Chromosome and Genetic Mapping

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The First Cytogenetic Map of the Tuatara, *Sphenodon punctatus*

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Cytogenetics · Microchromosome · Reptile · Tuatara

Abstract
Tuatara, *Sphenodon punctatus*, is the last survivor of the distinctive reptilian order Rhynchocephalia and is a species of extraordinary zoological interest, yet only recently have genomic analyses been undertaken. The karyotype consists of 28 macrochromosomes and 8 microchromosomes. A Bacterial Artificial Chromosome (BAC) library constructed for this species has allowed the first characterization of the tuatara genome. Sequence analysis of 11 fully sequenced BAC clones (~0.03% coverage) increased the estimate of genome wide GC composition to 47.8%, the highest reported for any vertebrate. Our physical mapping data demonstrate discrete accumulation of repetitive elements in large blocks on some chromosomes, particularly the microchromosomes. We suggest that the large size of the genome (5.0 pg/haploid) is due to the accumulation of repetitive sequences. The microchromosomes of tuatara are rich in repetitive sequences, and the observation of one animal that lacked a microchromosome pair suggests that at least this microchromosome is unnecessary for survival. We used BACs bearing orthologues of known genes to construct a low-coverage cytogenetic map containing 21 markers. We identified a region on chromosome 4 of tuatara that shares homology with 7 Mb of chicken chromosome 2, and therefore the orthologous region of the snake Z chromosome. We identified a region on tuatara chromosome 3 that is orthologous to the chicken Z, and a region on chromosome 9 orthologous to the mammalian X. Since the tuatara determines sex by temperature and has no sex chromosomes, this implies that different tuatara autosome regions are homologous with the sex chromosomes of mammals, birds and snakes. We have identified anchor BAC clones that can be used to reliably mark chromosomes 3–7, 10 and 13, some of which are difficult to distinguish based on morphology alone. Fluorescence in situ hybridization mapping of 18S rDNA confirms the presence of a single NOR located on the long arm of chromosome 7, as previously identified by silver staining. Further work to construct a dense physical map will lead to a better understanding of the dynamics of genome evolution and organization in this isolated species.

Tuatara have been described as the most distinctive surviving reptile in the world [Daugherty et al., 1990] and a species of extraordinary zoological interest [Groombridge, 1982]. Molecular cytogenetic characterization and a gene map for the species would therefore be of great value for bringing this species into the genomic era. Tuatara are the only living representatives of the reptilian order Rhynchocephalia (also known as Sphenodontia),

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which diverged from other reptilian orders approximately 270 million years ago [Hugall et al., 2007]. Rynchocephalids are regarded as the sister group of the squamates, based on morphological and genetic analyses [Rest et al., 2003; Hugall et al., 2007]. They were globally widespread until the late Cretaceous (65–80 million years ago) but now survive only on some off-shore islands of New Zealand. Until recently, 2 allopatric species of tuatara were recognized: *Sphenodon punctatus* and *Sphenodon guntheri*, [Daugherty et al., 1990]. However, recent genetic analyses by Hay et al. [2009] indicate that tuatara should be regarded as a single species *(S. punctatus)*, with 3 distinct genetic/geographic groups.

In addition to their unique phylogenetic placement, several aspects of tuatara biology have the potential to inform studies of genome evolution and development. Tuatara are extremely long-lived, probably surviving over 100 years [Dawbin, 1982; Castanet et al., 1988] and have a long generation time (sexual maturity at 14 years, mean generation interval 50 years) [Cree et al., 1992; Allendorf and Luikart, 2006]. Females reproduce only every 4 years on average [Cree et al., 1992], and a long period of egg incubation occurs prior to hatching (11–16 months in the wild). Tuatara have temperature-dependent sex determination (TSD), and are one of the few species to exhibit a female-to-male pattern of TSD, where males are produced at high temperatures [Mitchell et al., 2006]. No sex-specific differences in chromosomes or DNA have been found [Norris et al., 2004; Mitchell et al., 2006]. Tuatara also have unusual thermal biology, remaining active at extremely low temperatures (~5°C), and exhibiting the lowest optimal body temperature range of any reptile (16–21°C) [Werner and Whitaker, 1978; Thompson and Daugherty, 1998; Besson, 2009]. In accordance with this, they have a low metabolic rate [Thompson and Daugherty, 1998] and one of the largest reptilian genomes, with a C-value of 5.0 [Olmo, 1981, 2003; Vinogradov and Anatskaya, 2006].

Like most sauropsids, the tuatara karyotype is composed of macrochromosomes (M) and microchromosomes (m). A complete karyotype of tuatara (2n = 36, 28 M and 8 m) was first determined by Wylie et al. [1968], although earlier attempts had been made [Keenan, 1932; Hogben, 1921]. Norris et al. [2004] provided the first comprehensive analysis of *Sphenodon* karyology, including morphological descriptions of each chromosome, C-banding and silver staining of the nucleolus organizer (AgNOR). They examined 89 animals from across the range and reported no karyological differences between populations or between sexes. One animal from Ruamahua-iti Island possessed a peculiar chromosome 3, with additional material on the long arm of one member of the pair in all cells examined. To date, no other chromosomal aberration has been reported in this species.

On the basis of gross morphology of the macrochromosomes, Norris et al. [2004] suggested that the tuatara shares more similarity in karyology with turtles than with squamates. However, chromosome painting and gene mapping studies are much better able to reveal chromosomal homologies, and recent studies have shed light on the origin and evolution of sex chromosomes in particular [Marshall Graves, 2008]. Chromosome painting has shown the chicken Z to be entirely homologous with chromosome 5 in *Chelodina longicollis*, an Australian turtle [Marshall Graves and Shetty, 2001]. Chicken Z genes show homology to chromosome 6q in the Chinese soft-shelled turtle, *Pelodiscus sinensis*, and to chromosome 3 in the crocodile *Crocodylus siamensis*, [Kawai et al., 2007]. Among squamates, chicken Z is equivalent to chromosome 2p of snakes [Matsubara et al., 2006] and 2p of 2 agamid lizards [Ezaz et al., 2009; Srikulnath et al., 2009a]. Perhaps most remarkably, synteny, and even order, of 6 Z-borne genes in chicken and the gecko *Gekko hokouensis* Z chromosome is conserved [Kawai et al., 2009]. The Z chromosomes of other reptiles are not as well characterized; however, the snake Z shares homology with chicken 2p and agamid 6p, and the turtle Z with chicken chromosome 15 [Matsubara et al., 2006; Ezaz et al., 2009; Kawagoshi et al., 2009; Srikulnath et al., 2009a]. Given its unique phylogenetic position between archosaurs and squamates, tuatara is well placed to elucidate the mode of karyotype evolution among sauropsids.

The tuatara genome has received relatively little attention to date. Genetic studies have focused largely on the isolation of mitochondrial markers for phylogenetic and phylogeographical studies [Hay et al., 2003; Rest et al., 2003] and characterization of neutral (microsatellite) and adaptive (MHC) genetic markers for population analyses [Aitken et al., 2001; Miller et al., 2005, 2006; MacAvoy et al., 2007]. Although more than 1,500 papers have been published on topics ranging from physiology to phylogeny of the tuatara, only 4 deal specifically with karyology and 3 with genomic organization. However, interest in tuatara genomics is increasing and the availability of a tuatara BAC library has recently enabled the first investigations of its nuclear genome [Shedlock, 2006; Wang et al., 2006; Organ et al., 2008]. Here we present the first physical map of the chromosomes of tuatara and report our observations on genome evolution in this interesting and enigmatic reptile.

Material and Methods

Animals, Blood Culture and Chromosome Preparation

Blood samples were collected from animals held captive at Taronga Zoo, Sydney (RFID implant numbers 6306A5 and F75DAE). Both specimens were female and originated from Stephens Island, New Zealand but were hatched in captivity in 1986 [M. Thompson, pers. comm.]. Chromosomes were prepared from short-term culture of peripheral blood leukocytes. Up to 1 ml of blood was collected by caudal venipuncture into heparinized tubes. The tubes were centrifuged at 260 *g* for 5 min and the buffy coat collected. Cultures were established in 2 ml of Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% (v/v) fetal bovine serum (JRH Biosciences), 1 mg/ml L-glutamine (Sigma), 100 U/ml penicillin (Multicell), 100 mg/ml streptomycin (Multicell) and 3% phytohemagglutinin M (PHA M; Sigma). Cultures were incubated at 26°C for 6–7 d [Wylie et al., 1968; Norris et al., 2004] in 5% CO_2. Chromosomes were harvested 2 h after adding colcemid (75 ng/ml) by treatment with hypotonic solution (KCl, 0.075 mM) and fixation in methanol:acetic acid (3:1) according to standard protocols. The cell suspension was dropped onto slides and air dried. Slides were kept frozen at –80°C.

Probe Selection, Preparation and Fluorescence in situ Hybridization

We mapped clones from a tuatara BAC library [Wang et al., 2006] made available commercially (SymBio Corp, Menlo Park, Calif. USA). We chose clones that had been fully sequenced by the NIH Intramural Sequencing Center (www.nisc.nih.gov) as part of the NISC Comparative Sequencing Initiative. We also scanned the NCBI trace archives of paired BAC ends [Wang et al., 2006] for orthologues of genes found on chicken chromosomes Z and 2. The gene content of BAC clones was determined using BLAST after masking the query sequence using RepeatMasker [Smit et al., 2009]. CG content was estimated as by Shedlock et al. [2007]. We mapped the clones identified by Wang et al. [2006] as those containing *DMRT1*. To map 18S rDNA, we used a BAC clone containing this locus from the tammar wallaby [Haines, 2005]. Some ambiguous assignments were clarified by comparing them with BACs of known location [H. Miller, unpublished data]. Details can be found in table 1.

Clones were grown overnight in 15 ml cultures and BAC DNA extracted using the Promega Wizard Plus SV Miniprep DNA Purification System according to the manufacturer's protocol (with volumes scaled up). DNA concentration was measured on a NanoDrop (Thermo Scientific) and labeled by nick translation incorporating either Orange or Green-dUTP (Abbott Molecular). Labeled BAC DNA (200–500 ng) and boiled genomic DNA (1 μg) were co-precipitated and resuspended in hybridization buffer (50% v/v deionized formamide, 10% w/v dextran sulphate, 2× SSC, 1× Denhardt's solution and 40 mM sodium phosphate). Probes were added to slides under cover slips and sealed with rubber cement. DNA was denatured by heating the slide to 68.5°C for 5 min on a heat block and hybridized overnight at 37°C in a humidified chamber. Cover slips were removed by soaking for 5 min in 2× SSC. The slides were washed once in 0.4× SSC, 0.3% IGEPAL CA-630 (Sigma) at 60°C for 2 min, once in 2× SSC, 0.1% IGEPAL at room temperature for 1 min, then dehydrated through an ethanol series (1 min in each of a 70%, 90% and 100% solution), air dried, stained with DAPI (50 μg/ml DAPI solution in 2× SSC) for 45 s at room temperature and mounted with Vectashield (Vector Laboratories). Vernier co-ordinates of each metaphase were recorded and images of 3–10 cells were captured using a Zeiss Axioplan2 epifluorescence microscope. Images were analyzed using IPLab imaging software (Scanalytics Inc). Ambiguous chromosomal assignments were resolved by simultaneously hybridizing differentially labeled probes to a slide.

Telomere Peptide Nucleic Acid Probe

Telomeres were visualized following the protocol of Lansdorp et al. [1996] with some modifications. Briefly, 10 μl of hybridization mixture containing 70% formamide, 0.3 μg/ml Cy3-$(CCCTAA)_3$ peptide nucleic acid (PNA) probe (Biosynthesis, Inc, Texas) and 1× Denhardt's solution in 10 mM Tris pH 7.2 were added to the slide under a cover slip and sealed with rubber cement. The DNA was denatured by heating for 3 min at 80°C. After hybridization for 2 h at 37°C in a humidified chamber, the slides were washed at room temperature with 70% formamide, 1% BSA, 10 mM Tris pH 7.2 (2 times for 15 min) and then with 0.1 M Tris, 0.15 M NaCl, pH 7.5 containing 0.08% Tween-20 (3 times for 5 min). The slides were then dehydrated through an ethanol series, air dried, stained with DAPI and image capture and analysis was performed as described above.

Chromomycin A_3 Staining

For Chromomycin A_3 (CMA_3) staining, we used a modified CMA_3-methyl green method [Babu and Verma, 1995]. 300 μl of a solution containing CMA_3 (0.5 mg/ml) and 5 mM $MgCl_2$ in 0.5× McIlvaine's buffer (pH 7.0) was added to slides under a cover slip and incubated at room temperature for 1 h. After brief rinsing in water, the slides were placed in a Coplin jar containing a freshly prepared solution of methyl green (100 μM) in 0.1 M Tris (pH 7.0) for 10 min. The slides were rinsed again in water, mounted as above and stored at 4°C for 1 week before image capture and analysis.

Silver Staining (Ag-NOR)

Ag-NOR staining was carried out using the method of Howell and Black [1980]. Briefly, 2 drops of a 2% gelatin, 1% formic acid solution and 4 drops of a 50% $AgNO_3$ solution were added to a slide under a cover slip. The slide was incubated on a heat block at 70°C until the appearance of a golden brown color, then washed in distilled water and mounted as above. Images were captured under bright field microscopy.

Results

We chose 27 clones that contained orthologues of known genes, based on earlier full BAC sequencing, BAC end sequencing or library screening (table 1, fig. 1). At least 21 genes were represented. We used fluorescence in situ hybridization (FISH) to assign these BACs unambiguously to 11 tuatara chromosomes (fig. 1). The tuatara karyotype contains 16 pairs of macrochromosomes and 4 pairs of microchromosomes. Some tuatara chromo-

Table 1. Details of BAC libraries and clones mapped

Library ID	Reference	Clone ID	Sequence accession	Representative loci	Chromosomal location
AGI, *Macropus eugenii* (tammar wallaby)	Haines, 2005	329J14*		18S rRNA	7q
VMRC12 *Sphenodon punctatus* (tuatara)	Wang et al., 2006	69A1*	gnl|ti|908600346	*ACO1*	3p
		515D6; 162F5; 289C19; 557I22; 497H14; 82P24; 150B6; 269F20; 81B4; 58N13		*DMRT1*	16;18
	NISC Comparative Sequencing Initiative	40N7	AC154075	*T*	3q
		224G8	AC155214	*ARHGAP21*	4q
		239M1	AC153105	*PPP1R9A; PON2*	4q
		311J16	AC153757	*GATAD1; ANKIB1*	4q
		16O14*	AC154160	*GPHN*	5q
		462H11	AC154989	*RTF1; NDUFAF1*	5q
		178H23	AC155213	*NOTCH1*	6q
		436B16*	AC155215	*INPP5E; NOTCH1*	6q
		356P22	AC154074	*GRM8*	7q
		460J16	AC153758	*RSRC1; SND1*	7q
		475O6*	AC161716	*PHF6; HPRT1*	9p
	H. Miller (unpublished data)	44B3*		MHC-related	4q
		531J19*		MHC-related	13q
		437A11		MHC-related	13q;15–18
		448I11		MHC-related	4q;6p;13q

The genic content of fully or end sequenced clones was determined by BLAST after having masked the sequence for vertebrate retroelements, DNA transposons, simple repeats and low complexity motifs using RepeatMasker. Clones that uniquely identify chromosomes (anchor BACs) are indicated by an asterisk. The 10 clones thought to contain *DMRT1* were mapped both in pools (simultaneously) and individually with the same hybridization pattern obtained.

somes are difficult to distinguish by size and morphology, particularly chromosome pairs 9 and 10, as well as 13 and 14 [Norris et al., 2004]. We assigned BAC clones 475O6 to chromosome 9 and 531J19 to chromosome 13. The BACs we assigned to these chromosomes are useful as anchor markers because they produce single, clear signals that unambiguously identify the chromosomes. Similarly diagnostic clones for chromosomes 3–7 are identified in table 1. Chromosomes 1–2, 8, 10–12 and 14 remain without anchor BACs, but can be distinguished by size and morphology. The 4 microchromosomes (15–18) can be distinguished by size and the hybridization pattern of 2 clones (515D6 and 437J19; fig. 2C).

Some fully sequenced BAC clones contain an unusually large number of unique repetitive elements amounting to ~90/Mb [Shedlock, 2006]. We found that several BACs containing such repetitive elements hybridized to multiple chromosomes (table 1, fig. 2B, C and fig. 3D). For instance, the BAC clone 448I11 hybridizes to chromosomes 4, 6 and 13, in large domains which may represent up to 3% of the total chromosome length (TCL) (fig. 3D). Similarly, clone 437A11 hybridizes to 13q and to all 4 microchromosomes (~1% TCL). All clones identified by Wang et al. [2006] as containing *DMRT1* (table 1) hybridize to both tuatara chromosomes 16 and 18 in a diffuse pattern, suggestive of a high repeat content in these chromosomes (~1% TCL). The smallest microchromosome, 18, appears to consist predominately of repeats contained in clone 437A11 and those associated with *DMRT1*.

Using an 18S rDNA FISH probe, we were able to colocate the silver stained NOR and 18S rDNA hybridization signal, confirming the presence of a single active NOR on the distal end of the long arm of chromosome 7. In addition, in all cells examined, whether by AgNOR

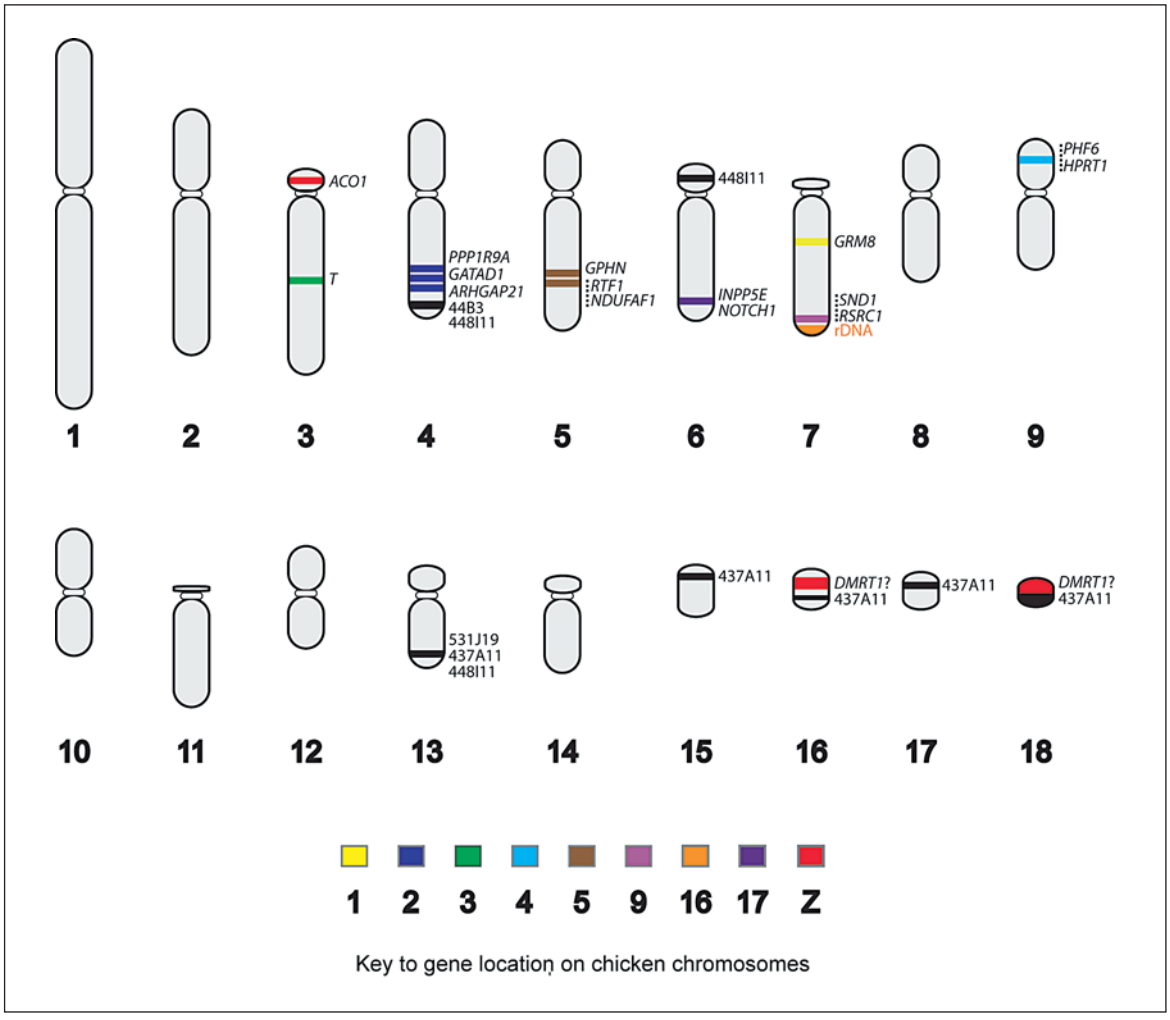

Fig. 1. Tuatara–chicken comparative gene map. Fully sequenced BAC clones were mapped by FISH to metaphase chromosomes and their gene content was determined by BLAST, or by library screens. Colors indicate homology to chicken chromosomes. Clone numbers are indicated in black where no genic data were available. Gene symbols are those recommended by the HUGO Gene Nomenclature Committee. Gene symbols joined by a dashed line indicate loci found in the same BAC clone but whose order on the chromosome is unknown.

staining or by FISH, one homolog consistently stained more heavily or gave a greater hybridization signal (fig. 3B). The PNA telomere probe produced clear and unambiguous hybridization signals identifying telomeres on each chromatid of all chromosomes (fig. 3C). No interstitial hybridization signals were observed on any chromosome. No general trend in arm length and signal intensity was apparent, but the microchromosomes appear to have disproportionately long telomeres.

To investigate the distribution of GC-rich sequences, we used CMA_3 methyl green staining (fig. 3A). No discernable bands were visible on any chromosomes, but more intense staining was invariably observed on the NOR. The microchromosomes also stained more heavily, indicative of a higher GC content in those elements. Using draft assemblies of fully sequenced BAC clones (table 1; ~0.03% genome coverage) we estimated the genome wide GC content to be 47.76% (SD = 0.63).

Of the 2 animals examined in this study, one animal (#6306A5) possessed a karyotype consistently deficient in one pair of microchromosomes (fig. 2). To investigate this further we took a second blood sample 6 months later and made new chromosome preparations. The PNA telomere probe (fig. 2A) revealed no intrachromosomal

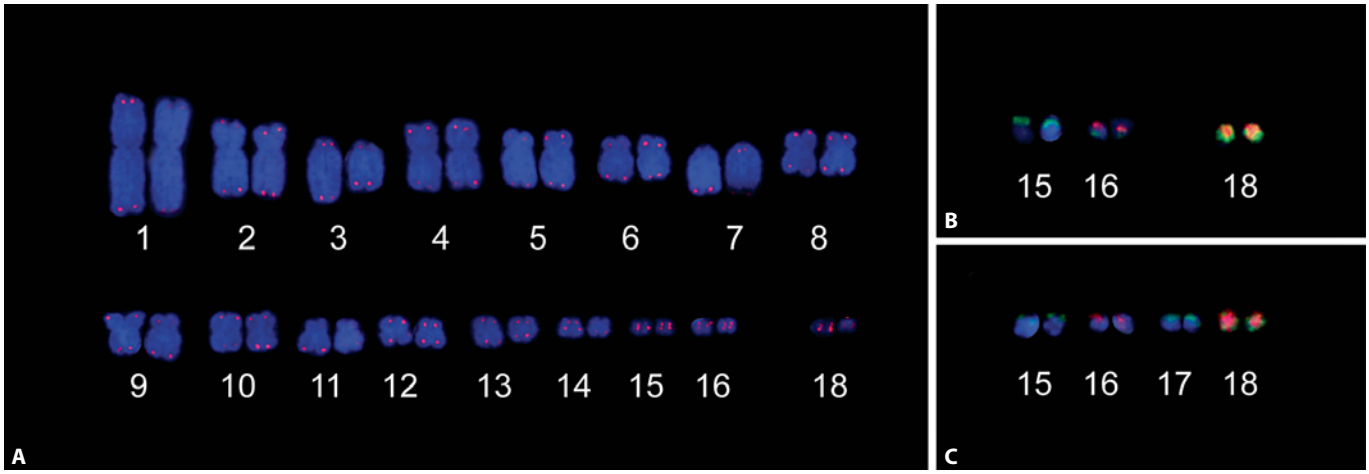

Fig. 2. A Karyotype of animal #6306A5, lacking the third largest pair of microchromosomes (chromosome 17). The telomeric probe reveals only 4 signals for each chromosome, indicating that the loss of this pair is not the result of a fusion event. **B** Using differentially labeled BAC clones (515D6 in red and 437A11 in green) that hybridize to all microchromosomes, we were able to determine that the 17th pair was missing by comparison with the karyotype of an animal with a full chromosomal complement (**C**).

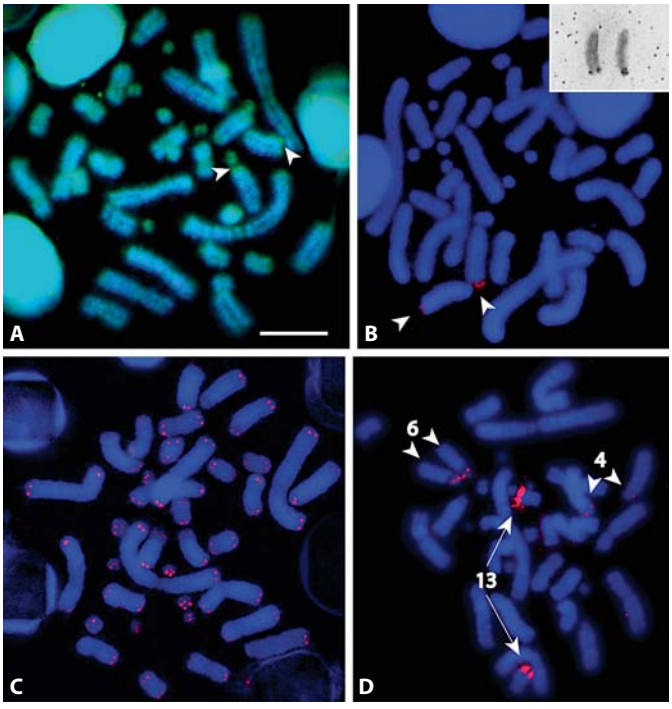

Fig. 3. A CMA_3 and methyl green staining of tuatara metaphase chromosomes. Arrows indicate the position of the NOR on chromosome 7, which is more heavily stained. Staining of the microchromosomes is also marginally more intense. **B** FISH mapping of 18S rDNA to chromosome 7 (arrowheads) is concordant with AgNOR staining (inset). **C** Telomeres are clearly visible on each chromosome and no interstitial signals were found, suggesting no recent rearrangements. **D** Some clones (e.g. 448I11) hybridize in a pattern consistent with discrete accumulation of repetitive sequences in large domains on multiple chromosomes. The scale bar represents 10 μM.

telomere sequence that could denote a fusion. Based on chromosome size and 2-color hybridization with clones 437A11 and 515D6, each microchromosome pair is distinguishable. We identified the missing element as chromosome 17 by comparing the abnormal karyotype (fig. 2B) with that of a normal animal (fig. 2C).

Discussion

BAC-Anchored Cytogenetic Map and Chromosome Homology

Even with this light coverage map, we were able to identify regions of conserved synteny between tuatara and other reptiles, as well as some rearrangements be-

tween tuatara and other species. The distal long arm of tuatara chromosome 4 corresponds to a 7-Mb gene-dense region of chicken chromosome 2, and is bounded by genes that are found on the snake Z chromosome [Matsubara et al., 2006]. The synteny of this region appears to be conserved in all vertebrates but is interrupted in eutherian mammals between *GATAD1* (human 7q21) and *ARHGAP21* (human 10p12). Synteny and even the order of genes found on macrochromosomes are highly conserved among sauropsids. Genes spanning the length of the snake Z map to a contiguous block of chicken 2p and chromosome 6 of the butterfly lizard [Srikulnath et al., 2009b]. Although no genes that have been located on the snake Z are present on our map, it is likely that tuatara chromosome 4 is equivalent to the snake Z chromosome, which, in all snakes examined to date, is the fourth or fifth largest pair.

Tuatara 5q shares homology with a 4.7 Mb region of chicken chromosome 5. Synteny of this block is conserved in all sequenced tetrapods with the exception of eutherian mammals (*GPHN* is on human 14q23 and *RTF1* on human 15q14). Two genes, *GRM8* and *SND1,* which in humans lie within 1.6 Mb on chromosome 7, are respectively located medially and distally on the long arm of chromosome 7 in tuatara. Of the 2 genes contained in clone 460J16 in tuatara, *SND1* is absent from the chicken assembly but *RSRC1* lies on chicken chromosome 9 and human 3, suggesting that tuatara chromosome 7 arose by fusion of ancestral chromosomes. Fission of ancestral chromosomes could also explain this composition, though it would require a greater number of rearrangements. *PHF6* and *HPRT1* (contained in the same BAC clone) map to chromosome 9 in tuatara, indicating homology with a 46 kb region of chicken chromosome 4 and a 127 kb region of the human X.

The genes *ACO1* and *DMRT1* are located on the Z chromosome in chicken, but in tuatara the BAC clones corresponding to these genes map to chromosomes 3, 16 and 18 (table 1). In all vertebrates studied to date, *DMRT* genes are arranged *DMRT1-DMRT3-DMRT2*, a region spanning 94 kb in zebra finch and up to 271 kb in opossum. To obtain tuatara clones containing *DMRT1*, Wang et al. [2006] screened the BAC library using conserved intronic and intergenic regions from *DMRT1* and DM domain sequences of *DMRT2*. They assembled 10 BACs into a 300 kb contig from which they were able to amplify the non-coding probe sequences, but failed to amplify the DM domain of any gene. This suggested to the authors that they had not identified *DMRT1* but had instead found a duplicated cassette of the non-coding regions. Hybridization of each of the 10 contiguous BAC clones to 2 chromosomes in a diffuse pattern strongly supports this suggestion, and our mapping of *DMRT1* to chromosomes 16 and 18 should be considered provisional.

Repeat Content and GC Composition

Our physical mapping data demonstrate discrete accumulation of repetitive elements in large domains on some chromosomes, particularly the microchromosomes. Three of the clones we mapped (437A11, 448I11, 515D6) account for approximately 5% of the total chromosome (genome) length (fig. 2C, 3D). Tuatara has a large genome [5.0 pg/haploid; Olmo, 1981] with more than twice the number of repeat types per megabase than *Anolis*; indeed, more than 20 times the zebra finch [Shedlock, 2006], yet the gene structure (exon and intron length) is much the same as for birds and other reptiles [Organ et al., 2008]. Genome survey sequencing of 89 BAC ends [~121 kb; Wang et al., 2006] and analyses of 11 fully sequenced BACs (1.6 Mb; table 1) [Shedlock, 2006] suggest that retroelements make up about 5–6% of the tuatara genome. By comparison, transposable elements make up about 9% of the minimal chicken genome and 40–50% in humans [Lander et al., 2001; ICGS Consortium, 2004]. A figure of 6% for tuatara must therefore be a gross underestimate of the true value, due to the low proportion (~0.03%) of the genome sampled and the poor representation of reptile repeats in databases used by RepeatMasker [Chapus and Edwards, 2009]. The tuatara's large genome, then, is almost certainly due to accumulation of repetitive DNA.

We used CMA_3-methyl green staining to visualize GC isochore structure on tuatara chromosomes. Previous attempts using G-banding have not been successful [Norris et al., 2004; and in our laboratory]. Notably, all microchromosomes and the NOR on the long arm of chromosome 7 were densely stained by CMA_3. This suggests that the microchromosomes have a higher GC content than the rest of the genome, as is the case in chicken and softshelled turtle [Auer et al., 1987; ICGS Consortium, 2004; Kuraku et al., 2006]. A high GC content on the microchromosomes implies an increased recombination rate [Fang et al., 2008; Marsolier-Kergoat and Yeramian, 2009] and also correlates with increased gene density [Costantini et al., 2006, 2007]. The lack of isochore structures on the macrochromosomes reveals a fairly homogeneous GC distribution on these chromosomes in tuatara, as is the case for many other reptiles, fish and amphibians [Bernardi, 2000; Hughes et al., 2002]. The nucleolus or-

Table 2. Genome-wide GC composition of representative amniotes

Species	%GC
Human	39.9
Platypus	45.5
Chicken	40.2
Alligator	42.5
Turtle	43.6
Anolis	41.5
Tuatara	47.8

The tuatara has the highest GC content of any vertebrate reported to date. Data are from this study, Shedlock et al. [2007] and Warren et al. [2008].

ganizer region, containing 18S rDNA, is GC rich in all animals [Varriale et al., 2008] so it was not surprising to see dense staining of CMA_3 in this region. Despite a homogenous GC distribution, the tuatara genome is composed of 47.76% GC (SD = 0.63), the highest known for any vertebrate [table 2, Olmo, 2008; Costantini et al., 2009]. This estimate of GC content is based on our analysis of 11 fully sequenced BAC clones representing only about 0.03% of the genome, so it may be an unrepresentative sample and may change as more in-depth sequence data become available for tuatara.

Telomeres

Telomere length has been found to be negatively correlated with age in snakes [Bronikowski, 2008], turtles [Hatase et al., 2008], alligators [Scott et al., 2006], birds [Haussmann and Vleck, 2002; Haussmann et al., 2003], humans [Tsuji et al., 2002] and in many other taxa, so as a species that is thought to live for over 100 years [Dawbin, 1982; Castanet et al., 1988] tuatara present a good model for examining telomere length and senescence. Indeed, by comparison with other vertebrates, tuatara appear to have relatively long telomeres [H. Bender pers. comm.]. Quantitative methods such as QFISH [Zijlmans et al., 1997; Slijepcevic, 2001] or qPCR [Cawthon, 2002; O'Donovan et al., 2009] are needed to confirm this observation. Long telomeres have been found in many long-lived vertebrates, including turtles [Hatase et al., 2008], birds and some mammals [Haussmann et al., 2003]. Some studies have suggested a positive correlation between telomere length and total chromosome or arm length [Zijlmans et al., 1997; Sridevi et al., 2002; Wise et al., 2009]; however, our results do not support this observation. In tuatara, the size of microchromosomal telomeres is equivalent to or longer than those of the macrochromosomes (fig. 2A, 3C). Expansion of the telomeric sequences may be due to the repetitive content of the microchromosomes.

Interstitial telomeric repeat sequences associated with constitutive heterochromatin (as revealed by banding techniques) may be indicative of ancestral chromosomal rearrangements in some vertebrates [Meyne et al., 1990; Ruiz-Herrera et al., 2008]. Vague C-banding, no demonstrable G-bands [Norris et al., 2004] and the absence of interstitial telomeric signals in all chromosomes suggest that the tuatara has a relatively stable and evolutionarily derived karyotype [Meyne et al., 1990; Nanda et al., 2002; Ruiz-Herrera et al., 2008]. Having no obvious interstitial telomeric sequences also makes this species amenable to a rapid and reliable qPCR test for telomere length [Nakagawa et al., 2004]. Determining the age of tuatara once they reach adulthood is currently problematic, and a lack of long-term (i.e. >50 year) longitudinal studies of marked animals means that many aspects of tuatara life history (such as maximum longevity, the age structure of populations and at what age they cease to be reproductively active) are unknown. Conservation management of tuatara would therefore benefit greatly from such an assay if a relationship between age and telomere length could be demonstrated.

An Abnormal Karyotype and Tuatara Microchromosomes

Our study identified a female with an abnormal karyotype that was consistently lacking in one pair of microchromosomes. The hybridization pattern of 2 BAC clones (437A11 and 515D6) differs between the odd animal and an animal possessing a normal chromosome complement only at chromosome 17, suggesting loss rather than fusion with another chromosome. If the disappearance of the chromosome had resulted from fusion with another pair, interstitial telomeric signals might have been observed, but they were not. This loss of a microchromosome is a particularly striking finding because the animal still survives and is apparently healthy at 23 years of age (although the effect on its reproductive capacity remains unknown). Chromosome 17 represents about 1–1.5% of the genome [Norris et al., 2004] but presumably it carries largely repetitive sequences and contains no genes that are essential for survival. The higher GC composition of microchromosomes may be due to the frequency of CR1-like LINE repeats and a greater recombination rate, rather than higher gene density in these chromosomes.

Although fewer in number, the 4 microchromosomes of tuatara are similar in many respects to the microchromosomes of birds and other reptiles. Our mapping data indicate a high repeat content on all microchromosomes, particularly chromosomes 17 and 18, explaining the viability of one animal which lacks chromosome 17. The microchromosomes of birds and turtles are also repeat rich [Stefos and Arrighi, 1974; Matzke et al., 1990; Fillon et al., 1998; Yamada et al., 2005] despite their abundance of genes [McQueen et al., 1998; Burt, 2002; Kuraku et al., 2006]. The size of tuatara microchromosomes relative to the rest of the genome (smallest 0.9% – largest 2%) [Norris et al., 2004] is comparable to the range found in chicken (smallest 0.4% – largest 2%) [Bloom et al., 1993]. In absolute terms, however, the microchromosomes of tuatara are very much larger, with a size range of 43–113 Mb (1 pg = 978 Mb) [Dolezel et al., 2003], compared to just 7–23 Mb in chicken [Bloom et al., 1993]. The gene content of these large tuatara microchromosomes remains unknown, and we were unable to examine replication timing explicitly. Squamates and archosaurs (with the exception of crocodilians) usually possess a large complement of microchromosomes. The reduction of microchromosomes in the tuatara karyotype probably proceeded by fusion events, as proposed for crocodilians and many birds [Burt, 2002; de Oliveira et al., 2005; Nie et al., 2009].

Conclusion

Tuatara has frequently been referred to as a 'living fossil' because it bears some skeletal features mistakenly interpreted as primitive (they are in fact derived) [Whiteside 1986; Mo et al., 2010]. It appears that many features of its genome are evolutionarily derived as well. These include the large genome size [Thomson and Muraszko, 1978; Burt, 2002], a high GC content [Wang et al., 2006], absence of interstitial telomeres [Meyne et al., 1990] and a small number of microchromosomes [Norris et al., 2004]. Our study presents a preliminary overview of genome evolution and organization in tuatara. A gene dense cytogenetic map covering all the chromosomes will elucidate the extent to which lineage-specific chromosomal rearrangements have occurred, and which are retained. Such a map is also essential should a tuatara genome project be undertaken [Lewin et al., 2009]. Even low coverage sequencing can be of great utility [Green, 2007] and the tuatara's large genome may harbor countless novel genes and regulatory elements [Peterson et al., 2009].

Our low-coverage cytogenetic map identified a region on chromosome 4 that shares homology with chicken chromosome 2, and therefore the orthologous region of the snake Z chromosome. We identified a region on chromosome 3 that is orthologous to the chicken Z, and a region on chromosome 9 homologous to the mammalian X. A cytogenetic map of higher resolution would be of great benefit in understanding the evolution of amniote sex chromosomes from autosomal pairs. Similarly, knowledge of the genic content of the microchromosomes will lead to a greater understanding of their evolution; our observation of one animal that lacked a pair suggests that not all microchromosomes are necessary for survival. Sequence analysis of a small genome sample increased the estimate of genome-wide GC composition to 47.8%, the highest reported among vertebrates to date. This is an interesting observation as high GC content has often been considered an adaptive response to high body temperature [Bernardi, 2000; Olmo, 2003], but tuatara has the lowest active body temperature of any amniote. The tuatara's large genome is doubtless due to accumulation of repetitive sequences. Such sequences are key agents of regulatory innovation, chromosome rearrangements and evolutionary change [Sharp et al., 2006; Jurka et al., 2007]. Perhaps this species' generous endowment of repetitive DNA has played a role in its continued survival over the last 60 m.y., despite dramatic changes in geology, climate and population size [Cooper and Cooper, 1995; Landis et al., 2008; Jones et al., 2009].

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