applied Ecology, University of Canberra,
Bruce ACT 2616, Australia

Introduction

The cane toad (*Bufo marinus*) is an iconic invasive species known for its ability to establish quickly and thrive in novel ecosystems. Cane toads were intro-

duced to Australia in 1935 in an effort to control the spread of sugarcane beetle (Easteal 1981). Since that time, the cane toad has become one of the most notorious animal pests in Australia. An estimated 200 million cane toads now occupy an area more than five times the size of the United Kingdom, with a migration front advancing at 65–100 km each year, and recent reports indicate that their potential range is much larger than previously estimated (Urban et al. 2007). Toxicity, voracity, and lack of natural predators in Australia are the key elements of cane toad success, and of the threat posed by this species in Australian ecosystems. Not surprisingly, the cane toad has become the subject of intense public and scientific interest. Despite this, we know little about many basic aspects of cane toad biology that are needed for effective control.

All amphibians studied to date have a genotypic sex determination (GSD) mechanism (Hayes 1998; Wallace et al. 1999; Eggert 2004). GSD is a common sex-determining mechanism in vertebrates, having manifested itself in a variety of different genetic systems within mammals, snakes, birds, fishes, and amphibians (Valenzuela 2008). By definition, a GSD system requires a genetic, hence, chromosomal difference between males and females. GSD systems can involve either female homogamety/male heterogamety (XX/XY systems, as in mammals) or female heterogamety/male homogamety (ZZ/ZW systems, as in birds).

Although the entire class (Amphibia) is considered to utilize GSD, reports of sex chromosomes in amphibian lineages have been sparse, even though more than 25% of known species, including all extant genera within the order Anura (frogs and toads), have been karyotyped (Duellman and Trueb 1994; Hayes 1998; Schmid and Steinlein 2001; Nakamura 2009). Moreover, cytologically recognizable sex chromosomes, when present, are often not heteromorphic (Schmid and Steinlein 2001). Despite cases of both ZZ/ZW and XX/XY species having been identified through a variety of cytological, molecular, and breeding experiments, many anuran genera apparently lack heteromorphic sex chromosomes (Hillis and Green 1990; Hayes 1998). Minimal differentiation may not necessarily suggest a recent evolution of sex chromosomes however, as is exemplified in various ancient bird and snake lineages which show homomorphism in their Z and W chromosomes (Bergero and Charlesworth 2009).

Like other GSD vertebrates, amphibians are thought to carry genes on their sex chromosomes

which control sex steroid production both in males and females (Nakamura 2009). Also, heterogametic sex in non-amniote vertebrates is sometimes not fixed permanently, having been observed to have switched relatively recently in evolutionary time (Miura 2007). For instance, in *Rana rugosa*, the sex-determining system has changed twice, independently from XX/XY to ZZ/ZW (Ogata et al. 2008): the Z chromosome is homologous to Y while the W is homologous to X, with multiple rearrangements causing the switch in heterogamety (Miura 2007; Nakamura 2009).

The relatively few sex chromosome systems that were identified in Anurans seem to be diverse and lineage-specific. Some frog species in the genus *Leiopelma*, are completely aberrant, evidently using 00/0W system (Green 1988), while still some other species such as *R. rugosa* have population-specific female or male heterogamety (Miura et al. 1998). The large amount of variation within known groups suggests that sex chromosomes in anuran lineages have arisen independently (Uno et al. 2008). Unfortunately, this diversity in sex-determining mechanisms renders comparative prediction of chromosomal composition ineffective, so that each group must be studied independently.

Breeding studies carried out in the early 1900s were the first to suggest that members of the genus *Bufo* utilize GSD. However, these studies disagreed on the question of male or female heterogamety. Hillis and Green (1990) list bufonids as having retained the putative ancestral ZZ/ZW system, largely due to the indirect evidence collected by one of the aforementioned early studies. More than 55 *Bufo* species have been karyotyped, with a typical organization of $2n=22$ chromosomes throughout the Americas, Eurasia and Africa (with the exception of the African *B. regularis* group, which has $2n=20$).

Cytological studies of *B. marinus* are relatively abundant in the literature, due to the wide geographic range and accessibility of this species. Previous studies of cane toad chromosomes have revealed a characteristic bufonid karyotype of $2n=22$ chromosomes (Ullerich 1967; Cole et al. 1968; Volpe and Gebhardt 1968). These studies have not reported heteromorphic sex chromosomes in either sex, despite the wide variety of cytological methods used, including quinacrine staining (Schmid 1978; Schmid and de Almeida 1988), silver staining (Schmid 1980; Baldissera et al. 1999), chromomycin A3 (Schmid 1980), Giemsa

staining (Schmid and de Almeida 1988; Baldissera et al. 1999), C-banding (Schmid and de Almeida 1988), mithramycin (Schmid and de Almeida 1988), and Giemsa staining after restriction endonuclease treatments (Schmid and de Almeida 1988).

NORs are found on the chromosomes of most species of *Bufo*, located on different chromosomes in different species, consistent with *Bufo* phylogeny (Bogart 1972). In the case of *B. marinus*, a NOR is present on both copies of the short arm of chromosome 7 (Ullerich 1967; Cole et al. 1968; Volpe and Gebhardt 1968; Miller and Brown 1969; Schmid 1978; Baldissera et al. 1999). Bogart (1972) mentions a secondary constriction on the short arm of chromosome 5, but this finding has not been corroborated by other workers and was not observed in the present study. The NOR location on chromosome 7 is thought to be one of two ancestral NOR positions in bufonids, the other being chromosome 1. The localization of an NOR on chromosome 7, coupled with the putative ancestral chromosome number $2n=22$, highlights the evolutionary conservation of the *B. marinus* karyotype.

The principal aim of this study was to determine whether sex chromosomes exist in *B. marinus*, and if so, what type of system is used to determine sex in this species. Recent advances in molecular karyotyping techniques, such as comparative genome hybridization (CGH), have allowed morphologically similar chromosomes to be searched for sex-specific sequences that identify “cryptic” sex chromosomes in various species (Traut et al. 1999; Ezaz et al. 2005; Ezaz et al. 2006; Kawai et al. 2007). Using CGH, coupled with more traditional karyotyping and staining methods, we have identified Z and W sex chromosomes in *B. marinus*. Our findings have practical implications in future research in establishing monosex populations of cane toad and therefore represent a significant first step in genetic bio-control of cane toad in Australia and other unique ecosystems where cane toads have become established.

Materials and methods

Animals

Adult *B. marinus* were collected from Townsville, Queensland and the grounds of The University of Queensland, St. Lucia campus, Brisbane, Australia.

Blood was then collected via cardiac puncture with a heparinized (heparin: Sigma, St Louis, MO, USA) 25-gage needle attached to a 1 to 2-ml disposable syringe, after euthanasia. Specimens were initially sexed upon capture by their skin texture and other morphological characters. Sex was then verified surgically after euthanasia.

Blood culture and chromosome preparations

Mitotic metaphase chromosome spreads of *B. marinus* were prepared from short-term culture of whole blood. Approximately 100–200 μ l was cultured in 2 ml of Dulbecco's Modified Eagle's Medium (GIBCO) supplemented with 10% fetal bovine serum (JRH Biosciences), 1 mg/ml L-Glutamine (Sigma), 10 μ g/ml gentamycin (Multicell), 100 units/ml penicillin (Multicell), 100 μ g/ml Streptomycin (Multicell), and 3% Phytohemagglutinin M (Sigma). Cultures were incubated at 30° for 96–120 h in a 5% CO₂-incubator. At 6 and 4 h prior to harvesting, 35 μ g/ml 5'-bromo-2'-deoxyuridine (Sigma) and 75 ng/ml colcemid (Roche) were added to the culture, respectively. Metaphase chromosomes were then harvested and fixed in 3:1 methanol: acetic acid following the standard protocol (Verma and Babu 1995). The cell suspension was dropped onto a glass slide and air-dried. For DAPI (4',6-diamidino-2-phenylindole) staining, slides were mounted with anti-fade medium Vectashield (Vector Laboratories) containing 1.5 μ g/ml DAPI.

DNA extraction and labeling

Total genomic DNA (gDNA) was extracted from whole blood following the protocol of Ezaz et al. (2005). Female gDNA was labeled with SpectrumGreen-dUTP (Vysis, Inc.) and male gDNA was labeled with SpectrumRed-dUTP (Vysis, Inc.) by nick translation.

Comparative genomic hybridization

CGH was performed according to Ezaz et al. (2005). Briefly, slides were denatured for 1.5–2 min at 70°C in 70% formamide with 2X SSC, dehydrated through an ethanol series, air-dried and kept at 37°C until probe hybridization. For each slide containing one drop of cell solution, 250–500 ng of SpectrumGreen-labeled female and SpectrumRed-labeled male

gDNA was co-precipitated with 5–10 µg boiled gDNA from the homogametic sex (as a competitor), and 20 µl glycogen (as a carrier). Since we worked under the assumption of an unknown homogametic sex, male and female DNA was used reciprocally as a competitor.

The co-precipitated probe DNA was resuspended in 20 µl hybridization buffer (50% formamide, 10% dextran sulfate, 2X SSC, 40 mmol/L sodium phosphate pH7.0, and 1X Denhardt's solution). The hybridization mixture was denatured at 70°C for 10 min, rapidly chilled for 2 min before 18 µl of probe mixture was placed on a single drop on a slide and hybridized at 37°C in a humid chamber for 3 days. Slides were washed once at 60±1°C in 4X SSC, 0.3% Tween 20 for 2 min followed by another wash at room temperature in 2X SSC with 0.1% Tween 20. Slides were then air-dried and mounted with anti-fade medium Vectashield (Vector Laboratories) containing 1.5 µg/ml DAPI. Gray scale image were captured using a fluorescence microscope, followed by superimposition of the source images into a color image.

Reverse fluorescence staining and C-banding

Reverse fluorescence chromosome staining was performed as described by Schweizer (1976) with minor modifications. Briefly, 200–300 µl of 0.5 mg/ml chromomycin A3 (CMA3) solution (in McIlvaine's buffer, pH7.0) was placed on slides and covered with a cover slip. Slides were incubated at room temperature 2–3 h, then rinsed in distilled water and air dried. After drying, slides were placed in 1 mg/ml DAPI solution for 2 min before being air dried and mounted with anti-fade medium Vectashield (Vector Laboratories). The slides were examined under a fluorescence microscope.

C-banding was performed as described by Ezaz et al. (2005). Briefly, slides were aged at room temperature for 2–3 days, soaked in 0.2 N HCl for 45 min, then treated with Ba(OH)₂ (Sigma) for 1.5 min at 50°C and finally washed in 2X SSC for 1 h at 60°C. Slides were then rinsed in distilled water and stained with 4% Giemsa in 0.1 M phosphate buffer for 10–20 min at room temperature. Slides were then rinsed in distilled water, air dried, and mounted with DPX mounting medium (Aldrich, Milwaukee, WI, USA).

Morphometric and statistical analysis of chromosomes

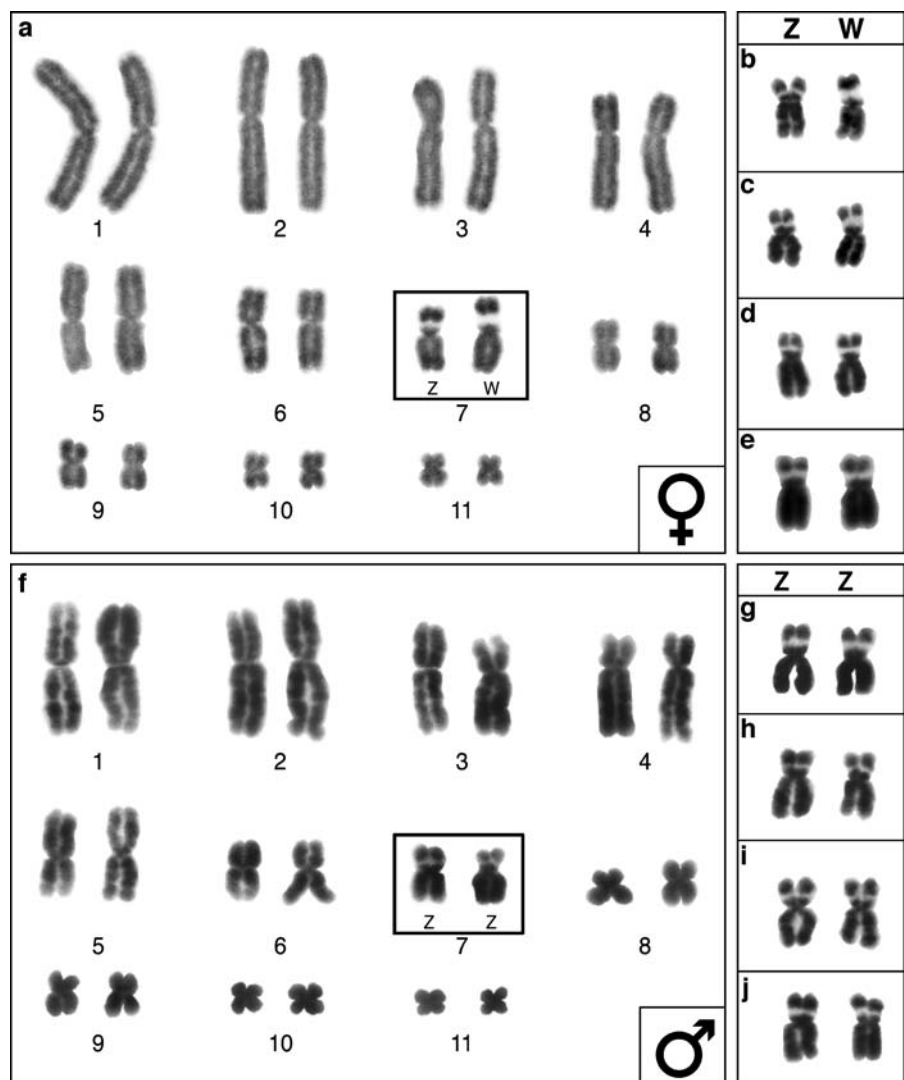
Quantitative analysis was performed by measuring the short arm and long arm of each chromosome using Adobe Photoshop® CS3 (Adobe Systems Inc., San Jose, CA, USA). One homologue of each somatic chromosome was analyzed from metaphase chromosomes of three male and three female specimens. Arm lengths were averaged from all specimens and ratios calculated accordingly. Sex chromosome measurements were performed on nine females and five males. Statistical significance values for differences between the long arms/short arm ratio of chromosome 7 homologues of males and females were calculated using an unpaired *t* test in Prism 5.0a (Graphpad Software, San Diego, CA, USA). Additionally, the relative total length, long arm length, and short arm length of each chromosome were calculated as fraction of the haploid complement of the male karyotype. Relative length percentages were rounded up to the nearest one hundredth.

Results

Karyotype of *B. marinus*

Mitotic karyotypes of 11 female and 11 male *B. marinus* were analyzed using DAPI staining of cultured cells. Karyotypes of both sexes were virtually identical, with a diploid chromosome complement of 2n=22. Chromosome pairs are numbered 1–11 in order of decreasing size, with five large, four medium, and two small chromosomes pairs (Fig. 1). Every pair was readily identifiable by size and morphology. The five largest consisted of two metacentric (1 and 5) and three sub-metacentric chromosomes (2, 3, and 4). Of the four medium-sized chromosomes, 6 and 7 were sub-metacentric, whereas 8 and 9 were metacentric. Of the two smallest chromosomes, 10 was metacentric and 11 was sub-metacentric (Table 1, Fig. 1). Furthermore, we observed differences in morphology as well as size between the chromosomes 7 pair in all female specimens. The NOR on one homologue of chromosome 7 in females was generally larger than the other (Table 1, Fig. 1a–e). Conversely, the chromosome 7 pair in all males is homomorphic and similar to the smaller homologue of the female

Fig. 1 DAPI stained inverted metaphase karyotypes from female (**a**) and male (**f**) *Bufo marinus* ($2n=22$). The nucleolus organizer region (NOR) is clearly visible on the short arm of chromosome 7 in both sexes (marked inside black box). Panels **b–e** represent chromosome 7 homologues from additional females. Panels **g–j** represent chromosome 7 homologues from four additional males



chromosome 7 pair (Table 1, Fig. 1f–j). The degree of difference in the NOR size of females varied intra-specifically; many specimens exhibited larger size difference than others between homologous NORs (Fig. 1b–c and d–e, respectively). Morphometric analysis, however, revealed a significant difference between the long arm to short arm ratio of female chromosome 7 homologues ($p=.0143$), while the male homologues showed no significant difference (Table 1).

Reverse fluorescence staining and C-banding

Using DAPI staining, the extended NOR region in the W chromosome was found to be DAPI faint,

suggesting a GC-rich region. Therefore, we decided to perform CMA3 staining (which stains GC rich regions commonly associated with heterochromatin), in order to analyze the composition of the chromosomes and the associated NORs. The chromosomes of three males and three females were analyzed in this study. The NORs of males and females generally stained brighter than other chromosomal regions (Fig. 2). Also, the long NOR on the W chromosome was consistently brighter than the short NOR of the Z chromosome in all female metaphases examined, even in specimens where the NOR size difference is minimal (Fig. 2f–h). The short NORs on both Z chromosomes in the males showed similar signal intensity (Fig. 2).

Table 1 *B. marinus* karyo-type arm ratios and percent haploid complement

Number		LA/SA±SEM	Type	% of male haploid complement		
				Total length	LA	SA
1		1.03±.003	m	16.42	8.32	8.10
2		1.40±.036	sm	16.14	9.42	6.72
3		1.53±.062	sm	13.69	8.28	5.41
4		1.98±.057	sm	12.46	8.25	4.21
5		1.01±.008	m	11.02	5.55	5.48
6		1.37±.029	sm	8.04	4.65	3.40
7	Fw	1.19±.049*	sm	6.38	3.23	2.37
	Fz	1.38±.044	sm	5.60	3.45	2.93
	Mz	1.32±.057	sm	6.10	3.43	2.66
8		1.18±.036	m	5.04	2.72	2.32
9		1.04±.019	m	4.78	2.43	2.34
10		1.05±.032	m	3.46	1.77	1.70
11		1.21±.106	sm	2.85	1.56	1.29

m metacentric ($r=1.0\text{--}1.18$), *sm* submetacentric ($r=1.19\text{--}2.0$), *LA* long arm, *SA* short arm, *SEM* standard error of mean, *Fw* female W, *Fz* female Z, *Mz* male Z

* $P<.05$

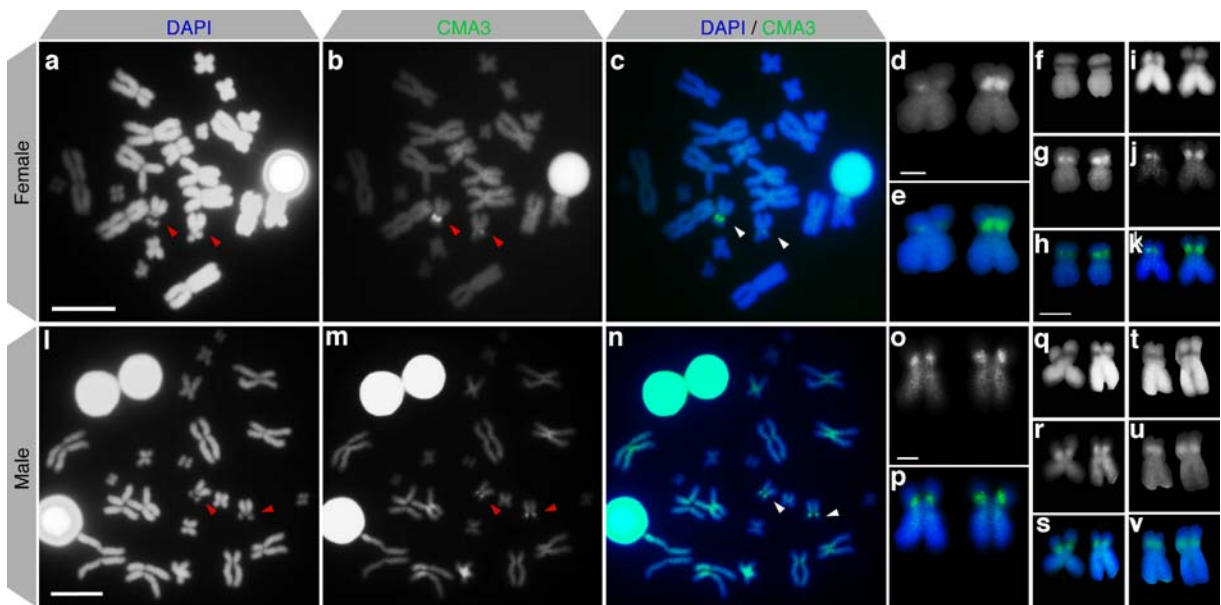


Fig. 2 Reverse fluorescence staining of female (top panels a–k) and male (bottom panels l–v) chromosomes using chromomycin A3 (CMA3). Panels a and l depict DAPI stained metaphase chromosomes. Panels b and m depict CMA3 stained metaphase chromosomes. Panels c and n depict overlaid images of DAPI and CMA3 stained chromosome spreads. Panels d and o depict CMA3 stained female and male chromosome 7 pair, respectively, while panels e and p depict overlaid DAPI and CMA3 stained female and male chromosome 7 pair, respectively. Panels f and i, g and j, and h and k

depict DAPI, CMA3, and overlaid images, respectively. f–k represent chromosome 7 pairs from two female specimens independent from that of panels a–e. Panels q and t, r and u, and s and v depict DAPI, CMA3, and overlaid images, respectively. Panels q–v represent chromosome 7 pairs from two independent male specimens from that of panels l–p. Arrowheads indicate nucleolus organizer regions (NORs) on chromosome 7 homologues. Scale bar indicate 10 μm (a, b, c, l, m, n), 2.5 μm (d, e, o, p), and 5 μm (f–k, q–v)

After identifying a putative GC-rich region using CMA3 staining, we next performed C-banding in order to obtain further evidence of the constitution of the putative NORs. Two female (Fig. 3) and two male (not shown) specimens were examined using C-banding, with 4–5 metaphase spreads examined from each. Centromeric bands were identified on all 22 chromosomes (Fig. 3a). The only non-centromeric banding was restricted to the short arm of the chromosome 7 pair and colocalized with the CMA3 signal within the putative NOR (Figs. 2, 3b,c)

Comparative genomic hybridization

Having established a potential female-specific chromosome, we next performed CGH to confirm and locate any sex-specific chromosomal regions, using chromosome spreads from three females and three males. Metaphase karyotypes (3–5) were analyzed from each individual. A signal was observed on only one of the two chromosome 7s in all observed cells from each of three females hybridized with male genomic DNA probe (Fig. 4). This female-specific signal was consistently observed on the chromosome 7 homologue with the extended NOR. The reciprocal hybridization of the male chromosome spreads with female genomic DNA probe resulted in no specific signal. Therefore, females possess, on one of the two members of the chromosome 7 pair, a region not found in males. The result obtained from CGH identifies the female-specific chromosome as the W,

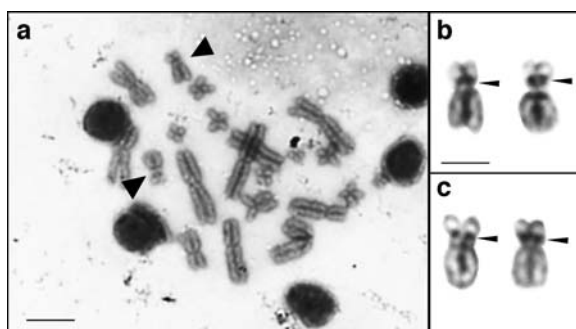


Fig. 3 C-banded karyotype of female *B. marinus*. Panel **a** represents metaphase chromosome spread from a female individual. Panels **b** and **c** represent the chromosome 7 pair from panel **a** and that of a second female, respectively. Arrowheads indicate the chromosome 7 homologues in panel **a** and nucleolus organizer regions (NORs) on chromosome 7 homologues in panels **b** and **c**. Scale bar indicates 10 μm (**a**) and 5 μm (**b,c**)

confirming the existence of a ZZ/ZW female heterogamety/male homogamety sex-determining mechanism in this species (Fig. 4).

Discussion

In this study, we demonstrated using various cytogenetic techniques that *B. marinus* has distinguishable sex chromosomes and female heterogamety involving chromosome pair 7, establishing that a ZZ/ZW sex-determining system operates in this species. This is the first time sex chromosomes that have been identified in any New World Bufonid.

Based on DAPI-stained mitotic karyotype analysis of *B. marinus*, we were able to establish a diploid chromosome complement of $2n=22$, in agreement with previous studies (Ullerich 1967; Cole et al. 1968; Volpe and Gebhardt 1968; Miller and Brown 1969; Schmid 1978; Baldissera et al. 1999). Of the entire karyotype, the chromosome 7 pair in this species was found to have a significant size variation in female specimens, and a female-specific signal was consistently observed on the longer member of the pair using both CGH and CMA3 staining. The difference is consistently associated with the chromosomal region previously identified by other investigators as an NOR on the chromosome 7 using silver staining (Schmid 1980; Baldissera et al. 1999), rRNA-DNA hybridization (Miller and Brown 1969), C-banding (Schmid 1978), and mithramycin staining (Schmid 1982). Thus, the male and female karyotypes of *B. marinus* can be distinguished even without CGH and CMA3 staining, on the basis of NOR morphology. In females, but not males, NOR length is dimorphic on the chromosome 7 pair. The female-specific region on the chromosome with the longer NOR identifies this chromosome 7 as a W chromosome.

In a previous study, the difference in the length of homologous NORs in cane toads has been attributed to heterochromatinization (Volpe and Gebhardt 1968; Schmid 1980). We were able to support the heterochromatin hypothesis by reverse fluorescence staining and C-banding of male and female chromosomes. C-banded chromosomes showed minimal staining with the only signal observed in the centromeres and NOR, suggestive of high GC content in the stained regions. Brighter CMA3 fluorescence was consistently observed on the longer NOR, implying that

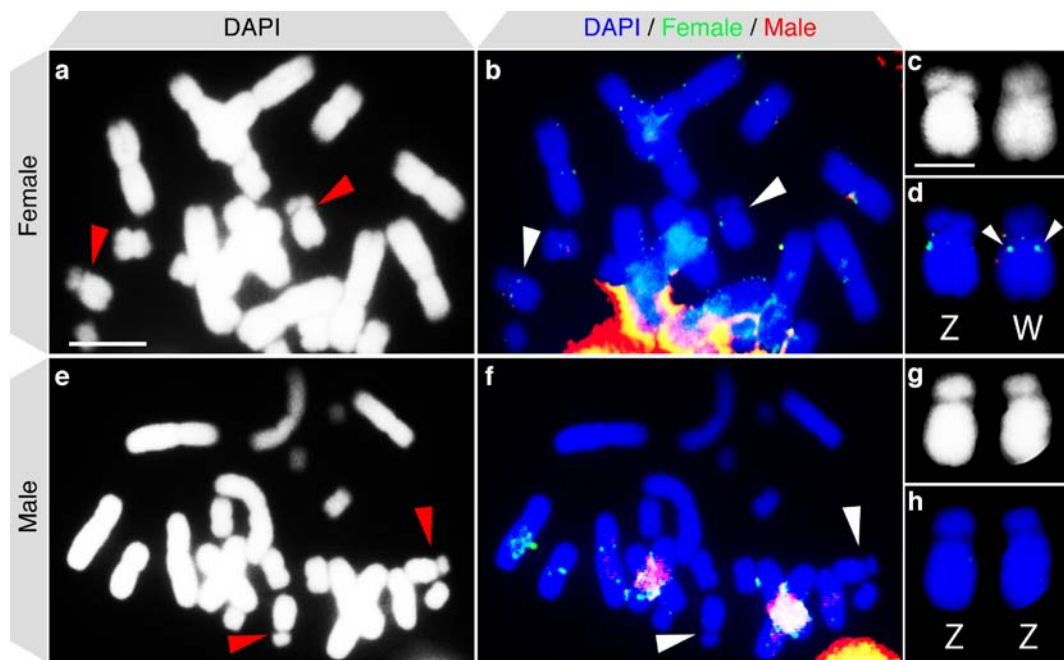


Fig. 4 CGH images of female and male metaphase chromosome spreads. Metaphase chromosomes were hybridized with SpectrumGreen-dUTP labeled female genomic DNA and SpectrumRed-dUTP labeled male genomic DNA. Subsequently, DAPI staining was used to show metaphase chromosome spreads (panel a and e). Panels b and f represent the DAPI-stained metaphase spreads hybridized with labeled genomic DNA from males and females. Two green signals below the

NOR and adjacent to the centromere can be observed on one of the chromosome 7 pairs (panel b and d). Panels g and c represent DAPI stained chromosome 7 pairs in female and male, respectively. Arrowheads indicate chromosome 7 homologues in panels a–b, e–f, and CGH signals on the W chromosome in panel d. Scale bars indicate 10 μ m (a–d) and 5 μ m (c,d,g,h)

there is significant NOR-associated heterochromatin accumulation on the W chromosome. Still, the presence of heterochromatin may not be exclusive of additional rDNA copies within the long NOR. Miller and Brown (1969) found no significant difference in genomic rDNA content between specimens with dimorphic NORs and those with equal-sized NORs, leading to the hypothesis that specimens with dimorphic NORs may have unequally distributed rRNA genes between the chromosome 7 pair. This difference may have arisen due to unequal crossing over and amplification of heterochromatin between sister chromatids carrying NORs (King et al. 1990; Watson et al. 1996; Boron et al. 2009). Additionally, Gall (1968) determined that ovarian DNA in *Xenopus laevis* is 20 to 30 times enriched in DNA coding for rRNA genes which are required in large quantities for oogenesis. If the W chromosome NOR is enriched in rDNA, an analogous system to *X. laevis* is likely to have evolved. The W-specific region identified through CGH is sex-specific and could potentially encourage

unequal crossing over and heterochromatin accumulation as well as rDNA amplification. Nevertheless, the signal observed with reverse fluorescence does not co-localize with that of CGH, indicating that the W-specific region identified by CGH is euchromatic, transcriptionally active, and separate from the NOR.

The location of the NOR and the accumulation of heterochromatin often associated with it, may play a key role in the evolution of sex chromosomes by favoring accumulation of sex-determining loci (Reed and Phillips 1997). Observation of nonpartnered NORs on chromosomes suggest that NORs in fact do not pair and recombine, and so are likely to accumulate heterochromatin which would serve to further reduce recombination (Charlesworth et al. 1986; Watson et al. 1996). The occurrence of NORs on sex chromosomes has been shown in other basal vertebrates such as the anuran species *Gastrotheca riobambae* (Schmid et al. 1983), *Leiopelma hamiltoni* (Green 1988), *Hyla femoralis* (Schmid and Steinlein 2003; Wiley 2003), and *Buergeria buergeri* (Schmid

et al. 1993), fish species *Hoplias malabaricus* (Born and Bertollo 2000) and *Salvelinus alpinus* (Reed and Phillips 1997), and several marsupial and eutherian mammal species (Goodpasture and Bloom 1975; Watson et al. 1996). W chromosome-specific NOR sequence alteration implies not only recruitment of novel sex-determining genes in this area but also very recent fixation of this phenotype in the Australian population of *B. marinus*. Interestingly, another aberrant karyotype where only one chromosome 7 had NOR was observed in South America but not in introduced regions, suggesting a previous loss of karyotype diversity due to anthropogenic relocation into the Caribbean (Miller and Brown 1969).

Our identification of a female-specific chromosome in the *B. marinus* resolves the discrepancies of earlier studies and confirms that this toad species has a ZZ/ZW chromosomal system. In addition to the discovery of the female-specific region on chromosome 7 that is likely to harbor the sex-determining gene, we have identified an NOR dimorphism on the W chromosome which makes the female karyotype readily identifiable by commonly used metaphase chromosome analyses. We were able to ascertain the nature of the NOR heteromorphism as being due to large amounts of heterochromatin accumulation rather than ribosomal gene copy multiplication, as had previously been hypothesized.

The ability to reliably sex cane toads prior to sexual maturity will be of great value in monitoring and control efforts in Australia and other countries with invasive *B. marinus*. Identification of a W chromosome is a major advance in the quest to discover a sex-specific marker in the cane toad. Sexual differentiation is a crucial part of organismal biology and has recently been recognized to be a plausible target for invasive species control (Gutierrez and Teem 2006; Cotton and Wedekind 2007). With technology now available for genetic modification of amphibians, methods such as the introduction of individuals carrying "trojan" sex chromosomes or specific sex-determining genes intended to disturb the sex determination pathway have become realistic undertakings (Ueda et al. 2005; Loeber et al. 2009). In combination with chromosome, microdissection and recent advancements in throughput DNA sequencing technology, gene mining on the sex chromosomes is a financially and technologically realistic goal. The discovery of a sex-determining gene in a toad would

reveal the method of sex determination in a member of one of the most specious and widespread group of vertebrates in the world.

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References

- Baldissera FA, Batistik RF, Haddad CFB (1999) Cytotaxonomic considerations with the description of two new NOR locations for South American toads, genus *Bufo* (Anura: Bufonidae). *Amphib-Reptil* 20:413–420
- Bergero R, Charlesworth D (2009) The evolution of restricted recombination in sex chromosomes. *Trends Ecol Evol* 24:94–102
- Bogart JP (1972) Karyotypes. In: Blair WF (ed) *Evolution in the genus Bufo*. University of Texas Press, Austin
- Born GG, Bertollo LA (2000) An XX/XY sex chromosome system in a fish species, *Hoplias malabaricus*, with a polymorphic NOR-bearing X chromosome. *Chromosome Res* 8:111–118
- Boron A, Porycka K, Ito D, Abe S, Kirtiklis L (2009) Comparative molecular cytogenetic analysis of three Leuciscus species (Pisces, Cyprinidae) using chromosome banding and FISH with rDNA. *Genetica* 135:199–207
- Charlesworth B, Langley CH, Stephan W (1986) The evolution of restricted recombination and the accumulation of repeated DNA sequences. *Genetics* 112:947–962
- Cole CJ, Lowe CH, Wright JW (1968) Karyotypes of eight species of toads (genus *Bufo*) in North America. *Copeia* 1968:96–100
- Cotton S, Wedekind C (2007) Control of introduced species using Trojan sex chromosomes. *Trends Ecol Evol* 22:441–443
- Duellman WE, Trueb L (1994) *Biology of amphibians*. Johns Hopkins University Press, Baltimore, Maryland
- Eastale S (1981) The history of introductions of *Bufo marinus* (Amphibia: Anura); a natural experiment in evolution. *Biol J Linn Soc* 16:93–113
- Eggert C (2004) Sex determination: the amphibian models. *Reprod Nutr Dev* 44:539–549
- Ezaz T, Quinn AE, Miura I, Sarre SD, Georges A, Marshall Graves JA (2005) The dragon lizard *Pogona vitticeps* has ZZ/ZW micro-sex chromosomes. *Chromosome Res* 13:763–776
- Ezaz T, Valenzuela N, Grutzner F, Miura I, Georges A, Burke RL, Graves JA (2006) An XX/XY sex microchromosome system in a freshwater turtle, *Chelodina longicollis* (Testudines: Chelidae) with genetic sex determination. *Chromosome Res* 14:139–150

- Gall JG (1968) Differential synthesis of the genes for ribosomal RNA during amphibian oogenesis. *Proc Natl Acad Sci U S A* 60:553–560
- Goodpasture C, Bloom SE (1975) Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. *Chromosoma* 53:37–50
- Green DM (1988) Cytogenetics of the New Zealand frog, *Leiopelma hochstetteri*; extraordinary supernumerary chromosome variation and a unique sex-chromosome system. *Chromosoma* 97:55–70
- Gutierrez JB, Teem JL (2006) A model describing the effect of sex-reversed YY fish in an established wild population: the use of a Trojan Y chromosome to cause extinction of an introduced exotic species. *J Theor Biol* 241:333–341
- Hayes TB (1998) Sex determination and primary sex differentiation in amphibians: genetic and developmental mechanisms. *J Exp Zool* 281:373–399
- Hillis DM, Green DM (1990) Evolutionary changes of heterogametic sex in the phylogenetic history of amphibians. *J Evol Biol* 3:49–64
- Kawai A, Nishida-Umehara C, Ishijima J, Tsuda Y, Ota H, Matsuda Y (2007) Different origins of bird and reptile sex chromosomes inferred from comparative mapping of chicken Z-linked genes. *Cytogenet Genome Res* 117:92–102
- King M, Contreras N, Honeycutt RL (1990) Variation within and between nucleolar organizer regions in Australian hylid frogs (Anura) shown by 18S+28S in-situ hybridization. *Genetica* 80:17–29
- Loeber J, Pan FC, Pieler T (2009) Generation of transgenic frogs. *Methods Mol Biol* 561:65–72
- Miller L, Brown DD (1969) Variation in the activity of nucleolar organizers and their ribosomal gene content. *Chromosoma* 28:430–444
- Miura I (2007) An evolutionary witness: the frog *Rana rugosa* underwent change of heterogametic sex from XY male to ZW female. *Sex Dev* 1:323–331
- Miura I, Ohtani H, Nakamura M, Ichikawa Y, Saitoh K (1998) The origin and differentiation of the heteromorphic sex chromosomes Z, W, X, and Y in the frog *Rana rugosa*, inferred from the sequences of a sex-linked gene, ADP/ATP translocase. *Mol Biol Evol* 15:1612–1619
- Nakamura M (2009) Sex determination in amphibians. *Semin Cell Dev Biol* 20:271–282
- Ogata M, Hasegawa Y, Ohtani H, Mineyama M, Miura I (2008) The ZZ/ZW sex-determining mechanism originated twice and independently during evolution of the frog, *Rana rugosa*. *Heredity* 100:92–99
- Reed KM, Phillips RB (1997) Polymorphism of the nucleolar organizer region (NOR) on the putative sex chromosomes of Arctic char (*Salvelinus alpinus*) is not sex-related. *Chromosome Res* 5:221–227
- Schmid M (1978) Chromosome banding in Amphibia I. Constitutive heterochromatin and nucleolar organizer regions in *Bufo* and *Hyla*. *Chromosoma* 66:361–388
- Schmid M (1980) Chromosome banding in amphibia. IV. Differentiation of GC- and AT-rich chromosome regions in Anura. *Chromosoma* 77:83–103
- Schmid M (1982) Chromosome banding in amphibia. VII. Analysis of the Structure of NORs in Anura. *Chromosoma* 87:327–344
- Schmid M, de Almeida CG (1988) Chromosome banding in Amphibia. XII. Restriction endonuclease banding. *Chromosoma* 96:283–290
- Schmid M, Steinlein C (2001) Sex chromosomes, sex-linked genes, and sex determination in the vertebrate class amphibia. *EXS* 91:143–76
- Schmid M, Steinlein C (2003) Chromosome banding in Amphibia. XXIX. The primitive XY/XX sex chromosomes of *Hyla femoralis* (Anura, Hylidae). *Cytogenet Genome Res* 101:74–79
- Schmid M, Haaf T, Geile B, Sims S (1983) Chromosome banding in Amphibia. VIII. An unusual XY/XX-sex chromosome system in *Gastrotheca riobambae* (Anura, Hylidae). *Chromosoma* 88:69–82
- Schmid M, Ohta S, Steinlein C, Guttenbach M (1993) Chromosome banding in Amphibia. XIX. Primitive ZW/ZZ sex chromosomes in *Buergeria buergeri* (Anura, Rhacophoridae). *Cytogenet Cell Genet* 62:238–246
- Schweizer D (1976) Reverse fluorescent chromosome banding with chromomycin and DAPI. *Chromosoma* 58:307–324
- Traut W, Sahara K, Otto TD, Marec F (1999) Molecular differentiation of sex chromosomes probed by comparative genomic hybridization. *Chromosoma* 108:173–180
- Ueda Y, Kondoh H, Mizuno N (2005) Generation of transgenic newt *Cynops pyrrhogaster* for regeneration study. *Genesis* 41:87–98
- Ullerich FH (1967) Weitere untersuchungen über chromosomen verhältnisse und DNS-gehalt bei Anuran (Amphibia). *Chromosoma* 8:345–368
- Uno Y, Nishida C, Yoshimoto S, Ito M, Oshima Y, Yokoyama S, Nakamura M, Matsuda Y (2008) Diversity in the origins of sex chromosomes in anurans inferred from comparative mapping of sexual differentiation genes for three species of the Raninae and Xenopodinae. *Chromosome Res* 16:999–1011
- Urban MC, Phillips BL, Skelly DK, Shine R (2007) The cane toad's (*Chaunus* [Bufo] *marinus*) increasing ability to invade Australia is revealed by a dynamically updated range model. *Proc Biol Sci* 274:1413–1419
- Valenzuela N (2008) Sexual development and the evolution of sex determination. *Sex Dev* 2:64–72
- Verma RS, Babu A (1995) Human chromosomes: principles and techniques. McGraw-Hill, Inc., New York
- Volpe EP, Gebhardt BM (1968) Somatic chromosomes of the marine toad, *Bufo marinus* (Linnae). *Copeia* 1968:570–576
- Wallace H, Badawy GM, Wallace BM (1999) Amphibian sex determination and sex reversal. *Cell Mol Life Sci* 55:901–909
- Watson JM, Meyne J, Graves JA (1996) Ordered tandem arrangement of chromosomes in the sperm heads of monotreme mammals. *Proc Natl Acad Sci USA* 93:10200–10205
- Wiley JE (2003) Replication banding and FISH analysis reveal the origin of the *Hyla femoralis* karyotype and XY/XX sex chromosomes. *Cytogenet Genome Res* 101:80–83