Cytogenet Genome Res 105:93–99 (2004)
DOI: 10.1159/000078014

Cytogenetic and Genome Research

Chromosomes of tuatara, *Sphenodon*, a chromosome heteromorphism and an archaic reptilian karyotype

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Abstract. We examined karyotypes of the endemic New Zealand reptile genus *Sphenodon* (tuatara) from five populations, finding a karyotype unchanged for at least one million years. Animals karyotyped were from five geographically distinct populations, representing three groups, namely *S. guntheri*, *S. punctatus* (Cook Strait group), and *S. punctatus* (northeastern North Island group). All five populations have a diploid chromosome number of 2n = 36, consisting of 14 pairs of macrochromosomes and four pairs of microchromosomes. Chromosomal differences were not found between the five populations nor between female and male animals, except for one animal with a structural heteromorphism. Similarity between *Sphenodon* and Testudine karyotypes suggests an ancestral karyotype with a macrochromosome complement of 14 pairs and the ability to accumulate variable numbers of microchromosome pairs. Our research supports molecular phylogenies of the Reptilia.

Sphenodon is the only surviving genus of the ancient diapsid order Sphenodontia and is represented by two species, *S. guntheri* and *S. punctatus*. *Sphenodon guntheri* (Buller, 1877) survives on North Brother Island, Cook Strait, New Zealand, and has recently been reinstated to species status (Daugherty et al., 1990). The other species, *Sphenodon punctatus*, is present on four islands in western Cook Strait, New Zealand (here represented by Stephens Island tuatara, *S. punctatus* Cook Strait group), and on 26 islands off the northeastern North Island of New Zealand (represented here by Ruamahua-iti, Aorangi and Stanley Island tuatara, *S. punctatus* northeastern group).

The evolutionary position of *Sphenodon* remains contentious. On the basis of morphological studies most authors group *Sphenodon* as a sister-group of Squamata within the superorder Lepidosauria (Olmo, 1986; Benton, 2000). With the development of molecular techniques for phylogenetic analysis, a number of phylogenetic trees have been produced with the aim of clarifying the position of *Sphenodon* within the Reptilia. Examination of combined nuclear protein coding gene sequences and mitochondrial DNA demonstrated that the morphological grouping of *Sphenodon* with squamates is not supported (Seutin et al., 1994; Hedges and Poling, 1999). Rather, the authors grouped *Sphenodon* with crocodiles, turtles and birds, a conclusion supported by examination of hemoglobin subunits (Brown, 1993). Phylogenies of the Reptilia based on αA, αD, and β globin chains (Gorr et al., 1998) did not support a monophyletic Lepidosauria. Indeed, these studies group *Sphenodon* closer to turtles or birds, rather than Squamata. Similar results were obtained from in-depth examination of αD, where *Sphenodon* placed closer to birds and turtles, well away from the Squamata (Shishikura, 2002). Also, examination of *Sphenodon* and turtle sperm demonstrated a high level of similarity, and did not support close relationships between Sphenodontia and the Squamata (Healy and Jamieson, 1994).

Two issues of contention over or with the molecular and morphological data are of special interest; namely how closely related *Sphenodon* is to the squamates, and which other reptilian orders belong in the Diapsida. In particular, the phylogenetic position of the Testudines (turtles) is in debate. Traditionally classified as anapsid (Lee, 1997; Wilkinson et al., 1997), recent molecular work (Platz and Conlon, 1997; Zardoya and Meyer, 1998; Hedges and Poling, 1999) prompted the suggestion that the question is no longer whether or not turtles are diapsid, but where in the Diapsida they fit (Rieppel, 1999).

Chromosomal examination of the other three reptilian orders has revealed limited variation within the Testudines and Crocodylia (crocodiles) and extensive variation in morphology

Received 27 May 2003; revision accepted 25 November 2003.

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and number within the Squamata (lizards and snakes). Testudine chromosome variation consists of changes in diploid number, usually related to microchromosome (group C chromosome) number, although some group A and B changes are seen between families (Bickham and Carr, 1983; Bickham, 1984). Karyotypic evolution in turtles has decelerated and is now virtually non-existent (Bickham, 1981). Crocodylia have two karyotypic forms, a predominantly submeta- and metacentric form, and a predominantly subtelo- and telocentric form. The chromosomal phylogeny suggested by Bickham (1984) from a re-analysis of Cohen and Gans (1970) suggests the primitive Crocodylian karyotype was predominantly meta- and submetacentric, with more derived species characterized by fission events. Squamata, on the other hand, are the most speciose of the reptilian orders, and exhibit greater chromosome morphology variability (Olmo, 1986; Olmo et al., 2002). Evidence exists for chromosomal differences within the Squamata leading to speciation (King, 1979; Moritz, 1986).

Previous reports of tuatara chromosomes are limited, predate contemporary methods, and do not state where the animals examined originated from. Two early authors reported low quality spreads obtained from preserved testis material (Hogben, 1921; Keenan, 1932). Wylie et al. (1968) produced the first high quality karyotypes of male and female tuatara using blood from animals of unknown origin. Wylie's work indicated a diploid number of 2n = 36 chromosomes containing 14 pairs of macrochromosomes and four pairs of microchromosomes, separated into four groups based on size and morphology (Wylie et al., 1968). Banding and nucleolus organizing region (NOR) studies were not performed.

The paucity of chromosomal data has so far prevented karyotype comparison between and within *Sphenodon* species, thereby limiting any consideration of chromosome evolution within these reptiles. The present study was initiated in light of knowledge of the time northern and Cook Strait islands became geographically isolated from each other and from mainland New Zealand. The aim was to assess *Sphenodon* chromosomal and banding pattern variation and compare it with other reptiles. In particular, we wanted to know how the chromosomal data fit with competing morphological and molecular phylogenies of the Reptilia.

Material and methods

We took blood samples from 37 specimens of known sex of *S. guntheri*, 12 specimens of known sex of *S. punctatus* Cook Strait group, and 40 specimens of known sex of *S. punctatus* northeastern group, for chromosome analysis.

Sphenodon guntheri from North Brother Island (41°07′S, 174°73′E) were bled at night by drawing 0.5 ml of whole blood from the caudal vein using a heparinized 25-gauge needle and 2.5-ml syringe. In some cases, where more blood could be obtained without stressing the animal, 1 ml of whole blood was taken allowing two cultures to be set up. Each 0.5-ml sample was placed in a sterile 50-ml culture tube containing 10 ml of PB-Max karyotyping medium (Lifetech, Gibco BRL). The preparations were kept at ambient air temperature (8–12 °C) and returned by helicopter to the New Zealand mainland within 12 h of sampling. *Sphenodon punctatus* Cook Strait group was represented by 12 tuatara from Stephens Island (40°40′S, 173°59′E) held in captivity, *S. punctatus* northeastern group was represented by one female Stanley Island (36°38′S, 175°53′E) tuatara held in captivity, 34 tuatara from Ruamahua-iti (Aldermen Island group, 36°58′S, 176°5′E), and five tuatara from Aorangi Island (Poor Knights Island group 35°28′S, 174°43′E). The procedure for blood sampling of all *S. punctatus* individuals was as described for *S. guntheri*.

Blood samples were cultured for six days at 26 °C, then 0.3 ml of 0.05 % colchicine (BDH) was added to the cultures and samples incubated for a further 50 min. Where a second culture from Ruamahua-iti samples was available, colcemid was used as a cell cycle inhibitor (0.5 ml of 10 μg/ml). Cells were then harvested using an adaptation of the human lymphocyte protocol (D. Romain, personal communication) as follows. After colchicine pretreatment, cultures were spun for 10 min at 1000 rpm, the supernatant was removed and cells resuspended in 6 ml of hypotonic solution (0.4 % KCl:0.4 % NaCl 1:1) for 20 min at 26 °C. After hypotonic treatment, cultures were centrifuged for 10 min at 1000 rpm and the supernatant was removed to 0.5 ml. The cell pellet was resuspended in the remaining solution and 2 ml of fresh fixative (3:1 ethanol:acetic acid) quickly added and mixed thoroughly. Additional fixative was added to bring the total volume to 6 ml. After 20 min incubation at room temperature, cells were centrifuged at 1000 rpm for 10 min, supernatant was removed to 1 ml, and the cell pellet resuspended in 5 ml of fresh fixative. Centrifuging, supernatant removal and cell pellet resuspension was repeated 3–5 times until the supernatant became clear. Finally, 1–2 ml of fixative was added and the suspension stored at –20 °C.

Chromosome preparations were made by dropping two to three drops of cell suspension from a height of 60–80 cm onto a slide. Slides were stained with 10 % Giemsa (BDH) in a phosphate buffer solution, pH 6.8, for 10 min. Good quality spreads were photographed on Kodak Tmax100 film using an Olympus AX70 photomicroscope. Karyotyping was done by aligning putative pairs by decreasing lengths. Chromosome arms were measured from the centromere to the tip of the arm, and a minimum of 10 chromosome spreads per animal used for analysis. Arm lengths were expressed as a proportion of the total chromosome length (TCL) in the set. The centromeric index (CI), using averaged TCL-derived arm lengths, allowed the chromosomes to be identified using the nomenclature of Green and Sessions (1991). For the structurally heterozygote animal, chromosome arm lengths were expressed as a proportion of the total chromosome length and their distribution graphed (following Patau, 1960; Craddock, 1975) to compare animals. A normal animal from Ruamahua-iti was used as a control for the presumed normal member of chromosome pair 3. Using TCL to normalize chromosome arm length potentially brings a source of bias when a structural heteromorphism is present. Expressing each arm as a proportion of the entire karyotype ([arm length of chromosome/TCL of all 36 chromosomes] × 100) minimizes bias and was used to prepare Fig. 6.

For C-banding, slides were placed in 0.02 N HCl for 1 h, rinsed, then treated with saturated $Ba(OH)_2$ at 60 °C for 30 s. After further rinses in distilled H_2O, slides were placed in 2× SSC at 60 °C for 1 h, rinsed and stained in a Giemsa solution as described above. Ag-NOR banding was performed using fresh slides stained with two drops of developer (0.5 ml formic acid in 50 ml of 2 % gelatine solution) and four drops of 50 % silver nitrate solution. The two solutions were mixed and spread by placing a large (22 × 50 mm) coverslip over the liquid. Slides were then heated on a heating block until the mixture turned dark brown, at which point the coverslip and liquid was rinsed off and the slide air-dried. For both C- and Ag-NOR banding, slides of human chromosomes were run in parallel with *Sphenodon* slides as a control.

Results

The karyotype of *Sphenodon* contains 18 pairs of chromosomes, consisting of 14 pairs of macrochromosomes and four pairs of microchromosomes (Fig. 1a). Ten of the 14 macrochromosome pairs of *Sphenodon* can be defined using a combination of total chromosome length (TCL) and centromeric index (CI). Data for the range of each chromosome TCL and CI (Table 1) have been used to prepare the idiogram of Fig. 3. Chromosome pairs 1 to 5 can be defined on the basis of TCL and CI as metacentric, submetacentric, telocentric, metacentric and

Fig. 1. Karyotypes of *Sphenodon*. (**a**) Giemsa-stained karyotype of a male *S. guntheri*; (**b**) Ag-NOR-stained karyotype of a female *S. punctatus* (northeastern group, Stanley Island).

Table 1. Quantitative description of *Sphenodon* chromosomes. The range of total chromosome length (TCL) and centromeric index (CI) is listed.

Chromosome	TCL	CI
1	15.80–17.11	1.29–1.37
2	10.11–10.75	1.79–2.10
3	8.18–9.08	7.05–11.61
4	8.16–8.79	1.44–1.60
5	6.71–8.01	1.73–2.76
6	6.21–6.64	4.91–6.63
7	5.85–6.87	10.47–20.00
8	5.20–5.65	1.80–2.07
metacentric 9	4.91–5.49	1.14–1.52
metacentric 10	4.41–4.76	1.08–1.50
11	4.35–4.83	8.69–22.90
12	3.19–3.66	1.07–1.25
submetacentric 13	3.84–4.25	1.89–2.57
submetacentric 14	3.27–3.74	1.73–2.19
15	1.62–2.29	
16	1.38–1.69	
17	1.01–1.53	
18	0.88–1.27	

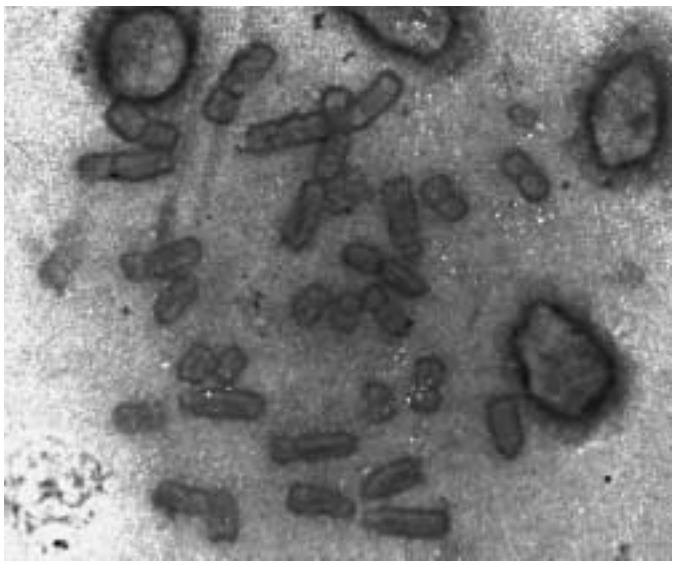

Fig. 2. C-banded metaphase of *Sphenodon*.

submetacentric respectively. Chromosome pairs 6 and 7 are very similar in TCL, but can be distinguished as subtelocentric and telocentric respectively by using CI. Further separation of the two pairs is possible as the telocentric pair always contains the Ag-NOR (Fig. 1b). Chromosome 8 is, in all spreads, a submetacentric chromosome and can be defined using CI, although it is of similar TCL as pairs 9 and 10. Chromosomes 9 and 10 are metacentric and comprise about 10% of TCL (each 5%). Due to similar TCL and CI unequivocal pairing of homologues is not always possible. Chromosome 11 can be defined on the basis of CI and TCL as telocentric. Chromosome 12 is metacentric. Chromosomes 13 and 14 are submetacentric and comprise 7.5% of TCL (about 3.5 and 4% TCL respectively). Their difference in TCL is too small to separate the two chromosome pairs consistently. The remaining four pairs of microchromosomes comprise about 6.5% of TCL. Their morphology cannot be accurately defined.

C-banding in the *Sphenodon* karyotype is shown in Fig. 2. Limited C-banding can be distinguished in the centromeric regions. No intercalary bands are seen, and there are no C-band differences between the sexes, populations or species. Ag-NORs are located on chromosome 7 of the *Sphenodon* karyotype (Fig. 1b). We noted incidences of both heteromorphic and homomorphic Ag-NORs (Fig. 4) which varied within populations, although within the same animal Ag-NOR morphology was consistent.

Twenty-seven spreads were examined from a Ruamahua-iti female tuatara, 18 with colchicine and 9 with colcemid as divi-

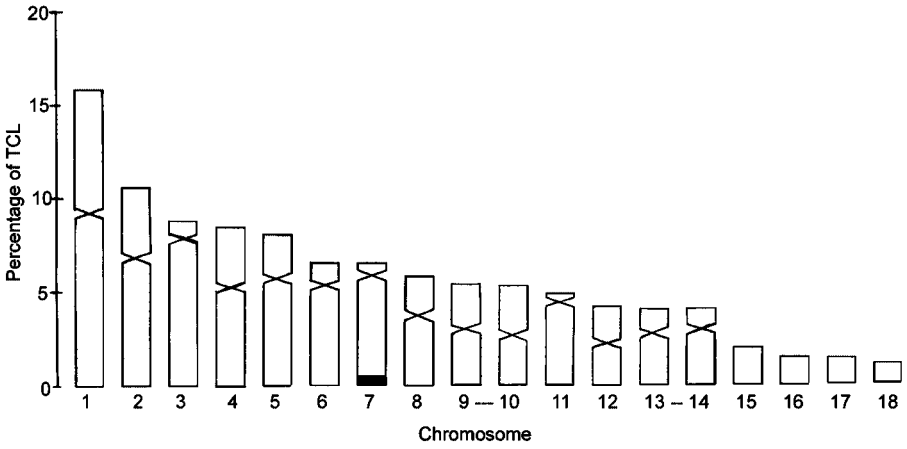

Fig. 3. Idiogram of *Sphenodon* indicating total chromosome length (TCL), centromere position and Ag-NOR location (black block).

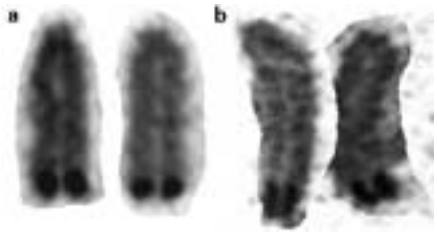

Fig. 4. Variation in Ag-NORs in *Sphenodon*. (**a**) Stanley Island (homomorphic); (**b**) Stephens Island (heteromorphic).

Fig. 5. Partial karyotypes (chromosomes 1–7) of a Ruamahua-iti animal with additional material on chromosome 3. (**a**) Giemsa staining; (**b**) C-banding; (**c**) Ag-NOR staining.

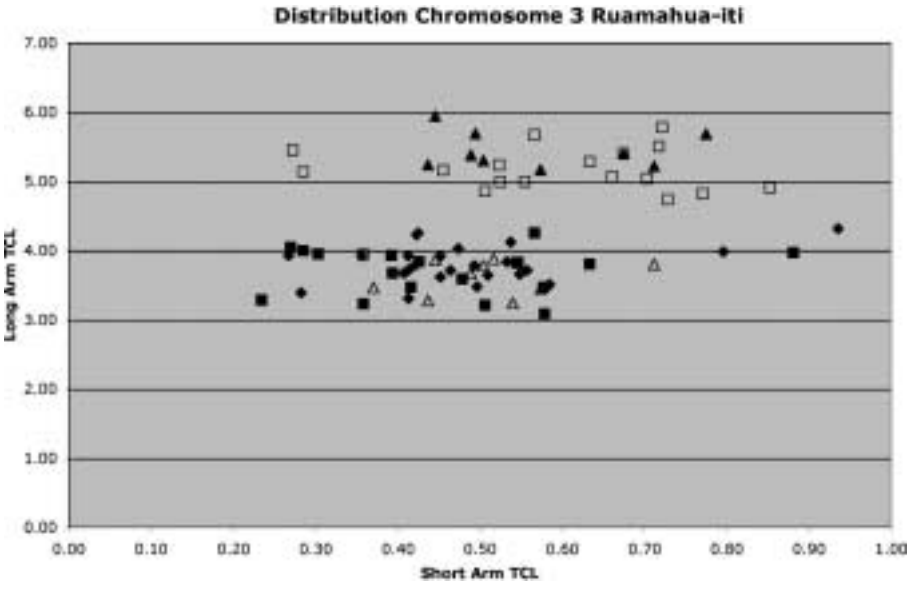

Fig. 6. Scatter diagram of mutant and normal chromosome 3 using colchicine and colcemid as cell cycle inhibitors, plotting TCL of the long arm against TCL of the short arm. (♦) Normal Ruamahua-iti chromosome 3; (□) chromosome 3 with additional material inhibited with colchicine; (■) other member of chromosome pair 3 inhibited with colchicine; (▲) chromosome 3 with additional material inhibited with colcemid; (△) other member of chromosome pair 3 inhibited with colcemid.

Fig. 7. Partial haploid karyotypes (chromosomes 1–14) of (**a**) *Sphenodon punctatus*, 2n = 36; (**b**) *Chelydra serpentina* (Chelydridae), 2n = 52; (**c**) *Clemmys guttata* (Emydidae), 2n = 50; (**d**) *Geochelone carbonaria* (Testudinidae), 2n = 52; (**e**) *Rhinoclemmys punctularia* (Bataguridae), 2n = 56; (**f**) *Carettochelys insculpta* (Carettochelyidae), 2n = 68; (**g**) *Trionyx spiniferus* (Trionychidae), 2n = 66; (**h**) *Kinosternon scorpiodes* (Kinosternidae), 2n = 56; (**i**) *Chelonia mydras* (Cheloniidae), 2n = 56; (**j**) *Chelodina expansa* (Chelidae), 2n = 54; (**k**) *Pelusios subniger* (Pelomedusidae), 2n = 34; (**l**) *Podocnemis unifilis* (Podocnemididae), 2n = 28; (**m**) *Alligator mississippiensis,* 2n = 32; (**n**) *Paleosuchus palpebrosus,* 2n = 42; (**o**) *Crocodylus johnstoni,* 2n = 32. This figure has been constructed with material from the current study, from Cohen and Gans (1970), Bickham (1975), Bickham and Baker (1976), Bickham et al. (1980), Bull and Legler (1980), Haiduk and Bickham (1982), and Bickham et al. (1983). Chromosomes have been resized to allow comparison.

sion inhibitor. In all spreads one chromosome 3 contained an extended long arm that was more than 40% larger than that of its homologue (Fig. 5a). Both cell cycle inhibitors, colcemid and colchicine, produced chromosomes with identical morphology, demonstrating the heteromorphism is not an artifact of preparation. All other chromosomes were present with a normal proportion of TCL, implying the mutation is a duplication or interstitial or terminal extension. The additional material did not stain C-band positive (Fig. 5b) and did not contain a silver-stained nucleolus organizing region (Ag-NOR) (Fig. 5c). A distribution diagram of chromosome 3 TCL (abnormal and normal) comparing colchicine and colcemid treatment with a normal *Sphenodon* karyotype is presented in Fig. 6. The normal chromosome 3 from this animal falls within a normal chromosome 3 TCL distribution for Ruamahua-iti animals. No meiotic material was available for investigation on how the heteromorphism behaved during meiosis.

Chromosomal similarities between *Sphenodon* and the Testudines are apparent in their macrochromosome complements. Haploid karyotypes from eight species of turtle, three species of Crocodylia and one species of *Sphenodon* are presented in Fig. 7.

Discussion

The karyotypes of *S. guntheri, S. punctatus* (Cook Strait group), and *S. punctatus* (northeastern group) analyzed in the present study are identical, with a diploid number of 36 consisting of 14 pairs of macrochromosomes and four pairs of microchromosomes with the exception of one animal. The chromosomes of males and females from the five populations are not distinguishable. C- and Ag-NOR banding has been demonstrated in the genus *Sphenodon*. C-bands are located in the centromeric regions and all chromosomes within the karyotype have a similar banding pattern. No variation in C-bands was seen between populations, indicating a very low level of constitutive heterochromatin accumulation within *Sphenodon*. Ag-NORs were consistently located on chromosome 7 in all populations. Unfortunately, our attempts at G-banding *Sphenodon* chromosomes have so far been unsuccessful. Since Olmo et al. (2002) have recently reviewed that there are "different karyotypical evolutionary rates and different G-banding structures in turtles and crocodiles on the one hand and squamates on the other", this most appropriate staining technique for evolutionary comparisons should be applied by future researchers on *Sphenodon* chromosomes.

Four locations examined, namely North Brother Island, Ruamahua-iti, Stanley Island, and Stephens Island, have been isolated from the New Zealand mainland since the last rise in sea level 8–12,000 years ago, and Aorangi has been isolated for 1 million years (Hayward, 1986) indicating that the tuatara karyotype has remained unchanged for at least 1 million years. Research examining allozymes, albumin, mitochondrial DNA, and nuclear DNA indicated unusually low variation (Hay et al., 2003), a situation reflected in our chromosomal data. The current study offers no contrary evidence to the division suggested of *S. guntheri*, *S. punctatus* (Cook Strait group), and *S. punctatus* (northeastern group) (Hay et al., 2003), but offers little support either as no chromosomal variation is present within *Sphenodon*.

In order to preclude preparatory techniques as the source of chromosome variation, a difference of 20% within the pair is required for a structural heteromorphism to be unambiguously identified (Bentzer et al., 1971). Using this definition, one animal from 89 *Sphenodon* examined karyotypically demonstrated a chromosomal heteromorphism. The additional material on one member of chromosome pair 3 is a duplication, or interstitial or terminal extension, or perhaps an inherited translocated chromosome, as the rest of the karyotype is identical to other *Sphenodon.* Unfortunately, all but 17 animals on Ruamahua-iti are unmarked, so the animal carrying the heteromorphic chromosomes 3 cannot be identified for subsequent study. The animal was an adult female and when measured and examined visually as part of a larger study (Sue Keall, personal observation) did not appear to differ from other Ruamahua-iti animals.

Absence of C-banding suggests the additional material is not heterochromatic, or has not yet transformed into heterochromatin as seen in *Litoria* (King, 1980). Limited distribution of C-bands in *Sphenodon*, and the absence of C-banding on the additional material, prohibits identification of where the extra material may have originated from if an internal duplication of chromosome 3 is not the source. The absence of Ag-NOR banding on the extra material demonstrated that a duplication of the NOR carrying region of chromosome 7 is not the source of the heteromorphism. Without breeding experiments, discussion on maintenance of the extra material is necessarily limited. The rarity and protection status of *Sphenodon* prevents investigation of meiotic material, even if we knew which animal was carrying the addition. The large size of the material, and absence of chromosomal variation in *Sphenodon*, suggests the animal may not be able to produce balanced meiotic products.

The karyotype of *Sphenodon* is significantly different from all of the Squamata (Olmo, 1986). Comparison of the *Sphenodon* karyotype with other reptilian orders allows consideration of karyotype evolution. Crocodylian karyotypes consist of two forms, a predominantly meta- and submetacentric chromosome form and a subtelo- and telocentric form (Cohen and Gans, 1970; Bickham, 1984). The Crocodylian diploid number ranges from 30 to 42, and the chromosomes are significantly different in morphology to *Sphenodon* (Fig. 7). The Testudines have a diploid number between 50 and 70, although high homology between turtle and *Sphenodon* macrochromosomes is apparent between these two ancient lineages (Fig. 7). Two of the nine species of turtles, namely *Trionyx spiniferus* (family Trionychidae) and *Carettochelys insculpta* (family Carettochelyidae), exhibit high chromosomal divergence compared to other Testudines and *Sphenodon* and these two families have been described as the two most divergent and distinctive turtle families (Bickham et al., 1983).

In *Sphenodon* the first 14 chromosomes account for approximately 85% of the TCL, which is comparable to the data presented for various species of *Clemmys*, *Mauremys*, and *Sacalia* (Bickham, 1975). Given the long period of divergent evolution between the Testudine and Sphenodontia and high level of karyotypic conservancy, we suggest that a karyotype for a hypothesized common ancestor is likely to have had a low diploid number, similar to that in *Sphenodon*, and a predisposition towards accumulating variable numbers of microchromosomes. Microchromosome accumulation could occur either through macrochromosome breakages or de novo formation. The first 14 chromosome pairs of the hypothesized ancestor would have been similar in morphology to the chromosomes seen currently in *Sphenodon* and most Testudine karyotypes. Evidence of chromosomal rearrangements can be seen in NOR positional changes between turtles and *Sphenodon*. No examples of a telomeric NOR on a large telocentric chromosome, as seen in *Sphenodon*, are present in turtles (Bickham and Rogers, 1985). A recent review of avian microchromosomes suggested a primitive avian karyotype had around 20 microchromosomes (Burt, 2002). This microchromosome number is similar to that of the Testudines, suggesting that either *Sphenodon* has lost microchromosomes from a primitive karyotype with around 20 microchromosomes, or that the turtle and bird lineages went through a period of rapid microchromosome accumulation since *Sphenodon* divergence.

The Testudine order split from a reptilian common ancestor around 300 million years ago (Benton, 2000) and the Sphenodontia are thought to have split from the Lepidosauria line dur-

ing the Triassic (Benton, 2000). The current study does not answer the question of the phylogenetic position of *Sphenodon*, but it does suggest a close chromosomal relationship with turtles, offering support to molecular (Brown, 1993; Seutin et al., 1994; Gorr et al., 1998; Zardoya and Meyer, 1998; Hedges and Poling, 1999; Shishikura, 2002) and spermatogonial work (Healy and Jamieson, 1994). It is presumed that significant DNA changes and inter-chromosomal rearrangements have accumulated during this time, but gross chromosome morphology has remained conserved.

Acknowledgements

We acknowledge the support of *Te Atiawa*, *Ngati Koata*, *Ngati Tamatera, Ngati Wai* and the New Zealand Department of Conservation, including that of Peter Gaze, Jason Roxburgh, Richard Parrish and Ian Hogarth. The following people provided assistance during the fieldwork: Bernard Goetz, Barbara Blanchard, Susan Keall, Nicky Nelson, Bruce Benseman, Brendan Daugherty, Gilly Adams, Leigh Bull, James Sainsbury, Karena Eton and Sarah Coddington-Lawson. Ann Wylie provided valuable information concerning her study; Dennis Romain (Genetic Services, Wellington Hospital, New Zealand) provided assistance on blood culture techniques, and John Bickham provided information on turtle chromosomes. Thanks are due to Frances Butcher for support during the research.

Research complied with relevant legislation, Department of Conservation permits: BRO 001, TAK 0021, 9/253 ROA, HAMRO 71042; VUW Animal Ethics Committee permit GR99R1.

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