Chromosomes, nuclear genes and the phylogenetic placement within the Reptilia of *Sphenodon* (tuatara)

By

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Abstract

Chromosomes were examined from five populations of *Sphenodon* (tuatara) using giemsa, Ag-NOR, C-, G- and RE- banding. There were no differences between species, populations or sexes, although one animal had a structural heteromorphism.

Chromosome morphology homology to Testudines (turtles), Aves (birds) and to a lesser extent Crocodylia (crocodiles) allowed reconstruction of a Reptilian proto-karyotype, dated to 300 million years ago.

DNA sequence was isolated from the WT1, AMH, DMRT1, FoxG1 and 28S. No variation was present in *Sphenodon* 28S, FoxG1 or AMH sequence. 28S could be dated to a common ancestor with Testudines, similar to the archaic karyotype. FoxG1 and AMH reflect an Oligocene divergence, WT1 divides north-eastern North Island and Cook Strait, and can be dated to the Pleistocene or the Pliocene, and DMRT1 appears a recent post-Pliocene divergence.

FISH localised DIG-labelled probes of AMH to chromosome 11 and WT1 to chromosome 13 or 14. Human telomeric probes localised to *Sphenodon* telomeric regions demonstrating the highly conserved nature of telomeric sequences. Comparative genomic hybridisation with chicken chromosomes did not produce any regions of homology, implying significant chromosomal and DNA changes since the Orders shared a common ancestor, although macrochromosome morphology has remained similar.

Sphenodon chromosomal and nuclear DNA analyses demonstrate evolutionary decoupling, supporting recent mtDNA work.

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This thesis would not have been possible with the support – both emotional and financial - and encouragement from my late wife, Frances Butcher. I dedicate this work to her. Thanks also to our respective families for support and encouragement.

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Chapter 1 : Chromosomes of *Sphenodon* (tuatara)

An in-depth examination of *Sphenodon* (tuatara) chromosomes was performed. Five populations, representing both *Sphenodon* species and both groups of *S. punctatus* were sampled. No sex, population or species differences were seen with Giemsa, C-, AgNOR-, G- and RE- banding although one animal from Ruamahua-iti possessed a structural heteromorphism on chromosome 3. Presence of an identical karyotype in the Poor Knights Island population demonstrates the *Sphenodon* karyotype has been unchanged for at least one million years.

Comparing *Sphenodon* chromosomes to other reptiles demonstrated high homology to Testudines, Aves and to a lesser extent the Crocodylia.

Introduction

The sole member of the reptilian order Sphenodontida in the superorder Rhynchocephalia, *Sphenodon* (tuatara) is endemic to New Zealand. The phylogenetic position of Sphenodontida is in question (addressed in greater detail in Chapter 2) with conflicting phylogenies produced from molecular and morphological evidence. Within *Sphenodon* recent work has clarified relationships between populations (Hay, Daugherty et al. 2003).

There are four extant orders of reptiles; namely Sphenodontida, Testudines (turtles), Crocodylia (crocodiles and alligators) and Squamata (lizards and

snakes). With the exception of the Sphenodontida, karyotypes of the reptilian orders have been extensively examined.

The orders Sphenodontida, Testudine, and Crocodylia are significantly older than Squamata. Sphenodontida separated around 210-230 million years ago (MYA), Crocodylia 250 MYA, and the Testudines during the lower Permian (290 MYA) (Benton 2000) so that karyotypes have had a long period in which to evolve and differentiate.

The most recent order, Squamata, is thought to have diverged during the late Jurassic (around 150 MYA) (Benton 2000). Chromosomal variation is much higher in this Order, with no obvious similarities between karyotypes of the infraorders Scincomorpha (skinks), Gekkota (geckos), Iguania (iguanas), Diplogossia (includes glass lizards), Platynota (monitor lizards) and the suborder Ophidia (snakes) (Bickham 1984; Olmo 1986; Zug, Vitt et al. 2001).

Two major karyology reviews have been published (Bickham 1984; Olmo 1986), and one summary on chromosome banding techniques performed on reptiles (Olmo 2005). These reviews form the basis of this introduction and have been updated where appropriate.

Reptilian Chromosomes: An Overview

Turtle chromosomes

The most speciose of the three archaic reptilian lineages, the Order Testudine is divided into two suborders, 13 families, and over 285 species (Zug, Vitt et al. 2001). Diploid numbers range from 26-68 (Bickham 1984; Olmo 1986), with most of the variability resulting from changes in numbers of microchromosomes.

The suborder Pleurodira

Pleurodira, the side-necked turtles (where the head cannot be drawn into the carapace), contains the superfamilies Pelomedusoides, Podocnemididae, and Chelidae with over 65 species between them (Zug, Vitt et al. 2001).
All species of *Pelusios* (superfamily Pelomedusoides) have an identical karyotype of 34 chromosomes, species of *Pelomedusa* (superfamily Pelomedusoides) differ from *Pelusios* by having a uniarmed chromosome 5 and two extra microchromosomes (Bull and Legler 1980). The first 11 chromosomes in the superfamily Pelomedusoides are nearly indistinguishable (Bull and Legler 1980), the only major variation occurring in microchromosome number.

Within *Podocnemys* (superfamily Podocnemididae) all species have identical 2n=28 karyotypes; *Erymnochelys madagascariensis* differs from *Podocnemys* by two metacentric chromosomes (rather than two subtelocentrics) and *Peltocephalus dumeriliana* has a diploid number of 2n=26 with all chromosomes biarmed.
The family Chelidae has over 50 species in 11 genera, and exhibits very high levels of karyotypic homogeneity, with diploid numbers ranging between 2n=50 and 64.

Two competing hypotheses have been advanced to explain chromosome evolution in the Pleurodira. If the Pelomedusoides are the most archaic and primitive members of the suborder (and there is morphological evidence to support this), the archaic condition is a low diploid chromosome number, and the Chelids are derived via duplication mechanisms (Killebrew 1975). The

alternative hypothesis proposed suggests that Chelids have a primitive karyotype and the Pelomedusids have lost a high number of microchromosomes (Bull and Legler 1980).

The suborder Cryptodira

The suborder Cryptodira, or hidden-neck turtles, retract their neck and head into the body cavity (Zug, Vitt et al. 2001).

The family Chelydridae contains three genera, *Chelydra*, *Macrochelys*, and *Platysternon*. *Chelydra* and *Macrochelys* have a diploid number of 2n=52, and *Platysternon* a diploid number of 2n=54. The karyotype of *Chelydra* consists of 12 macrochromosome and 14 microchromosome pairs, whereas *Macrochelys* has 13 macrochromosome and 13 microchromosome pairs. The *Macrochelys* karyotype can be derived from *Chelydra* by translocation of the NOR-bearing region in *Chelydra* (Bickham and Baker 1976; Haiduk and Bickham 1982). The karyotype of *Platysternon* consists of 13 macrochromosomes and 14 microchromosome pairs (Haiduk and Bickham 1982), gross macrochromosomes morphology is identical to *Chelydra* and *Macrochelys*, the diploid number difference results from the addition of one microchromosome pair.

The superfamily Chelonioidea contains the families Cheloniidae (five genera), and Dermochelyidae (one species, *Dermochelys coriacea*).

Karyotypes from the Cheloniidae all show a diploid number of 2n=56, consisting of 12 macrochromosome and 16 microchromosome pairs (Bickham, Bjorndal et al. 1980; Bickham 1981). Comparative chromosome banding demonstrated high homology between *Chelonia mydras*, *Dermatemys mawii* (superfamily

Kinosternoidea) and the Emydidae (superfamily Testudinoidea) (Bickham, Bjorndal et al. 1980; Carr, Bickham et al. 1981).

The Trionychoidea superfamily contains two families, the Carettochelyidae (one species), and the Trionychidae (two subfamilies, 14 genera, and more than 26 species).

Carettochelys insculpta has a diploid number of 68, 6 pairs of macrochromosomes and 28 pairs of microchromosomes (Bickham, Bull et al. 1983). All species karyotyped in the Trionychidae have 66 chromosomes, 6 pairs of macrochromosomes and 27 pairs of microchromosomes (Bickham, Bull et al. 1983). The superfamily Trionychoidea is regarded as the most divergent and distinctive of the Testudines on the basis of morphological and molecular (Shaffer, Meylan et al. 1997), serological (Frair 1983), and karyological (Bickham, Bull et al. 1983) studies.

Two families have been identified in the Kinosternoidea, the Dermatemydidae (one species) and the Kinosternidae (2 subfamilies, 4 genera and more than 23 species) (Zug, Vitt et al. 2001).

Dermatemys mawii has a diploid number of 2n=56 consisting of 12 macrochromosome and 16 microchromosome pairs. The karyotypic homology of this ancient family to many other Cryptodira suggests the karyotype is similar to an ancestral form (Carr, Bickham et al. 1981). Within the Kinosternidae family a diploid number of 2n=54 is seen in all Staurotypins and 2n=56 in all Kinosternins, resulting from the addition of one extra microchromosome pair (Sites, Bickham et al. 1979).

The remaining superfamily, Testudinoidea, contains three families, the Emydidae (10 genera with at least 40 species), the Bataguridae (23 genera and a minimum of 65 species), and the Testudinidae (11 genera and at least 45 species) (Zug, Vitt et al. 2001).

The Emydidae have a diploid number of 2n=50 with 13 macrochromosome and 12 microchromosome pairs (Bickham 1975; Bickham and Baker 1976; Bickham and Baker 1976). High chromosomal homology is seen in the Testudinidae with a diploid number of 2n=52 consisting of 14 macrochromosome and 12 microchromosome pairs in most genera, except the derived *Gopherus* and one species of *Testudo*. The karyotype is very similar to the Bataguridae and the Emydidae.

The Bataguridae are a diverse group of turtles, a situation that has led to phylogenetic confusion. Chromosomally, diploid numbers range from 2n=50-56, with 11-13 macrochromosome and 12-17 microchromosome pairs. The major differences appear to be numerical changes in the number of microchromosomes. Male heteromorphic chromosomes have been observed in *Siebenrockiella crassicollis* and *Staurotypus salvinii* and *Staurotypus triporcatus* (Bull, Moon et al. 1974; Sites, Bickham et al. 1979; Carr and Bickham 1981), although the karyotype of other closely related *Siebenrockiella* species is very similar to a presumed ancestral karyotype (Bickham and Baker 1976).

Both major reviews of Testudine karyology (Bickham 1984; Olmo 1986) emphasise high levels of similarity between the suborders, superfamilies and families. Previously published Testudine karyotypes representing each family

were rearranged in descending height, resized to facilitate comparison and presented as Figure 1.1. Comparison of the various karyotypes demonstrates high homology particularly in the first 11-14 pairs of macrochromosomes. Two families stand out as having significantly different karyology, namely the Carettochelyidae and Trionychidae, a situation supported by studies that describe these families as the most derived Testudines (Bickham, Bull et al. 1983).

Figure 1.1 : Haploid turtle karyotypes. Key: 1=Chelydra serpentina, 2=Macroclemmys temminckii, 3=Platysternon megacephalum, 4=Clemmys guttata, 5=Sacalia bealei, 6=Siebenrockiella crassicolis, 7=Maureyms caspica, 8=Rhinoclemmys punctularia, 9=Rhinoclemmys pulcherrima**,** 10=Geochelone denticulata, 11=Geochelone carbonaria, 12=Chinemys reevesi, 13=Carettochelys insculpta, 14=Trionyx spiniferus, 15=Kinosternon scorpiodes, 16=Chelonia mydras, 17=Batrachelys dahli, 18=Chelus fimbriatus, 19=Hydromedusa tectifera (first 11 chromosomes), 20=Phyrnops geoffroanus, 21=Platemys platycephala (first 13 chromosomes), 22=Chelodina expansei, 23=Chelodina steindachneri (first 11 chromosomes), 24=Elseya dentate, 25=Rheodytes (first 11 chromosomes only), 26=Emydura australis, 27=Pseudomydura umbrina, 28=Pelusios subniger, 29=Pelomedusa subrufa, 30=Podocnemis unifilis, 31=Erymnochelys madagascariensis (adapted from previously published material Bickham 1975; Killebrew 1975; Bickham 1976; Bickham and Baker 1976; Bickham, Bjorndal et al. 1980; Bull and Legler 1980; Carr and Bickham 1981; Haiduk and Bickham 1982; Bickham, Bull et al. 1983; Bickham and Carr 1983; Bickham, Tucker et al. 1985).

Crocodylian chromosomes

The order Crocodylia is the sole extant reptilian representatives of the superorder Archosauria that originated in the late Permian or early Triassic (around 250 MYA), and contains 23 species (Benton 2000; Zug, Vitt et al. 2001). Diploid numbers range from 2n=30-42 with two major karyotypic forms seen, a predominately meta- and submeta- centric chromosome form, and a predominately telo- and subtelo- centric chromosome form. Figure 1.2a summarises Crocodylian karyology and is ordered following a traditional phylogeny (Bickham 1984; Poe 1996; Zug, Vitt et al. 2001), whereas Figure 1.2b reflects recent reordering of the Crocodylia based on mtDNA (Janke, Gullberg et al. 2005). Similar to the Testudines, low levels of chromosomal variation are present in the Crocodylia with *Alligator*, *Tomistoma* and *Gavialis* possessing a presumed basal karyotype (Bickham 1984).

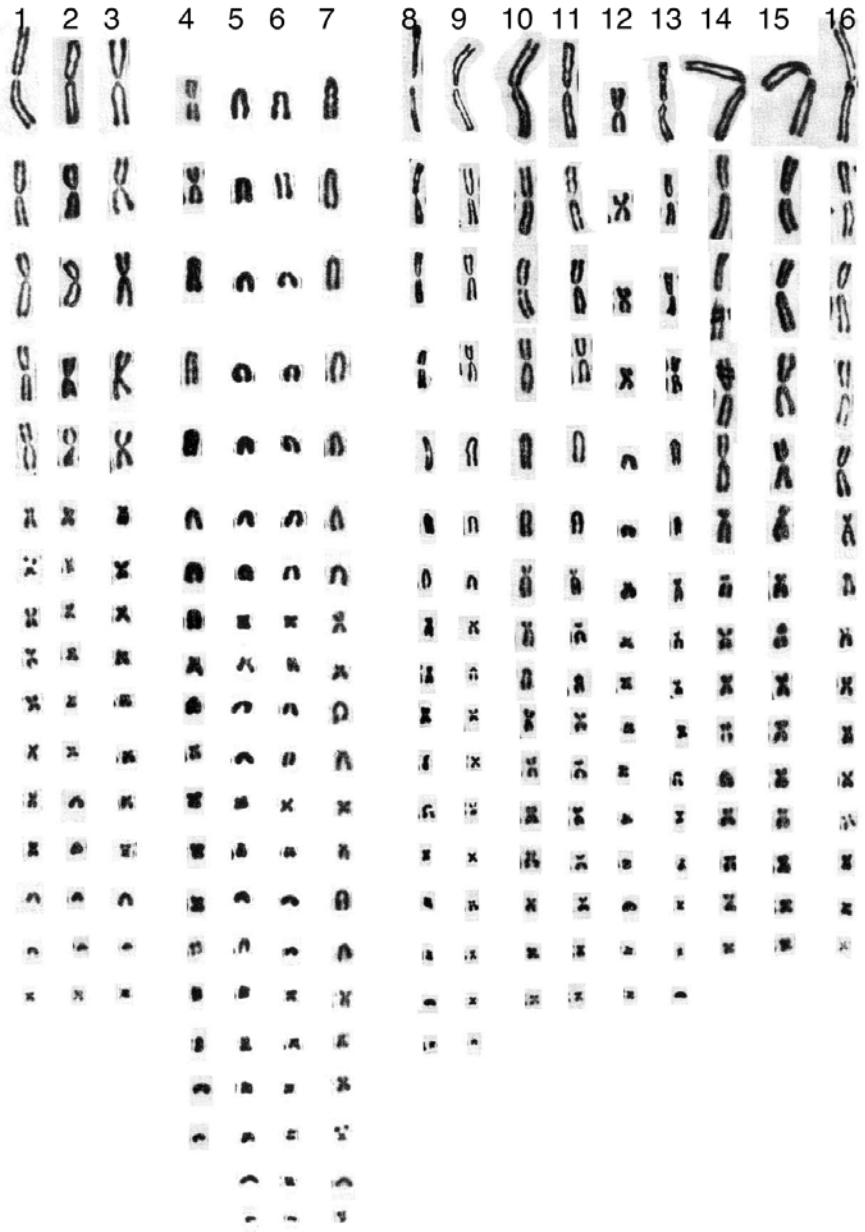


Figure 1.2a: Haploid crocodilian karyotypes arranged using traditional crocodilian phylogenies. Key 1=*Alligator mississippiensis*, 2=*Tomistoma schlegelii*, 3=*Gavialis gangeticus*, 4=*Ostelaemus tetraspis*, 5=*Paleosuchus palpebrosus*, 6=*Melanosuchus niger*, 7=*Caiman latirostris*, 8=*Crocodylus siamensis*, 9=*Crocodylus porosus*, 10=*Crocodylus intermedius*, 11=*Crocodylus johnsoni*, 12=*Crocodylus novaeguineae*, 13=*Crocodylus moreletti*, 14=*Crocodylus cataphractus*, 15=*Crocodylus palustris*, 16=*Crocodylus rhombifer*. Adapted from previously published material (Cohen and Gans 1970).

Figure 1.2b: Haploid Crocodylian karyotypes arranged following an alternate phylogeny (Janke, Gullberg et al. 2005). Key 1=*Alligator mississippiensis*, 2=*Ostelaemus tetraspis*, 3=*Paleosuchus palpebrosus*, 4=*Melanosuchus niger*, 5=*Caiman latirostris*, 6=*Gavialis gangeticus*, 7= *Tomistoma schlegelii*, 8=*Crocodylus siamensis*, 9=*Crocodylus porosus*, 10=*Crocodylus intermedius*, 11=*Crocodylus johnsoni*, 12=*Crocodylus novaeguineae*, 13=*Crocodylus moreletti*, 14=*Crocodylus cataphractus*, 15=*Crocodylus palustris*, 16=*Crocodylus rhombifer*. Adapted from previously published material (Cohen and Gans 1970).

Squamata chromosomes

The Squamata are the most recent divergence of the Reptilia. A diploid chromosome number of 36 is common, although significant variation occurs in both diploid number and morphology within families, and often between closely

related species (Bickham 1984; Olmo 1986). Squamata, in addition to the being the most recent reptilian radiation, are also the most speciose with well over 6000 species described (Apesteguia and Novas 2003). There are also higher levels of heterozygous sex chromosomes within the Squamata, although some authors have perpetuated a misinterpretation of the presence of sex chromosomes within the New Zealand genus *Cyclodina* (Donnellan 1991; Shine, Elphick et al. 2002), contrary to work done on the genus (O'Malley 1971; Norris 2003).

Sphenodon chromosomes

Chromosomes of the tuatara have been demonstrated in three papers prior to the current study. Early twentieth century research used testicular material, producing poor quality karyotypes resulting from technical limitations in karyotype preparation. Hogben identified 26 chromosomes and a number of "granules" (Hogben 1921), which subsequent studies have identified as microchromosomes. Keenan identified a chromosome number of 36 (Keenan 1932), although chromosome morphology was impossible to distinguish**.** The first example of tuatara chromosomes using modern blood culturing cytological methods examined one male and one female from an unknown location (Wylie, Veale et al. 1968). A diploid number of 2n=36 was confirmed, consisting of 14 pairs of macrochromosomes, and four pairs of microchromosomes. High quality chromosome preparations allowed chromosome morphology to be accurately determined, and no sex related heteromorphisms were seen.

*The Tuatara (*Sphenodon*)*

Distribution and description of archaic sphenodontians

The inclusive clade Rhynchocephalia comprising Gephyrosaurus and Sphenodontia (Evans and Sigogneau-Russell 1997) has never been as speciose as other reptilian lineages. The peak of Rhynchocephalia diversification occurred during the late Triassic and Upper Jurassic eras (Benton 2000). During this time Sphendontians had a worldwide distribution (Figure 1.3). At least eight genera were present in Europe during the Triassic, including insectivory and herbivory sphenodontians and by the late Jurassic this number had increased to around 30 sphenodontian forms (Reynoso 1996), including aquatic forms in France (Evans and Sigogneau-Russell 1997).

The first fossil records of sphenodontians are from Scotland and Germany during the Triassic (Fraser and Benton 1989). By the Late Triassic/Early Jurassic sphenodontians were present throughout much of Europe, China, North America, and Mexico (Figure 1.3). The first recorded Gondwanic presence of sphenodontians is from early Cretaceous fossils found in Morocco. Two species have been identified, *Tingitana anovalae* and an unnamed genus and species "Sphenodontian B", both are similar to European, American and Chinese species although *Tingitana* appears distinct on the limited material available (Evans and Sigogneau-Russell 1997).

An early Mesozoic sphenodontian of the *Clevosaurus* genus was found in South Africa, the first record of *Clevosaurus* in the southern hemisphere. The partial

skull appeared similar to Canadian and Chinese specimens supporting a Pangean distribution (Sues and Reisz 1995).

Although not as plentiful as in Europe, sphenodontians were present in the southwest USA during the Upper Triassic. Identification has proven difficult as only fragments have been found, and is complicated by sphenodontian ontogenetic tooth differentiation (Harris, Lucus et al. 1999).

Cynosphenodon huizachalensis was described from mid Jurassic fossil material found in the Huizachal Canyon, Mexico, and contained many shared characteristics with extant *Sphenodon* (Reynoso 1996). The Huizachal Canyon has provided a wealth of sphenodontid material including a 'dwarf' form, *Zapaton ejidoensis*, which appears very similar to *Sphenodon* and *Cynosphenodon* (Reynoso and Clark 1998). Recent work describing sphenodontids from the Huizachal Canyon includes a middle Jurassic species which may have had venom apparatus, and again is probably a close relative of *Sphenodon* and *Cynosphenodon* (Reynoso 2005).

Reynoso constructed a Rhynchocephalian lineage demonstrating that *Sphenodon* although archaic, is a highly derived genus. The number of shared characteristics with the presumed sister genus, *Cynosphenodon*, imply that the shared characters were present by the Middle Jurassic (Reynoso 1996). An in-depth examination of dental and jaw formation between *Cynosphenodon* and *Sphenodon* supported the monophyly of the sister grouping of the two genera (Reynoso 2003).

By the late Jurassic and into the Cretaceous, specialisation was occuring in the American sphenodontians. In particular the Tlayua formation in Central Mexico appears to have been a hotspot of reptilian diversification during the Jurassic. Two unique sphenodontians have been found in this area, a "beaded" sphenodontian and a semi-aquatic sphenodontian. Protective covering via beadlike osteoscutes have been identified on *Pamizinsaurus tlayuaensis*, a new species closely related to extant *Sphenodon* (Reynoso 1997). *Ankylosphenodon pachystosus* is similar morphologically to *Sphenodon*, particularly in the number and location of ribs and dental arrangement, however the skeleton suggests a semi-aquatic lifestyle, the first description of such a sphenodontian, although obligatory aquatic sphenodontians are known (Reynoso 2000).

Patagonian fossil discoveries strongly suggest that Sphenodontians were radiating very successfully during the Cretaceous, and were a major part of the South American ecosystem (Apesteguia and Novas 2003). Evidence from Laurasia suggests sphenodontians were being out-competed by lizards (Carroll 1988), but the quantity and variety of fossils from South America support the view that Gondwanic sphenodontians played a significant ecological role. For instance, the largest sphenodontian ever found, *Priosphenodon avelasi*, is from late Cretaceous era Patagonia with adults measuring up to 1m in length (Apesteguia and Novas 2003).

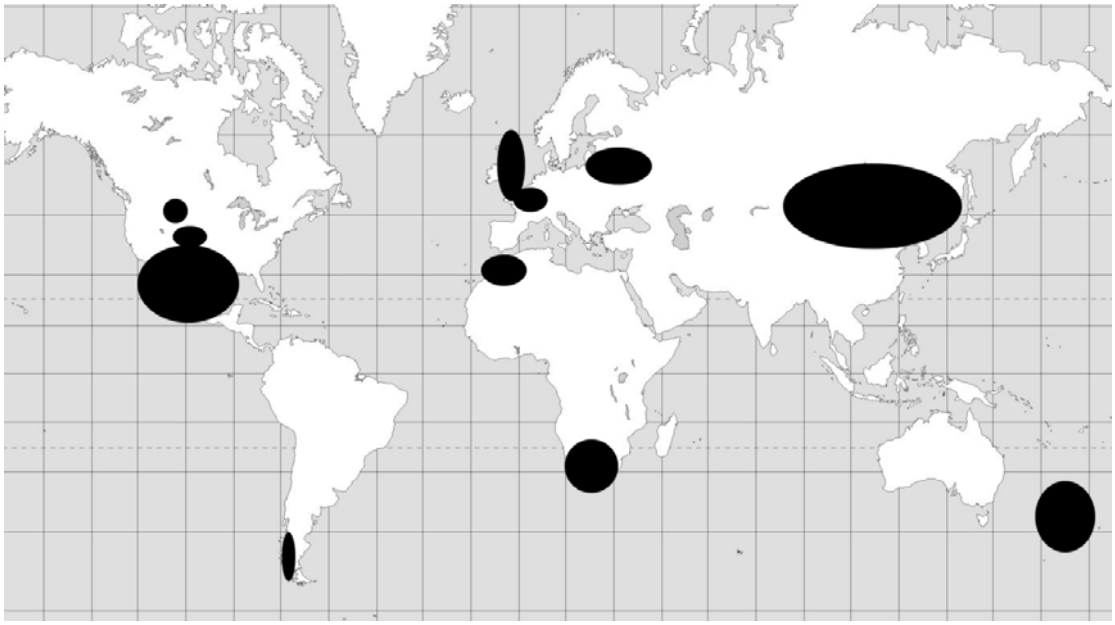


Figure 1.3 : Distribution of fossil Sphenodontian material on a modern world map. Created using a variety of sources (Sues and Reisz 1995; Reynoso 1996; Reynoso 1996; Evans and Sigogneau-Russell 1997; Reynoso 1997; Reynoso and Clark 1998; Benton 2000; Reynoso 2000; Apesteguia and Novas 2003).

Location of extant tuatara and relationships between populations

Tuatara survive on 35 New Zealand predominantly rodent free offshore islands (Gaze 2001; Hay, Daugherty et al. 2003) (Figure 1.4). Recent work clarified extant *Sphenodon* phylogeny. The trinomial *Sphenodon punctatus punctatus* was dropped from the description of the north-eastern North Island populations, leaving *Sphenodon punctatus* with two groups, namely; *Sphenodon punctatus* (Cook Strait group), and *Sphenodon punctatus* (north-eastern North Island group) (Hay, Daugherty et al. 2003). *Sphenodon guntheri* (Buller 1877) is found on North Brother Island (and two islands containing transplanted populations) and was recently restored to species status (Daugherty, Cree et al. 1990). Very low variation in *Sphenodon* allozymes, mtDNA, nuclear DNA, and albumin (Hay, Daugherty et al. 2003) suggests a stable genome.

All of the islands containing *Sphenodon* were isolated from the New Zealand mainland during the last glacial period 8-10,000 years ago, except for the Poor Knights Island group, which has been isolated for up to one million years (Hayward 1986).

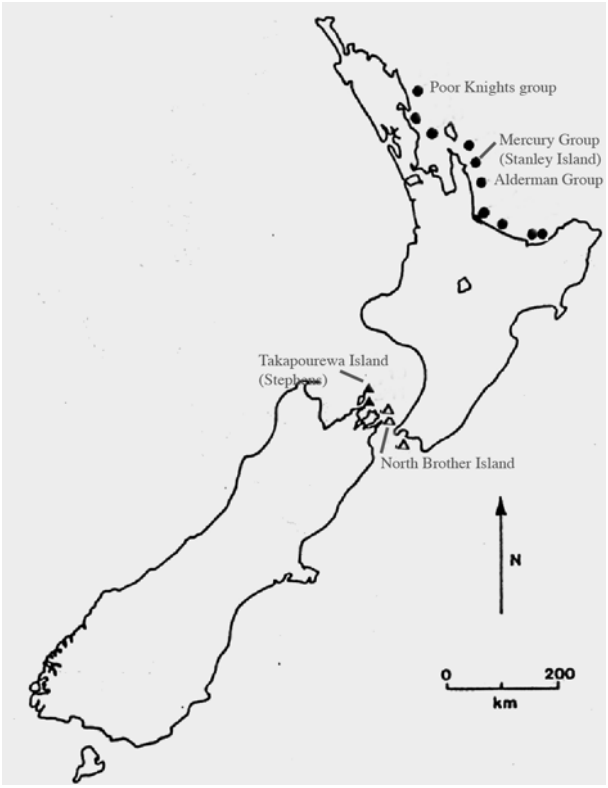


Figure 1.4: Present distribution of tuatara (Gaze 2001).

Northern Tuatara (solid circles)	**Cook Strait Tuatara (solid triangles)**	**Brothers Tuatara (open triangle)**
Poor Knights Group (5 islands)	Takapourewa (Stephens) Island	North Brother Island
Hen and Chickens Group (5 islands)	Trios Group (3 islands)	Tito Island
Little Barrier Island		Matiu/Somes Island
Cuvier Island		
Mercury Group (4 islands)		
Alderman Group (7 islands)		
Karewa Island		
Plate Island		
Moutoki Island		
Moutohora Island		

Reptilian chromosome banding and use of banding for evolutionary studies

Banding of chromosomes started in the late 1960s when it was demonstrated that action of repetitive DNA sequences, heat, alkali, trypsin and detergents induced individual reproducible bands on human chromosomes (Pardue and Gall 1970; Seabright 1971; Sumner, Evans et al. 1971). Banding studies allowed human karyotypes to be further separated, morphologically identical chromosomes could be distinguished by the presence or absence of bands, allowing unambiguous identification of chromosome pairs. The range of techniques gradually increased, each revealing different cytological aspects of the chromosomes. Human banding techniques were adapted by animal and plant karyologists, allowing comparisons of relationships between various species or populations to be made. For example banding studies on the Australian Gekkonidae revealed significant chromosomal variation, including marker chromosomes (basic karyomorph) and C- and G- banding differences (second karyomorph) (King and Rofe 1976; King 1979; King 1984). The accumulated data allowed King (1984) to suggest a primitive karyotype for the Australian Gekkonidae of 2n=44, a karyotype mirrored in distantly related species in Fiji. High levels of chromosomal repatterning had occurred leading to 2n=38, 40 and 42 within derived genera. The hypothesised relationships correlated well with known land and climatic changes.

Identification of sex-linked differences and/or sex chromosomes is possible using banding, and delineation of specific karyotype races can support

conservation focus on particular populations. If populations differ significantly in chromosome banding patterns then this can be interpreted as differences that could lead (or may already have led) to reproductive isolation.

Within the Reptilia, around 30% of all species have been karyotyped, although within particular orders this percentage varies considerably. All species of Crocodylia and Sphenodontia have been karyotyped, 70% of all extant Testudines, and around 25% of all Squamates (Bickham 1984; Olmo 1986; Olmo, Capriglione et al. 2002; Norris, Rickards et al. 2004).

C-banding

The term C-banding refers to the digestion of constitutive heterochromatin by barium hydroxide during the banding process (Arrighi and Hsu 1971). The technique has proven quick, cheap, and adaptable to a wide range of species. Results from C-banding demonstrate dark stained regions of heterochromatin (Craig and Bickmore 1996), generally located in centromeric and telomeric regions, but some intercalary bands (within chromosome arms) do appear. Analysis of variation in C-bands was summarised in five general rules for heterochromatin distribution (Schweizer and Loidl 1986):

1. When present, C-bands are preferentially located in telomeres, centromeres and nucleolus organising regions (NORs).
2. All chromosomes show similar banding patterns, except in chromosomes with special functions such as sex chromosomes.

3. Pronounced telomeric bands are associated with short chromosomes or chromosome arms, whereas longer chromosomes or chromosome arms tend to develop intercalary bands.
4. C-banding in non-homologous members of the diploid set tend to be located at similar sites with respect to centromere-band distance.
5. For each intercalary band there exists a centromere arm whose telomere is the same distance from the centromere as the proximal border of the band. Thus telomeres appear to define location of intercalary bands.

Exceptions are seen, however the rules appear to hold in the majority of cases (Schweizer and Loidl 1986).

Most species exhibit C-banding to some degree. C-banding has proven useful in identifying morphologically undifferentiated primitive sex chromosomes. Both sexes of the endemic New Zealand frog *Leiopelma hamiltoni* have morphologically identical chromosomes. When C-banded, a heteromorphism was observed between male and female karyotypes on the smallest chromosome, a result interpreted as a morphologically undifferentiated sex chromosome (Green 1988).

Nucleolous organiser region banding

Nucleolous organiser regions (NORs) are the site of 18S and 28S ribosomal cistrons, and the nucleolous is a structure formed whenever the NOR is active (Schwarzacher and Wachtler 1986). As such, all animal and plant karyotypes have at least one pair of NORs. Some organisms, including humans, pigs, amphibians and fish (King, Contreras et al. 1990; Mellink, Bosma et al. 1994; Daga, Thode et al. 1996) have multiple copies of NORs, thought to be the result of

gene amplification and replication. It is quite common for NORs to be of differing size, for example heteromorphic NORs were seen in the majority of *Bufo* and *Hyla* animals examined (Schmid 1978).

There are two staining techniques in use for NOR identification. The more traditional of the two, silver stained NORs (Ag-NORs), involves placing silver nitrate on chromosomes and heating until the mixture reacts with the chromosomes. Under a microscope NORs stain much darker than the rest of the karyotype. Ag-NOR staining locates NOR genes active in the preceding interphase (Cheng, Heneen et al. 1995). To provide a more quantitative analysis of NORs, *in situ* hybridisation techniques can be used. The technique hybridises copies of the 18S and 28S ribosomal cistrons on to karyotypes resulting in a quantifiable value of 'grains' of probe hybridised to the NOR carrying chromosome. Although not exact, it allows an estimation of the number of NOR gene copies on the NOR carrying chromosomes. Hybridisation using 18S and 28S rRNA cistrons onto chromosomes of crocodile species demonstrated that one species, *Crocodylus johnstoni*, probably has variation in the copy number of the NOR genes, producing a heteromorphic secondary constriction (which mapped to the NOR) (King, Honeycutt et al. 1986). Similar *in situ* work was performed on Australian hylid frogs (King, Contreras et al. 1990), again using 18S and 28S RNA probes, the objective was to explore previous results from Ag-NOR staining. Previously observed heteromorphic NORs could be explained as absolute differences of rDNA within the NOR (King, Contreras et al. 1990) and not, as suggested by Ag-NOR staining, the product of chromosome packing (King 1980).

The two techniques provide different information, Ag-NOR stains active NOR sites and *in situ* hybridisation stains all sites of NOR genes. For example, within the species *Brassica campestris* two Ag-NORs are present (one on each homologue), representing one pair of active rDNA loci, however *in situ* work reveals five pairs of NOR-gene carrying sites suggesting four of these sites are inactive (Maluszynska and Heslop-Harrison 1993; Cheng, Heneen et al. 1995).

G-banding

Demonstrated in the early 1970's by authors using trypsin digestion or heated SSC (Seabright 1971; Sumner, Evans et al. 1971) G-banding was widely hailed as an ideal tool to individually identify human chromosomes. Permanence of chromosome preparations and use of standard laboratory and microscopy equipment, has ensured G-banding is still in use today with few modifications. The technique has proven very successful on many mammalian karyotypes, including monotremes, but has had limited use in plants with standard techniques only recently being developed (Murtagh 1977; Wrigley and Marshall Graves 1988; Song, Liu et al. 1994). Monotremes (the echidna and the platypus) are classified within the Mammalian subclass Prototheria, but exhibit a unique combination of reptilian and mammalian features (Grutzner, Deakin et al. 2003). Monotremes diverged from the mammalian lineage around 200-210 MYA (Killian, Buckley et al. 2001; Belov, Zenger et al. 2002; Woodburne, Rich et al. 2003), and the divergence between the platypus and the echidna occurred between 21-25 MYA (Westerman and Edwards 1992; Killian, Buckley et al. 2001; Grutzner, Deakin et al. 2003). Chromosomally the monotremes have been extensively examined, all three species have a unique chain arrangement in male

meiosis formed by several chromosomes with no obvious homologues in the karyotype (Wrigley and Marshall Graves 1988; Grutzner, Deakin et al. 2003). A number of microchromosomes are seen, typical of avian and reptilian karyotypes (with the exception of the Crocodylia who lack microchromosomes). The presence of clear, reproducible G-bands in monotremes supports a closer relationship with mammalian chromosomes, given the difficulty of reptilian G-banding.

G-banding has been demonstrated in reptiles. Within Testudines G-banding has been produced from sacrificial techniques using heart muscle cell cultures, limiting its use for endangered species (Sites, Bickham et al. 1979; Bickham, Bull et al. 1983). G-banding has been demonstrated in Crocodylia using cells grown from embryonic tail tips (King, Honeycutt et al. 1986; Valleley, Harrison et al. 1994). Some evidence of G-banding within New Zealand skinks has been seen in late prophase mitotic stomach wall cells (Norris 1997). G-banding patterns appear to reflect the stability of reptilian karyotypes. Testudines and Crocodylia exhibit large blocks of undifferentiated G-banding (Bickham 1984; King, Honeycutt et al. 1986; Olmo, Odierna et al. 1991; Olmo, Capriglione et al. 2002; Olmo 2003), whereas the Squamata have more G-positive and G-negative bands, similar to 'typical' mammalian chromosome organisation (Olmo, Capriglione et al. 2002; Olmo 2003).

Restriction enzyme digestion banding

Restriction endonucleases (REs) cleave DNA from metaphase chromosomes resulting in banding patterns (Sahasrabuddhe, Pathak et al. 1978). Dark bands

induced by Type II REs reflect regions of the chromosome resistant to the enzyme (Lorite, Garcia et al. 1999), due to levels of condensed chromatin preventing REs access for digestion (Mezzanotte, Bianchi et al. 1983). REs often induce banding patterns similar to C-bands, although REs digest chromosomes in a different manner and allow higher resolution of bands (Lloyd and Thorgaard 1988).

A recent review of reptilian karyology, including banding patterns, revealed that the only Reptilia to be RE-banded are the Squamates. RE-banding has been demonstrated in *Tarentola mauritanica* (two subspecies), *Sphenops sepsoides*, *Chalcides chalcides*, *C. ocellatus* and *Podarcis sicula sicula* (Capriglione, Olmo et al. 1989; Caputo, Odierna et al. 1993; Odierna, Aprea et al. 1994; Olmo 2005 see reference to Chromorep). A study looking at RE digestion of DNA in the lizard *P. s. sicula* demonstrated three RE groupings, namely; (i) extensive cutters *Alu*I (AGCT), *Hae*III (GGCC), *Hinf*I (GANTC), *Mbo*I (GATC), *Sau*3A I (GATC); (ii) moderate cutters *Dra*I (TTTAAA), *Ava*I (CpyCCpuG), *Pvu*II (CAGCTG), *Eco*RII (CCA/TGG); and (iii) less extensive cutters *Eco*RI (GAATTC), *Hind*III (AAGCTT), *Msp*I (CCGG) and *Taq*I (TCGA). Digesting chromosomes using an extensive cutter, *Alu*I, and a less extensive cutter, *Hind*III, produced dark banded centromeric regions (Capriglione, Olmo et al. 1989). RE-banding patterns obtained using *Alu*I and *Hae*III in *T. mauritanica* were described as 'peculiar' (Odierna, Aprea et al. 1994). Chromosomes appeared extremely digested, with only some regions of pericentromeric heterochromatin staining darkly. These patterns resembled C-banding. *Alu*I chromosome digestion in the skinks *S. sepsoides* and *C. ocellatus* produced patterns identical to C-bands. The closely related species *C. chalcides*

produced no *Alu*I banding, a result interpreted as significant differences in heterochromatin distribution between the two *Chalcides* species (Caputo, Odierna et al. 1993). RE banding has been used in many mammalian species and some avian species. *Alu*I digestion in the eagle *Aquila adalberti* demonstrated sex related differences between the Z and W chromosomes (Padilla, Martinez-Trancon et al. 1999). No Testudines or Crocodylians have been RE-banded.

Use of banding studies

A goal of cytogenetics is to provide unambiguous identification of each chromosome pair. Although basic morphology (size and centromere position) can be used to identify many pairs of chromosomes, there are many cases where chromosomes are of similar morphology creating difficulty in consistent identification and pairing. An example of this are the *Leiopelma hamiltoni* chromosomes where both sexes have identical karyotypes with many chromosomes of similar size and centromere position. Specific banding techniques separated these chromosomes, for example unique banding patterns between male and female *L. hamiltoni* allowed identification of sex chromosomes (Green 1988).

Arm ratio, centromeric index, and chromosome analysis

Measurement of chromosome arms allows calculation of the centromeric index (CI) and subsequent description of chromosomes using standard nomenclature. Traditional nomenclature divides chromosome morphology into six categories, including Metacentric and Telocentric for those chromosomes with absolute CI values of 1.00 and ∞, that is, centromere at the exact midpoint and no proximal

arm respectively (Levan, Fredga et al. 1964). Hybridisation of telomeric repeat sequences $(TTAGGG)_n$ has revealed that chromosomes with no apparent proximal arm still contain telomeric sequences and so possess very short proximal arms (Lizarralde, Bolzan et al. 2005).

For this report I have chosen to follow chromosome nomenclature where chromosome morphology is divided into four categories. The term acrocentric is rejected on the basis that the root word of acrocentric, *akros*, has the same meaning as the root of telocentric, *telos* (Green and Sessions 1991). In the interests of removing ambiguity, the redundant term acrocentric has been replaced by subtelocentric (Figure 1.5). The term Telocentric (CI=∞) is also rejected on the basis that all chromosomes have a telomeric sequence and therefore a proximal arm, albeit indistinguishable cytologically.
The nomenclature and CI from Green and Sessions (1991) is reproduced here as Table 1.1, and graphically represented as Figure 1.5. The categories defined by CI used to divide chromosomes are arbitrary, in much the same way as the term species is an arbitrary divider of organisms. Chromosome morphology groups have proven useful, allowing analysis and comparison between chromosomes using a standard description.

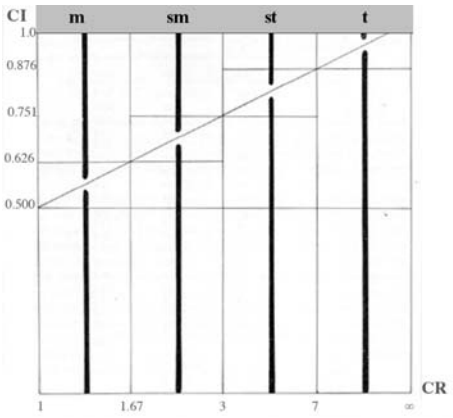


Figure 1.5: The range of centromeric positions divided into four regions, 'm'=metacentric, 'sm'=submetacentric, 'st'=subtelocentric, 't'=telocentric (modified from Levan, Fredga et al. 1964).

Table 1.1: Nomenclature for centromeric position on mitotic chromosomes (Green and Sessions 1991)

Chromosome type	Abbreviation	Centromeric ratio[a]	Centromeric index[b]
metacentric	m	1.00-1.67	0.500-0.375
submetacentric	sm	1.68-3.00	0.374-0.250
subtelocentric	st	3.01-7.00	0.249-0.125
telocentric	t	7.01-∞	0.124-0.000

[a] Length of long arm / length of short arm.
[b] Length of short arm / total chromosome length.

Aims of the current study

The primary aim of the current study was to attempt to answer the question 'do *Sphenodon* exhibit variation in chromosome morphology?' Data obtained examining the primary question would allow comparison between males and females and species. Comparison of *Sphenodon* chromosomes with other members of the Reptilia would allow construction of phylogenies and possibly proto-karyotype reconstruction.

Methods

Blood samples were collected 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* north-eastern 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 heparinsed 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 hours 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* north-eastern 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 *S. punctatus* individuals was the same as *S. guntheri*.

The following technique is based on previous work (Wylie, Veale et al. 1968) with optimisation of culture media and cell inhibitor timing. Blood samples were cultured for six days at 26°C, at which point 0.3 ml of 0.05% colchicine (BDH)

was added to the cultures and samples incubated for a further 50 minutes. 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 *pers comm.*). After colchicine pre-treatment, cultures were spun for 10 minutes at 1000 rpm, the supernatant removed and cells re-suspended in 6 ml of hypotonic solution (1:1 0.4% KCl : 0.4% NaCl) for 20 minutes at 26°C. After hypotonic treatment cultures were centrifuged for 10 minutes at 1000 rpm and the supernatant removed to 0.5 ml. The cell button 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 to the total volume to 6 ml. After 20 minutes incubation at room temperature, cells were centrifuged at 1000 rpm for 10 minutes, supernatant removed to 1 ml, and the cell button resuspended in 5 ml of fresh fixative. Centrifuging, supernatant removal and cell button 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 cell suspension on to microscope slides and air-drying.

Giemsa staining

Slides were stained in 5% giemsa (BDH) in a phosphate buffer (Appendix 1) for eight minutes.

C-banding

Slides were placed in 0.02N HCl for one hour, rinsed in dH_2O, and treated with saturated $Ba(OH)_2$ at 60°C for 30 seconds. After two further rinses in distilled

H_2O slides were placed in 2 x SSC (Appendix 1) at 60°C for one hour, rinsed with dH_2O and stained in a giemsa solution as described above.

Ag-NOR banding

Ag-NOR banding was performed by placing two drops of developer (0.5 ml formic acid in 50 ml of 2% gelatine solution) and four drops of 50% silver nitrate solution on air-dried slides. The two solutions were mixed and spread by placing a large (22 x 50 mm) coverslip on the liquid and heated on a heating block until the mixture turned a dark brown, at which point the coverslip and liquid were rinsed off and the slide air dried.

Restriction endonuclease banding

A number of protocols were found to be unsuccessful in producing RE-banding in *Sphenodon* (de la Torre, Mitchell et al. 1991; Schmid, Feichtinger et al. 2002), although reproducible banding was achieved using the following protocol (adapted from Chaves, Adega et al. 2002). Slides were airdried and aged for five hours at 65^{0}C. After cooling to room temperature, 40 µl of RE solution was applied. RE solution consisted of 1 µl (10 U) of RE, 9 µl of appropriate buffer and 90 µl of UltraPure Water (Invitrogen 10977-015). Three TypeII REs were used, namely *Hae*III + REact buffer 3 (Invitrogen); *Eco*RI + REact buffer 2 (Invitrogen); and *Alu*I + Buffer A (Roche). The RE solution was spread by a 22x50mm coverslip and incubated at 37^{0}C for 12-14 hours in a humid chamber. Following digestion, slides were washed in three changes of water, air dried and stained in 5% giemsa for eight minutes.

G-banding

G-banding was performed using standard techniques (Seabright 1971; Sites, Bickham et al. 1979). Airdried slides were incubated in 2x SSC for 90 minutes at 65^0C, or baked overnight at 65^0C. Slides were treated with trypsin (2.5% of a stock solution Gibco 12604) in NaCl or PBS for 2-5 minutes, rinsed in distilled water, 70% ethanol, 95% ethanol and stained in a 1% Giemsa solution for 10 minutes.

Preparation and analysis of karyotypes

Good quality chromosome spreads were photographed on Kodak Tmax100 film using an Olympus AX70 photomicroscope. Negatives were scanned at 1500 dpi (dots per inch) using a negative scanner and saved as Adobe Photoshop files. Images so obtained were either printed out using a 1440 dpi printer, or manipulated into karyotypes using Adobe Photoshop or GIMP (www.gimp.org). Putative chromosome pairs were aligned in descending order by length and chromosomes measured. A minimum of 15 chromosome spreads were used for analysis of an animal. Arm lengths were expressed as a proportion of the total chromosome length (TCL) of the chromosome set. Centromeric index (CI), derived from averaged TCL arms lengths, allowed chromosomes to be identified using morphological nomenclature (Green and Sessions 1991).

Results

Karyotypes suitable for analysis were obtained from all five populations. Not all blood cultures produced chromosomes.

All five populations have an identical karyotype with a 2n=2x number of 36 consisting of 14 pairs of larger chromosomes and 4 pairs of small microchromosomes (Figure 1.6a and b). There were no differences between male and female karyotypes.

The data in Table 1.2 provides a summary of the variation in TCL and CI between each chromosome pair, from which an idiogram (Figure 1.7) has been created. The variation in TCL and CI allows consistent identification of most of the tuatara karyotype.

Table 1.2: Quantitative description of Sphenodon *chromosomes. The range of total chromosome length (TCL) and centromeric index (CI) is listed.*

Chromosome	TCL Range	CI Range
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	

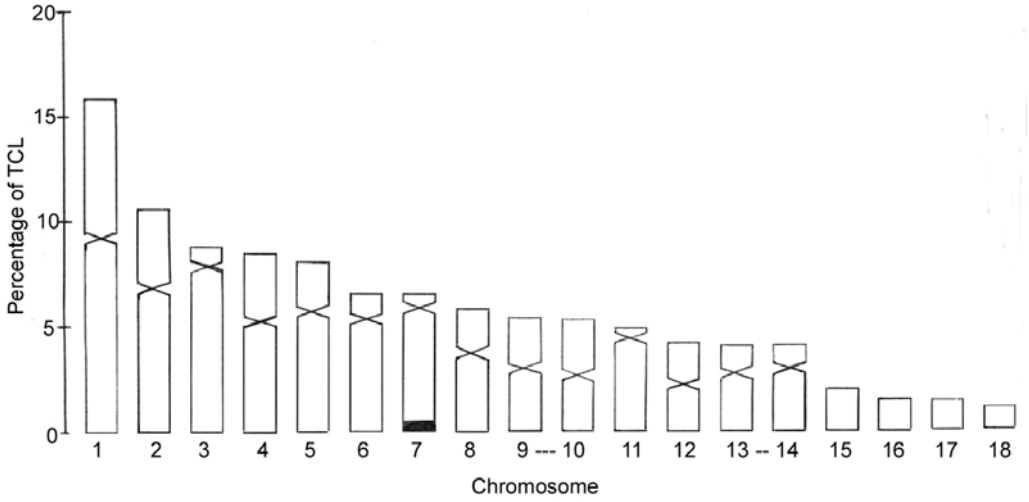


Figure 1.7: Idiogram of *Sphenodon* indicating total chromosome length (TCL), centromere position and Ag-NOR location (black block).

Karyotype description

The karyotype of *Sphenodon* contains 18 pairs of chromosomes, consisting of 14 pairs of macrochromosomes and four pairs of microchromosomes (Figure 1.6a). Ten of the 14 macrochromosome pairs of *Sphenodon* can be defined using a

combination of TCL and CI. Data for the range of each chromosome TCL and CI (Table 1.2) has been used to prepare the idiogram (Figure 1.7). Chromosome pairs one to five can be defined on the basis of TCL and CI as metacentric, submetacentric, telocentric, metacentric and submetacentric respectively. Chromosome pairs six and seven are very similar in TCL, but can be distinguished using CI as subtelocentric and telocentric chromosomes respectively. Further separation of the two pairs is possible as the telocentric pair always contains the Ag-NOR (Figure 1.6b). Chromosome pair eight is, in all spreads, a submetacentric chromosome and can be defined using CI, although it is of similar TCL to pairs nine and ten. Chromosomes nine and ten are metacentric and comprise about 10% TCL (each 5%). They cannot be consistently paired as unequivocal homologues due to similar TCL and CI. Chromosome 11 can be defined on the basis of CI and TCL as telocentric. Chromosome 12 is metacentric. Chromosomes 13 and 14 are sub-metacentric and comprise 7.5% TCL (about 3.5-4% TCL respectively). The difference in TCL is too small to separate the two chromosome pairs consistently. The remaining four pairs of micro-chromosomes comprise around 6.5% TCL. Their morphology cannot be accurately defined.

One animal from Ruamahua-iti contained a heteromorphic chromosome pair 3. Twenty-seven chromosome spreads were examined, 18 with colchicine and 9 with colcemid as the cell division inhibitor. In all spreads, one member of the chromosome 3 pair contained an extended ‘p’ arm that was more than 40% longer than the other member of the pair. Both inhibitors produced chromosomes with an identical heteromorphism demonstrating that the

heteromorphism was not an artefact of preparation. All other chromosomes within the karyotype fell within normal TCL values, implying the heteromorphism results from either a duplication or growth (either interstitial or telomeric). The extra material does not C-band or contain an Ag-NOR (Figure 1.8). A distribution diagram of chromosome 3 TCL using the mutant animal (colchicine and colcemid inhibited) and a standard Ruamahua-iti animal is presented as Figure 1.9 following standard chromosome mutation analysis (Patau 1960; Craddock 1975). The 'normal' member of chromosome 3 from the mutant animal falls within normal TCL for chromosome 3 from a Ruamahua-iti animal. No meiotic material was available for investigation on how the heteromorphism behaves during meiosis.

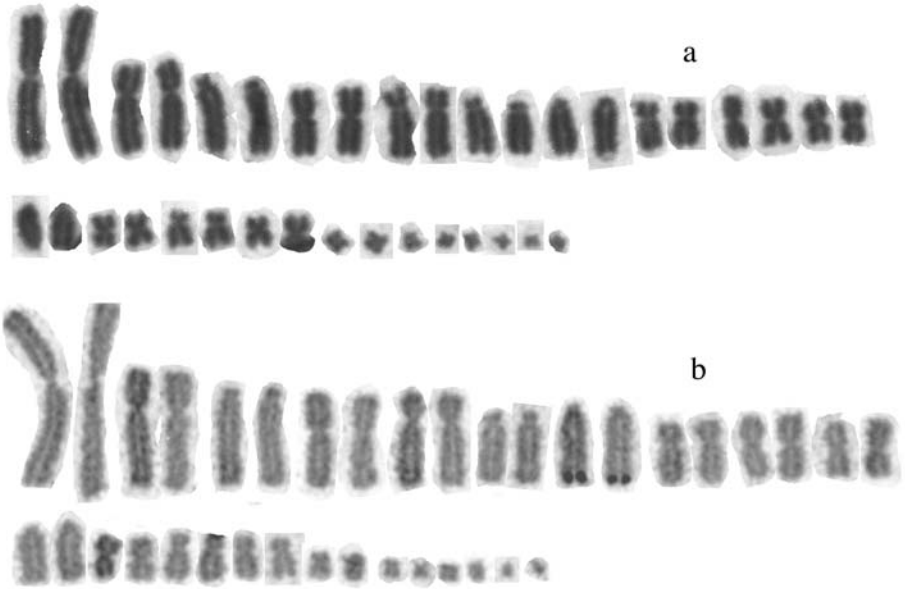


Figure 1.6: Karyotypes of *Sphenodon*. (a) Giemsa stained karyotype of a male *S. guntheri*; (b) Ag-NOR stained karyotype of a female *S. punctatus* (north-eastern group, Stanley Island)

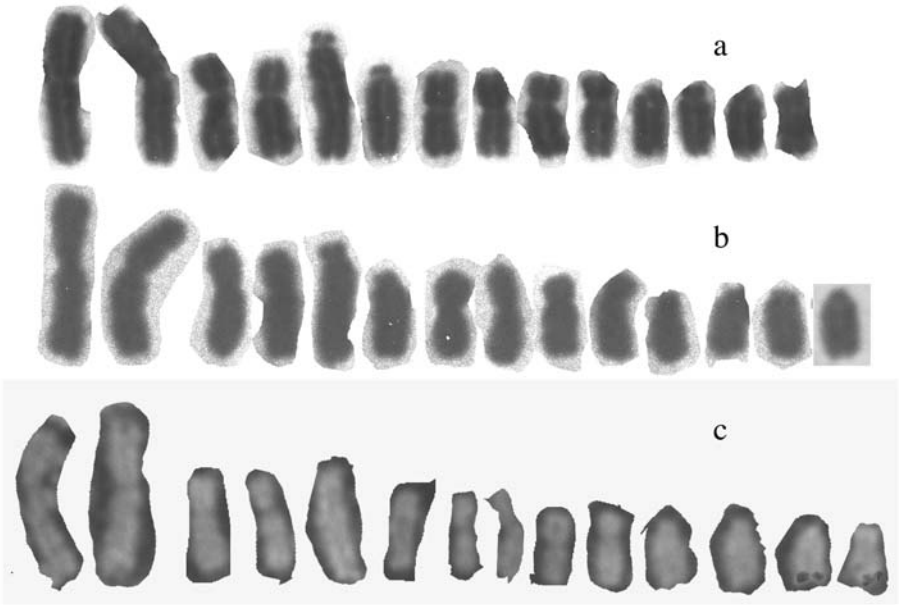


Figure 1.8: Partial karyotypes (chromosomes 1 – 7) of a Ruamahua-iti animal with additional material on chromosome 3. (a) Giemsa staining; (b) C-banding; (c) Ag-NOR staining.

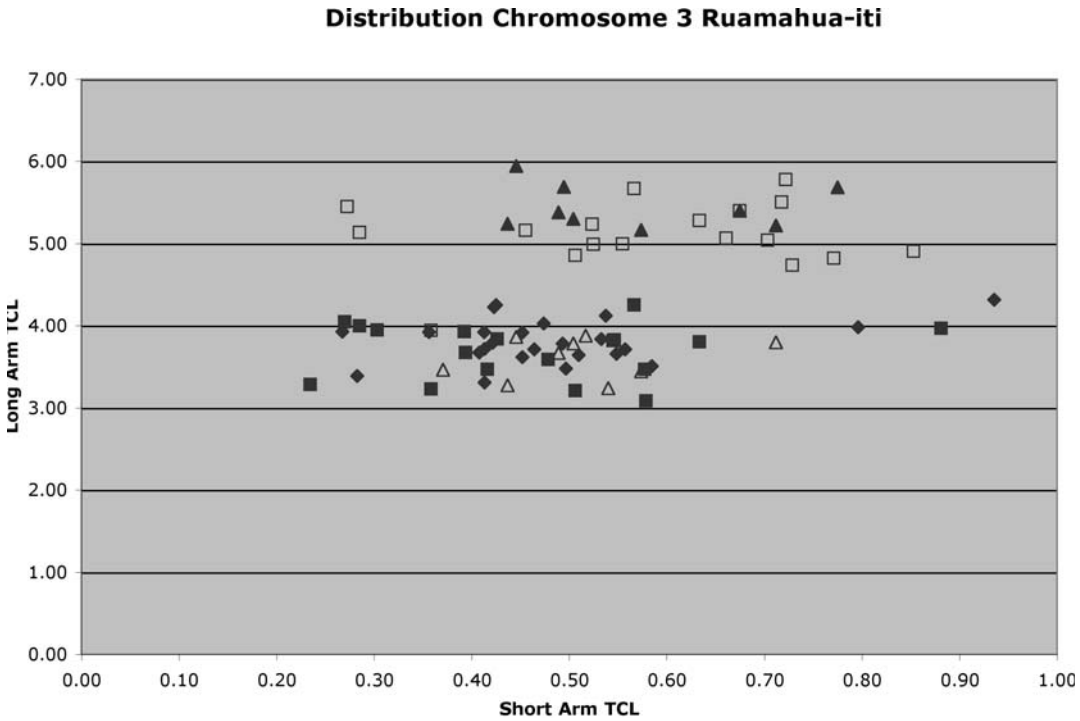


Figure 1.9: Scatter diagram of mutant and normal chromosome 3 using colchicine and colcemid as cell cycle inhibitors, plotting TCL of the long arm against TCL of the short arm. (◆) Normal Ruamahua-iti chromosome 3; (□) chromosome 3 with additional material inhibited with colchicine; (■) other member of chromosome pair 3 inhibited with colchicine; (▲) chromosome 3 with additional material inhibited by colcemid; (△) other member of chromosome pair 3 inhibited with colcemid.

Banding patterns within Sphenodon

C-banding in *Sphenodon* is restricted to centromeric regions (Figure 1.10), no banding within chromosome arms was noted in any animal.

Ag-NORs are located on chromosome 7 and allow chromosomes 6 and 7 to be unambiguously identified. No evidence for secondary NOR sites was seen.

Variation in Ag-NOR size is present with both heteromorphic and homomorphic sized pairs observed in all populations (Figure 1.11). Within the same animal Ag-NOR morphology was consistent, either heteromorphic or homomorphic.

*Alu*I and *Hae*III RE banding produced highly digested chromosomes, resulting in characteristic RE chromosome morphology. Identical RE patterns were seen in male and female karyotypes, and these patterns did not resemble C-bands. Some dark staining regions were seen (Figure 1.12a,b) on the telomeric regions and a

small region of one member of the chromosome 7 pair, and the centromeric region of chromosome 11. The telomeric bands on chromosome 7 reflect NOR location in *Sphenodon*. *Eco*RI digestion produced no banding in *Sphenodon* (Figure 1.12c). G-banded *in situ* chromosomes of *Sphenodon* are presented as Figure 1.13. G-banding appears restricted to telomeric regions.

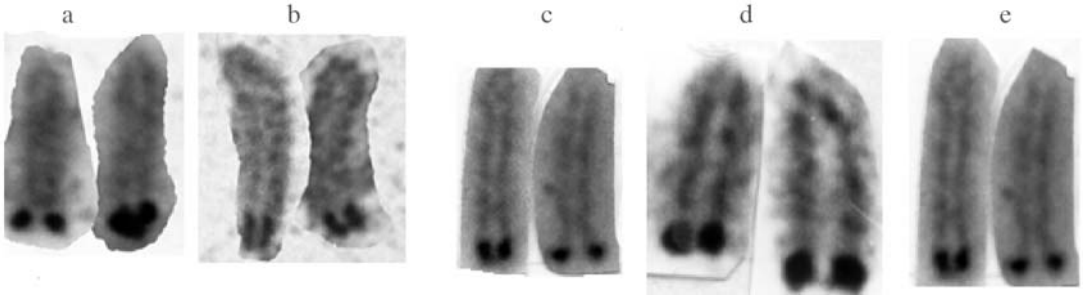


Figure 1.11: Variation in Ag-NORs in *Sphenodon*. (**a**) Stanley Island (heteromorphic); (**b**) Stephens Island (homomorphic); (**c**) Stephens Island (heteromorphic); (**d**) male Stephens Island (homomorphic); (**e**) North Brothers Island (heteromorphic).

Partial haploid karyotypes of *Sphenodon*, Testudines and Crocodylia when aligned (Figure 1.14) allowed comparison between the archaic reptilian orders.

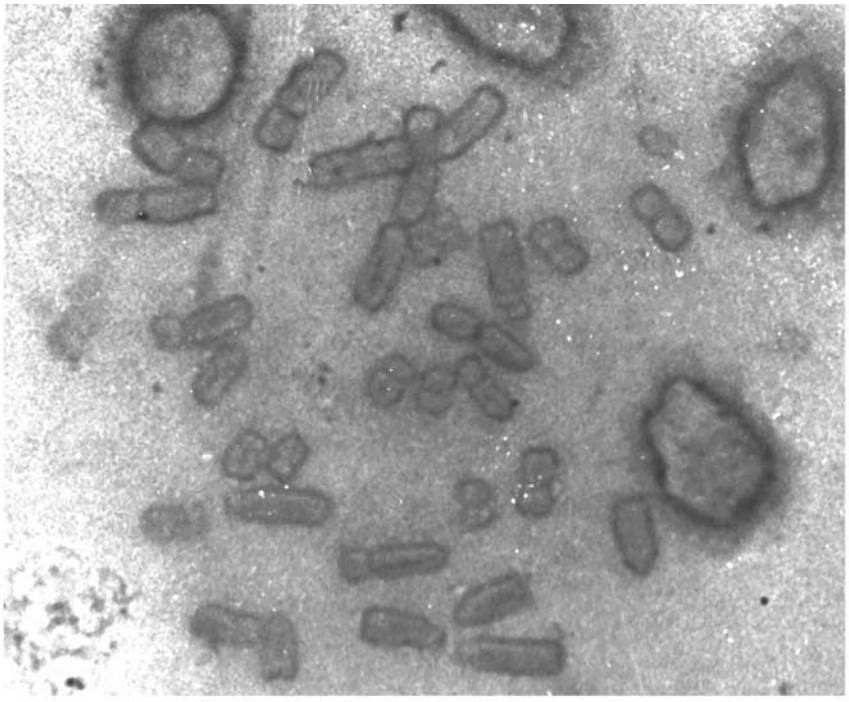


Figure 1.10: C-banded metaphase in situ spread of *Sphenodon*.

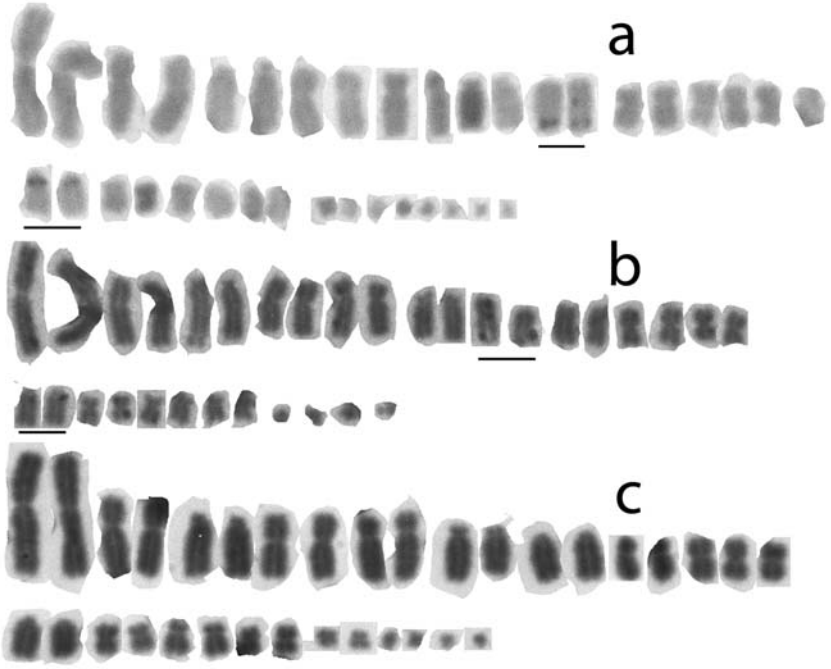


Figure 1.12: Restriction endonuclease banding in *Sphenodon*; a) *Alu*I RE digestion; b) *Hae*III RE digestion; c) *Eco*RI RE digestion.

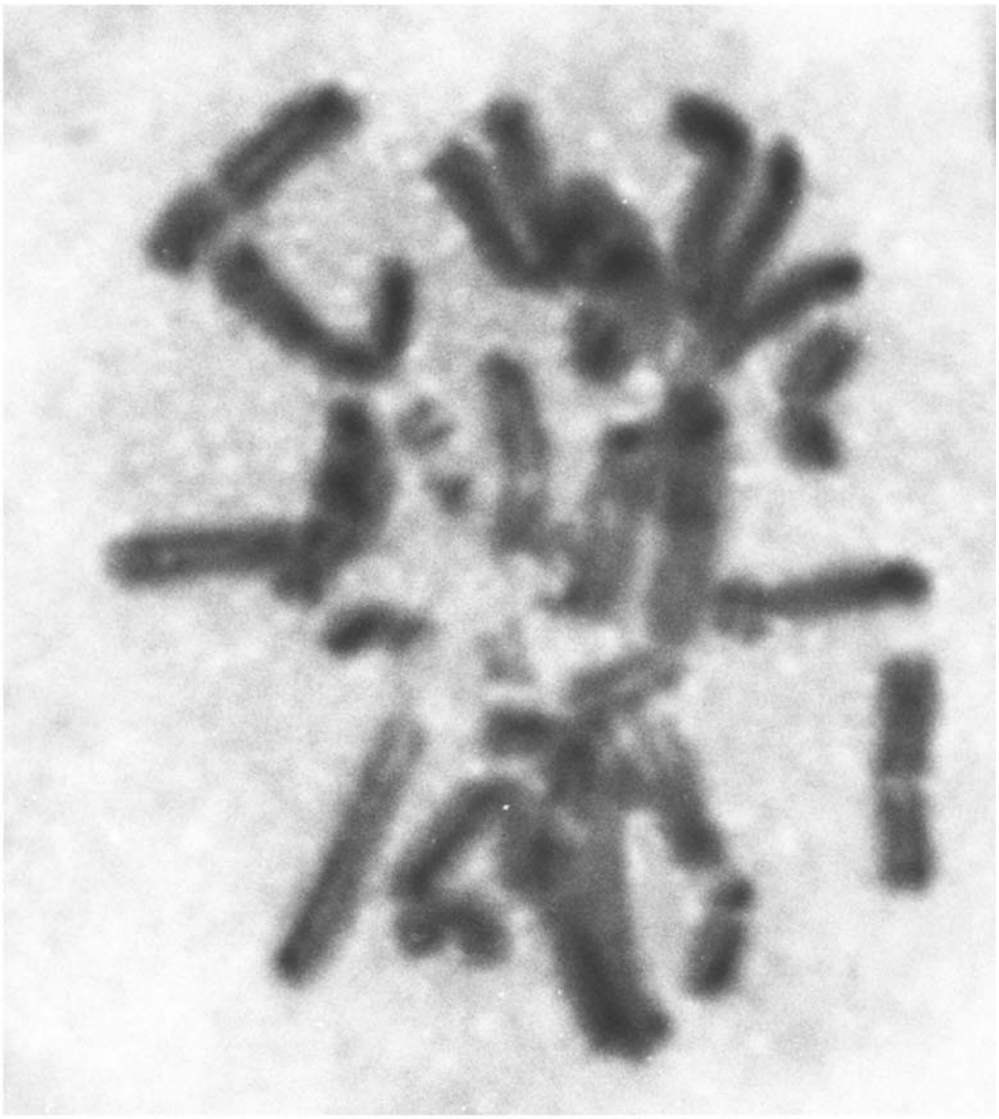


Figure 1.13: G- banded in situ chromosomes of *Sphenodon*.

Figure 1.14: 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. Chromosomes have been resized from original material to allow comparison (prepared from the current study and Cohen and Gans 1970; Bickham 1975; Bickham and Baker 1976; Bickham, Bjorndal et al. 1980; Bull and Legler 1980; Haiduk and Bickham 1982; Bickham and Carr 1983).

Blood cultures resulting in no chromosomes

Not all blood cultures produced chromosomes. Some cultures were contaminated, although the contamination appeared after three to four days in culture suggesting antibiotics in the culture media had lost efficacy. Other blood cultures, when prepared slides were examined under a microscope, appeared to have no dividing white blood cells (WBCs). WBCs are fragile, more so than red blood cells, and could have been damaged at some stage in culture or during transport from the islands. Discussions with others involved in blood culture work (Dr. D. Romain Capital Coast Health Cytogenetics Lab; Dr. J. Masabanda, Brunel University) suggested a failure rate up to 45% is within expected ranges for animals where there is no knowledge of the cell cycle and blood culturing requirements.

Comparison of the current study and Wylie et al. (1968)

The first high quality tuatara karyotype divided chromosomes into four groups on the basis of similar morphology (Wylie, Veale et al. 1968). The format followed the convention of dividing karyotypes into morphological groupings on the basis of CI. The current study follows the convention ordering chromosomes by decreasing TCL. The relationship between the chromosome numbering and grouping (Wylie, Veale et al. 1968) and the current study is shown as Table 1.3. Three groups remain where identification of pairs is impossible due to similarity in TCL and CI, namely chromosomes 9 and 10, chromosomes 13 and 14 and the four very small chromosome pairs (numbering follows current study). The division of the last four pairs of chromosomes as 'very small chromosomes' by Wylie et al. can now be validated using an argument based on CI. Within the last

four pairs it is, in almost all chromosome spreads, impossible to distinguish the centromere position, and therefore the CI cannot be calculated. For this reason I have termed the last four pairs of chromosomes ‘microchromosomes'.

Table 1.3: Comparison of Wylie et al (1968) and the current study chromosome numbering

Grouping of Wylie et al.	Wylie et al. 1968	Current Study
Group A	1	1
	2	2
	3	5
	4	4
Group B	5	3
	6	6
	7	7
	8	11
Group C	9	8
	10	9
	11	10
	12	12
	13	13
	14	14
Group D	15	15
	16	16
	17	17
	18	18

Discussion

Sphenodon has a diploid chromosome complement of 36, consisting of 14 pairs of larger macrochromosomes where the TCL and CI can be established, and four pairs of small microchromosomes where the CI cannot be accurately determined. Of the 14 pairs of large chromosomes, ten pairs can be definitely identified whereas the remaining four chromosomes are too similar in TCL and CI to unambiguously identify homologues. There are no sex-related heteromorphisms and no differences between the populations, sexes or individuals, with the exception of one animal with a structural heteromorphism from Ruamahua-iti. This was unexpected as karyotypic examination of other New Zealand herpetofauna suggests that chromosomal variation is the norm. Extensive variation is seen within the Gekkonidae with chromosomal changes correlating well with speciation (Hardy 1975; Norris 1997). Chromosomes of the two Scincidae families Cyclodina and Oligosoma are defined by a fixed heteromorphism (O'Malley 1971; Hardy 1979; Norris 1997; Norris 2003), and extensive variation is seen in the archaic frog *Leiopelma*, particularly *L. hochstetteri* (Green 1988; Green, Zeyl et al. 1993).

Four of the *Sphenodon* populations examined were isolated between 8-10,000 years ago from the New Zealand mainland, whereas the Poor Knights Island group separated around a million years ago (Hayward 1986), implying the tuatara karyotype is at least as old as the Poor Knights separation. The two groupings of *S. punctatus,* north-eastern North Island group and the Cook Strait group (Hay, Daugherty et al. 2003), are separated by 600 km in a direct line. At

least three major population and range contractions have taken place within New Zealand. During the Oligocene Drowning (27 MYA), the New Zealand landmass was reduced to a number of low relief islands, with a total landmass of around 20% of what is currently seen (Cooper and Cooper 1995). The Pliocene/Pleistocene (5-1 MYA) is thought to have dramatically affected *Sphenodon* populations (Hay, Daugherty et al. 2003), and the third most recent population perturbation was caused by human arrival. During the last 1-2000 years *Sphenodon* have become extinct from mainland New Zealand (Towns and Daugherty 1994). So a previously widespread and presumably interbreeding genus is now limited to a few islands where genetic exchange between populations is impossible.

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 growth, but is not a translocation 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 mutation 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 *pers. obs.*) did not appear to differ from other Ruamahua-iti animals. The length of the additional chromosome material may mean that the animal is unable to breed successfully. As the animal was

unmarked examination of breeding success is not possible and meiotic study was not possible due to the highly protected status of *Sphenodon*. The extra material on chromosome 3 may mean the animal is unable to produce balanced meiotic products. Although no studies on tuatara meiosis have been performed, the stability of the *Sphenodon* karyotype for at least one million years, suggests that chromosomal change is uncommon. The tuatara population on Ruamahua-iti is sizeable enough to not be in danger of extinction, so leaving the mutant animal on the island is unlikely to cause any problems.

The Ag-NOR was located on chromosome 7 and both homomorphic and heteromorphic NORs were seen in the tuatara karyotype, although the Ag-NOR form was consistent in all karotypes from an animal. Heteromorphic NORs suggests that there are some replication errors or gene duplications present in the telomeric regions of chromosome 7. Telomeric and subtelomeric regions are likely to experience breakage or mispairing due to the presence of repetitive DNA clusters, which appear to induce breakages (Rubtsov, Rubtsov et al. 2002).

C-banding produced dark stained centromeres, but no intercalary bands or NOR region staining. *Sphenodon* therefore contains limited heterochromatin.
REs banding produced different results to C-banding. The NOR region, one area of one chromosome of chromosome 7 and the centromeric regions of chromosome 11 were undigested by REs. Differences between RE banding and C-banding appear typical of reptilian and some avian species (Caputo, Odierna et al. 1993; Odierna, Aprea et al. 1994; Padilla, Martinez-Trancon et al. 1999).

G-banding in *Sphenodon* produced dark staining blocks around the centromeres and telomeres, without distinct mammalian style banding patterns. Comparison with Testudines and Crocodylia is difficult as resolution of banding in *Sphenodon* is poor compared with the cell cultures used for G- banding in other reptiles. Successful G-banding in *Sphenodon* may require a tissue culture technique as used in Testudines and Crocodylia (Sites, Bickham et al. 1979; Bickham, Bull et al. 1983; King, Honeycutt et al. 1986; Valleley, Harrison et al. 1994).

Macro-chromosome comparisons within the Reptilia

The karyotype of *Sphenodon* is significantly different to all of the Squamata (Olmo 1986). Although Squamata have high levels of chromosomal variation, and many species or families retain some level of homology, none resemble *Sphenodon*. The high degree of karyotype conservatation for at least one million years, is at odds with other members of the New Zealand reptilian fauna. Within the New Zealand Scincidae a fixed heteromorphism separates the two genera (O'Malley 1971; Hardy 1979; Norris 2003), and significant chromosomal variation within the New Zealand Gekkonidae correlates well with speciation events (Norris 1997). Both skinks and geckos are recent, post-Oligocene, arrivals to New Zealand.

Comparing *Sphenodon* to other reptilian orders; the Crocodylia has two karyotypic forms, a predominantly meta- and submeta- centric form, and a telo- and subtelo- centric form (Cohen and Gans 1970; Bickham 1984). The diploid number ranges from 2n=30-42 and chromosome morphology differs slightly from *Sphenodon* (Figures 1.2 and 1.14). The Testudines, although with a diploid

number between 2n=50-70 (Figure 1.1), show high homology with *Sphenodon* macrochromosomes, except for the highly divergent families Trionychidae and Carettochelyidae.

The high level of morphological similarity suggests a strong, ancient relationship with a common ancestor of Sphenodontia and Testudines. The alternative interpretation, of convergent karyotypic evolution, seems difficult to sustain given the high numbers of chromosomes, turtle species and families involved. The percentage of TCL contained within the first 14 macrochromosomes in both Testudines (Bickham 1975) and *Sphenodon* (current study) is approximately 85%, further supporting a relationship between the two orders. The constitution of the DNA will have changed significantly over the 350 MYA since a common ancestor, but chromosome morphology appears to have remained, with the major variation being numbers of microchromosomes.

RE digestion in *Podarcis* produced darkly stained centromeric regions using *Alu*I and *Hind*III. These two enzymes had been characterised as extensive, and less extensive cutters of DNA (Capriglione, Olmo et al. 1989). Results from the current study utilised two of the extensive cutters *Alu*I and *Hae*III, and a less extensive cutter *Eco*RI. *Sphenodon* chromosomes have limited regions resistant to *Alu*I and *Hae*III, whereas *Eco*RI left no chromosome regions undigested. Absence of homology with C-banding is in agreement with other reptilian RE studies (Caputo, Odierna et al. 1993; Odierna, Aprea et al. 1994). No homology in RE banding patterns is seen between *Sphenodon* and the Squamata (*Sphenops*, *Chalcides*, *Tarentola* and *Podarcis*), which was expected given the highly divergent chromosome morphology between the two Orders. A more

appropriate comparison would be with a Testudine, unfortunately no RE banding has been performed within the Testudines.

Successful G-banding in reptiles used chromosomes prepared from cell cultures derived from tail tips in *A. mississippiensis* (King, Honeycutt et al. 1986; Valleley, Harrison et al. 1994), or heart muscle from Testudines (Sites, Bickham et al. 1979). I have been unable to find reports of lymphocyte derived G-banding in the Reptilia. This may explain the low resolution of banding seen in *Sphenodon* chromosomes. Length of trypsin treatment was varied from 30 s – 6 minutes as was slide pre-treatment (baking overnight at 65^0C or 90 minutes in 2x SSC at 65^0C), neither made any difference to G-band resolution.

Comparison between *Sphenodon* and Testudine Ag-NORs reveals no homology in location. NOR location exhibits variation within and between Families and Subfamilies of Testudines (Bickham and Rogers 1985). The closest Ag-NOR in Testudines to chromosome 7 in *Sphenodon* is the *Chelydra serpentina* NOR on chromosome 6. In this species the NOR is located on the proximal arm, demonstrating significant change from the telomeric region of chromosome 7q location in *Sphenodon*. Of 45 Testudines representing 27 species, 15 of them possessed heterozygous sized NORs (Bickham and Rogers 1985), a similarity shared with *Sphenodon*. No homology was seen with the Crocodylia where NOR and secondary constrictions are located on chromosome 10 (King, Honeycutt et al. 1986).

Reptilian microchromosomes

Changes in microchromosome number appear to be the most dramatic form of variation between the *Sphenodon* and the Testudines.

Microchromosomes are ubiquitous in Aves (Burt 2002), Testudines (Olmo 1986), *Sphenodon* (current study and Norris, Rickards et al. 2004) and are present in the Squamata (for examples see Olmo 1986; Norris 1997; Bertolotto, Pellegrino et al. 2002; Yonenaga-Yassuda, Rodrigues et al. 2005), but absent in the Crocodylia (Cohen and Gans 1970). Primitive amphibians and lower fish have microchromosomes suggesting a common ancestor of birds, amphibians and reptiles possessed microchromosomes (reviewed in Burt 2002). Changes in microchromosome numbers have occurred within these lineages, implying mechanisms for accumulation and removal from the karyotype are present. A common ancestral microchromosome number of 20 pairs has been advanced (Burt 2002), meaning *Sphenodon* has lost 16 pairs, and that some lineages of Testudines have lost or gained pairs. The macrochromosome complement appears stable, but the microchromosome complement demonstrates variation between species and Orders. Changing numbers of microchromosomes may be linked to morphological changes or speciation due to microchromosome gene content. For example, up to 50% of all avian genes are located on microchromosomes (Smith, Bruley et al. 2000). Similar microchromosome variation exists in the Reptilia and may explain speciation events in Testudines. Very little sequencing of microchromosomes in Testudines, and none in *Sphenodon*, has been performed, making linking of microchromosome number, genes and speciation within reptiles speculative. The one exception is recent comparative FISH work, demonstrating that the sex chromosomes in *Chelodina longicollis* (Testudine) and *Pogona vitticeps* (Squamata) are microchromosomes (Ezaz, Quinn et al. 2005; Ezaz, Valenzuela et al. 2006).

Similarities exist between turtle and avian chromosomes, namely macrochromosomes homology and the large number of microchromosomes. Isolation of novel turtle repetitive DNA sequences from *Pelodiscus sinensis*, including microchromosome only sequence, demonstrate that turtle and avian chromosomes possess molecular as well as morphological similarities (Yamada, Nishida-Umehara et al. 2005).

Crocodylia are the only reptile lineage lacking microchromosomes, although birds who are most closely related to the Crocodylia, possess microchromosomes. There is evidence for karyotypic changes within the Crocodylia, firstly gross chromosome morphology differs from Testudine and *Sphenodon* karyotypes. Secondly, within Crocodilian karyotypes banding patterns appear derived compared to other reptiles (King, Honeycutt et al. 1986; Burt 2002). From this evidence there are two hypotheses to explain avian microchromosome evolution:

1) birds evolved from a common ancestor with crocodiles who had no microchromosomes and subsequently re-gained microchromosomes
2) the common ancestor of crocodiles and birds had microchromosomes, and subsequently the progenitor of modern crocodiles lost all microchromosomes leading to the modern crocodile karyotype

The parsimonious option (2) is the more likely. The last common ancestor of crocodiles and birds (Archosauria) *Protorosaurus*, has been dated to 255 MYA, and the last common ancestor of the Crocodylia, *Stagonosuchus*, to 240 MYA (Benton 1990; Janke, Gullberg et al. 2005). Therefore loss of microchromosomes

in the Crocodylia occurred in a common ancestor to all extant crocodiles in the last 240 million years.

Conclusions

Fully described tuatara karyotypes, prepared from five populations using whole blood, revealed no variation between populations, sexes, or species.
C-, Ag-NOR, G-, and RE- banding has been performed. Limited centromeric C-banding is seen, Ag-NORs are located on chromosome 7. *Alu*I and *Hae*III RE banding produced banding on chromosome 11, the NORs and a small interstitial region of chromosome 7. There were no sex related differences. *Eco*RI did not band *Sphenodon*.
G-band digestion produced indistinct telomeric region banding. Use of cell culture lines, as used in Testudines and Crocodylia, may demonstrate clearer G-banding in *Sphenodon*.

The identical karyotype of Poor Knights Island *Sphenodon* and all other island populations dates the karyotype to at least one million years. Similarities between the Testudines and *Sphenodon* support an ancient relationship between the two Orders, to the exclusion of Squamata and partial exclusion of the Crocodylia. *Sphenodon* and the Testudines shared a common ancestor around 300 MYA, therefore karyotypic similarities are ancient and reflect a primitive reptilian karyotype. With a great deal of recent work being done on reconstructing archaic karyotype evolution (Svartman, Stone et al. 2004; Svartman, Stone et al. 2006), the turtle and tuatara karyotypes provide an ideal

study with their known divergence times. This is explored further in Chapter 4 to develop a reptilian proto-karyotype.

Chapter 2 : A multigene approach to phylogenetic relationships of *Sphenodon* and the Reptilia

The close chromosomal relationship between Testudines and *Sphenodon* demonstrated in Chapter 1 was arrived at independently of characters and methods commonly used for phylogenetic reconstruction, namely DNA sequencing and morphological traits. In the current chapter DNA sequences of the genes FoxG1, 28S rRNA, and the sex determining genes AMH, WT1 and DMRT1, were isolated from *Sphenodon*. No variation was seen in FoxG1, 28S and AMH DNA sequences. WT1 sequence analysis clearly separated the north-eastern North Island and Cook Strait populations, supporting a unique allele of WT1 within Cook Strait populations. DMRT1 possessed no obvious population differentiation. Testudines and *Sphenodon* did not show the same close relationship reported in Chapter 1.

Introduction

Phylogenetic reconstruction using morphological characteristics can obtain data from fossil specimens, unlike molecular analysis where limits exist on obtaining samples from which DNA can be successfully extracted and sequenced (Noonan, Hofreiter et al. 2005; Rogaev, Moliaka et al. 2006). Therefore, one of the advantages of morphological analysis is the ability to collect data from extant and extinct species (Benton 1999; Lee 2001; Jenner 2004).

DNA phylogeny and molecular research on reptiles

Regions of the Tree of Life (http://tolweb.org/tree/phylogeny.html) pertaining to the Reptilia are reasonably well defined. The four extant and many extinct Orders have been defined and aligned using morphological evidence. Over the last 20 years fine-scale resolution using molecular data has accumulated, clarifying relationships between closely related species and occasionally questioning established morphological relationships. Descriptions of Reptilia relationships have been the subject of much discussion in the literature, and there are still regions within the Reptilia that are controversial, in particular the placement of turtles (Rieppel and deBraga 1996; deBraga and Rieppel 1997; Zardoya and Meyer 1998; Rieppel and Reisz 1999; Rieppel 2000; Lee 2001; Zardoya and Meyer 2001; Modesto and Anderson 2004). Recent review work on the definition of the Reptilia advanced removal of the formal name Anapsida, due to the limited use outside of referring to morphological structures (Figure 2.1) (Modesto and Anderson 2004). The current study follows the definition of Reptilia "…all members of the synapsid sister-group, regardless of the interrelationships of turtles and other extant non-synapsid amniotes" (Modesto and Anderson 2004).

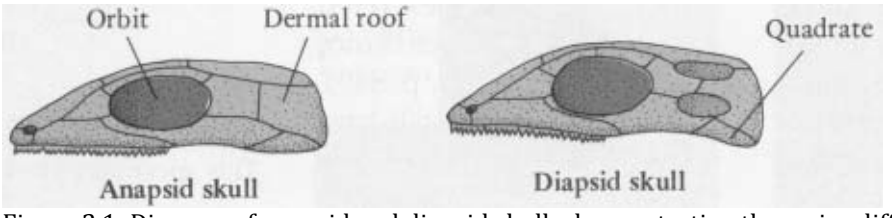


Figure 2.1: Diagram of anapsid and diapsid skulls demonstrating the major difference between the two (adapted from Dorit, Walker Jr et al. 1991).

One aim of phylogenetic reconstruction is to organise species into monophyletic groupings. Within the Reptilia, the two clades Archosauria (crocodiles, dinosaurs and

birds) and Lepidosauria (*Sphenodon* and Squamata) are generally agreed to be monophyletic (Rieppel and deBraga 1996; Rieppel 2000; Lee 2001). Both clades are diapsid, with two temporal openings (Figure 2.1). Although members of the Reptilia, Testudines (turtles) possess a derived anapsid skull (Figure 2.1) (Rieppel and Reisz 1999). Four categories for placement of the Testudines within the Reptilia are possible: (1) as an outgroup to the Lepidosauria and Archosauria; (2) as a sistergroup to the Lepidosauria; (3) as a sistergroup to the Archosauria; or (4) within one of the two clades. Depending on what is being analysed, molecular research has supported all four categories.

1. Outgroup to Lepidosauria and Archosauria

Traditionally supported by morphological studies using the diagnostic temporal openings (Lee 2001).

2. Sister group to the Lepidosauria

Early work using mtDNA and α-enolase sequences grouped the Archosauria and Lepidosauria as clades, with Testudines nearer to the Lepidosauria (Hedges 1994).

3. Sister group to the Archosauria

Sequencing of iguana and caiman mitochondrial DNA established that the iguana genome evolves more slowly than that of birds and mammals, whereas alligator and caiman mitochondrial genomes are evolving at a higher rate, a result contradicting the correlation between rate of molecular evolution and generation time (Janke, Erpenbeck et al. 2001). Rapidly evolving mtDNA is a problem for phylogenetic analysis as ordering and rates of evolution are not constant between all species.

Analysis of mtDNA placed turtles at the base of the archosaurian branch, supporting the hypothesis that the Testudine anapsid condition is secondarily derived from a diapsid state (Rieppel and Reisz 1999; Rieppel 2000; Janke, Erpenbeck et al. 2001).

Studies using incomplete mtDNA and short tRNA sequences produced ambiguous results (Seutin, Lang et al. 1994; Zardoya and Meyer 1998). Comparison of full mtDNA sequences from tuatara (*Sphenodon punctatus*), turtles (*Chrysemys picta, Chelonia mydas, Pelomedusa subrufa*), crocodiles (*Caiman crocodylus, Alligator mississippiensis*), birds (*Vidna chalybeata, Buteo buteo, Rhea americana*), lizards (*Eumeces egregius, Iguana iguana, Dinodon semicarinatos*) and mammals (*Didelphis virginiana, Mus musculus*) revealed similarities between *Sphenodon*, birds and crocodiles. Phylogenetic analysis supported a monophyletic Testudines as a sistergroup to the monophyletic Archosauria, with that clade a sistergroup to a monophyletic Lepidosauria (Rest, Ast et al. 2003).

4. Within one of the Lepidosauria and Archosauria clades

Reptilian relationships using molecular studies often split the traditional Lepidosauria and Archosauria. Haemoglobin (Hb) chains placed tuatara within the aves and turtles (α^{A}-Hb), with crocodiles as a sister-group. Squamata were well outside this grouping. β^{i}- and β^{ii}- Hb chains showed a similar pattern, although resolution was poor (Brown 1993). Pancreatic polypeptide sequence data placed turtles as diverging after snakes. The authors stated that the results were the same regardless of topology constraints used during analysis (Platz and Conlon 1997).

Recent work examining the two forms of the giant tortoise *Geochelone gigantea* α-D globin genes (α-D and α) supported a phylogeny where tortoises and birds were the closest living relatives of each other (Shishikura 2002). Phylogenies created using the α-D globin gene grouped tuatara and birds (rhea and chicken) with turtles as a sister-group. Within the α genes, turtles and aves grouped together, with *Homo sapiens* as an outgroup. It should be noted that there were no representatives of the Crocodylia in the phylogeny, which may have changed relationships within the Reptilia. The Komodo dragon and a snake grouped some distance from the aves, turtles and tuatara, although they possess α-D globins. This was interpreted as indicating the Squamata began diverging around 335 MYA, much earlier than other α type globins (Shishikura 2002). I suggest that the placement is more evidence for a rapidly evolving squamate genome compared to that of other reptiles. Firstly, most evidence suggests the Squamata diverged around 150 MYA (Olmo, Capriglione et al. 2002; Benton 2005). Secondly, high recombination rates, smaller G-banding regions, smaller chromosomes, and smaller effective deme sizes (Olmo, Capriglione et al. 2002) all provide opportunity for rapid evolution within the Squamata, compared to the other reptilian Orders.

Spermatozoa studies emphasised strong similarities between tuatara, turtle, crocodilian, and to a lesser extent non-passerine avian spermatozoa (Healy and Jamieson 1992; Jamieson and Healy 1992; Healy and Jamieson 1994). Reptilian eye structure analysis placed Crocodylia as a sister clade to two groupings, Testudines and a Lepidosauria and Aves clade (Caprette, Lee et al. 2004).

Molecular phylogenies appear more likely than traditional morphological analysis to support paraphyletic reptilian clades. DNA phylogenetics have been less successful in determining where in the Reptilia the Testudines fit, as gene sequences frequently give results at odds with other gene sequences. Larger datasets provide increased resolution, a situation seen with mtDNA where full sequence analysis gave significantly different results to earlier partial analysis by supporting the monophyly of Testudines, Archosauria and Lepidosauria (Hedges 1994; Seutin, Lang et al. 1994; Heise, Maxson et al. 1995; Gorr, Mable et al. 1998; Janke, Erpenbeck et al. 2001; Zardoya and Meyer 2001; Rest, Ast et al. 2003). Other methods of phylogenetic analysis including sperm morphology and ophthalmic evolution supported a strong relationship between turtles and *Sphenodon*.

Mitochondrial DNA variation and rates of change within the archaic Reptilia

Mitochondrial DNA has been analysed within the Reptilia for inter- and intra-Order relationships, providing information on rates of mtDNA evolution.

The Testudines are a highly speciose Order with over 285 species (Zug, Vitt et al. 2001). Analysis of five clades of Cryptodire turtles suggested a rapid radiation 100 MYA, although resolution of species was not possible using 12S and CytB sequences (Shaffer, Meylan et al. 1997). The low level of mtDNA variation is adequate for resolving deeper clades but is poor at resolving intrageneric relationships (FitzSimmons, Moritz et al. 1995; Shaffer, Meylan et al. 1997). The limited amount of mtDNA variation is not reflected in turtle morphology where extensive variation is present (Feldman and Parham 2004). Rates of mtDNA

change in turtles are very low, with values between 0.25%-0.46%/MY/lineage observed in Testudines compared with an average in other vertebrates of 2%/MY (Avise, Bowen et al. 1992; Lamb, Lydeard et al. 1994), a situation ascribed to the long generation time in Testudines. Non-coding nuclear DNA from the Testudine *Glyptus muhlenbergii* also demonstrated low variation (King and Julian 2004). Low variation, although apparently the norm, is not ubiquitous. Exceptions have been found in Galapagos Island giant tortoises and the *Chrysemys dorsalis/picta* complex. The Galapagos Islands date from <2.5 – 0.5 MYA, and the populations of Testudines exhibit approximately 30 times greater mtDNA variation compared to nucDNA (Caccone, Gentile et al. 2004). Another recent study also demonstrated high turtle mtDNA diversity, around 2-2.5%/MY in the *Chrysemys dorsalis/picta* complex thought to have diverged around 3.47-2.7 MYA (Starkey, Shaffer et al. 2003). Both populations diverged recently suggesting that release from selective constraints, and subsequent intensive inbreeding, increased mtDNA variation.

Although the Crocodylia is an archaic reptilian order, the rate of mtDNA evolutionary change is high (Janke, Erpenbeck et al. 2001; Janke, Gullberg et al. 2005). Full mtDNA sequences from seven species of crocodile, representing all three traditional morphological lineages, demonstrated only two mtDNA lineages, namely: Alligatoridae containing *Alligator* and *Caiman* and Crocodylidae containing *Crocodylus* and the previously separate *Gavialis/Tomistoma*. The authors noted that Crocodylian mtDNA appears to be evolving significantly quicker than other amniotes, although the rate appears consistent within the Crocodylia (Janke, Gullberg et al. 2005). Calibrating a

molecular clock derived from mtDNA suggested modern crocodiles originated around 140 MYA.

Low levels of mtDNA variation were demonstrated between *Sphenodon* populations (Hay, Daugherty et al. 2003). No island or sex related differences were observed, although some population structure is present, namely: north-eastern North Island tuatara, and Cook Strait tuatara. This result did not support the species status of *S. guntheri* on North Brother Island in Cook Strait, with *S. guntheri* grouping within Cook Strait *S. punctatus* populations. Mitochondrial DNA phylogenies placed Poor Knights Island within the Bay of Plenty samples (Hay, Daugherty et al. 2003), demonstrating mtDNA stability and low levels of variation as the Poor Knights Islands became isolated one million years ago (Hayward 1986). Recent work comparing mtDNA sequence from sub-fossil *Sphenodon* (8750-650 years before present) to extant samples, found that *Sphenodon* has a very high rate of mtDNA change (Hay, Subramanian et al. 2008). The study compared ancient and modern samples from various regions, suggesting a high rate of change is present in all populations, and not unique to one region. This result did contradict earlier findings (Hay, Daugherty et al. 2003) where genetic variation was found to be low. A 312bp nucDNA aldolase sequence isolated was identical among ten tuatara populations (Hay, Daugherty et al. 2003). *Sphenodon* microsatellites demonstrated significantly higher levels of variation than isolated turtle populations. This was interpreted as tuatara populations were holding on to historic genetic variation present prior to kiore (rat) introduction. Differentiation of populations was possible, but creation of robust phylogenetic trees was problematical due to some microsatellites being

fixed, probably resulting from bottlenecks (McGibbon 2003; MacAvoy, McGibbon et al. 2007).

Reptilian sex determination

Phenotypic sex results from two distinct processes, namely sex determination and sex differentiation. Sex determination refers to the interaction between genes or chromosomes and the environment in the organism, causing irreversible commitment to either a male or female pathway (Sarre, Georges et al. 2004), and sex differentiation is the development of gonadal tissue from primitive sex cells.

There are two major forms of sex determination, Epigenetic Sex Determination (ESD) and Genotypic Sex Determination (GSD) (Bull 1980). GSD occurs when the sex of the progeny is determined solely by genetic factors. For example, in humans (and most mammals) the female sex has homomorphic (XX) sex chromosomes whereas the male has heteromorphic (XY) sex chromosomes. It is the presence, or rather the action, of genes on the Y chromosome that cause the XY embryo to develop as male. Epigenetic sex determination is where environmental influences affect sex. The most common example, and the one most relevant to the current study, is Temperature Sex Determination (TSD). During a critical period of incubation, known as the Thermo-Sensitive Period, ambient temperature causes embryonic sex determination. TSD is present to some degree in all four Reptilian Orders suggesting TSD should be considered basal for the reptile group (Bull 1980) (Figure 2.2), although the incidence of

TSD varies between the Orders, as does the Transitional Range Temperature where both sexes are produced.

Aside from five Testudine species, *Kachuga smithii* (Emydidae, ZW/ZZ system), *Siebenrockiella crassicollis* (Emydidae, XX/XY system), *Staurotypus salvinii* (Kinosternidae, XX/XY system), *Staurotypus triporcatus* (Kinosternidae, XX/XY system) and *Chelodina longicollis* (Chelidae, XX/XY system) (Sharma, Kaur et al. 1975; Sites, Bickham et al. 1979; Bull and Legler 1980; Carr and Bickham 1981; Ezaz, Valenzuela et al. 2006), all known cases of reptilian sex chromosomes are in the Squamates. Squamate sex chromosomes appear to have arisen multiple times with some species possessing sex chromosomes and other closely related species having homomorphic chromosomes.

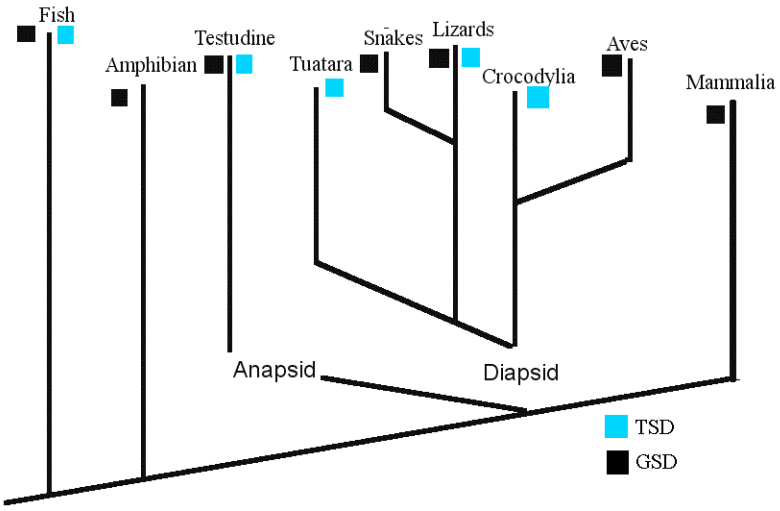


Figure 2.2: Incidence of GSD and TSD using a traditional morphological division of anapsids and diapsids (Smith and Sinclair 2004).

Incidence of TSD and GSD within Reptilian Orders

Order: Squamata

Snakes: GSD is present in all snake species. Increasing derived morphology in snakes is correlated with increasingly heteromorphic sex chromosomes. For this reason snakes are often cited as an example of sex chromosome evolution (Bickham 1984; Olmo 1986).

Lizards: Within the lizards both GSD and TSD are present. Due to the huge number of lizard species, it has not been possible to analyse karyotypes for evolution of GSD from a presumed basal TSD.

Order: Testudine

TSD is the most common mechanism of sex determination within the Testudines, but at least five species exhibit GSD (Sharma, Kaur et al. 1975; Sites, Bickham et al. 1979; Bull and Legler 1980; Carr and Bickham 1981; Bickham 1984; Olmo 1986; Ezaz, Valenzuela et al. 2006).

TSD occurs in all marine turtles, but individual populations differ in their response to temperatures (Chevalier, Godfrey et al. 1999).

Order: Crocodilia

All crocodile and alligator species exhibit TSD (Lang and Andrews 1994).

Order: Sphenodontidae

All tuatara populations have TSD (Cree, Thompson et al. 1995).

TSD in Reptiles

There are three patterns of TSD: FM (low temperature ♀, high temperature ♂); MF (low temperature ♂, high temperature ♀); and FMF (low and high temperatures ♀,

midrange temperatures ♂). FMF patterns occur in all reptilian lineages with the possible exception of *Sphenodon*, suggesting it is basal (Lang and Andrews 1994; Mitchell, Nelson et al. 2006). MF patterns occur only in turtles although some evidence suggests that FM and MF patterns result from female mortality at the 'missing' temperature (Ewert, Jackson et al. 1994). Only two species are known with the apparently rare form of FM TSD. The first species identified with FM was also the first reptile to have its sex ratio associated with incubation temperature (Charnier 1966; Ewert, Jackson et al. 1994). The rarity of the FM pattern has led some authors to suggest that a repeat of Charnier's pioneering work using modern temperature-shift experiments would reveal a FMF or MF pattern (Ewert, Jackson et al. 1994). However, recently published work on *Sphenodon* also demonstrated a FM pattern, suggesting that although rare, it does exist (Mitchell, Nelson et al. 2006).

Even at optimal male producing temperatures, a 100% male clutch is unlikely. For example, in *C. johnstoni* the largest percentage of males in a clutch is 56-88% (Webb, Beal et al. 1987; Webb, Manolis et al. 1990; Webb, Manolis et al. 1992). The lack of absolutes observed in all TSD species suggests a genetic component in TSD, or that within a TSD system there is a second system of GSD, although frame-shift experiments are required to test this (Ewert, Jackson et al. 1994; Rhen and Lang 1998).

Molecular Biology of Sex Determination

The current study focuses on three genes known to be involved in sex determination, namely AMH, DMRT1 and WT1. An overview of their roles in sex determination follows.

General background

Preceding sexual differentiation, embryos develop two pairs of undifferentiated genital ducts, namely the Müllerian, which develops into the female reproductive tract, and the Wolffian which develops into the male reproductive tract (Werner, Huth et al. 1996).

Sex determining gene: WT1

The Zinc Finger Proteins SF1 and WT1 have early roles in sex determination (Ramkissoon and Goodfellow 1996). WT1 is critical to kidney developmental regulation (Hastie 1994; Murdock and Wibbels 2006) and is highly conserved within the amniotes (Schmahl, Yao et al. 2003). The multiple effects of WT1 mutations is indicative of an early role in sex determination, as is the high level of conservation in the alternative splice site between chicken, alligator, marsupial and human genes (Kent, Coriat et al. 1995).

Within the Reptilia, both sequence analysis and expression profiles of SF1 and WT1 have been published. Sequence comparison of alligator, chicken, mouse and human SF1, DAX1 and WT1 showed high levels of conservation. Expression of WT1 and SF1 occurs at approximately the same time, supporting an early role in sex determination, and expression profiles suggest SF1 and WT1 interact to activate testis-specific gene expression (Nachtigal, Hirokawa et al. 1998; Western, Harry et al. 2000). Turtle WT1 showed high homology to alligator WT1 (88.8%) (Spotila, Spotila et al. 1998), and differed in product levels depending on the incubation temperature of the embryo. All animals examined have demonstrated alternative splicing of WT1, reflecting an archaic regulating mechanism. WT1 studies

in *T. scripta* showed a size increase in gonads at stages 16-17 before other morphological differences were apparent, presumably due to an increase of pre-Sertoli proliferating cells (Schmahl, Yao et al. 2003).

Sex determining gene: AMH

Expression of AMH in male embryos suppresses the Müllerian duct. After initial testes development, two simultaneous pathways are initiated, stimulatory (high levels of androgen and androgen receptors) and inhibitory (MIS = müllerian inhibiting substance also known as AMH = anti-müllerian hormone, also known as MIF = müllerian inhibiting factor) promoted by SRY and SOX9 (Werner, Huth et al. 1996; Western, Harry et al. 1999). Mutations in AMH leads to ‘normal’ males with uteri and fallopian tubes, therefore AMH is an important inhibitor of female development rather than a promoter of male development (Erickson 1997). Mammalian AMH is partially regulated by SOX9, differing from alligators where AMH expression precedes SOX9 (Western, Harry et al. 1999; Morrish and Sinclair 2002). Sequence analysis demonstrated alligator AMH to be closer to chicken AMH than to human or mouse. A situation reflected in AMH expression profiles where alligator AMH is expressed in developing testes from the early TSP similar to chicken expression patterns (Oreale, Pieau et al. 1998; Western, Harry et al. 1999). The potential of AMH to be the alligator Male Determining Factor is weakened by its expression profile, where AMH is most prolific at Stage22 rather than Stage21 when male specific development occurs. There is evidence that immature Sertoli cells express AMH, as low levels of AMH are detectable in undifferentiated male cell lineages. SF1 in testes in humans,

turtles and mice suppress oestrogen expression and promote AMH (Western, Harry et al. 2000).

Sex determining gene: DMRT1

DMRT1 (**D**oublesex and **M**ab3 **R**elated **T**ranscript) has a dimorphic expression pattern in humans and was initially isolated from sex reversed males. Human DMRT1 acts too late to be a testicular determining factor, although in birds DMRT1 is located on the avian Z chromosome and is expressed before other sex determining genes at higher levels in males (ZZ) than females (ZW) (Raymond, Kettlewell et al. 1999; Raymond, Parker et al. 1999; Smith, McClive et al. 1999; Ellegren 2000; Nanda, Zend-Ajusch et al. 2000; Shan, Nanda et al. 2000; Nanda, Haaf et al. 2002; Pask, Behringer et al. 2003).

DMRT1 has dimorphic expression within gonadal tissue in mice, chickens, alligators and turtles (Torres Maldonado, Landa Piedra et al. 2002; Koopman and Loffler 2003). It appears that there is consistency in DM factors being involved in male development, but little consistency in how they act between species (Hodgkin 2002; Ferguson-Smith 2007).

DMRT1 expression levels in *A. mississippiensis* are lower at female promoting temperatures than male promoting temperatures (Smith, McClive et al. 1999; Torres Maldonado, Landa Piedra et al. 2002) during and after the thermo-sensitive period. DMRT1 has been shown to act before SOX9 in species with TSD (*A. mississippiensis*, *T. scripta*, and *L. olivacea*) (Kettlewell, Raymond et al. 2000;

Torres Maldonado, Landa Piedra et al. 2002) positioning DMRT1 as acting upstream to maintain SOX9 expression in male embryos.

In addition to gene analysis, extensive work on effects of exogenous estrogen on TSD has been performed as estrogen is thought to be the temperature sensitive event of TSD (Pieau, Dorizzi et al. 1999; Miller, Summers et al. 2004). Exogenous estrogen results in reduced DMRT1 during the thermo-sensitive period at male temperatures (Murdock and Wibbels 2006). Patterns of expression suggest exogenous estrogen acts on, or before, DMRT1 expression during sex determination, indicating that suppression of DMRT1 is one of the effects of estradiol. Whether estrogen has a direct or indirect action is unclear (Murdock and Wibbels 2006).

Reptilian sex determination summary

Different reptilian Orders and species are likely to have evolved different sex determination mechanisms, although pivotal sex determining genes will have sexually dimorphic expression prior to the TSP. For example, a combination of WT1 and DMRT1 appear critical to sex determination in *T. scripta*. It appears that WT1 precedes DMRT1 expression so the mechanism of sex determination maybe different from birds, where DMRT1 is the sex determining gene. Crocodilian sex determination is more ambiguous as expression studies have provided no clear indication of the genes determining sex.

Nuclear genes used in the current study

Two nuclear genes, 28S rRNA and FoxG1, were selected to provide a comparison with sex determining gene phylogenies, as sex determining and standard genes are presumed to be under differing selection pressures.

28S rRNA subunit

The 28S rRNA subunit is over 4000 bp long and is present in all eukaryotic nuclear genomes. Compared to other rRNA subunits, 28S possesses increased variation and has been successfully used to create phylogenies from disparate species (Hillis and Dixon 1991). The increased variation does not appear to affect phylogenetic reconstruction with work comparing small subunit rRNA (SSU rRNA) and large subunit rRNA (LSU rRNA, 5S, 5.8S and 28S) over 28 taxa (Winchell, Sullivan et al. 2002) concluding:

1. LSU tree was very similar to that of the SSU
2. Adding LSU to the SSU data strengthens bootstrap support for groups above the SSU-only values
3. Some slight disagreement observed between LSU and some SSU trees.

These conclusions were in addition to previous work (Hillis 1987; Hillis and Dixon 1991) demonstrating rRNA phylogenies were generally consistent with other data sources.

In addition to 28S data in Genbank, sequences of 28S from hagfish, lampreys, sharks, batoids (skates and rays), turtle, lizard and the brittlestar allowed analysis of 28S sequence from 46 taxa (Mallatt and Winchell 2007). The large dataset was analysed using maximum-likelihood and maximum-parsimony methods, revealing phylogenies very similar to 18S and other phylogenetic trees (Mallatt and Winchell 2007). Within the Reptilia section of the phylogeny, turtles placed as an outgroup to a mammal, bird and lizard clade. The authors were more interested in the basal levels of the tree (placement of the lampreys), although they did note the Amniota (reptiles, birds and mammals) and the

placement of the turtles were well supported. 28S analysis supported the traditional morphological position of turtles (Mallatt and Winchell 2007).

FoxG1 nuclear gene

Reconstruction of phylogenies requires genes that are reasonably conserved between disparate species, and in the current study, Orders. Transcription factors, including the recently described FoxG1 (Forkhead Box G1), contain enough variation to resolve trees from various Orders and have been used extensively for phylogenetic analysis. FoxG1 is highly conserved in vertebrates and has an important role in forebrain size by regulating proliferation and cell factor specialization (Hanashima, Shen et al. 2002; Pauley, Lai et al. 2006).

Recent work sequencing FoxG1 from a number of mammalian (Old World monkey, New World monkey, microbat, macrobat, zebra and rhino) and reptilian (crocodile, lizard and tortoise) sources combined with existing sequence data (chimpanzee, human, mouse, rat, chicken, *Xenopus* and Zebrafish) allowed creation of an extensive phylogeny (Bredenkamp, Seoighe et al. 2007). The reptiles sequenced included crocodiles, with the most complex brains of the diapsid reptiles, and the tortoise as the anapsids have the smallest forebrain of the Reptilia (Bredenkamp, Seoighe et al. 2007). No phylogeny incorporating the reptiles was produced.

The aims of this chapter

Obtaining sequence from the five genes (WT1, AMH, DMRT1, FoxG1, 28S) will enable answering the following questions:

- How much variation is there between *Sphenodon* populations based on nuclear DNA sequences?
- What is the phylogenetic position of *Sphenodon* and the Testudines among the reptiles?
- Are there differences between phylogenies created using mtDNA, nuclear DNA and morphology?
- Are there differences between sex determining genes and other nuclear gene phylogenies?
- Where do *Sphenodon* sex determining genes fit within the Reptilia phylogeny, and do they provide information on which genes could be early stage sex determination in *Sphenodon*?

Methods

DNA isolation

DNA was isolated from stored frozen whole blood using a Qiagen DNeasy tissue kit (Qiagen 69504) and the supplied protocol. A small piece of whole blood was placed into a 1.5 ml microcentrifuge tube with 180 μl Buffer ATL and 20 μl proteinase K, mixed by vortexing and incubated at 55^0C for 1-3 hours until the sample was completely lysed. 200 μl Buffer AL at 55^0C was added to the sample, mixed by gentle inversion, and incubated at 70^0C for 10 minutes. 200 μl ethanol (96-100%) was added to the sample, vortexed and pipetted into a DNeasy column and collection tube assembly. The assembly was centrifuged at 8000 rpm for one minute and the collection tube (with flow-through) discarded. The DNeasy tube was placed in another collection tube, 500 μl Buffer AW1 added, and centrifuged at 8000 rpm for one minute. Again the collection tube and flow-through were discarded. The column was placed in another collection tube, 500 μl Buffer AW2 added and centrifuged at maximum speed for three minutes to dry the DNeasy membrane. The flow-through and collection tube were discarded. The DNeasy column was placed in a 2 ml micro-centrifuge tube and 200 μl Buffer AE added on the membrane. A one minute incubation at room temperature was followed by a one minute centrifugation at 8000 rpm to elute the DNA. The elution was repeated into a new micro-centrifuge tube. A summary of isolated DNA samples is given in Table 2.1.

Some DNA samples were a gift from Dr. E. MacAvoy (EM in Table 2.1).

Table 2.1: Samples used for DNA sequence analysis

Sample	Island	Sex	Species	Source
Coppermine 2791 M	Coppermine Island	Male	*S. punctatus*	EM
Coppermine 2793 M	Coppermine Island	Male	*S. punctatus*	EM
North Brother 1 F	North Brother Island	Female	*S. guntheri*	
North Brother 2 M	North Brother Island	Male	*S. guntheri*	
North Brother 3 M	North Brother Island	Male	*S. guntheri*	
North Brother 4 M	North Brother Island	Male	*S. guntheri*	
Poor Knight 1 M	Poor Knights Island	Male	*S. punctatus*	
Stephens 1 F	Stephens Island	Female	*S. punctatus*	
Stephens 2 M	Stephens Island	Male	*S. punctatus*	
Stephens 3 F	Stephens Island	Female	*S. punctatus*	
Stephens 4 F	Stephens Island	Female	*S. punctatus*	
Stephens 5 M	Stephens Island	Male	*S. punctatus*	
Stephens 6 F	Stephens Island	Female	*S. punctatus*	
Stephens 7 M	Stephens Island	Male	*S. punctatus*	
Tawhiti Rahi 2521 M	Tawhiti Rahi	Male	*S. punctatus*	EM
Tawhiti Rahi 2525 F	Tawhiti Rahi	Female	*S. punctatus*	EM
Tawhiti Rahi 2526 M	Tawhiti Rahi	Male	*S. punctatus*	EM
Tawhiti Rahi 2529 F	Tawhiti Rahi	Female	*S. punctatus*	EM

DNA probe design

Molecular primers were designed using Oligo6 (http://www.oligo.net/) or online (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) from sources detailed below, and constructed by Invitrogen. All primers were diluted to a working solution of 10 μM.

DMRT1 primers

The primers DMRT1 76F and DMRT1 250R are from the turtle *Trachemys scripta* DMRT1 (Murdock and Wibbels 2003), primers BN DMRT1 562U24 and BN DMRT1 751 L25 were designed from *T. scripta* published sequence (Genbank Accession Number AY316537). From *Sphenodon* sequence extracted using DMRT1 76F and 250R, BNDM_TUT primers were designed to have higher specificity to *Sphenodon* DMRT1 sequence.

DMRT1 76F: AGCAGGCTCAAGAAGAGGAAC
DMRT1 250R: ATTCCTTCCATCACCAGCAGA

BN DMRT1562 U24: CCGCAGATCTTCACTTTCGAGGAC
BN DMRT1751 L25: TTGCTTATTCGCCATCTTCTATGAC

BNDM_TUT_F: ACCGGGTGGTCGTTCACTGAT
BNDM_TUT_R: GCTGTCAGCCGCAGGTTT

WT1 primers

Primers S+H 1.1 and S+H 1.3 are previously published (Spotila, Spotila et al. 1998) and the sequence those authors obtained from the turtle *T. scripta* was used to design primers BN WT1 281U23 and BN WT1 705L25. The turtle primers extract a 512 bp sequence, and the BN WT1 primers were designed to extract a 449 bp sequence.

S+H WT 1.1: CCATTTATGTGTGCTTAYCCNGG
S+H WT 1.3: CAGGTTTTGCATTGGAANGGYTT

BN WT1 281U23: AGATGGGCTCCGACGTGCGAGAT
BN WT1 705L25: GTGAGAGGGAGTGTGGCCGTAACTT

AMH primers

Primers were designed for AMH from a 999 bp alligator sequence (Genbank Accession Number AF180294) (Western, Harry et al. 1999), and a 339 bp sequence from the turtle *T. scripta* (Genbank Accession Number AY235424) (Takada, DiNapoli et al. 2004).

Croc_amh_f_bn: CTCGCTTCCTGTCGGTGGC
Croc_amh_r_bn: CGGCACGGCAATGAACTTGTA

Amhturt_f: CCGGCTGCAGGAGCTGACGAT
Amhturt_r: GCAAGCCCTCCCCGGTGA

FoxG1 primers

Published primers for FoxG1 (Bredenkamp, Seoighe et al. 2007) designed to extract the full-length coding region were used.

FoxG1 Sense: 5′-GTGATG(CT)TGGA(CT)ATGGG(AG)GA(TA)AG-3′
FoxG1 Antisense: 5′-GGTGTAAAA(CT)GTTCACTTACAGTCTG-3′

28S primers

Previously published primers (Hillis and Dixon 1991) were used to extract a 748 bp sequence from *Sphenodon*.

28S AA (jj) AGGTTAGTTTTACCCTACT
28S V (gg) AAGGTAGCCAAATGCCTCGTCAT

PCR protocols

Various protocols were trialled for the five genes. The following were found to be successful in obtaining high quality PCR product.

PCR protocol WT1, 28S and FoxG1

The PCR mastermix was made up using as per Table 2.2. Each mastermix was set up using N (number of samples), plus C (control), plus one extra (to run four samples, the mastermix was made for six). From the mastermix, 24 μl was aliquotted into PCR tubes and 1 μl DNA added. For WT1 the most successful primers were BN WT1 281U23 and BN WT1 705L25.

PCR protocol DMRT1 and AMH

The PCR mastermix was made up as per Table 2.2. DMRT1 76F and DMRT1 250R primers were used and from the subsequently isolated sequence, *Sphenodon* specific primers were designed; BNDM_TUT_F and BNDM_TUT_R. For AMH, alligator primers worked however the concentration of DNA was poor, so turtle primers (amhturt_f and amhturt_r) with greater specificity were used. *Taq* mastermix was added using the hot start protocol described below.

After aliquotting into PCR reaction tubes, 1 μl of DNA was added. Tubes were heated at 94^{0}C for two minutes then 2.5 μl of *Taq* mastermix added.

Table 2.2: PCR mastermix for sequence extraction.

PCR Mastermix solution	**Quantity in μl**				
	WT1	**AMH**	**DMRT1**	**FoxG1**	**28S**
Water	13.35	14.5	14.5	16.9	13.4
Q Solution	5				5
10x Buffer	2.5	2.5	2.5	2.5	2.5
$MgCl_2$		1.5	1.5	1.5	
dNTP	1	1	1	1	1
Forward Primer	1	1	1	1	1
Reverse Primer	1	1	1	1	1
Q *Taq*	0.15			0.1	0.1
DNA	1	1	1	1	1
Total	**25**	**22.5**	**22.5**	**25**	**25**
***Taq* Mastermix solution**					
Water		2.15	2.15		
10x Buffer		0.25	0.25		
Q *Taq*		0.1	0.1		
Total		**2.5**	**2.5**		

PCR touchdown protocol

For all PCR reactions the following touchdown 55/45 protocol was used. An initial incubation at 94^{0}C for two minutes (at which point the *Taq* mastermix was added if the samples were DMRT1 or AMH), twenty-one cycles of 94^{0}C for 20 seconds, 55^{0}C for 30 seconds, and 72^{0}C for 30 seconds, followed by twenty-five cycles of 94^{0}C for 20 seconds, 45^{0}C for 30 seconds, and 72^{0}C for 30 seconds. A 15 minute incubation at 72^{0}C completed the protocol. The samples were held at 4^{0}C.

For all reactions, PCR products were run out on a 1% agarose gel (0.5 g agarose, 50 ml TAE buffer, 1 μl ethidium bromide), with a 100 bp ladder (BioRad EZ Load 170-8352) to estimate PCR product size. When high quality bands of the correct size were seen on the 1% agarose gel, PCR products were run out on a low

melting point NuSieve agarose gel (0.7 g Low Melting Point NuSieve agar in 60 ml TAE buffer + 1 μl ethidium bromide) with a 100 bp ladder. Bands of interest were cut out under ultraviolet light and placed in previously weighed 1.5 ml Eppendorf tubes. Band weight was calculated by re-weighing the Eppendorf and subtracting the initial tube weight.

The Qiagen MinElute Gel Extraction Kit (Qiagen 28704) was used to isolate the DNA from the excised band. Three volumes of Buffer QG was added to the gel fragment (for example 100 mg of gel required 300 μl of Buffer QG) and incubated for 10 minutes at 50^0C. The sample was vortexed every three minutes to aid in dissolving the gel. One gel volume of isopropanol was added, mixed by inversion and the sample added to a MinElute column and collection tube. The sample was centrifuged for one minute (all centrifugation was at 13,000 rpm in a standard benchtop micro-centrifuge) and the flow-through discarded. The column was replaced in the collection tube, 500 μl of Buffer QG added and the column centrifuged for one minute, the flow-through discarded and the column returned to the collection tube. The sample was washed by placing 750 μl of Buffer PE in the column, standing for five minutes at room temperature and centrifuging for one minute. After discarding the flow-through, the column and collection tube were again centrifuged for one minute. The column was removed, placed in a 1.5 ml microcentrifuge tube and 10 μl of Buffer EB (elution buffer) added to the centre of the membrane. After a one minute incubation at room temperature the sample was centrifuged for one minute, the elution labelled and stored at -20^0C.

Once isolated, 2 μl of gel product + 2 μl ddH2O + 1 μl loading buffer (Bio-Rad) was run on a 1% gel with a Low DNA Mass Ladder (LML) (Invitrogen 10068-013) to confirm DNA isolation and estimate DNA concentration.

If the gel extraction was successful, samples were sequenced using the Allan Wilson Centre (AWC, http://awcmee.massey.ac.nz/index.htm) Big Dye Termination Protocol v3.1 (BDT). Primers were freshly diluted 1:10 from the 10 μM stock (5 μl of stock + 45 μl ddH_2O). The BDT mastermix used the AWC ¼ X protocol (summarised in Table 2.3), and generally 3-4 μl of DNA template was used for the required 20ng DNA concentration for the PCR protocol.

Table 2.3: AWC protocol for Big Dye Termination protocol ¼X.

Chemical	**Quantity**
Big Dye Terminator mix	2 μl
5x Sequencing Buffer	3 μl
Primer (diluted)	3.2 μl
Template	1-4 μl depending on DNA concentration
Water	Up to 20 μl

It was found that the primers BN WT1 705L25, DMRT1 250R, BNDM_TUT_F, amhturt_r, 28SAA and FoxG1sense gave the best sequencing results.

The BDT cycle sequencing reaction followed the recommended protocol of 94^0C for one minute, twenty five cycles of 96^0C for ten seconds, 50^0C for five seconds, and 60^0C for four minutes, followed by holding the sample at 4^0C.

Following BDT, PCR samples were precipitated following the AWC protocol. All incubation and centrifugation was at room temperature unless otherwise noted. The cycle sequencing reaction, 50 μl ethanol, and 4 μl 1:1 3M sodium acetate

(ph5.2) : 125 mM EDTA were added to a 1.5 ml eppendorf tube and mixed by inversion. Following a 15 minute incubation, the tube was spun at 13,000 rpm for 15 minutes, the supernatant removed and 70 μl of 70% ethanol added. The tube was spun at 13,000 rpm for 10 minutes, the supernatant removed and a second 70 μl of 70% ethanol added. Again the sample was centrifuged for 10 minutes at 13,000 rpm, and the supernatant removed with a narrow-bore pasteur pipette. Tubes were air dried for 30-60 minutes, sealed and stored at 4^0C before couriering to the AWC.

Chromatograms and sequences were examined using FinchTV (http://www.geospiza.com/finchtv/), high quality sequence was highlighted and pasted into a text file. Sequence obtained was checked for quality and edited by hand so that only high quality sequence was used for analysis. Sequence alignment used ClustalX (http://bips.u-strasbg.fr/fr/Documentation/ClustalX), CLC Sequence Viewer v5 (http://www.clcbio.com) or MEGA4 (Tamura, Dudley et al. 2007). Alignments were examined and limited phylogenies created for tuatara samples, and samples representing the Mammalia, Reptilia and Aves. Alignments were checked by comparing ClustalX and Sequence Viewer results.

Gene confirmation

Sphenodon sequence was confirmed as being the gene of interest by using Blast (http://blast.ncbi.nlm.nih.gov/). Blast parameters were nucleic acid (BlastN); nucleotide collection (nr); and either megablast or discontiguous megablast.

Phylogenetic analysis

Maximum parsimony was used for phylogenetic analysis. Alignment was performed using CLC Sequence Viewer or MEGA4 with the following parameters

alignment gap cost 25; Gap extension cost 1. Maximum parsimony analyses were performed in MEGA4 using a Max-mini Branch and Bound algorithm and a bootstrap value of 1000.

Results

Sphenodon sequence was obtained for all five genes. Table 2.4 summarises the Blast results with the most relevant species included. The lower the E-value the less likely the similarity occurs by chance.

Table 2.4 Blast results from Sphenodon *sequences*

Gene	Accession	Species	Max Score	Total Score	Query Coverage	E Value	Max Identity
28S	DQ283487.1	Ichthyophis cf. peninsularis MW 375 28S ribosomal RNA gene, partial sequence	1199	1199	99.00%	0	99.00%
	AY859626.1	Chrysemys sp. JM-2004 internal transcribed spacer 2 and 28S ribosomal RNA gene, partial sequence	1184	1184	100.00%	0	98.00%
	DQ283651.1	Chelydra serpentina 28S ribosomal RNA gene, partial sequence	1184	1184	99.00%	0	98.00%
	DQ283622.1	Pelomedusa subrufa 28S ribosomal RNA gene, partial sequence	1184	1184	99.00%	0	98.00%
	DQ283650.1	Alligator sinensis 28S ribosomal RNA gene, partial sequence	1181	1181	99.00%	0	98.00%
FoxG1	BC092710.1	Danio rerio forkhead box G1, mRNA complete cds	42.8	42.8	9.00%	0.91	96.00%
	NM_131067.1	Danio rerio forkhead box G1 (foxg1), mRNA complete cds	42.8	42.8	9.00%	0.91	96.00%
DMRT1	EU526604.1	Crocodylus palustris doublesex mab-3 related transcription factor 1 isoform a1 (Dmrt1) mRNA, complete cds, alternatively spliced	39.2	39.2	11.00%	6	100.00%
	AF192560.1	Alligator mississippiensis doublesex and Mab3 related transcription factor 1 (DMRT1) mRNA, partial cds	39.2	39.2	11.00%	6	100.00%
WT1	AF019779.1	Trachemys scripta Wilms tumor 1 protein (TWT1) mRNA, complete cds	42.8	42.8	7.00%	0.89	100.00%
AMH	AY235424.1	Trachemys scripta anti-Mullerian hormone mRNA, partial cds	41	41	4%	4.8	100.00%

DNA Sequences

A summary of *Sphenodon* DNA sequences obtained is presented as Table 2.5, where the base pair length (bp) refers to the edited sequence length. Table 2.6 lists the Genbank sequences used during analysis. Figures 2.3-2.8 demonstrate the PCR bands of interest, and subsequent DNA concentration estimation for AMH, WT1 and DMRT1 (two forms), 28S and FoxG1.

Table 2.5 Summary of sequence data

Gene	Sample	Island	Sex	Base pairs (edited)
AMH	Stephens 1 F	Stephens	F	448
	Coppermine 2791	Coppermine	M	445
	North Brother 1 F	North Brother	F	421
	Tawhiti Rahi 2526	Tawhiti Rahi	M	227
	Tawhiti Rahi 2525	Tawhiti Rahi	F	224
	Tawhiti Rahi 2529	Tawhiti Rahi	F	161
	Tawhiti Rahi 2521	Tawhiti Rahi	M	221
WT1	Tawhiti Rahi 2526	Tawhiti Rahi	M	337
	Tawhiti Rahi 2525	Tawhiti Rahi	F	286
	Tawhiiti Rahi 2529	Tawhiti Rahi	F	346
	Coppermine 2793	Coppermine	M	277
	North Brother 2 M	North Brother	M	297
	Stephens 3 M	Stephens	F	296
	Stephens 2 M	Stephens	M	297
	Stephens 4 F	Stephens	F	297
	Stephens 5 M	Stephens	M	211
DMRT1	Tawhiti Rahi 2529	Tawhiti Rahi	F	342
	Stephens 4 F	Stephens	F	134
	Stephens 6 F	Stephens	F	179
DMRT1	Stephens 8 F	Stephens	F	93
(BN	North Brother 4 M	North Brother	M	318
Primers)	North Brother 1 F	North Brother	F	149
	North Brother 2 M (Fwd)	North Brother	M	419
	North Brother 2 M (Rev)	North Brother	M	357
	Stephens 1 F	Stephens	F	154
	Stephens 1 F (2)	Stephens	F	154
	Coppermine 2795	Coppermine	F	97
FoxG1	Stephen 7 M	Stephens	M	252
	North Brother 1F	North Brother 1	F	304
	Poor Knights 1 M	Poor Knights	M	299
	Poor Knights 1 M	Poor Knights	M	296
	North Brother 4 M	North Brother	M	303
	North Brother 3 M	North Brother	M	303
28S	Poor Knights 1 M	Poor Knights	M	542
	North Brother 3 M	North Brother	M	668
	North Brother 2 M	North Brother	M	158
	Stephens 4 F	Stephens	F	668
	Poor Knights 1 M	Poor Knights	M	652
	North Brother 1 F	North Brother	F	492
	Stephens 7 M	Stephens	M	642

Table 2.6 Genbank accession numbers for DNA sequences used in analysis

Gene	Species	Genbank Accession	Reference (if applicable)
FoxG1	*Cebus capucinus*	DQ387961	(Bredenkamp, Seoighe et al. 2007)
	Chlorocebus pygerythrus	DQ387962	(Bredenkamp, Seoighe et al. 2007)
	Pipstrellus rusticus	DQ387963	(Bredenkamp, Seoighe et al. 2007)
	Epomophorus gambianus	DQ387964	(Bredenkamp, Seoighe et al. 2007)
	Ceratotherium simum	DQ387966	(Bredenkamp, Seoighe et al. 2007)
	Crocodylus niloticus	DQ387967	(Bredenkamp, Seoighe et al. 2007)
	Psammobates geometricus	DQ387968	(Bredenkamp, Seoighe et al. 2007)
	Agama atra	DQ387969	(Bredenkamp, Seoighe et al. 2007)
	Homo sapiens	NM_005249	
	Mus musculus	NM_008241	
	Rattus norvegicus	NM_012560	
	Danio rerio	NM_131067	
	Gallus gallus	NM_205193	
28S	*Homo sapiens*	NR_003287.1	
	Mus musculus	NR_003279.1	
	Xenopus laevis	X59734.1	(Ajuh, Heeney et al. 1991)
	Anolis carolinensis	AY859623	(Mallatt and Winchell 2007)
	Iguana iguana	DQ283590	(Frost, Grant et al. 2006)
	Pelomedusa subrufa	DQ283622	(Frost, Grant et al. 2006)
	Chelydra serpentina	DQ283651	(Frost, Grant et al. 2006)
	Alligator sinensis	DQ283650	(Frost, Grant et al. 2006)
	Crocodylus porosus	EF063685	(Seebacher and Murray 2007)
	Danio rerio	AF398343	
	Gallus gallus	DQ018756.1	

Phylogenetic trees were constructed using Sequence Viewer v5 or MEGA4 (Figures 2.9, 2.10, 2.11).

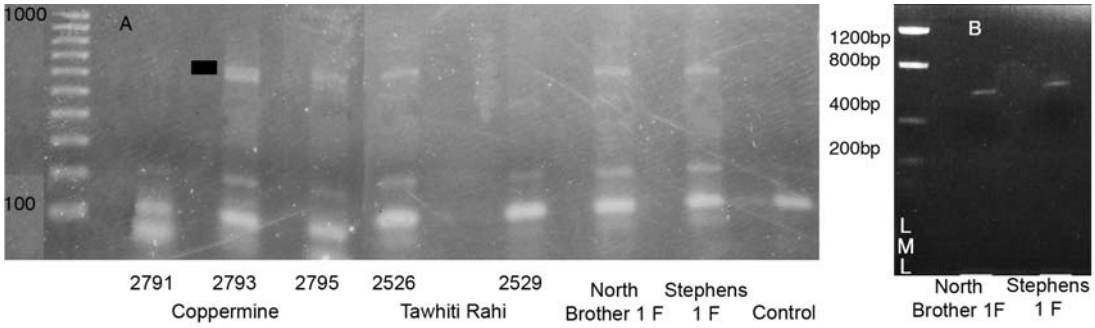


Figure 2.3*: **(a)*** AMH isolation, black bar indicates band of interest in various tuatara, ***(b)*** LML to estimate DNA concentration from two samples.

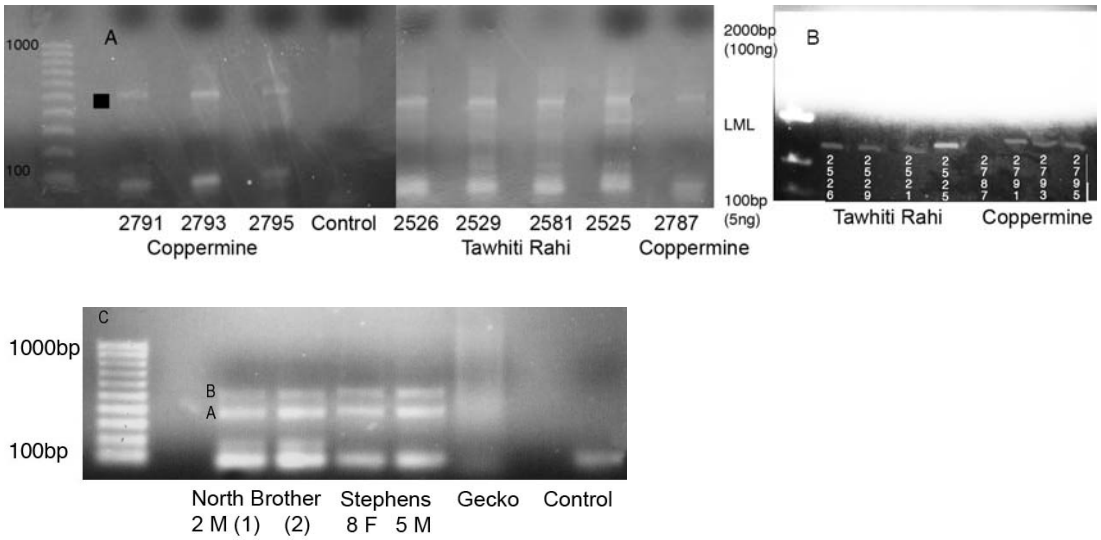


Figure 2.4: **(a)** WT1 isolation, black bar indicates bar of interest in various tuatara (Coppermine and Tawhiti Rahi samples from two gels aligned), **(b)** LML gel to estimate isolated DNA concentration, **(c)** WT1 isolation from Cook Strait samples demonstrating two bands labelled *A* and *B*.

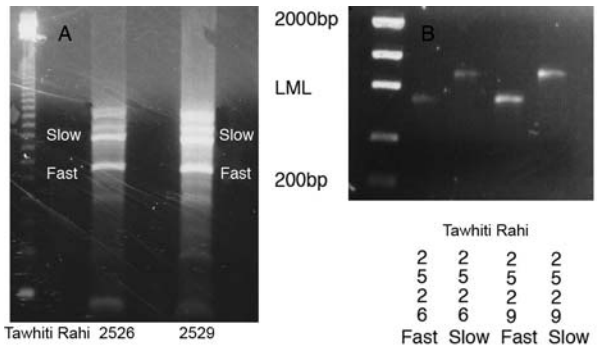


Figure 2.5: **(a)** DMRT1 isolation, the two bands cut out are labelled Fast and Slow based on their migration distances, **(b)** Estimation of DNA concentration.

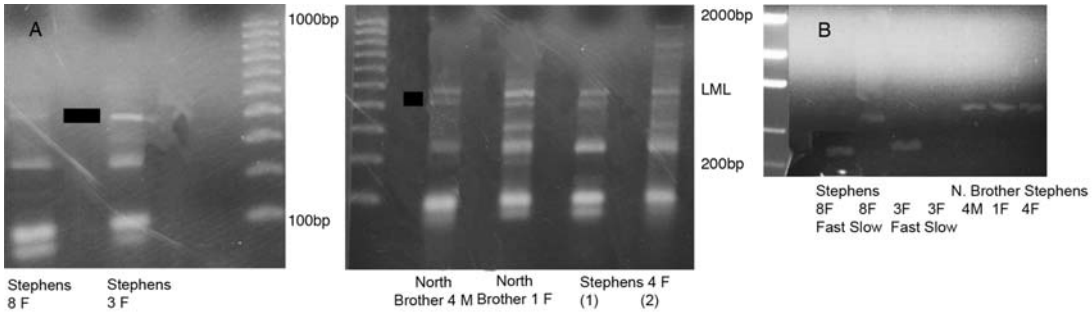


Figure 2.6: **(a)** DMRT1 alternative isolation, bands of interest are marked with a black bar, **(b)** LML to estimate DNA concentration for BDT sequencing.

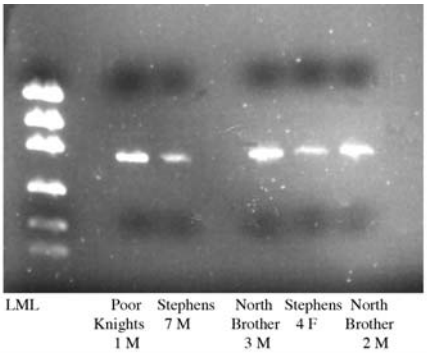


Figure 2.7: Estimation of DNA concentration from extracted 28S sequence.

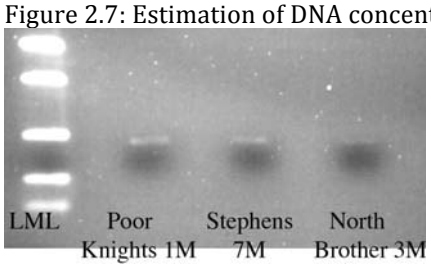


Figure 2.8: Estimation of DNA concentration from extracted FoxG1 sequence.

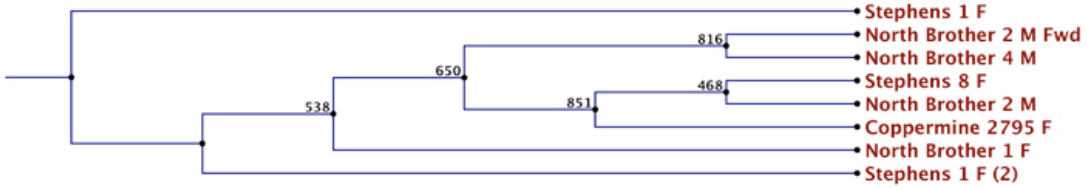


Figure 2.9: Phylogeny created from sequence extracted by BN DM TUT F/R primers, Neighbour-Joining 1000 bootstrap.

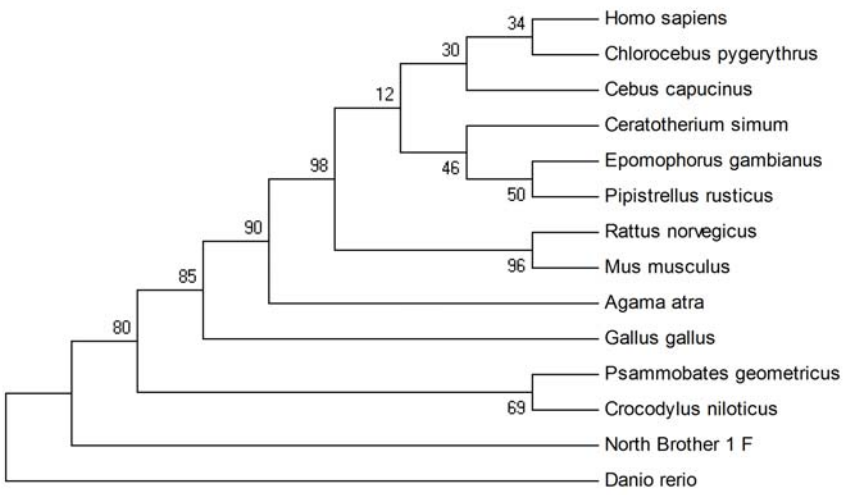


Figure 2.10: Maximum Parsimony phylogeny created using FoxG1 sequence, 1000 bootstrap.

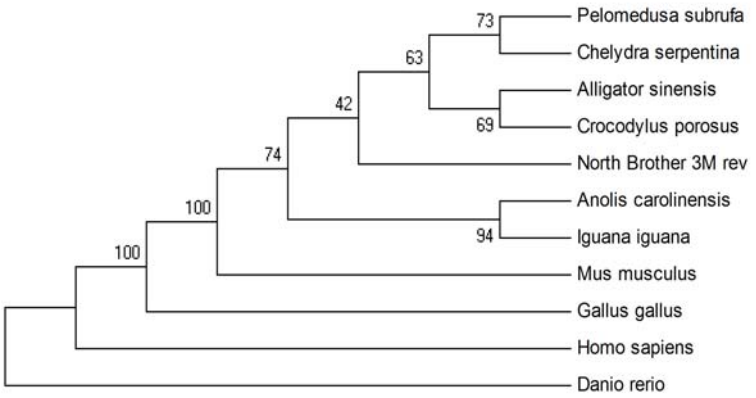


Figure 2.11 Maximum Parsimony phylogeny created using 28S sequences, 1000 bootstrap.

DNA analysis summary

The low homology between *Sphenodon* and other reptiles, and the small number of base-pairs, prevented phylogenetic reconstruction for AMH, WT1 and DMRT1, reflecting the long period of isolation since a common ancestor.

No variation was observed in *Sphenodon* AMH sequence (Appendix 3). WT1 groups *Sphenodon* into north-eastern North Island and Cook Strait groups, neither group shows variation between individuals (Appendix 3). DMRT1 isolated in the current study can be grouped into two forms. The first of these was demonstrated to be DMRT1 using BlastN analysis. This form used the *T. scripta* probes 76F/250R (Murdock and Wibbels 2003). The other DMRT1 sequence (Appendix 3) was isolated using BN_DM_TUT_F/R primers. This sequence exhibited no homology to any gene when run through BlastN, although homology was seen to mammalian whole chromosome sequences. No population or sex related structure was seen in the phylogenetic tree (Figure 2.9).

FoxG1 possess no sequence variation in *Sphenodon* (Appendix 3). The maximum parsimony tree (Figure 2.10) groups the Reptilia together and places the Avian sample within the Reptilia, although branches have low support. The Archosauria is paraphyletic, with the Testudine placing between the Crocodylia and the Aves. The Squamate *Agama* is outside the Archosauria and Testudines group, with *Sphenodon* and the Zebrafish as outgroups to the rest of the Reptilia. Testudine placement supports Category 4 where the Archosauria is not supported.

There is no variation present in *Sphenodon* 28S sequence, and very little within the Reptilia (Appendix 3). The 28S maximum parsimony tree (Figure 2.11)

divided species into traditional groupings. The Testudines, Crocodylia, Squamates and Mammalia all group with other members of their respective Orders. Crocodylians and Testudines group with *Sphenodon*, with the Squamates appearing the most unique of the reptile sequences. The basal positioning of the chicken sequence creates a paraphyletic Archosauria, supporting Category 4 for Testudine placement. Strong support (100) for the branch separating the Reptilia and mice was observed, but resolution of individual reptile branches is low, with the exception of the Squamates (94). These low levels of resolution reflect the low levels of variation seen in the alignment (Appendix 3).

Discussion

Phylogenetic relationships of the Reptilia

AMH phylogenetics

AMH sequence analysis showed no variation in DNA between sexes or populations (Appendix 3). Tawhiti Rahi (Poor Knights Island group) has been isolated for 1 million years (Hayward 1986), and the similarity of the Tawhiti Rahi sequence to Cook Strait samples demonstrated AMH is highly conserved within *Sphenodon*.

WT1 phylogenetics

WT1 sequence from *Sphenodon* separated into Cook Strait and north-eastern North Island groups.

The differentiation of the two tuatara groups by WT1 sequence is informative. The north-eastern North Island sequence is at least one million years old, and could date back to the Pliocene/Pleistocene (5 MYA), the Oligocene Drowning (25 MYA) or Gondwanic separation (80 MYA). No species differences are present, as demonstrated by North Brother 2 M (*S. guntheri* from North Brother Island) and *S. punctatus* (Cook Strait) having identical sequences (Appendix 3). This suggests two potentially linked options:

1. Cook Strait tuatara are relics of another lineage.
2. Cook Strait tuatara have evolved a different form or function for WT1.

There is support for both positions. Mitochondrial DNA does separate north-eastern North Island and Cook Strait tuatara (Hay, Daugherty et al. 2003),

implying a degree of differentiation. There is no reason why different regions of New Zealand could not have had different tuatara lineages. Indeed, one theory of tuatara population evolution strongly supports this (see Chapter 4).

Cook Strait WT1 sequence is distinct from north-eastern North Island WT1, suggesting the role of WT1 may have changed in Cook Strait tuatara. The gene may have moved within the sex determination cascade, either gaining or losing function, leading to the sequence change. WT1 is known to be regulated by alternative splicing (Kent, Coriat et al. 1995; Spotila and Hall 1998; Western, Harry et al. 2000) which could explain the *Sphenodon* differences, although alternative splicing occurs during translation meaning the base DNA gene sequence is retained. This does not appear to be the situation in *Sphenodon*. Analysis of Cook Strait sequence trace demonstrated no evidence of a second sequence underneath, a situation that would suggest two alleles of WT1. Detailed examination of the gels that the bands were cut from revealed a single WT1 band in north-eastern North Island (Figure 2.4a), and a double band in all Cook Strait samples (Figure 2.4c). Both Cook Strait bands were sequenced using forward and reverse primers, although the only readable sequence was from the 'A' band. These results provide support for a unique version of WT1 in Cook Strait *Sphenodon*, although the sequence is similar enough to other reptilian WT1 to be isolated using WT1 primers. Sequence length also supports the uniqueness of Cook Strait WT1 with sequence ranging between 211-297 bp, and the north-eastern North Island populations between 277-346 bp, using the same primers.

DMRT1 phylogenetics

Three *Sphenodon* DMRT1 sequences were isolated in the current study. These were similar to published *Sphenodon* DMRT1 (Wang, Miyake et al. 2006).

Sequences obtained from BNDMTUTF/R primers show no homology to any gene when run through BlastN, although limited homology was seen to mammalian whole chromosome sequences. This can be interpreted as either (i) contamination of one primer (but not the DNA as other primers successfully extracted expected sequences) by human DNA, or (ii) the extracted tuatara sequence is a pseudogene with limited homology to the DMRT gene. Analysis provided limited sequence resolution (Figure 2.9), and if the primers were contaminated I would expect all sequences to be identical, which was not the case. Contamination of other reagents can be excluded, as PCRs for other genes were being run at the same time using the same reagents, protocols and equipment.

Three populations of tuatara have this sequence, suggesting it has been present within *Sphenodon* for at least 10,000 years when the islands became separated from the mainland. Poor quality sequence, which was unable to be analysed, was obtained from Tawhiti Rahi samples, implying the sequence may be present in that population and therefore dating the sequence to 1 MYA. If the sequence is present within the Tawhiti Rahi population then it appears to have accumulated more mutations than more recently isolated populations, typical of a pseudo-gene. An alternative explanation of the very low quality Tawhiti Rahi sequence is that the sequence was absent, and the primers were amplifying non-specific

product, dating the evolution of the potential pseudo-gene to after Tawhiti Rahi isolation one million years ago.

FoxG1 phylogenetics

No variation in *Sphenodon* FoxG1 sequence was seen. The Archosauria are paraphyletic with the Testudines grouping between the Crocodylia and the Aves, and the grouping of the mammals reflects a previous study (Bredenkamp, Seoighe et al. 2007). The Testudine location supports a Diapsid affinity within Category 4. The very low levels of support for the reptilian branches reflects the long time since a common ancestor. FoxG1 is not a good choice for reptilian phylogenetic reconstruction, a situation also observed in previous work (Bredenkamp, Seoighe et al. 2007).

28S phylogenetics

No variation was seen between the *Sphenodon* populations, and the Poor Knights sample dates the sequence to at least one million years ago (Appendix 3). Comparison of all reptile sequence demonstrated that 28S is a highly conserved gene (Appendix 3). Although often regarded as a good gene for deep phylogeny analysis (Hillis and Dixon 1991; Winchell, Sullivan et al. 2002), it appears the highly conserved nature of 28S within the Reptilia, causes it to be of limited use when assessing phylogenetic relationships. The paraphyletic Archosauria places Testudines within Category 4.

Comparison to recent work on tuatara and reptilian phylogenies

No sex related differences were seen, and no sequence variation was present in the aldolase (Hay, Daugherty et al. 2003), AMH, FoxG1 and 28S gene sequences, demonstrating sequence conservation for over one million years. Differentiation of Cook Strait and north-eastern North Island groups was possible with WT1 where Cook Strait tuatara appear quite distinct from a north-eastern North Island tuatara. No population structure was observed in the DMRT1 sequence, due to the limited numbers of sequences obtained.

The current genomic DNA work allows comparison between mtDNA analysis (Hay, Daugherty et al. 2003; Rest, Ast et al. 2003; Hay, Subramanian et al. 2008), chromosomal analysis (Chapter 1 and Norris, Rickards et al. 2004), and allozyme analysis (Daugherty, Cree et al. 1990; Hay, Daugherty et al. 2003).

In contrast to the results from the chromosome analysis, tuatara gene sequence analysis demonstrated few similarities with Testudines, with the exception of 28S.

Most recent *Sphenodon* phylogenetic work has used mtDNA, so the nuclear sequences in the current study allow comparative analysis. Mitochondrial DNA is maternally inherited and haploid, so mtDNA genes have an effective population size one quarter that of a nuclear gene (Moore 1995). For that reason evolutionary rates of mtDNA and nuclear DNA change can differ, leading to conflicting phylogenetic trees.

A slow growing, infrequent breeding species such as *Sphenodon* would be expected to have low levels of genetic variation, particularly after the major bottlenecks the species has been through (Oligocene Drowning and the Pliocene/Pleistocene ice-age). In this respect the absence of chromosomal variation was expected. However recent work on microsatellites (McGibbon 2003; MacAvoy, McGibbon et al. 2007), and a comparative study of ancient and modern *Sphenodon* mtDNA (Hay, Subramanian et al. 2008), suggest a rapid rate of DNA change in *Sphenodon*.

Testudines are also slow growing, infrequent breeders where some species exhibit rapid rates of mtDNA change (Starkey, Shaffer et al. 2003; Caccone, Gentile et al. 2004). In fact, rates of mtDNA change seem to be high in the archaic reptiles, with recent work revealing significant rates of change in all three Orders (Janke, Erpenbeck et al. 2001; Starkey, Shaffer et al. 2003; Caccone, Gentile et al. 2004; Janke, Gullberg et al. 2005; Hay, Subramanian et al. 2008).

One result from the current gene study is that although within *Sphenodon* there may be little variation, comparing tuatara sequences to the rest of the Reptilia revealed extensive divergence. The gene fragments for aldolase, AMH, DMRT1 (including the published *Sphenodon* sequences) and WT1 were so divergent that phylogenetic reconstruction was impossible due to the limited number of base-pairs in common. FoxG1 analysis had low levels of support within the Reptilia, suggesting it too is a poor choice for deep phylogenetic reconstruction. The 28S sequence demonstrated high levels of conservation with other reptiles.

The low levels of similarity with other reptiles supports recent work demonstrating a rapidly evolving *Sphenodon* mtDNA genome (Hay, Subramanian et al. 2008). The mtDNA result also suggests that the microsatellite DNA

variation is not a relict, but reflects a rapidly evolving genome. If this is correct then in addition to mtDNA and microsatellite DNA, the current study suggests that nuclear DNA genes are also evolving rapidly.

The current work, although on extant populations, does provide some level of comparison with recent mtDNA analysis (Hay, Daugherty et al. 2003; Hay, Subramanian et al. 2008), as well as allowing comparison between sex determining and standard nuclear genes.

Sex determining genes are under different selection pressures compared to standard nuclear genes. The absence of variation in AMH either reflects a highly conserved early stage sex determination gene, or a gene that is involved so late in the process that it is effectively a standard nuclear gene similar to aldolase, FoxG1 and 28S. AMH does not appear to be an early stage sex determining gene in other reptiles (Western, Harry et al. 1999; Takada, DiNapoli et al. 2004) suggesting in *Sphenodon* it is similar to a nuclear gene. WT1 or DMRT1 appear better candidates for early stage sex determination within *Sphenodon* and the Reptilia.

These results do not necessarily support the recent work examining mtDNA change in *Sphenodon* (Hay, Subramanian et al. 2008). Genes in the current study, although in some cases demonstrating significant divergence from other reptiles, show no variation between populations of *Sphenodon* (AMH, FoxG1, 28S). The authors of the ancient and modern comparative mtDNA study described significant change within *Sphenodon* populations. Analysis of aldolase (Hay, Daugherty et al. 2003), FoxG1 and AMH suggests that significant DNA changes have occurred within the *Sphenodon* lineage, but that they occurred much earlier (Gondwanic separation or Oligocene drowning) and that following a severe

bottleneck, and subsequent recolonisation, the genes have been under stabilising selection.

The two sex determining genes that demonstrated variation, WT1 and DMRT1, are also significantly different from other reptilian sequences. The two genes may reflect a similarly rapid divergence as seen in the mtDNA, although there is evidence that the divergence is deeper than the 8000 years seen within the mtDNA. In particular WT1 where the island group separation is at least one million years old. DMRT1 is the only nuclear gene in the current study that could support the mtDNA data, although the number of samples will need to be increased to confirm this.

The results from the genes in the current study suggest *Sphenodon* exhibits differing rates of change within chromosomes, mtDNA (Hay, Subramanian et al. 2008), microsatellites (MacAvoy, McGibbon et al. 2007) and between various nuclear DNA genes. This adds support to the recent suggestion of decoupling of evolutionary processes within *Sphenodon* (Hay, Subramanian et al. 2008). Evidence supporting gene divergence times is explored in Chapter 4.

Testudine placement within the Reptilia

The degree of sequence divergence meant that not all genes could be assessed for placement of the Testudines. FoxG1 and 28S sequence analysis support Category 4.

To summarise:

- Morphology generally has a monophyletic Lepidosauria and Archosauria (Category 1)
- Full mtDNA sequence supports a monophyletic Lepidosauria and the

Testudines as a sister-group to Archosaurs (Category 3)

- Chromosome morphology supports an ancient relationship between tuatara and Testudines to the exclusion of the Squamata (Category 4)
- Sperm morphology supports a tuatara and turtle relationship to the exclusion of the Squamata (Category 4)
- FoxG1 and 28S nuclear gene analyses supports diapsid (Category 4).

Potential for in situ *work using sex determining genes*

Most studies of sex determining genes, after sequence isolation, attempt to hybridise the gene to developing gonadal tissue. This *in situ* work provides information on when and where the gene is expressed, establishing the position in the sex determination cascade. In ESD reptiles the gene needs to be expressed near the beginning of the TSP, leading the developmental cascade down a pathway causing embryonic sex differentiation. Presumably at male determining temperatures a gene (or promotor, or gene complex) is activated during the TSP inhibiting the default female pathway and causing the embryo to develop as male.

Unfortunately the highly protected status of the tuatara precludes embryonic *in situ* work. So identification of where and when the three sex determination genes are expressed, and in which order, is not possible. For that reason I have chosen to perform FISH with the isolated sequences to identify which chromosomes are involved. This work is described in Chapter 3.

Conclusions

Sphenodon gene sequence was successfully isolated for three sex determining genes and two nuclear genes. To summarise:

- There was no variation present in *Sphenodon* FoxG1, 28S and AMH sequences,
- WT1 sequence separates north-eastern North Island and Cook Strait *Sphenodon* populations,
- DMRT1 revealed some population differences between *Sphenodon* island groups,
- The genes analysed do not clarify Testudine position due to significant genetic changes between the Orders,
- Sex determining genes (WT1 and DMRT1) reveal more population structure within *Sphenodon* than standard nuclear genes,
- Full sequencing of genes may allow phylogenetic reconstruction and protein translation.

Chapter 3 : Comparative FISH in *Sphenodon*

To bring the chromosomal and molecular work together, probes developed from sex determining gene DNA fragments were localised to *Sphenodon* chromosomes. Human telomeric probes were hybridised to *Sphenodon* chromosomes to investigate homology between the disparate Orders, and chromosome painting was used to examine relationships between *Sphenodon* and the Aves.

Introduction

Chromosome evolution using FISH

Comparative FISH, also known as ZOO-FISH or comparative genomic hybridisation (CGH), allows reconstruction of ancestral karyotypes. Chromosome paints used for CGH are a labelled mixture of DNA sequences derived from fluorescent activated chromosome flow sorting or micro-dissection of chromosomes. Fluorescent-labelled chromosomes from one species can be hybridised onto another species karyotype to examine homology between the two, thereby examining cytogenetic evolutionary theories. For example CGH confirmed the origin of human chromosome 2 as a fusion of the old world monkey telocentric chromosomes 14 and 15. This one rearrangement is the main karyotypic change between the great apes (Wienberg and Stanyon 1997).

Most CGH work has involved painting human chromosomes on to other mammalian species. Examination of placental animals allowed reconstruction of

an ancestral mammalian karyotype of 2n=48 or 50 (Wienberg, Frönike et al. 2000). Recent work on the Afrotheria painted human chromosomes on to aardvark and elephant chromosomes, finding no cytogenetic support for a monophyletic Afrotheria although the data demonstrated strong evidence for an ancestral mammalian karyotype of 2n=44 (Yang, Alkalaeva et al. 2003). CGH between the Asian Squirrel (*Mentes berdmorei*) and *H. sapiens* using CGH demonstrated high homology between the two species. An interesting result as it demonstrates *M. berdmorei* has an archaic karyotype, rather than the highly derived karyotype typical of rodents. The high levels of conservation allowed reconstruction of an ancestral Sciurinae karyotype (Richard, Messaoudi et al. 2003). Extension of this work using another squirrel species, *Sciurus carolinensis*, demonstrated that the human karyotype is also highly conserved and is similar to the ancestral eutherian karyotype (Li, O'Brien et al. 2004). Rodents, with the exception of the squirrels, possess highly derived karyotypes. CGH work between various murid (mice) species isolated a number of shared chromosomal homologies (or syntenies), although extrapolation to a common ancestor with squirrels is difficult due to the large number of rearrangements (Stanyon, Yang et al. 2004).

CGH is also used to examine relationships between groups of related species. The tammar wallaby possesses a presumed ancestral karyotype 2n=2x=14, whereas the rock wallaby possesses greater chromosomal variation, with a presumed ancestral karyotype 2n=2x=22 present in some species. CGH confirmed classical cytogenetic theories of how chromosome fission in the tammar wallaby

karyotype lead to the rock wallaby karyotype (Waugh O'Neill, Eldridge et al. 1999).

Another example of resolving taxonomic relationships involves the subfamily Atelinae (spider and woolly monkeys). Comparative mapping using human, *Saguinus oedipus* and *Lagothrix lagothricha* chromosome specific probes supported the Atelinae as a monophyletic clade. CGH information allowed development of an ancestral karyotype and reconstruction of phylogenetic relationships (de Oliveira, Neusser et al. 2005).

Variation within orders can also be compared using CGH. Hybridisation in Testudines of Chromosome 1 from *T. scripta* (Emydidae) on to *Terrapene carolina* (Emydidae), *Chrysemys picta* (Emydidae), *Gopherus agassizii* (Testudinidae) and *Caretta caretta* (Cheloniidae) karyotypes demonstrated the karyotype similarity is reflected in the chromosomes genetic content. Chromosome 1 of *T. scripta* hybridised exclusively to Chromosome 1 in other species, which would only happen if no chromosomal rearrangements had occurred. Successful hybridisation demonstrated high levels of DNA sequence homology between species separated between 66-144 MYA (Muhlmann-Diaz, Ulsh et al. 2001), supporting previously observed G-band homology (Bickham 1981).

Chicken chromosome 4 hybridises to emu (ratite) chromosome 4 and a microchromosome pair, and paints turtle chromosome 4 plus the small arms of chromosomes 7 and 8 (Marshall Graves and Shetty 2000). Evidence for chicken and turtle chromosome homology was demonstrated using FISH and EST clones from cDNA libraries. Higher homology was seen between the turtle and the

chicken than between the chicken and a snake, supporting the previously observed highly conserved avian and Testudine karyotype and divergent Squamata karyotype (Matsuda, Nishida-Umehara et al. 2005). CGH using 31 turtle genes on to chicken chromosomes localised 17 genes to macrochromosomes, and 14 genes to microchromosomes. Genes from turtle chromosomes 1-5 were located on the homologous chicken chromosome. The genes from turtle chromosomes 6, 7 and 8 hybridised to chicken chromosomes Z, 7 and 6 respectively. These results demonstrate a close synteny between turtle and avian chromosomes (Kuraku, Ishijima et al. 2006), a synteny supported by other characteristics of avian and reptile genomes. High GC_3 gene content is associated with microchromosomes in reptile and avian lineages, where macrochromosomes are two to three times more likely to be GC_3 poor (Kuraku, Ishijima et al. 2006).

The next stage in karyotype evolution studies is to use chromosome probes derived from regions of various chromosomes rather than whole chromosome probes. These partial chromosome paints allow more precise identification of homologous regions, and can demonstrate internal chromosome rearrangements (Wienberg, Frönike et al. 2000). Another technique providing greater resolution of physical gene mapping involves lampbrush chromosomes, visible in amphibians, insects and birds. Lampbrush chromosomes are about 30 times larger than standard metaphase chromosomes, increasing precision of gene location using FISH. Comparative work between chicken and Japanese Quail karyotypes demonstrated low levels of rearrangements (Galkina, Deryusheva et al. 2006).

CGH can also be used to isolate the location of particular genes. This can be useful in comparing gene rearrangements, chromosome rearrangements or identifying potential sex chromosomes. CGH between human and chicken chromosomes using SOX gene probes, demonstrated that rearrangements between avian and human chromosomes occurred in the mammalian lineage post avian divergence, although sequence homology remains high (Kuroiwa, Uchikawa et al. 2002).

Sex chromosome evolution using CGH

CGH has been used to identify sex chromosomes. Within the Testudine Order both TSD and GSD are present, often with homomorphic sex chromosomes. Differentially labelled male and female total genomic DNA was used as probes in *Chelodina longicollis*, demonstrating the Y microchromosome in males consistently produced a low intensity signal compared to its homologue. These results were supported by C-banding and reverse fluorescent staining. Such a minimal difference, and absence of large chromosome changes, suggested a recent conversion to GSD. This was also the first report of a microchromosome pair acting as sex chromosomes in a Testudine, opening the possibility that they may exist in other species (Ezaz, Valenzuela et al. 2006). The same technique had been previously used to identify sex chromosomes in a GSD lizard, *Pogona vitticeps*, again identifying a microchromosome pair as the sex chromosomes (ZZ/ZW) (Ezaz, Quinn et al. 2005). The Squamata and Testudine examples suggest that microchromosomes may be a more common location for sex chromosomes in reptiles than previously thought.

Sex specific, or sex determining sequences, can be isolated and hybridised on to karyotypes to examine evolution and origin of the sex chromosomes. Absence of homology between the chicken Z chromosome and the mammalian X, implies independent autosomic origins for the two GSD systems. Human X chromosome paints hybridise to chicken chromosomes 1 and 4, and chicken Z chromosome paints mainly hybridise to human chromosomes 5 and 9. Chicken Z chromosome paints hybridised to the emu Z chromosome and chromosome 6 of the turtle (Shetty, Griffin et al. 1999; Marshall Graves and Shetty 2000).

Various methods can be used to isolate sex specific probes. Random Amplified Polymorphic DNA (RAPD) assays were carried out to find male specific markers in Rainbow trout (*Oncorhynchus mykis*). These markers were then localized onto the karyotype to identify a Y chromosome (Iturra, Medrano et al. 1998). In addition to differentiating sex chromosomes, sex chromosome probes can also demonstrate similarities. In the fish *Oreochromis niloticus* sex chromosome specific probes hybridise to all of the X and Y chromosomes demonstrating that the sex chromosomes are in very early stages of differentiation, although hybridisation differences between XX and YY genotypes indicate some changes between the chromosomes have occurred (Harvey, Masabanda et al. 2002).

Telomere hybridisation

Telomeric regions of chromosomes retain homology between disparate species. Human telomeric sequences hybridised to 91 species including fish, reptiles, amphibians, avian and mammalian chromosomes, demonstrating sequence

homology over a 400 MY period (Meyne, Ratliff et al. 1989). FISH using human telomeric paints demonstrated hybridisation to *T. scripta* (Testudine) telomeres (Muhlmann 2002).

Proto-karyotype reconstruction using CGH

Development of chromosome paints and CGH allows reconstruction of proto-karyotypes. In comparison to the extensive work in eutherians, there are very few studies on reptilian proto-karyotypes. The comparative work between reptilian species in Chapter 1 of the current study provides a guide for construction of a reptilian proto-karyotype.

Mammalian and eutherian proto-karyotypes

Some reconstructions aim for major events in chromosome evolution. For example an ancestral bony vertebrate proto-karyotype, with a suggested 12 chromosomes, was established from pairing results between *Tetraodon* (puffer fish) and human chromosomes (Jaillon, Aury et al. 2004). Most studies focus on various mammalian groups, generally comparing a particular species with human chromosomes. An attempt to clarify which of the four super-orders: Afrotheria, Xenarthra, Euarchontoglires and Laurasiatheria, separated first from a common Eutherian ancestor suggested representatives of the Xenarthra appeared most similar to a proposed 2n=48 ancestral eutherian karyotype (Svartman, Stone et al. 2004; Svartman, Stone et al. 2006). In this way archaic karyotypes can be extrapolated from extant animals.

Reptilian proto-karyotypes

Most CGH work in reptiles has been performed in turtles. The current study demonstrated high macrochromosome homology between the Orders Testudine and Sphenodontia, with the only major difference being numbers of microchromosomes. Crocodylia show some similarity to Testudines and *Sphenodon*, whereas Squamata possess quite distinct karyotypes. The Aves share a common ancestor with the Crocodylians, although microchromosomes have been lost in the Crocodylian lineage. Karyotypically the Aves, represented by the chicken (*Gallus domesticus*), are similar to the Testudine and Sphenodontia karyotype (Figure 3.1).

Comparison of the karyotype morphology of *Sphenodon*, Testudines and Aves certainly supports a close relationship, suggesting the common ancestor of Crocodylians and Aves was karyotypically similar to the Testudines and Sphenodontia lineages, although the Aves possess a more conserved karyotype than the crocodiles. CGH supports the archaic nature of the turtle and avian karyotype (Marshall Graves and Shetty 2000; Matsuda, Nishida-Umehara et al. 2005; Kuraku, Ishijima et al. 2006). For example, hybridising chicken chromosome paints onto turtle chromosomes demonstrated almost complete homology between the six largest chromosomes (Matsuda, Nishida-Umehara et al. 2005). Hybridising chicken chromosomes onto snake chromosomes did not demonstrates the same levels of homology.

Figure 3.1: 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; **(p)** *Gallus domesticus* 2n=78. Chromosomes have been resized from original material to allow comparison (prepared from the current study and Cohen and Gans 1970; Takagi and Sasaki 1974; Bickham 1975; Bickham and Baker 1976; Bickham, Bjorndal et al. 1980; Bull and Legler 1980; Haiduk and Bickham 1982; Bickham and Carr 1983).

Methods

Slide preparation and probe visualisation were the same for all three techniques.

Slide preparation

Airdried slides were passed through an ethanol series of 70%, 90% and 100% for two minutes to remove acetic acid from the cell fixative. Slides were airdried then baked at 65^0C for 15 minutes.

Probe visualisation

Slides were air dried in the dark and a drop of Prolong Gold antifade reagent with DAPI (Invitrogen) placed on the chromosomes, a coverslip spread the DAPI and was sealed with nail polish. After curing for 24 hours the slide was examined using fluorescent microscopy. Fluorescent microscopy used DAPI and FITC illumination and images were merged using Photoshop (www.adobe.com) or Gimp (www.gimp.org). Green and red channels were removed from the DAPI layer, and the opacity decreased so hybridisation from the FITC layer could be seen.

Single gene fragment FISH

Probe construction

Probes for *in situ* work were made using gene fragments described in Chapter 2 with the following modifications. In order to visualise fragments on chromosomes the PCR product requires incorporation of ddUTP (DIG labelled,

Roche). A ddNTP mixture was made from 1 μl ddCTP; 1 μl ddGTP; 1 μl ddATP; 0.8 μl ddTTP; 0.2 μl ddUTP and used in the following PCR mastermix (Table 3.1).

Table 3.1: PCR labelling mastermix for DIG labelling

Reagent	Quantity
ddH_2O	16.65 μl
Buffer	3.75 μl
$MgCl_2$	1.5 μl
Fwd Primer	1 μl
Rev Primer	1 μl
ddNTP	1 μl
Taq	0.1 μl
DNA	1 μl
TOTAL	25 μl

PCR protocols were as described in Chapter 2. Bands of correct size were cut out under UV light and isolated as per Chapter 2. Extracted product was checked by running a small quantity on a gel, allowing estimation of the DIG-labelled DNA probe concentration.

Probe hybridisation

The following protocol is adapted from the Roche DIG manual (http://www.roche-applied-science.com/PROD_INF/MANUALS/InSitu/InSi_toc.htm). Following gel isolation of the DIG labelled probe, the probe was resuspended at a concentration of 10 ng/μl in a stock solution containing 50% deionised formamide, 2x SSC, 10% dextran sulphate and 50 mM sodium phosphate (ph7). The working solution was made by diluting 2-5 μl of stock to 10 μl with a solution containing 50% deionised formamide, 2x SSC, 10% dextran sulphate and 50 mM sodium phosphate (ph7) and mixing. Two *in situ* techniques were used, the first protocol following the Roche DIG manual where 10 μl of working solution probe was applied to a pre-aged slide, a coverslip placed over the probe and sealed with

rubber cement. The probe was denatured at 80^{0}C for two minutes and placed in a moist chamber at 37^{0}C overnight for hybridisation. In the second protocol, the probe was denatured at 80^{0}C for two to five minutes and placed on ice. A 17 x 17mm *in situ* frame (Eppendorf) was placed on the slide around the chromosomes and the probe placed within the frame. The coverslip provided with the frame was used to spread the probe.

Following hybridisation the coverslip or *in situ* frame was removed and the slide washed (all reactions are at room temperature unless otherwise noted) in three five minute washes at 45^{0}C in 2x SSC containing 50% formamide, five two minute washes in 2x SSC, and one five minute wash with the buffer used during detection.

Probe detection

Detection of the probe was performed by washing the slide briefly with TNT buffer (Appendix 1), then 100 μl of TNB buffer (Appendix 1) was pipetted onto the slide, covered with a 24 x 50 mm coverslip and incubated for 30 minutes at 37^{0}C. Unless noted, all incubation steps were performed in a moist chamber. The slide was soaked in TNT for five minutes to remove the coverslips, during which time fresh working solutions of the three antibodies were made up.

Antibody 1 (WS): mouse monoclonal anti-DIG, 0.5 μg/ml in TNB

Antibody 2 (WS): DIG-conjugated sheep anti-mouse Ig, 2 μg/μl in TNB

Antibody 3 (WS): Fluorescein-conjugated sheep anti-DIG, 2 μg/ml in TNB

Following TNT incubation the slide was rinsed with ddH_2O and 100 μl of Antibody 1 placed on the slide, covered with a 24 x 50 mm coverslip and incubated for 30 minutes at 37^{0}C. The slide was rinsed in three five minute

washes of TNT at room temperature, 100 μl of Antibody 2 pipetted onto the slide, covered with a 24 x 50 mm coverslip, and incubated at 37^0C for 30 minutes. Again the slide was washed in three five minute washes of TNT. Following the wash 100 μl of Antibody 3 was placed on the slide, a coverslip applied and the slide incubated for 30 minutes at 37^0C. After the final incubation the slide was washed in three five minute washes of TNT.

Visualising probes

After hybridisation and conjugation, slides were dehydrated through an ethanol series, five minutes each in 70%, 90%, and 100% ethanol, and visualised as described above.

Human telomeric FISH

FISH using a human Pan Telomeric Chromosome Paint (Cambio, Starfish, Cat. #1696) followed the supplied protocols for slide pre-treatment and *in situ* hybridisation.

Slides were prepared as above, allowed to cool, soaked in acetone for 10 minutes, air dried, and incubated for one hour at 37^0C with 200 μl of 2x SSC + RNase (100 μg/ml) with a parafilm coverslip. The slides were washed in 2x SSC for five minutes, followed by a five minute wash in PBS (Appendix 1). Slides were dehydrated through an ethanol series (70%, 90%, 100%) for two minutes and air dried. Cellular DNA was denatured by placing slides in 70% formamide and 2x SSC (15 ml 2x SSC and 35 ml formamide) at 70^0C for three minutes, followed by an ethanol dehydration series for two minutes each at 70%, 90%, 100% and

air dried. A 17 x 17 mm *in situ* frame (Eppendorf) was placed on the slide around the chromosomes to prevent the probe evaporating during incubation.

Probe preparation followed supplied protocols. The probe was warmed to 37^0C for five minutes and mixed well. For each slide 12.5 μl of probe was removed and placed in a microcentrifuge tube. The probe was denatured by placing the tube in a 85^0C heating block for 10 minutes, and immediately placed on ice. Each slide received 12.5 μl of probe in the middle of the *in situ* frame, and the supplied Eppendorf coverslip applied to spread the probe. Hybridisation was in a humidified chamber at 37^0C for 16 hours.

Following hybridisation the coverslip was removed by two five minute washes in 2x SSC at 37^0C. Slides were then washed three times in 0.5x SSC at 37^0C for three minutes each, and then twice in 2x SSC for five minutes. Probes were visualised as described above.

Whole chromosome painting

Slides were prepared as above. Cooled slides were washed in 2x SSC for five minutes, followed by a five minute wash in PBS. Slides were dehydrated through an ethanol series (70%, 90%, 100%) for two minutes each and air dried. Cellular DNA was denatured by placing slides in 70% formamide and 2x SSC (15 ml 2x SSC and 35 ml formamide) at 70^0C for three minutes, followed by an ethanol dehydration series (70%, 90%, 100%) for two minutes and air dried. A 17 x 17 mm *in situ* frame (Eppendorf) was placed around the chromosomes to prevent the probe evaporating during incubation.

The chicken whole chromosome paints were a gift from Prof. M. Ferguson-Smith (Department of Veterinary Science, University of Cambridge, UK). For each slide 2.5 μl of chromosome paint and 10 μl of hybridisation buffer (50% deionized formamide, 10% dextran sulphate, 2x SSC, 0.5 M phosphate buffer pH 7.3, 1x Denhardt's solution) was denatured at 65^0C for 10 minutes, and placed on ice. The mixture was applied in the middle of the *in situ* frame, and the supplied coverslip applied to spread the solution. Hybridisation was in a humidified chamber at 40-42^0C for two days.

Post hybridisation washes were all done at the incubation temperature (40-42^0C) unless otherwise noted. Two five minute washes in 50% deionized formamide and 2x SSC (25 ml 2x SSC and 25 ml of formamide) were followed by two five minute washes in 2x SSC, and a five minute wash in 4x SST (Appendix 1). The biotin labelled paints were visualised using Streptavidin alexa flouro 488 (Invitrogen) at a concentration of 1:750 (streptavidin:4xSST), and incubated for 20-30 minutes at 37^0C. Three final washes of three minutes each in 4x SST completed the post-hybridisation wash protocol. Probes were visualised as above.

Results

Sex determining gene hybridisation

Running the product of the DIG labelled DNA probes on a gel produced bands of the expected length (Figure 3.2). DIG-labelled WT1 and AMH concentration was estimated using a LML (WT1 demonstrated in Figure 3.3). DIG-UTP incorporation into a DMRT1 probe was unsuccessful with no bands observed. FISH using WT1 and AMH DIG labelled probes demonstrated hybridisation (Figures 3.4 and 3.5), the results of which are summarised in Table 3.2 (WT1), and the AMH probe in Table 3.3.

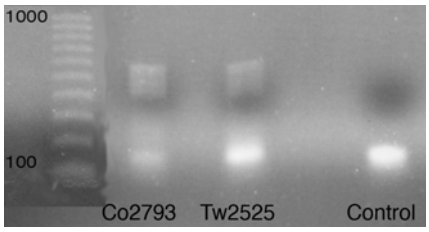


Figure 3.2: DIG labelled ddUTP incorporation using 1:500 dilutions of previously isolated tuatara WT1.

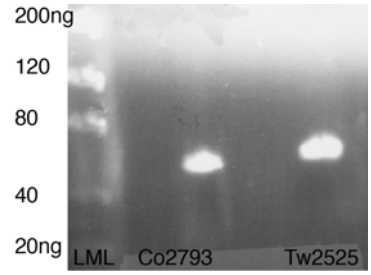


Figure 3.3: Estimation of DIG labelled WT1 probe concentration using a LML.

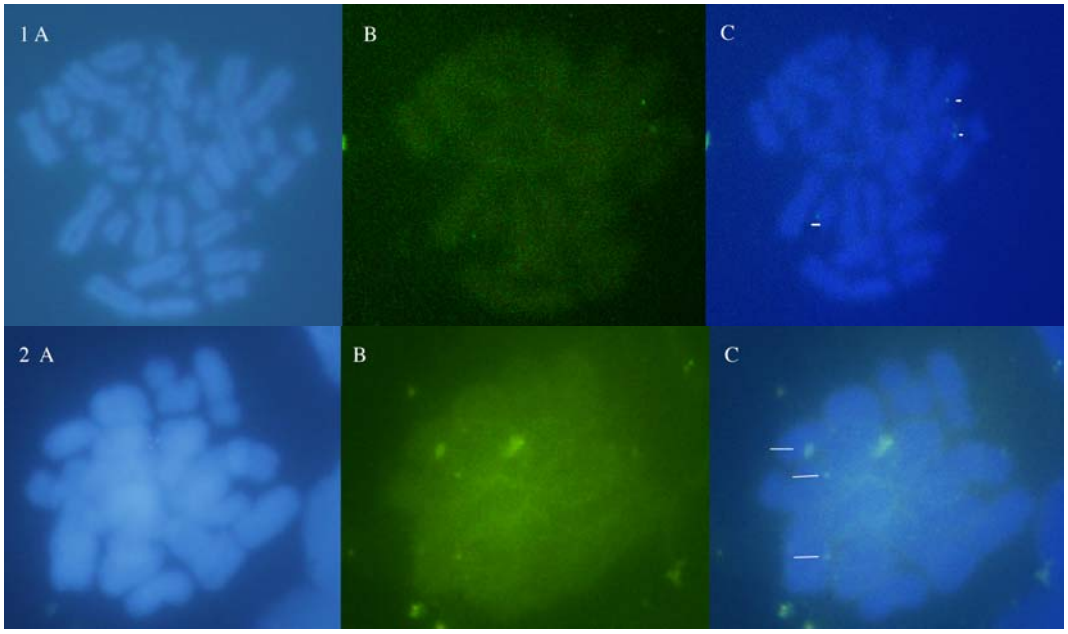


Figure 3.4: Examples of WT1 probe hybridisation in *Sphenodon*. (A) DAPI illumination, (B) FITC illumination and (C) composite image from DAPI and FITC images. Possible hybridisation indicated by white bars.

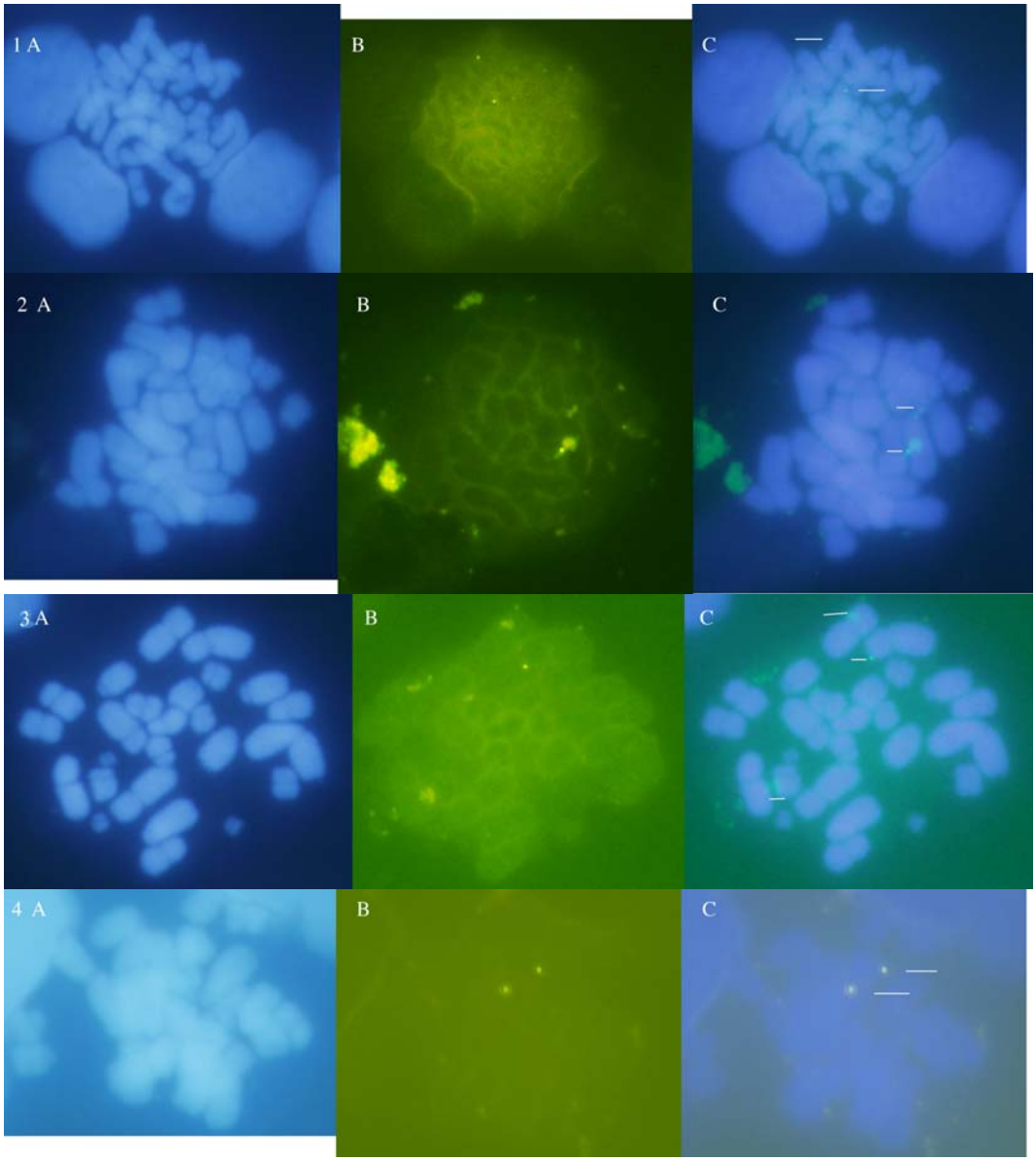


Figure 3.5: Examples of AMH probe hybridisation in *Sphenodon*. (A) DAPI illumination, (B) FITC illumination and (C) composite image from DAPI and FITC images. Possible hybridisation indicated by white bars.

Table 3.2: WT1 probe hybridisation summary

Image	Chromosomes identified
Figure 3.4(1)	Small submetacentric end of 'q' arms x2; 'p' arm of chromosome 5
Figure 3.4(2)	Small chromosome, morphology difficult to assess may be submetacentric (2 dots); large chromosome morphology unable to be determined

Table 3.3: AMH probe hybridisation summary

Image	Chromosomes identified
Figure 3.5(1)	Chromosome 11 x2
Figure 3.5(2)	Two dots on possible chromosome 11; possible hybridisation on small chromosome unknown morphology maybe chromosome 11
Figure 3.5(3)	'p' arm chromosome 5; the lower mark appears to be background
Figure 3.5(4)	Small chromosome morphology difficult to assess, one appears to be chromosome 11, the other is covered by other chromosomes

Telomeric FISH

Human telomeric chromosome paints were successfully hybridised onto *Sphenodon* chromosomes. A comparative figure using DAPI excitation (Figure 3.6a) and FITC excitation (Figure 3.6b) demonstrates chromosome location (DAPI) and telomeric probe hybridisation (FITC). A composite image where the FITC layer is merged with the DAPI layer demonstrates telomeric hybridisation (Figure 3.6c).

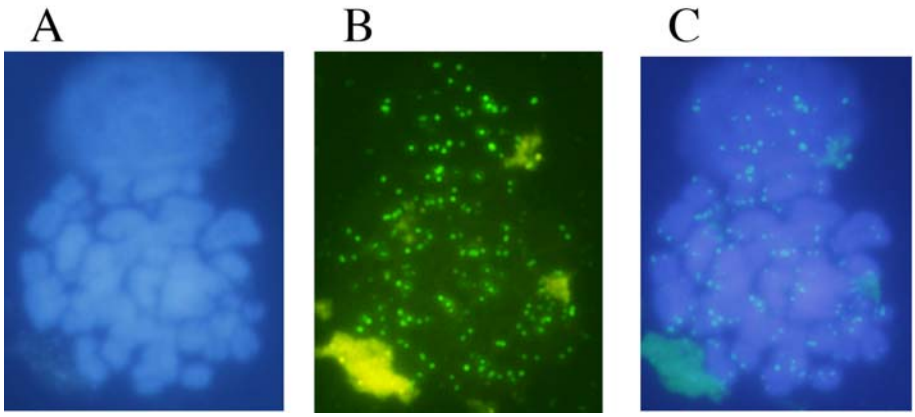


Figure 3.6: Human telomeric chromosome paint FISH on *Sphenodon* chromosomes. **(a)** DAPI excitation demonstrating chromosomes, **(b)** FITC excitation demonstrating locations of hybridisation, and **(c)** composite image with FITC overlaid on the DAPI image showing location of the telomere probe.

Whole chromosome FISH

Biotin labelled chicken chromosomes 1-5, W and Z were hybridised to *Sphenodon* chromosomes (Figures 3.7-12). Results were inconclusive. Chicken chromosome 1 shows some evidence of hybridisation on a microchromosome (Figure 3.7(1)), the larger marked area is non-specific. A large area of hybridisation is seen in Figure 3.7(2), although chromosome resolution is poor preventing identification of a particular chromosome. Figure 3.7(3) again demonstrates microchromosome hybridisation. Chicken chromosome 2 hybridised in Figure 3.8(1) onto a small submetacentric chromosome (lower white line) and a region of chromosome 1. Figures 3.8(2 and 3) show some hybridisation on a region of chromosome 3, although Figure 3.8(3) also demonstrates hybridisation on a small metacentric. Figures 3.8(4 and 5) have poor chromosome morphology but do appear to demonstrate evidence of hybridisation.

Chicken chromosome 3 shows hybridisation to *Sphenodon* chromosomes 3, 1 and a large sized chromosome of unknown morphology (Figure 3.9(1)).

Figure 3.10(1) shows hybridisation of chicken chromosome 4 on *Sphenodon* chromosome 4 (lower mark on picture), and another large chromosome. Figure 3.10(2) has some hybridisation on a large chromosome (possibly chromosome 4) plus two ambiguous regions on a small chromosome and the terminal region of chromosome 3. No hybridisation was seen with *G. domesticus* chromosome 5. Chicken Z chromosome hybridises (Figure 3.11) to the 'q' arms of a large submetacentric chromosome, possibly *Sphenodon* chromosome 5 or 8. Chicken W chromosome demonstrated evidence for hybridisation on the 'p' arms of a medium submetacentric and a large submetacentric possibly chromosome 4 (Figure 3.12).

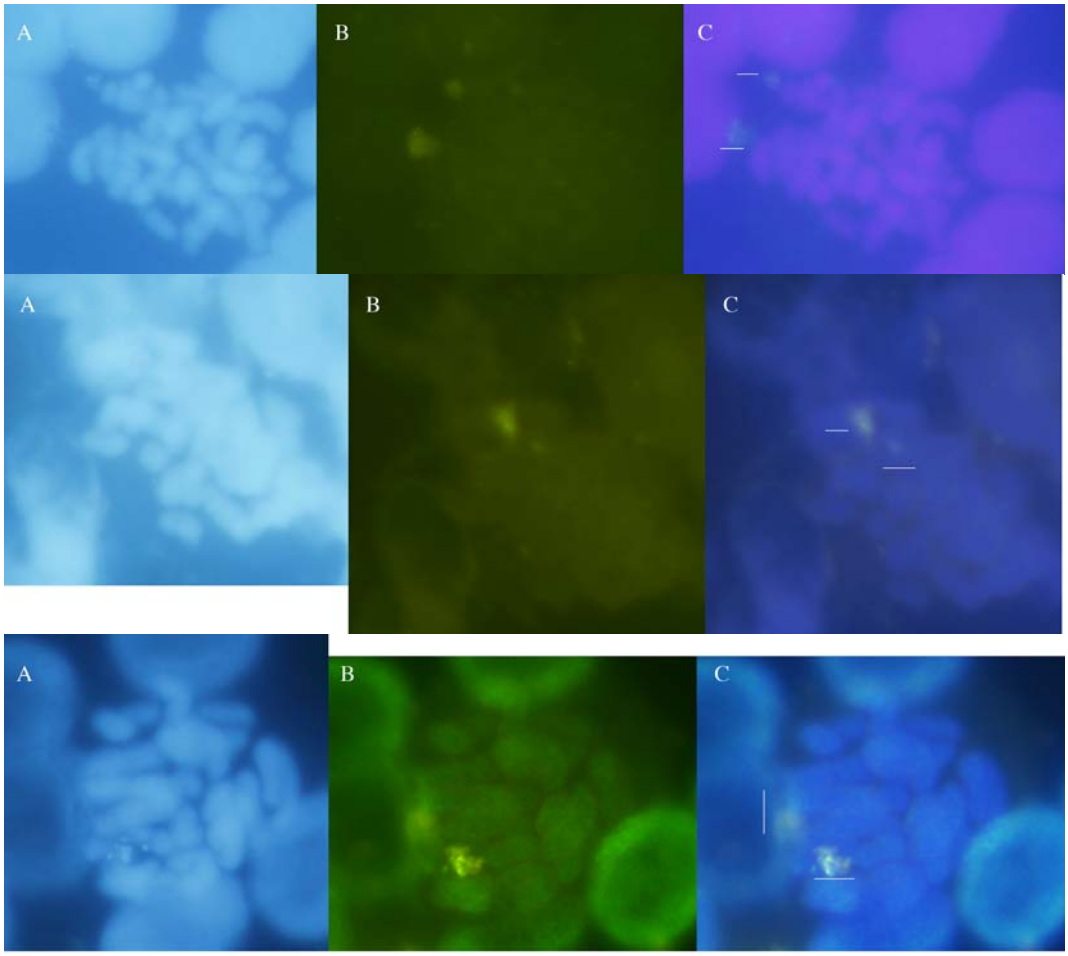


Figure 3.7: *Gallus domesticus* chromosome 1 hybridisation to *Sphenodon* chromosomes (**a**) DAPI, (**b**) FITC and (**c**) composite image. Possible hybridisation indicated by white lines.

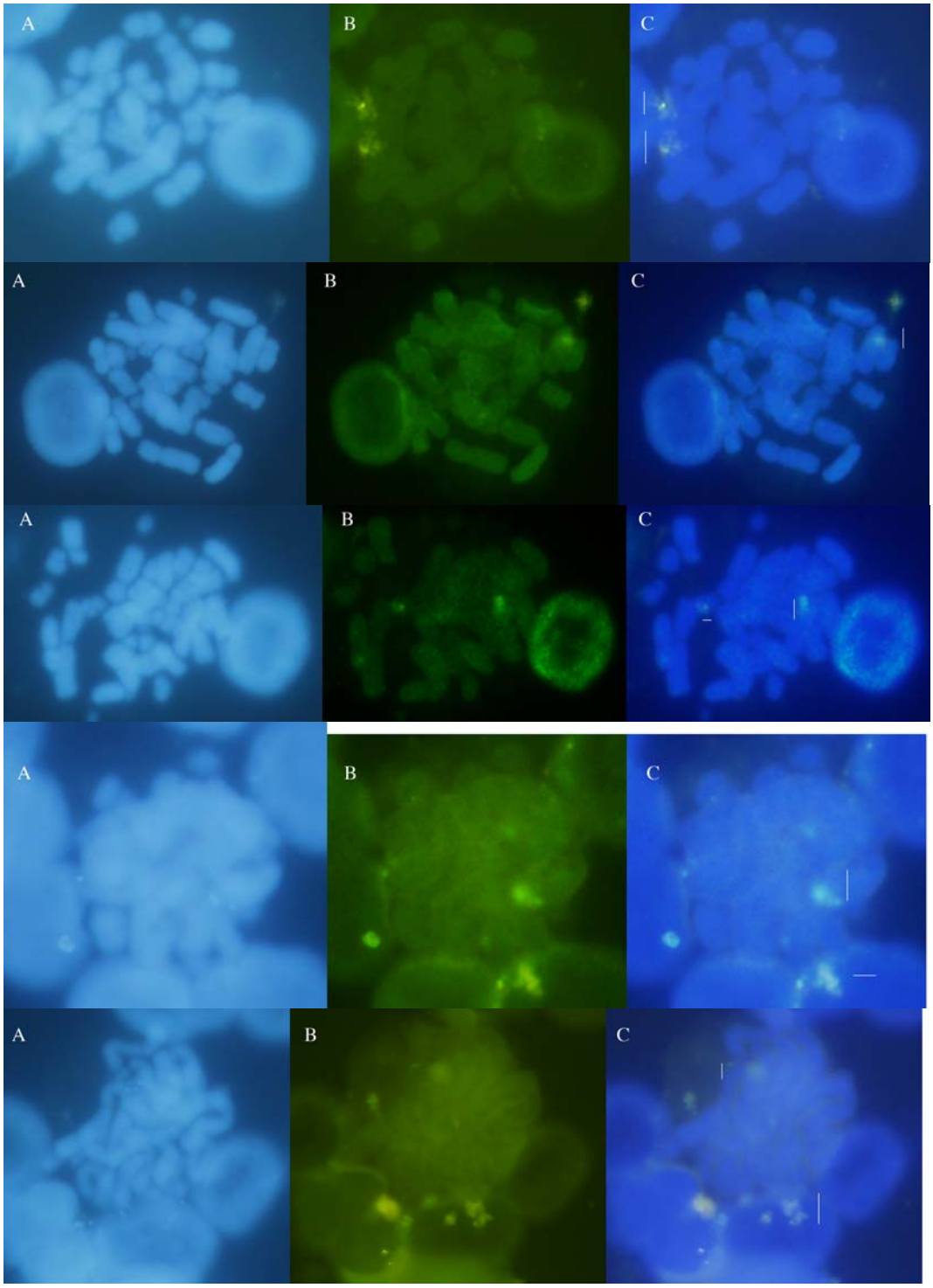


Figure 3.8: *Gallus domesticus* chromosome 2 hybridisation to *Sphenodon* chromosomes (**a**) DAPI, (**b**) FITC and (**c**) composite image. Possible hybridisation indicated by white lines.

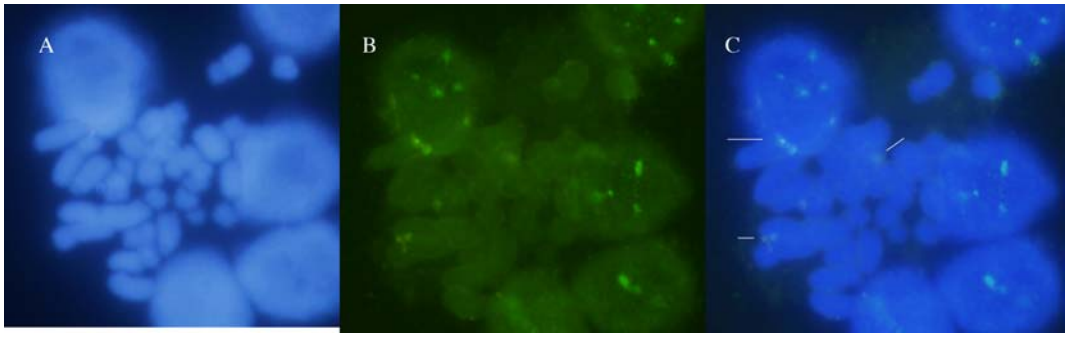


Figure 3.9: *Gallus domesticus* chromosome 3 hybridisation to *Sphenodon* chromosomes (**a**) DAPI, (**b**) FITC and (**c**) composite image. Possible hybridisation indicated by white lines.

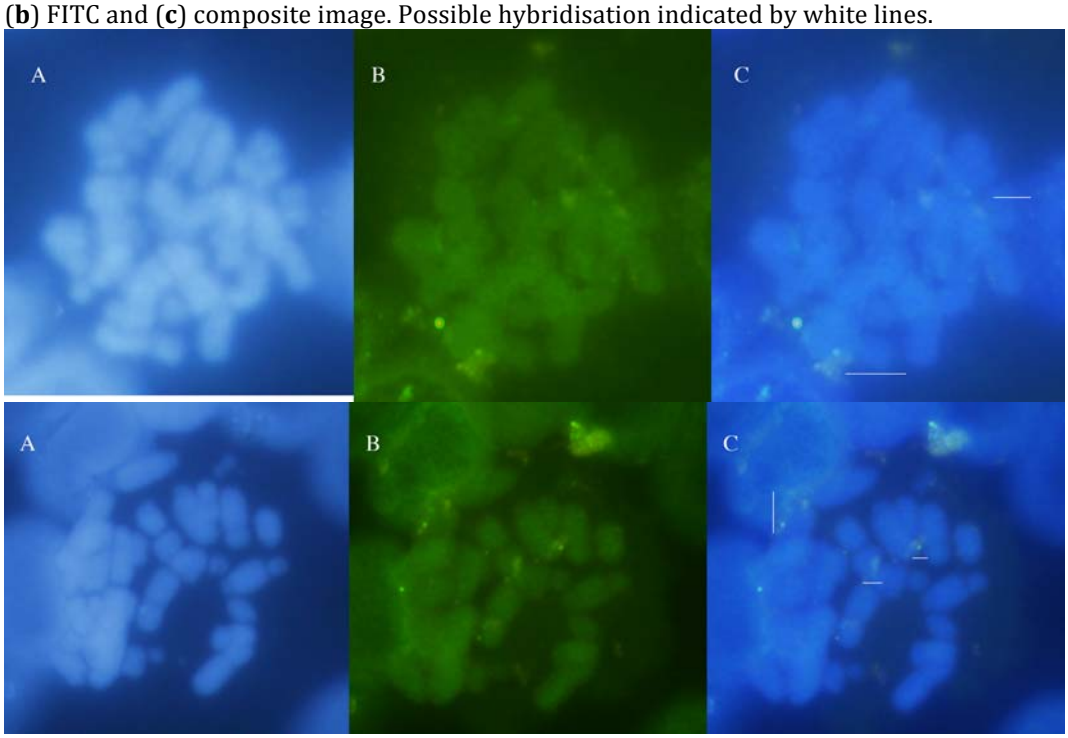


Figure 3.10: *Gallus domesticus* chromosome 4 hybridisation to *Sphenodon* chromosomes (**a**) DAPI, (**b**) FITC and (**c**) composite image. Possible hybridisation indicated by white lines.

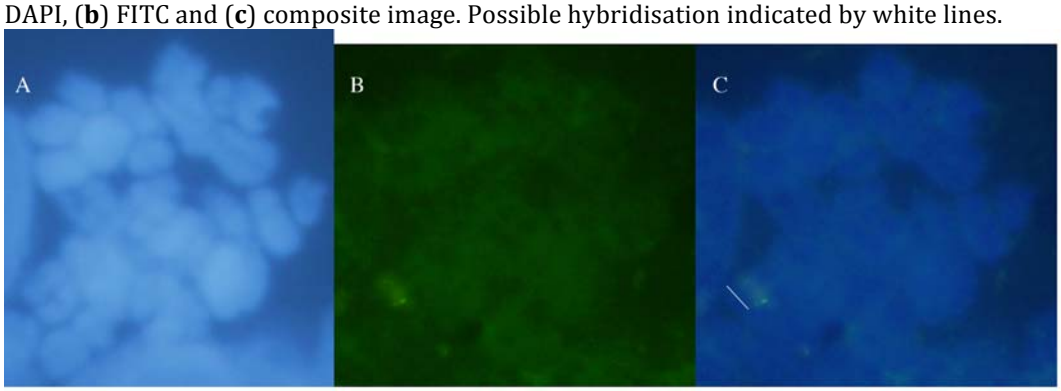


Figure 3.11: *Gallus domesticus* chromosome Z hybridisation to *Sphenodon* chromosomes (**a**) DAPI, (**b**) FITC and (**c**) composite image. Possible hybridisation indicated by white lines.

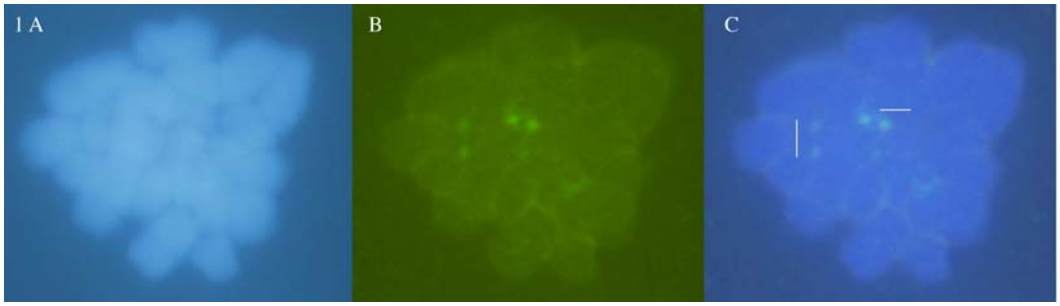


Figure 3.12: *Gallus domesticus* chromosome W hybridisation to *Sphenodon* chromosomes (**a**) DAPI, (**b**) FITC and (**c**) composite image. Possible hybridisation indicated by white lines.

Discussion

Single gene fragment FISH

Evidence for hybridisation of WT1 and AMH probes was seen. Discussions with various researchers performing single-copy gene fragments FISH (Dr's Maria Mulhmann, Al Rowland and Patricia O'Brien), suggested that hybridisation of DIG labelled probes may not be successful. Generally extracting an entire gene, cutting it with REs and labelling fragments with DIG-UTP is recommended. Signal was seen in the current study from a WT1 probe, constructed from north-eastern North Island *Sphenodon* DNA and hybridised to north-eastern North Island *Sphenodon* chromosomes. Hybridisation was seen to a small submetacentric chromosome (chromosomes 13-14) and possibly chromosome 5. Similar results were seen in all spreads examined suggesting the small DIG-labelled probe (approximately 300 bp) was being consistently identified. DIG-labelled AMH (approximately 350-400 bp) hybridisation was seen on chromosome 11 in most cells, with one hybridisation signal appearing on chromosome 5 (Figure 3.5(3)). The signals for WT1 and AMH are not strong, but the preparations had low background suggesting the two probes were being successfully located on *Sphenodon* chromosomes. Hybridisation results were from multiple slides using probes constructed at different times, providing further evidence that hybridisation of the gene fragments was occurring.

Gene localisation has not been performed in many species, however AMH and WT1 have been localised to: *Homo sapiens* WT1 chromosome 11p13, AMH

chromosome 19p13.3, AMHR chromosome 12p13 (www.atlasgeneticsoncology.org) and *Gallus domesticus* WT1 chromosome 2q21 (Delany and Daniels 2004), AMH microchromosome 28 (Oreale, Pieau et al. 1998; Smith, Paton et al. 2002). *Sphenodon* AMH and WT1 are located on different chromosomes to Aves and humans reflecting the long time period since a common ancestor. WT1 is located on chicken chromosome 2 and *Sphenodon* chromosome 13 or 14, and AMH is located on a chicken microchromosome and *Sphenodon* chromosome 11. The eleventh largest chicken chromosome is also a microchromosome, as is the AMH carrying chromosome 28 (Smith, Paton et al. 2002) (Figure 3.1). Chicken microchromosomes contain a high proportion of genes (Burt 2002), suggesting that they may be derived from the smaller sized macrochromosomes in a common ancestor, for example an ancestral chromosome 11 containing AMH. Comparison with other reptiles would provide more information on the evolution of gene location and chromosome rearrangements

Human telomeric probe FISH

Successful FISH using human telomeric paints demonstrated the highly conserved nature of telomeric sequences. *Sphenodon* and *Homo sapiens* last shared a common ancestor around 350 MYA when the diapsid and synapsid lineages diverged (Benton 1990; Benton 2005).

Chicken chromosome CGH

Evidence for CGH between *Sphenodon* and chickens is limited. CGH usually hybridises across a whole chromosome, or at least a significant proportion of a

chromosome. This was not seen in the current study. The two orders last shared a common ancestor when *Sphenodon* and the Archosauria diverged around 290 MYA (Benton 2005), and large evolutionary distances inhibits successful CGH. For example human chromosome paints have been shown to hybridise poorly to non-primate species (Wienberg, Frönike et al. 2000).

Chicken chromosomes 1, 2 and 3 do not show any clear evidence of hybridisation (Figures 3.7, 3.8 and 3.9). Some evidence for hybridisation is seen with chicken chromosome 4 where homology is seen to *Sphenodon* chromosome 4. Although the evidence for hybridisation is limited, as signal would be expected from both members of the chromosome pair. *G. domesticus* chromosome 5 produced no hybridisation signal.

CGH with *Gallus domesticus* Z chromosome is a more intriguing hybridisation result. Depending on how much divergence has occurred between the chicken Z and W sex chromosomes from their autosomic origin, the *Gallus domesticus* Z chromosome would be expected to hybridise to only one chromosome in non-avian species. A result observed in Figure 3.11. Chromosome morphology was difficult to establish but hybridisation was seen on 'q' arms of *Sphenodon* chromosome 5 or 8. In comparison, chicken Z chromosomes have hybridised to Testudine chromosome 6 (Matsuda, Nishida-Umehara et al. 2005). Some evidence for *G. domesticus* W chromosome hybridisation to *Sphenodon* chromosomes was seen on the 'p' arms of a medium submetacentric and possibly chromosome 4. If the hybridisation signal is correct, and it should be emphasised the results are not convincing, then it suggests that chromosomal

rearrangements occurred between chicken and *Sphenodon* before sex chromosomes differentiated in chicken.

In the current study various slide preparations, timings and temperatures were varied without increasing CGH success, supporting a suggestion of low levels of chromosome synteny between *Gallus domesticus* and *Sphenodon*.

Overall there was little evidence for successful *Gallus domesticus* CGH to *Sphenodon* chromosomes, suggesting the vast evolutionary distance between the two species may be too large for successful hybridisation. The chromosomes, although retaining morphological similarities, may have become so divergent through internal rearrangements and DNA changes that no homology remains.

Conclusions

Sex determining genes WT1 and AMH from Chapter 2 were labelled with DIG-UTP and hybridised on to *Sphenodon* chromosomes. WT1 localised to a small submetacentric chromosome 'q' arm (chromosome 13 or 14), and possibly chromosome 5 'p' arm. AMH localised to chromosome 11 near the centromeric region. These locations demonstrate no homology with either chicken or human gene localisations.

Human telomeric sequences were successfully hybridised onto *Sphenodon* chromosomes. The two species have been isolated for approximately 350 MYA demonstrating telomeric regions are highly conserved.

Chapter 4 : Summary of *Sphenodon* chromosomes, nuclear gene sequences, FISH and reptilian relationships.

The current study explored chromosomal and molecular variation within *Sphenodon* and the Reptilia. Use of chromosomal and molecular data allowed testing of evolutionary relationships using two largely independent methods.

This summary chapter is divided into three broad sections, namely (i) a discussion on chromosomal evolution in reptiles, (ii) a discussion on molecular variation in reptiles, and finally (iii) consideration of issues concerning *Sphenodon* and the Reptilia, including evidence for evolutionary decoupling within *Sphenodon*.

Sphenodon *and reptilian karyotypes*

Analysis of *Sphenodon* chromosomes demonstrated an identical karyotype in all populations and species. Even though the Poor Knights Island group (north-eastern North Island) was isolated one million years ago it has a karyotype identical to other *Sphenodon*, demonstrating a highly conserved karyotype. One Ruamahua-iti (north-eastern North Island group) animal possessed a chromosomal heteromorphism where one member of the chromosome 3 pair was lengthened due to interstitial or telomeric growth.

With such low chromosomal variation in *Sphenodon* it was interesting to compare its karyotype with that of other reptiles. Squamates are the most recent divergence of the Reptilia, and traditional morphological analysis groups them with *Sphenodon* as the Lepidosauria. This position is not reflected in chromosomal analysis where no homology was seen. The high levels of chromosomal variation seen in the Squamata has been associated with speciation in small isolated populations (King 1979). This has not happened in small island populations of *Sphenodon*.

Comparisons with the other two archaic reptilian lineages, Testudines and Crocodylia, were more productive. Very high homology in macrochromosome morphology between *Sphenodon* and the Testudines was seen, with the exception of two highly derived Testudine families. Microchromosome number differs between *Sphenodon* and the Testudines, with the Testudines having significantly higher numbers than the four pairs observed in *Sphenodon*.
Some level of homology between the *Sphenodon* karyotype and the proposed ancestral Crocodylian karyotype of *Alligator*, *Tomistoma* and *Gavialis* (Bickham 1984) was observed. Chromosomes 1,4,6,8,9,10,12,13 of *A. mississippiensis* show morphological homology to *Sphenodon* and the Testudines. *A. mississippiensis* chromosomes 2 and 3 are metacentric, whereas in the *Sphenodon* karyotype they are submetacentric and telocentric, respectively. Alligator chromosome 7 is similar to the telocentric of *Sphenodon*, although the centromeric index suggests the alligator chromosome 7 is subtelocentric. The telocentric marker chromosome 11 in *Sphenodon* and Testudines is subtelocentric in *A. mississippiensis*. Microchromosomes appear to be absent in the Crocodylia.

The Aves, as part of the Archosauria, shared a common ancestor with the Crocodylia, although the chicken karyotype has greater homology with the *Sphenodon* macrochromosome complement, similar to the Testudine lineage. Aves, unlike the Crocodylia, possess microchromosomes in greater numbers than *Sphenodon*, demonstrating homology with Testudine microchromosome number.

Reptilian proto-karyotype

Homology in chromosome morphology between *Sphenodon*, Testudines, Aves and to a lesser extent Crocodylia, supports an ancient relationship, allowing advancement of a proto-karyotype for a common ancestor of the Reptilia. Few authors now group the Testudines with the Parareptilia (Modesto and Anderson 2004), so dating the proto-karyotype becomes easier. The proposed vertebrate proto-karyotype of puffer fish and the common ancestor of mammals and reptiles 450 MYA had 12 macrochromosomes (Jaillon, Aury et al. 2004). From this significant chromosome rearrangements occurred before the proposed reptilian proto-karyotype of 28 macrochromosomes, which can be dated to 300 MYA.

An idiogram of the proto-karyotype morphology is presented as Figure 4.1.

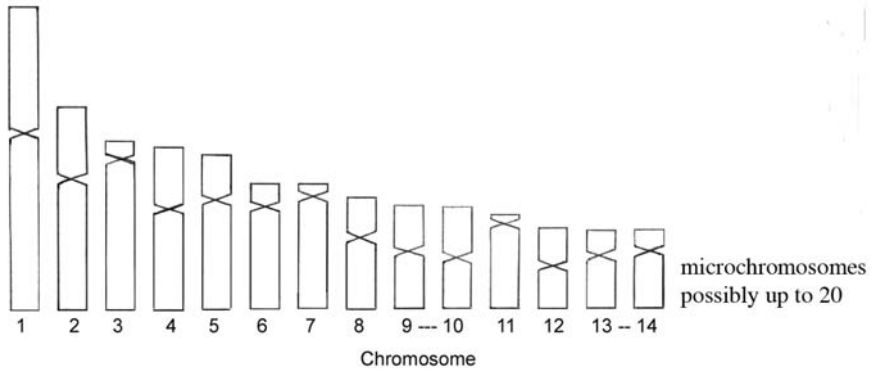


Figure 4.1: Reptilian proto-karyotype idiogram based on the *Sphenodon* karyotype.

The proto-karyotype is identical to the macrochromosome complement of *Sphenodon*, Testudines and the macrochromosomes of *Gallus domesticus*. As shown in other diagrams (Figure 1.14; Figure 3.1), the Crocodylia differ slightly from the proposed proto-karyotype. It is unlikely that Crocodylia possess a basal karyotype and that all other reptiles are derived, as this position has no support from either DNA or morphological data, and karyotypic synteny of the other three Orders suggest a derived origin for the Crocodylia.

Accepting that crocodiles are derived implies that their karyotype demonstrates reorganisation from the proposed reptilian proto-karyotype.
Without comparative genomic hybridisation, the following statements remain conjecture, but it is possible to derive the *A. mississippiensis* karyotype from the proto-karyotype. Thus chromosomes 2 and 3 have had pericentric inversions (or interstitial growth) causing a submetacentric chromosome to become metacentric (chromosome 2) and a telocentric chromosome to become metacentric (chromosome 3). Chromosomes 11 and 14 in *A. mississippiensis* are reversed from the proto-karyotype (submetacentric and telocentric whereas the proto-karyotype has telocentric and submetacentric). The group of alligator

chromosomes 11-14 are small and of similar total chromosome length, and the karyotype appears contracted, suggesting that ordering of these four chromosomes may be difficult, as chromosome arms and regions of arms are known to contract at differing rates (Bajer 1959; Sasaki 1961; Bentzer, v. Bothmer et al. 1971; Ho and Kasha 1974), causing ambiguous chromosome identification. Alternatively, *A. mississippiensis* chromosome 11 has had a pericentric inversion or telomeric growth, changing the centromeric index from telocentric to subtelocentric, and chromosome 14 lost the 'p' arm changing its morphology from submetacentric to telocentric. The morphology of these two chromosomes could be interpreted as suggestive of the 'p' arm of chromosome 14 translocating to chromosome 11. This hypothesis could be tested with comparative genomic hybridisation using other reptilian chromosomes. Deriving other Crocodylian karyotypes from the basal form has already been described (Bickham 1984).

The similarities between Testudines, *Sphenodon* and Avian macrochromosomes suggests a common ancestor for the three Orders, although the microchromosome number varies within the Reptilia and Aves. Microchromosomes are seen in high numbers in Testudines and Aves, low numbers in *Sphenodon* and some Squamata, and absent in the Crocodylia. So how many microchromosomes did the proto-karyotype possess? There are two possibilities: firstly, the common ancestor had a low number of microchromosomes with a predisposition for accumulation in the Avian and Testudine lineages; or secondly, the common ancestor had a high number of

microchromosomes (as seen in Aves and Testudines) with subsequent loss in the *Sphenodon,* Squamata and Crocodylian lineages.

The significant evolutionary distance between the Aves and the Testudines suggest the high number of microchromosomes with subsequent loss the more likely explanation. Other authors provide support for this position by suggesting the primitive avian karyotype had 20 pairs of microchromosomes (Burt 2002). If it is accepted that the proto-karyotype had a high number of microchromosomes then two major losses have occurred in extant Reptilia. Firstly, the *Sphenodon* lineage lost microchromosomes, reducing the proto-karyotype to the four pairs seen in *Sphenodon*. The second major loss occurred in the Crocodylia, removing microchromosomes completely.

Absence of microchromosomes in extant crocodiles implies a Crocodylian ancestor post-avian divergence had lost all its microchromosomes. Dating this event is difficult, however, the crocodile-bird divergence has been dated to <251 ->243 MYA, and the alligator-caiman split to <71 ->66 MYA (Muller and Reisz 2005), so the common ancestor existed at some point between those two date ranges.

FISH evidence supporting a reptilian proto-karyotype

The high level of chromosome conservation in macrochromosome morphology is reflected in comparative genomic hybridisation studies. Chromosome 1 of *T. scripta* hybridised exclusively to chromosome 1 in four other Testudine species, which would happen only if no chromosomal rearrangements had occurred

(Muhlmann-Diaz, Ulsh et al. 2001). The next logical step, given the apparent similarities between Aves, Testudines and Sphenodontia, was to hybridise chicken chromosome paints to Testudines. Again the morphological similarity was reflected, with chromosomes 1-6 'almost equivalent to each other', a situation not seen in hybridisation between a chicken and a Squamate (Matsuda, Nishida-Umehara et al. 2005). Absence of hybridisation using *G. domesticus* chromosome paints to *Sphenodon* chromosomes suggests the chromosomes have diverged significantly. Internal rearrangements of the chromosomes, since a common ancestor, have been sufficiently extensive that no homology is seen using comparative genomic hybridisation. This reflects the DNA differences seen in Chapter 2, although contrary to homology in chromosome morphology.

Evolutionary position

Variation in chromosomal morphology can be used to create phylogenetic trees which can be compared to both the proto-karyotype and phylogenies created using more traditional methods.

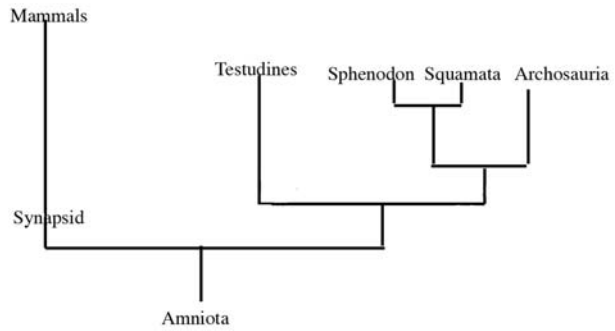


Figure 4.2: Traditional morphological phylogeny of Synapsids, Diapsids and Anapsids (modified from Lee 2001).

A traditional morphological phylogeny (Figure 4.2) groups *Sphenodon* and Squamata as the Lepidosauria, Testudines as the first to separate from the reptilian lineage, and the Crocodylia with the Aves as the Archosauria. A

phylogeny derived from chromosome morphology is presented as Figure 4.3. From a proto-reptilian karyotype with around 20 microchromosomes two phylogenies are possible. If substantial weight is given to morphological characters, where crocodiles and birds group as the Archosauria, and *Sphenodon* and Squamata as the Lepidosauria, then Figure 4.3a reflects changes from a proto-karyotype. It is possible, however, to represent chromosome change in the Reptilia using a more parsimonious tree. Figure 4.3b groups Testudines and Aves with their microchromosome similarities to the proto-karyotype. The other branch reflects groups that lost microchromosomes, the Lepidosauria diverging first, and retaining some microchromosomes, with the Crocodylia subsequently losing all microchromosomes.

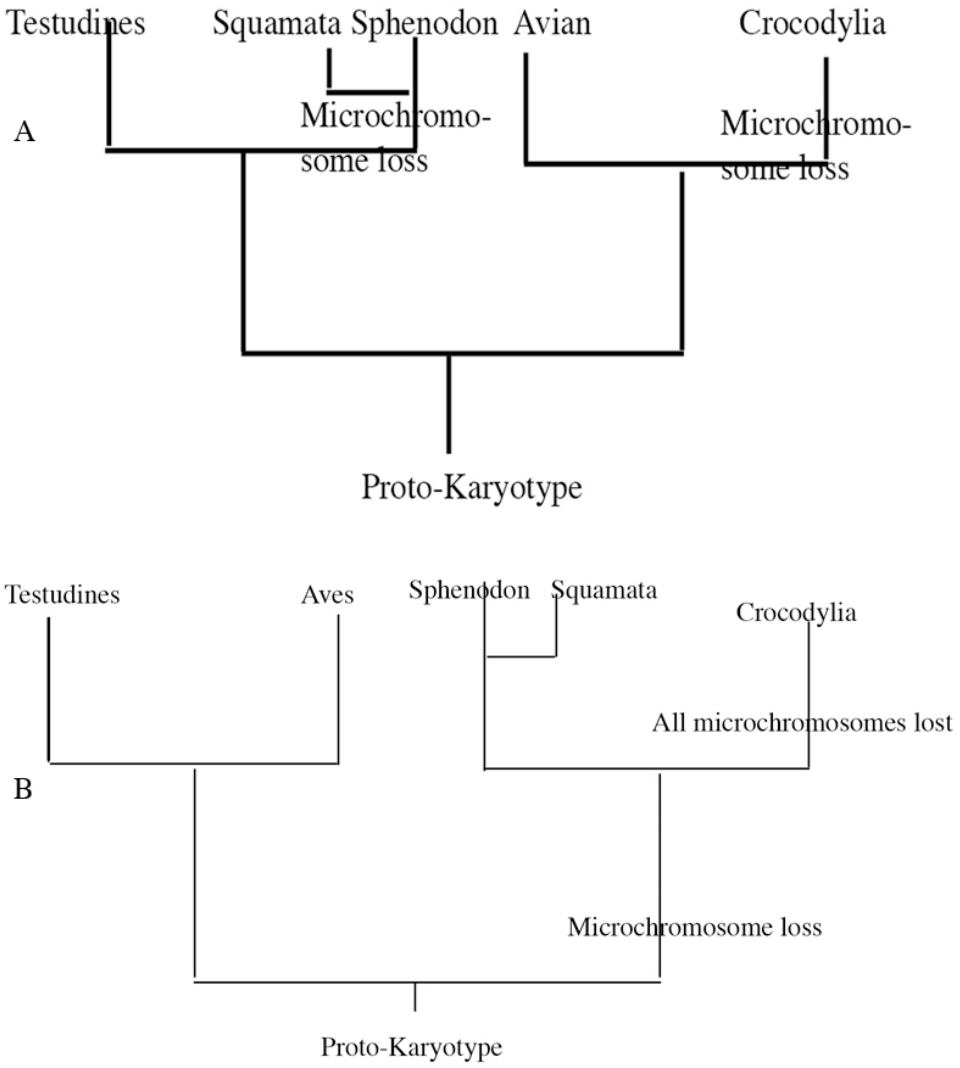


Figure 4.3: Phylogeny based on chromosome morphology (**A**) with a traditional Lepidosauria and Archosauria and (**B**) based purely on chromosome morphology and presence/absence of microchromosomes.

Molecular summary

The three sex determining and two nuclear gene fragments sequenced in the current study, with the addition of the previously published aldolase fragment (Hay, Daugherty et al. 2003), allowed comparison between the two types of nuclear genes. Four of the genes (AMH, aldolase, FoxG1 and 28S) were identical in all populations of *Sphenodon*. The two island groupings previously identified (Hay, Daugherty et al. 2003) were observed in WT1, and some population variation was present within DMRT1 sequence.

Absence of homologous sites, reflecting the significant time since a common ancestor, prevented phylogenetic analysis of AMH, WT1 and DMRT1. *Sphenodon* FoxG1 placed as an outgroup with Zebrafish, a position reflecting significant sequence divergence, implying FoxG1 is not a good choice for deep phylogeny within the Reptilia. 28S grouped *Sphenodon* within the Reptilia with no resolution, reflecting absence of variation within reptilian 28S.

Comparative work using mtDNA demonstrated rapid rates of evolutionary change in *Sphenodon*, and the decoupling of neutral and phenotypic evolution (Hay, Subramanian et al. 2008). The current study provides more evidence for evolutionary decoupling.

Sphenodon chromosomes reflect a reptilian proto-karyotype, demonstrating the chromosomes are highly resistant to morphological change. The divergent phylogenetic trees from molecular sequences in the current study suggests the genes examined are evolving at different rates. The similarities between *Sphenodon* populations from diverse locations dates sequences to over one million years old, far older than the sequences examined in the mtDNA study. The current data allows dating of the variation, which in some cases can be linked to major geological events. *Sphenodon* has been isolated since New Zealand separated from Gondwana (80 MYA) and has undergone at least one major (Oligocene Drowning, 25 MYA), and at least one minor (Pliocene/Pleistocene, 5 MYA) range contraction that may have contributed to the divergent phylogenetics.

Sequence from the 28S gene possesses high homology with other reptiles. The gene is identical within *Sphenodon* populations and demonstrates minimal sequence variation within the Reptilia (Appendix 3). The sequence homology with other reptiles dates 28S to prior to Gondwanic isolation, and the homology between *Sphenodon* and Testudines suggests sequence conservation back to a common ancestor. 28S reflects the archaic homology seen in the karyotypes.

The other genes with identical sequence within *Sphenodon* populations (aldolase, AMH and FoxG1) differ from other reptiles. The sequence divergence can be dated to prior to the Poor Knights Island group separation one million years ago. Precise dating is not possible with the current data, although the sequence divergence is post-Gondwana isolation. After sequence divergence one of the major bottlenecks (Oligocene or Pliocene) caused the population size to be drastically reduced, resulting in the absence of variation in *Sphenodon* seen today. Following the reduction in population and genetic diversity *Sphenodon* recolonised New Zealand. This hypothesis implies that sequencing the genes from fossil *Sphenodon* would demonstrate an identical sequence, as these samples date to 8000 years ago (Hay, Subramanian et al. 2008).

The two genes that exhibit variation within *Sphenodon*, WT1 and DMRT1, also demonstrate significant divergence compared to other reptiles. The Cook Strait and north-eastern North Island separation seen in WT1 sequence can be explained by one of two hypotheses.

1. During the Oligocene a refugium resulted in reduced genetic variation. Recolonisation was followed by some form of physical separation during the Pliocene of the north-eastern North Island and Cook Strait

populations, possibly by glaciation, leading to the Cook Strait refugium population developing a unique form of the gene.

2. During the Oligocene or Pliocene two refugium populations diverged. One became the progenitor of the extant north-eastern North Island populations, the other the progenitor of extant Cook Strait populations.

The problem with the second hypothesis is the identical sequence of aldolase, AMH and FoxG1 between the population groups. Perhaps the WT1 sequence diverged after the other genes were fixed, with the refugia and subsequent divergence of WT1 occurring during the Pliocene. If the first hypothesis is correct then sequencing of South Island fossil *Sphenodon* WT1 should reveal a sequence similar to north-eastern North Island.

Unfortunately, with the limited number of sequences obtained from DMRT1 evolutionary hypotheses are limited. However, the divergence seen suggests DMRT1 may be evolving rapidly within *Sphenodon* populations. Sequence differences were seen between three populations (Brothers Island, Stephens Island and Poor Knights Island group) so variation has arisen since the Cook Strait islands became isolated from each other around 10,000 years ago. This dates DMRT1 sequence divergence to the same time frame as that of the mtDNA.

Sex determination in reptiles

Most sex determination work has been done in mammals, although recent work in Aves, Testudines and Crocodylia is beginning to elucidate reptilian pathways. In birds, the Z chromosome linked gene DMRT1 is dosage dependent, with males (ZZ) having twice the dose of females (ZW).

The current study isolated *Sphenodon* sequence from three early stage sex determining genes (AMH, WT1 and DMRT1). All three demonstrated significant sequence variation from other reptiles, making hypotheses concerning *Sphenodon* sex determination difficult. In other reptiles, DMRT1 acts early during sex determination, and is probably the sex determining gene in birds. Early expression is seen in *T. scripta* and *C. versicolor* although not in *L. olivacea*. WT1 expression is dimorphic in *T. scripta*, but not in the Crocodylia. These two genes appear good candidates for full sequencing and expression studies (if possible) in *Sphenodon*.

Expression studies on *Sphenodon* embryos are unlikely to be granted research permits, so other methods of expression study will need to be utilised. Firstly *in situ* hybridisation of full DMRT1 and WT1 genes will allow localisation to a particular chromosome. Comparative labelling of male and female DNA, similar to work on other reptiles (Ezaz, Quinn et al. 2005; Ezaz, Valenzuela et al. 2006), may also reveal sex related differences.

Testudine relationships within the Reptilia

With the Anapsida being relegated to a description of temporal fenestrae (Modesto and Anderson 2004), the placement of Testudines with the rest of the Reptilia has become less contentious, with discussion shifting to where to place the Testudines. Chromosome analysis, resulting in the reptilian proto-karyotype, supports a close relationship between Testudines, Sphenodontia, Crocodylia and Aves.

No consistency was seen in Testudine placement within the Reptilia with the phylogenies created from gene sequences in the current study. This reflects the differing selective pressures on the genes, and the limited lengths of sequences used for analysis, from both *Sphenodon* and the Testudines.

Origin of tuatara populations

Tuatara populations have been isolated from the mainland for 8-12,000 years since the last glaciation (Pleistocene). The one exception, the Poor Knights Island (PKI) group, became isolated from the mainland 1 MYA (Hayward 1986). New Zealand has a depauperate fossil record, resulting from significant tectonic changes over the last 80 MY. However, fossil records demonstrate that tuatara were previously widespread throughout the Quaternary period New Zealand, from Northland to Otago and Canterbury, and in Otago during the Miocene (Worthy and Holdaway 1996; Worthy 1998; Brook 1999; Worthy, Tennyson et al. 2006).

During the Oligocene Drowning, New Zealand was reduced to an archipelago approximately 15% of the current landmass (Cooper and Cooper 1995). An event of this magnitude would necessarily create a bottleneck situation for surviving flora and fauna. A number of unique endemics including *Sphenodon* did survive, suggesting one of two hypotheses; namely (i) there was adequate refugia available during the Oligocene Drowning or (ii) New Zealand endemism and species variety was significantly greater than what is recognised today, and what survived is a remnant of that diversity. Hay et al (2003) dismiss the Oligocene Drowning as occurring too early to account for current reduced

genetic variation in *Sphenodon*. Rather they suggest that during the Pliocene/Pleistocene periods (5 MYA-10,000 years ago) volcanic and tectonic (Pliocene) and glaciation (Pleistocene) processes in New Zealand caused genetic reduction in *Sphenodon*. The implication is that although there may have been more species of *Sphenodon* post-Oligocene Drowning, the Pleistocene/Pliocene period reduced variation to the groupings seen today (Hay, Daugherty et al. 2003).

Although the current work is not directly related to methods of distribution patterns, some suggestions can be advanced. The hypothesis that most of the tuatara variation was lost during the Pleistocene/Pliocene period seems weak. Various lines of evidence including DNA (current study, Hay, Daugherty et al. 2003; Rest, Ast et al. 2003), chromosomal (current study, Norris, Rickards et al. 2004) and allozymes (Daugherty, Cree et al. 1990; Hay, Daugherty et al. 2003) demonstrate very low levels of variation within *Sphenodon*. If it is assumed that the Poor Knights Island population is a relict, rather than the result of humans moving animals between islands, then a last possible date for all variation reduction in tuatara is 1 MYA (when the Poor Knights Islands became isolated). Hay et al (2003) suggest that tuatara experienced a severe bottleneck leading to reduced genetic variation during the Pleistocene/Pliocene, and then dispersed over the entire extant range from Poor Knights Island to Cook Strait. This seems unlikely for three reasons:

1. Tuatara are slow breeders, reaching reproductive maturity around 15 years, have a low clutch size, and four years between clutches (Newman, Watson et al. 1994; Gaze 2001).
2. The presumed low vagility of tuatara (although this is difficult to assess

on isolated islands).

3. The distance involved (around 600 km in a straight line, assuming there were no tuatara populations further north than the Poor Knights Islands or further south than Cook Strait).

In defence of Hay et al., a 600 km dispersal from the Poor Knights Islands to Cook Strait is possible within the Pleistocene/Pliocene timeframe. From the beginning of the Pliocene to isolation of the Poor Knights Island group, a breeding population would need to become isolated and undergo a severe bottleneck, reducing genetic variation in mtDNA, nuclear DNA, allozymes, then recolonise within the current distribution. A distance of 600km in 3.5 MY (Pliocene, 5 MYA, assuming 0.5 MYA for the bottleneck, and the Poor Knights Island group was isolated 1 MYA) averages out to 17 cm/year. Assuming a breeding age of 15, the average movement per 15 years (or one generation) is 2.5 m. This is not unrealistic, although it does not take into account any dispersal throughout the mainland. It also assumes firstly that tuatara dispersed following the coastline (if it is assumed the refugia was around the Poor Knights Island group) to Cook Strait; and it secondly that the bottleneck was of relatively limited duration, allowing the population to recover quickly. Note that this rough calculation does not take into account what population size is required for tuatara to become distributed through the current range.

One problem with the assumption is that fossil *Sphenodon* have been found from the top of the North Island to the bottom of the South Island (Worthy and Holdaway 1996; Worthy 1998; Brook 1999; Worthy, Tennyson et al. 2006). Factoring in this distribution using the approximate calculations above, gives a

distance of 1210 km in a straight line, over a period of 3.5 million years and a generation time of 15 years, resulting in a dispersal measure of 5.2 m/generation. Again, this assumes tuatara dispersed down the coastline, and were not distributed throughout the mainland, although fossil evidence clearly demonstrates they were.

Alternative tuatara distribution theory

I accept that severe bottlenecks are the likely cause of the low genetic variation within tuatara. However, I do not accept that three and a half million years is sufficiently long for reduction of genetic variation and subsequent recolonisation throughout the North Island. If the Oligocene Drowning did provide a long and effective bottleneck, then it is possible that tuatara survived in one or more of the available refugia, and subsequently radiated throughout the rest of the range. This hypothesis gives a period of approximately 20 MY for subsequent recolonisation. During the most extreme period of the Oligocene Drowning (27 MYA), refugium were available in the north-eastern North Island (where islands with tuatara are now), a large region of the southern South Island (where tuatara fossils have been found), and a small area around Nelson, South Island (near to the Cook Strait islands) (Cooper and Cooper 1995). If the north-eastern North Island region acted as a refugium then subsequent bottlenecks before the landmass increased could have reduced mtDNA, nuclear DNA and allozyme variation.

If the Oligocene Drowning refugia hypothesis is correct then current low levels of variation result from 20 MY of evolution since the bottleneck, with possible secondary Pleistocene/Pliocene effects. The recent work describing rapid change

in mtDNA uses recent (less than 10,000 year old) samples (Hay, Subramanian et al. 2008) reflecting rapid changes following the last ice age (Pleistocene). The nuclear DNA sequences from the current study, in particular aldolase, AMH, FoxG1 and WT1, support an early time for the bottleneck. This argues for the Oligocene alternative *Sphenodon* distribution theory, rather than the later Pleistocene/Pliocene divergence recently advanced as the timeframe for genetic reduction (Hay, Daugherty et al. 2003).

Potential for fossil *Sphenodon*

Isolation and comparison of ancient mtDNA to extant samples demonstrated significant rates of mtDNA change using material representing the full *Sphenodon* distribution, from the top of the North Island to the bottom of the South Island (Hay, Subramanian et al. 2008). Sequencing of the control regions of mtDNA (HVR I and HVR II) demonstrated high levels of variation, although apparently not enough to suggest other species of *Sphenodon*. The mtDNA HVR data provide evidence that neutral evolution and phenotypic evolution are decoupled. If nuclear gene sequences can be obtained from fossil *Sphenodon*, then Otago, Canterbury and Northland populations can be compared to extant populations to assess nuclear DNA evolution rates. If the extinct South Island populations were found to possess genetically distinct nuclear DNA then this would support the Oligocene Drowning model, as regions of the southern South Island were available for refugium (Cooper and Cooper 1995) allowing survival of other *Sphenodon* lineages.

If the Otago populations were Oligocene Drowning relicts, and not post-Pleistocene/Pliocene dispersals, then sequence analysis could potentially provide information in relation to the following questions:

- Were other lineages or species of *Sphenodon* present in pre-historic New Zealand? Sequence data is available from both *S. punctatus* and *S. guntheri*, so comparison to fossil tuatara nuclear DNA could answer this question. Mitochondrial DNA has already demonstrated rapid rates of change without species differentiation.
- What rates of change are seen within tuatara species? The fossil material spans a 15 million year period and considerable geographical distance. There are limits on the age from which DNA can be extracted (Noonan, Hofreiter et al. 2005; Rogaev, Moliaka et al. 2006), limiting *Sphenodon* fossils to the Quaternary period around 10,000 YA. Sequence data should allow inter- and intra- population rates of change to be assessed. Although not involving a period as long as recent fossil Antarctic penguin studies, a longitudinal study would provide information on how much variation was present in an archaic population, and how this changed over time. Recent work estimated population changes over time for a vole species (Chan, Anderson et al. 2006). The authors used ancient (10,000 – 3,000 years old) and current genetic variation to estimate effective population size from both ancient and modern vole populations, and estimated both timing and severity of bottlenecks. The age of the vole samples and tuatara fossils are similar, so appropriate tuatara sequence data could be used to estimate effective population size of archaic tuatara. This information would also allow modelling of tuatara distribution,

bottlenecks and subsequent redistribution; and may also be of use in estimating how quickly extant island populations will lose or gain genetic variation. As seen in the current study, WT1 and DMRT1 appear to provide some resolution of groups or islands. For this reason, if nuclear DNA can be isolated from fossils, use of sex determining genes may provide more information than other nuclear DNA sequences. Fossil DNA sequence from WT1 and DMRT1 can be compared with other fossil deposits and extant samples, giving a good longitudinal study of sex gene evolution.

The differentiation in WT1 sequence may also be reflected in the fossil material. My suggestion that Cook Strait tuatara have a recently evolved allele of WT1 could be tested using southern tuatara samples. If the north-eastern North Island samples reflect the archaic form of the gene, then South Island fossils should be closer to north-eastern North Island sequence than to the Cook Strait sequence.

Status of species and populations of *Sphenodon*

The two species of tuatara can be separated using allozyme studies. However, mtDNA, WT1, allozymes and immunology (current study, Hay, Daugherty et al. 2003), differentiate Cook Strait and north-eastern North Island groups, supporting a well established genetic differentiation between the two groups, rather than differentiating between species. *S. guntheri* (North Brother Island) is differentiated by a fixed allele, an odd collection of polymorphic loci (alternatively supporting Cook Strait or north-eastern North Island *S. punctatus*), and four unique mtDNA control region sites. Is this sufficient to define a species? Or should tuatara species, if we are going to have two, be split into Cook Strait

tuatara and north-eastern North Island tuatara, a position supported by a greater range of data. The same question was discussed in some detail in an earlier paper (Hay, Daugherty et al. 2003), where the authors concluded that the subspecies status of north-eastern North Island *S. punctatus* be dropped, and *S. guntheri* remain as a separate species. The current study demonstrates no chromosomal differences, no variation in AMH, 28S or FoxG1; Cook Strait separation with WT1, and no clear differentiation with DMRT1. There is no support for the unique status of North Brother Island tuatara in the current study. A case could be made for the separation of north-eastern North Island and Cook Strait (including North Brother Island) *Sphenodon* using the current study and previous work (Hay, Daugherty et al. 2003). Hay et al considered this, and recommended against the elevation to species status. Instead, their focus was on Management Units of individual island stocks of *Sphenodon*. This seems to be the most reasonable approach to understanding tuatara populations, and I see no reason to argue against this on the basis of the current study.

Conclusions and future directions

1. *Sphenodon* exhibit no chromosomal variation, possessing a karyotype unchanged for at least one million years when the Poor Knights Island group became isolated from the mainland. Comparison with other Reptilia and the Aves demonstrated very high homology to the Testudines and Aves, and moderately high homology to the ancestral Crocodylia karyotype. Macrochromosome homology allowed extrapolation of a reptilian proto-karyotype, similar to both *Sphenodon* and Testudine macrochromosomes.

2. Although *Gallus domesticus* chromosomes did not hybridise successfully to *Sphenodon*, I suggest obtaining *Sphenodon* and Testudine chromosome paints would provide a useful resource. Aside from reciprocal painting to explore chromosome homology, *Sphenodon* paints would allow comparative work between *Sphenodon* populations. Painting *Sphenodon* chromosomes onto *Gallus domesticus* may prove more successful, as *Sphenodon* has a low mitotic rate whereas the chicken cell cycle can be optimised to produce a higher number of spreads for use in hybridisation work. If permits can be obtained, *Sphenodon* cell cultures would provide a good resource with which to optimise cell cycles. Reptilian G-banding has been demonstrated in cultured cell lines and maybe more successful in cultured *Sphenodon* cells.
3. Two nuclear sex determining genes isolated from *Sphenodon* demonstrated more variation and island resolution than chromosomes did. Full DNA sequence from the genes in the current study should be used to increase resolution of nuclear DNA phylogenies.
4. Including DNA from fossil *Sphenodon* should provide context concerning historical variation. Microsatellite and mtDNA work indicates that *Sphenodon* has higher levels of variation than that currently seen in some nuclear DNA genes, chromosomes and allozymes. Subfossil *Sphenodon* DNA may also demonstrate other lineages, allowing conclusions to be drawn on refugia location and population structure. With the WT1 separation seen in the current study, subfossil nuclear DNA could also enable one to explore the origin of *Sphenodon* WT1, answering the question 'is the Cook Strait WT1 gene of recent origin?'.

5. Chromosomes and 28S sequence reflect archaic relationships with a common ancestor of the Reptilia; mtDNA demonstrates significant Pleistocene divergence; aldolase, AMH and FoxG1 probably represent an Oligocene bottleneck. WT1 diverged through the Pleistocene or Pliocene and DMRT1 may be a recent divergence, although limited data exist to support this conclusion.
6. As a relict of Gondwanic New Zealand, the survival of *Sphenodon* is surprising given the dramatic tectonic and climatic changes that occurred over the 80 million years since separation. To have a conserved karyotype representing a reptilian proto-karyotype, contradictory DNA results of high variability of microsatellites, high mtDNA variation, low nuclear DNA in 28S and highly divergent WT1, AMH, DMRT1 and FoxG1 sequence, and low allozyme variation, all suggest decoupling of evolutionary processes and therefore potential for testing evolutionary theories using *Sphenodon*.

Appendix 1 : Chemical Solutions

SSC
8.76 g sodium citrate, 4.40 g NaCl in 1 L ddH_2O;
n * SSC refers to quantities of the salts, 2xSSC would have 17.52 g sodium citrate and 8.80 g NaCl in 1 L ddH_2O

SST
n * SSC + 0.05% Tween20, for example 4X SST = 4X SSC + 0.05% tween20

PBS
10x PBS: Dissolve 80 g NaCl, 2 g KCl, 26.8 g $Na_2HPO_4 – 7H_2O$ and 2.4 g KH_2PO_4 in 800 ml ddH_2O. Adjust pH to 7.4 with HCl. Adjust volume to 1 L with ddH_2O, aliquot and sterilise by autoclaving. Store at room temperature.

Phosphate buffer solution for giemsa staining
1.0 g KH_2PO_4, 0.8 g Na_2HPO_4, pH6.8 in 1 L ddH_2O

TNT buffer
100 mM Tris-HCl pH 7.5; 150 mM NaCl; 0.05% Tween 20

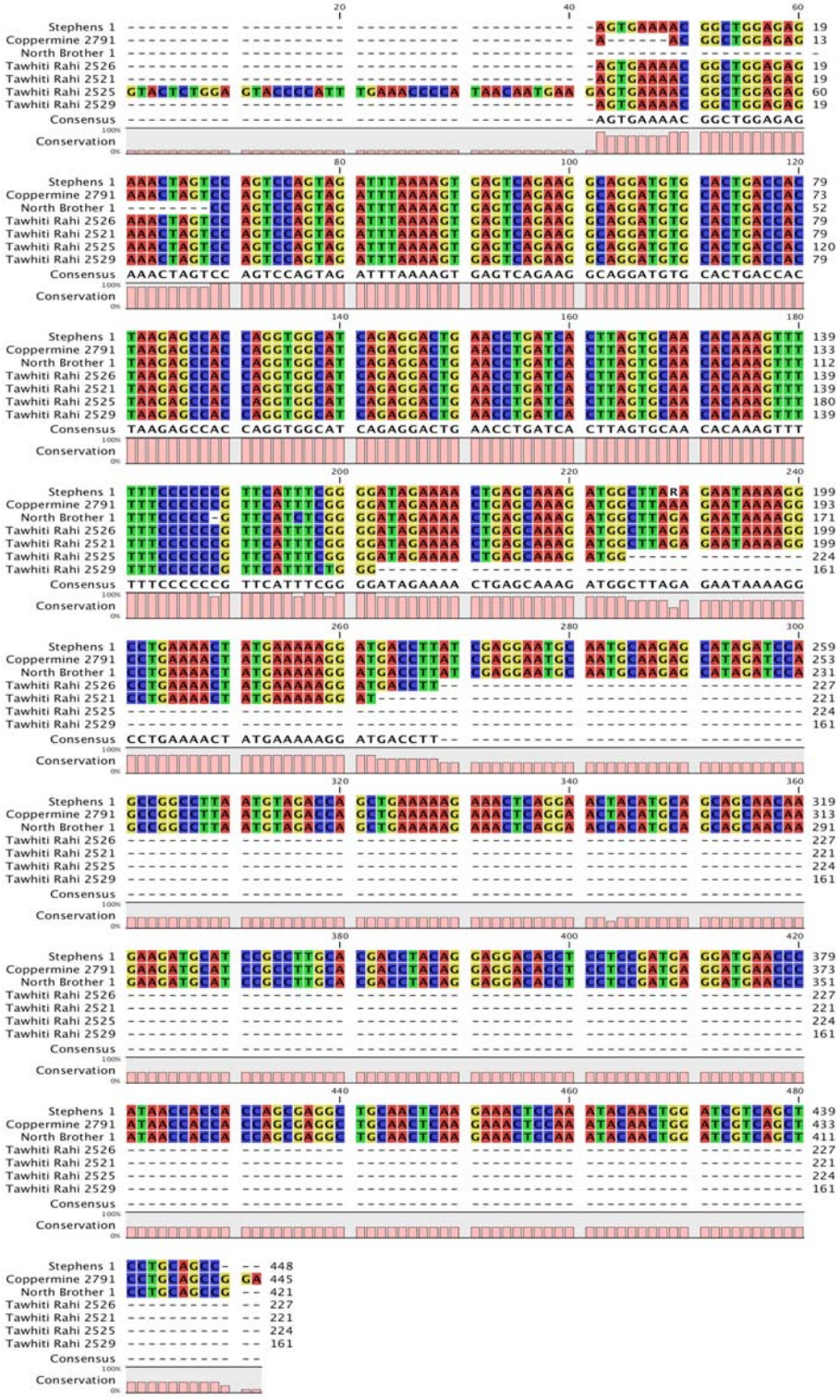
TNB buffer
100 mM Tris-HCl pH 7.5; 150 mM NaCl; 0.5% bovine serum albumin

Appendix 2 : List of abbreviations used

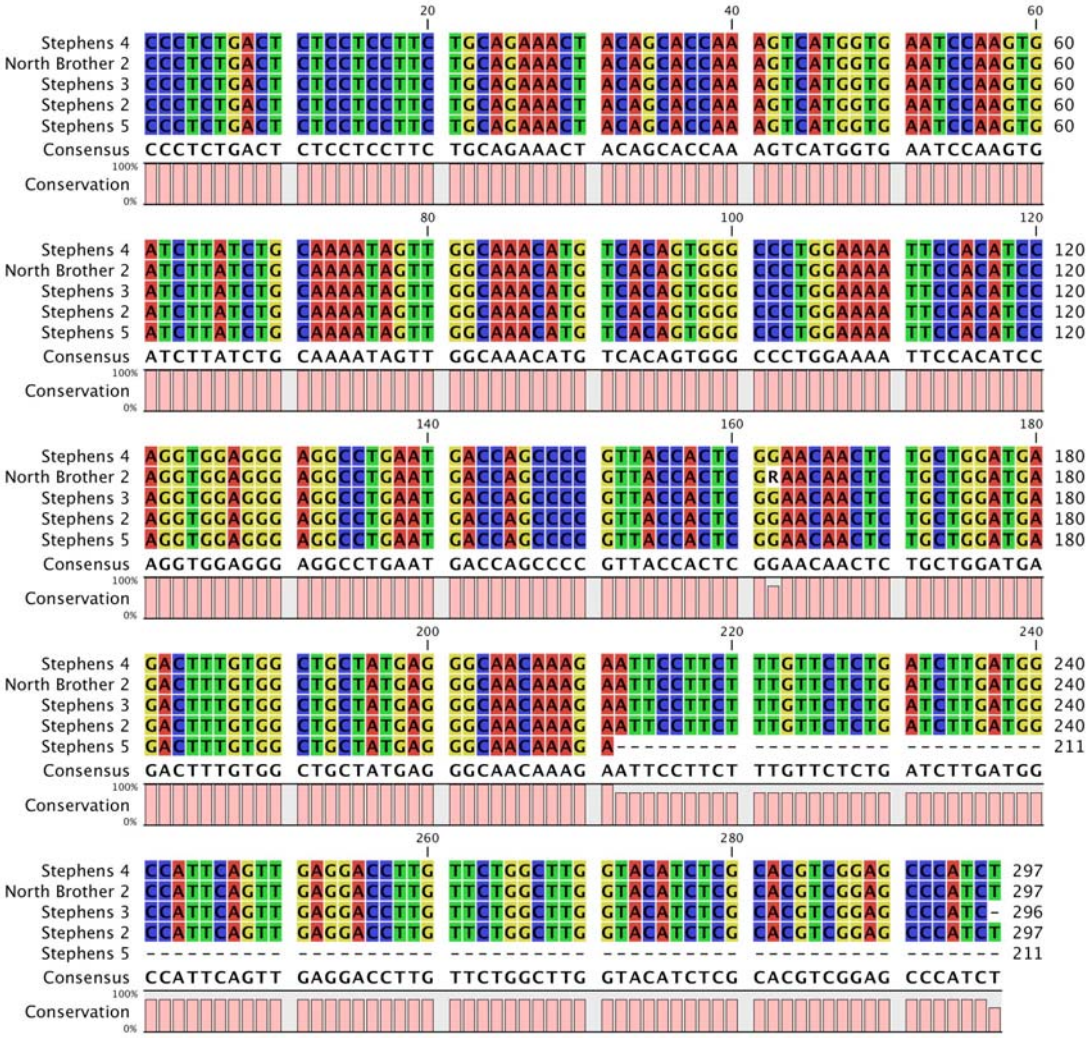
AWC – Allan Wilson Centre
BDT – big dye termination protocol v3.1
CA – common ancestor
CGH – comparative genomic hybridisation, or ZOO-FISH
CI – centromeric index
CR – centromeric ratio
DAPI – fluorescent, 4',6-diamidino-2-phenylindole
DIG – Digoxigenin
DPC – days post coitum
ESD – Epigenetic or environmental sex determination
EST – expressed sequence tag
FDF – female determining factor
GSD – genotypic sex determination
FISH – fluorescent *in situ* hybridisation
FITC - fluorescent, fluorescein isothiocyanate
LML – low DNA mass ladder
MDF – male determining factor
MYA – million years ago
NOR – nucleolous organising region
OD – Oligocene drowning
ORF – open reading frame
PAR – pseudo autosomal region
PP – Pliocene/Pleistocene
RAPD – randomly amplified polymorphic DNA
RE – restriction endonuclease
TCL – total chromosome length
TDF – testicular determining factor, or MDF
TSD – temperature dependent sex determination
TRT – transitional range temperatue
TSP – thermosensitive period

Appendix 3 : DNA sequence alignments

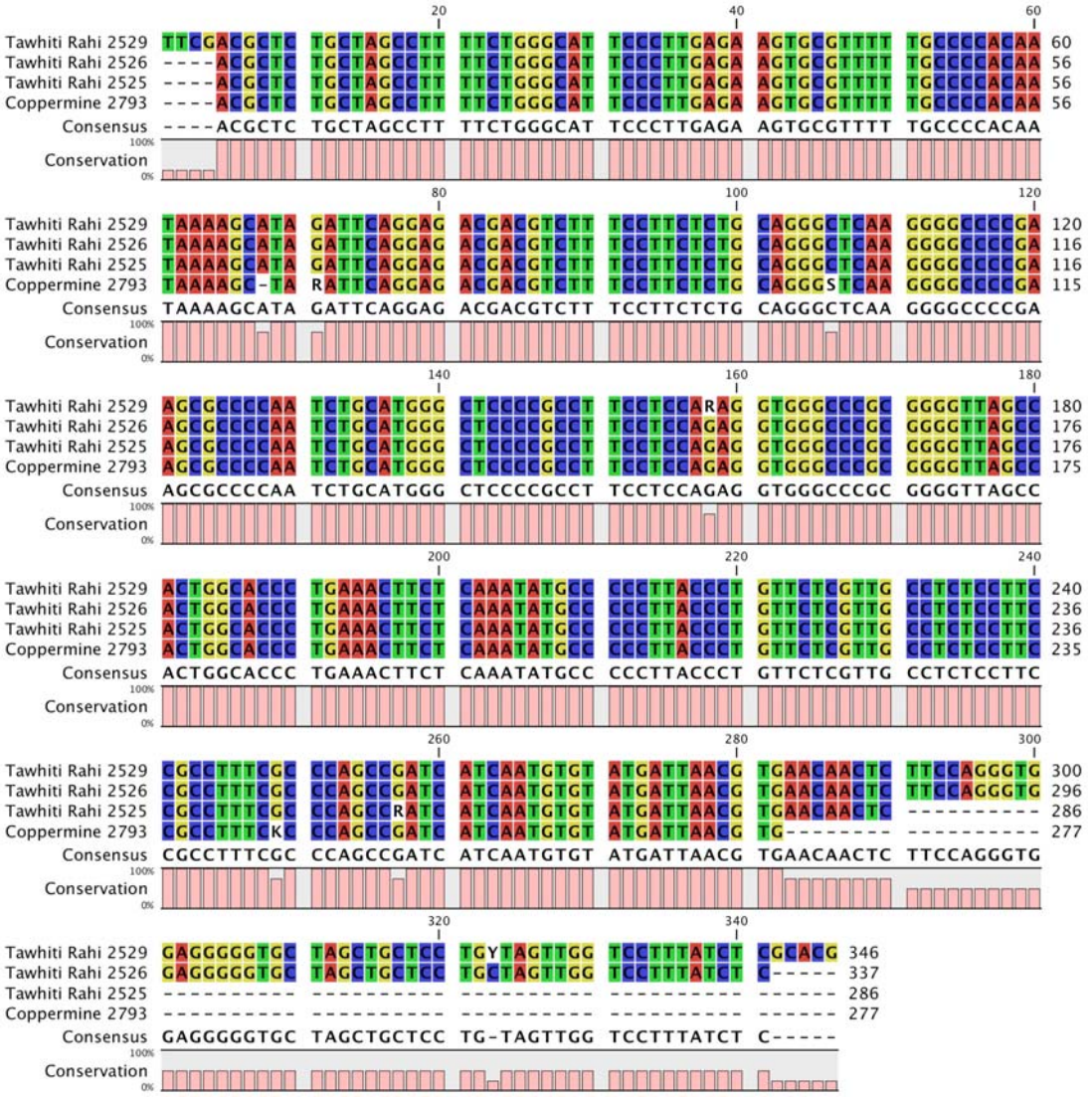
AMH Sphenodon *samples*



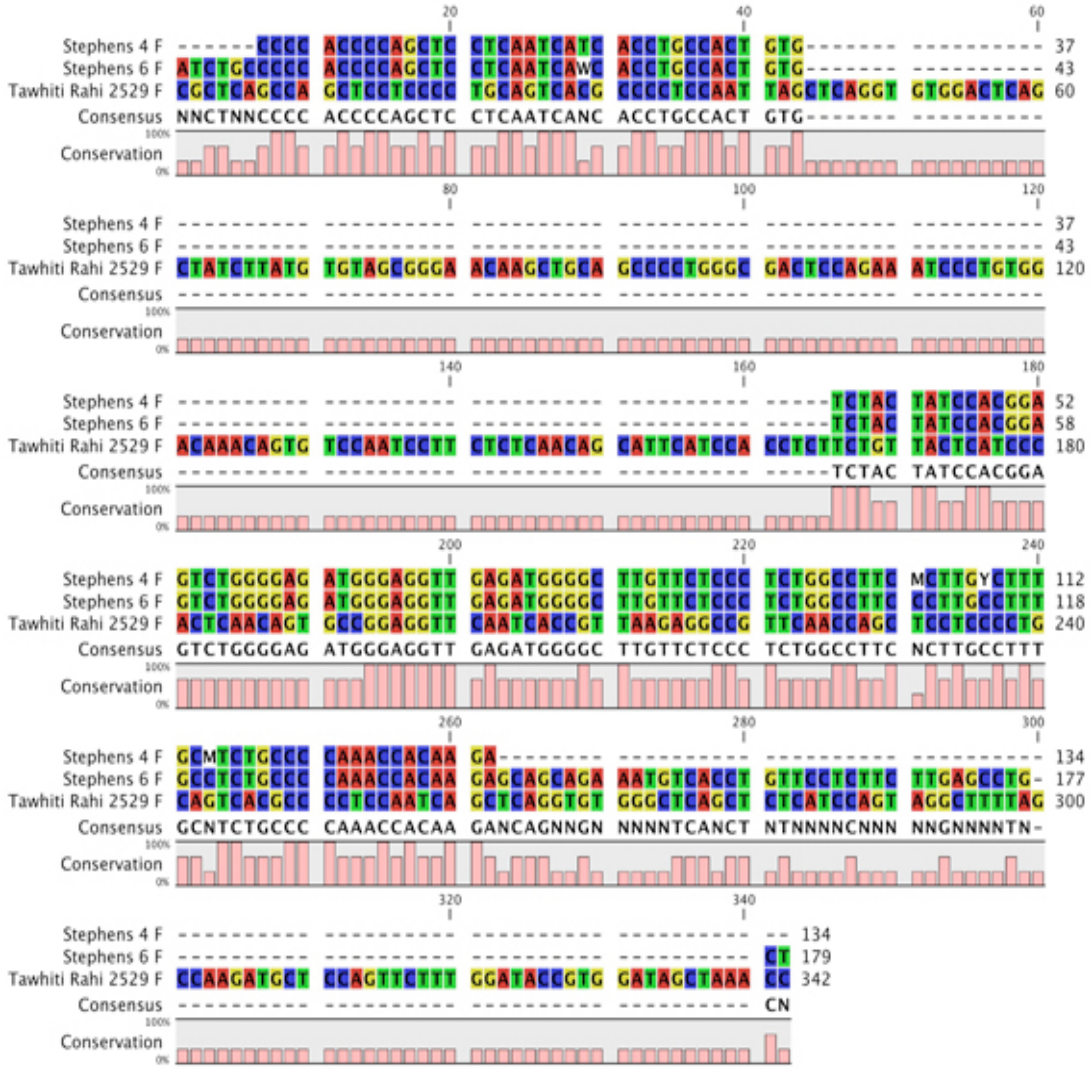
WT1 Sphenodon *sequence : Cook Strait*



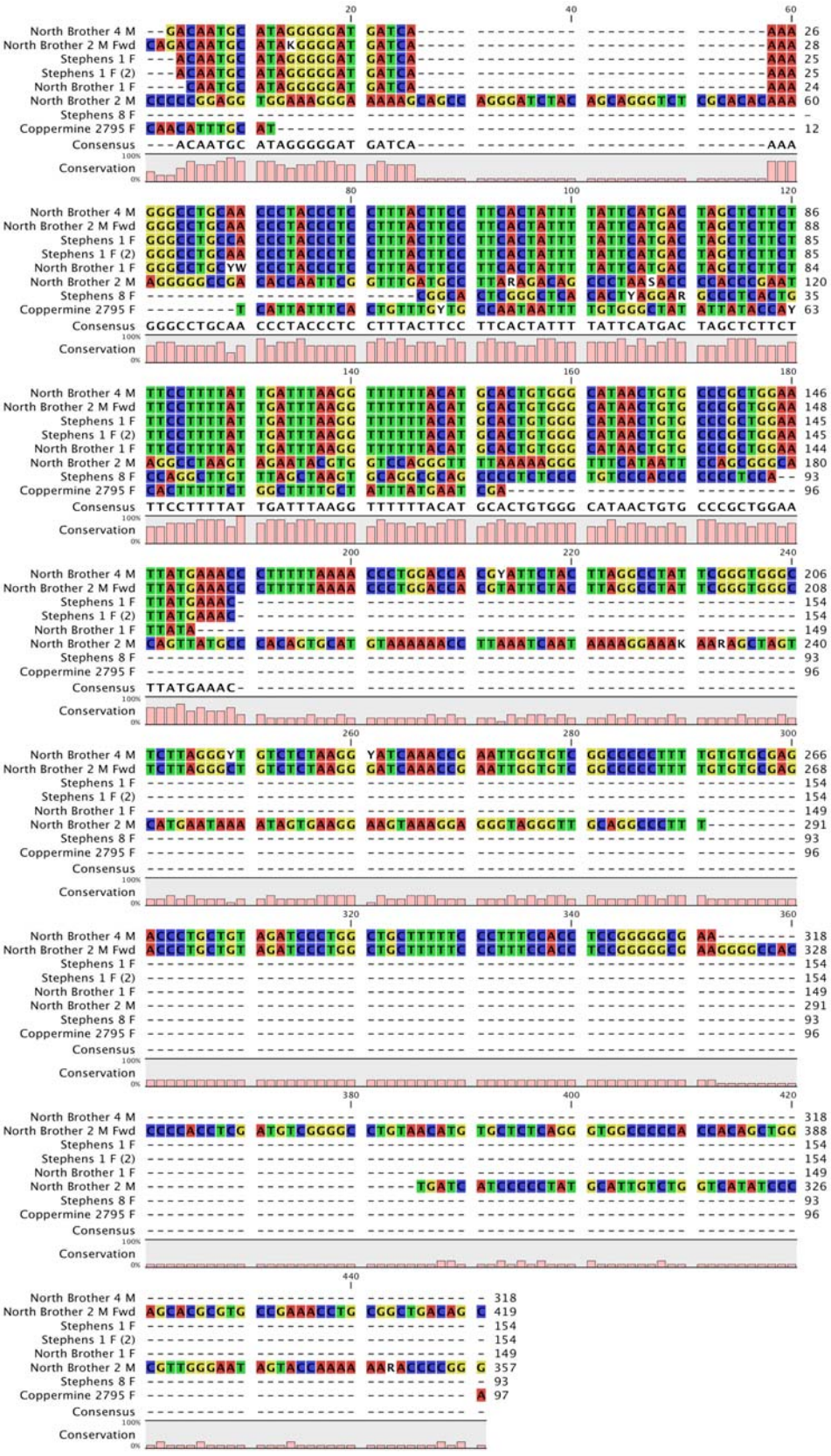
WT1 Sphenodon *sequence : North-eastern North Island*



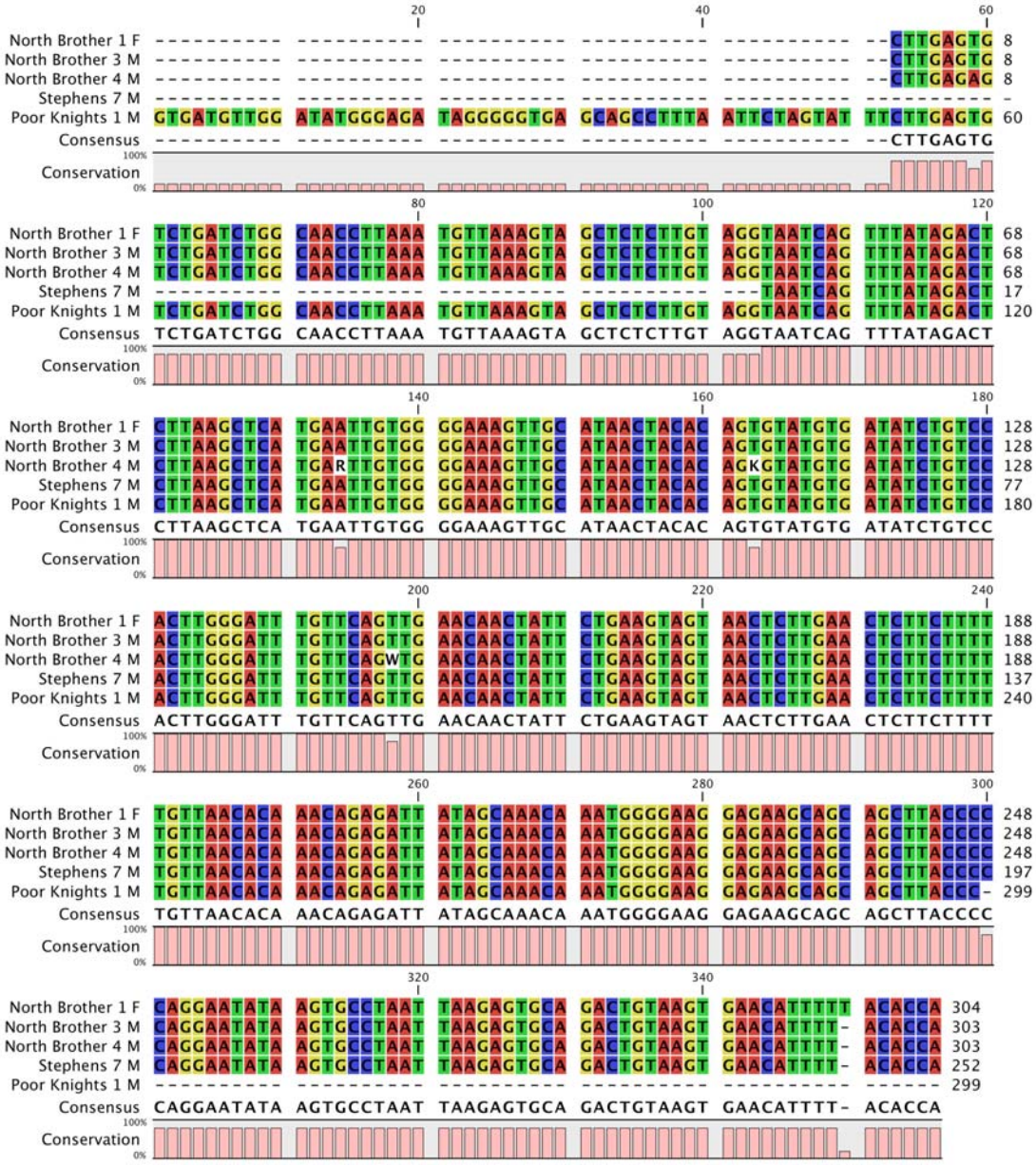
DMRT1 Sphenodon sequence



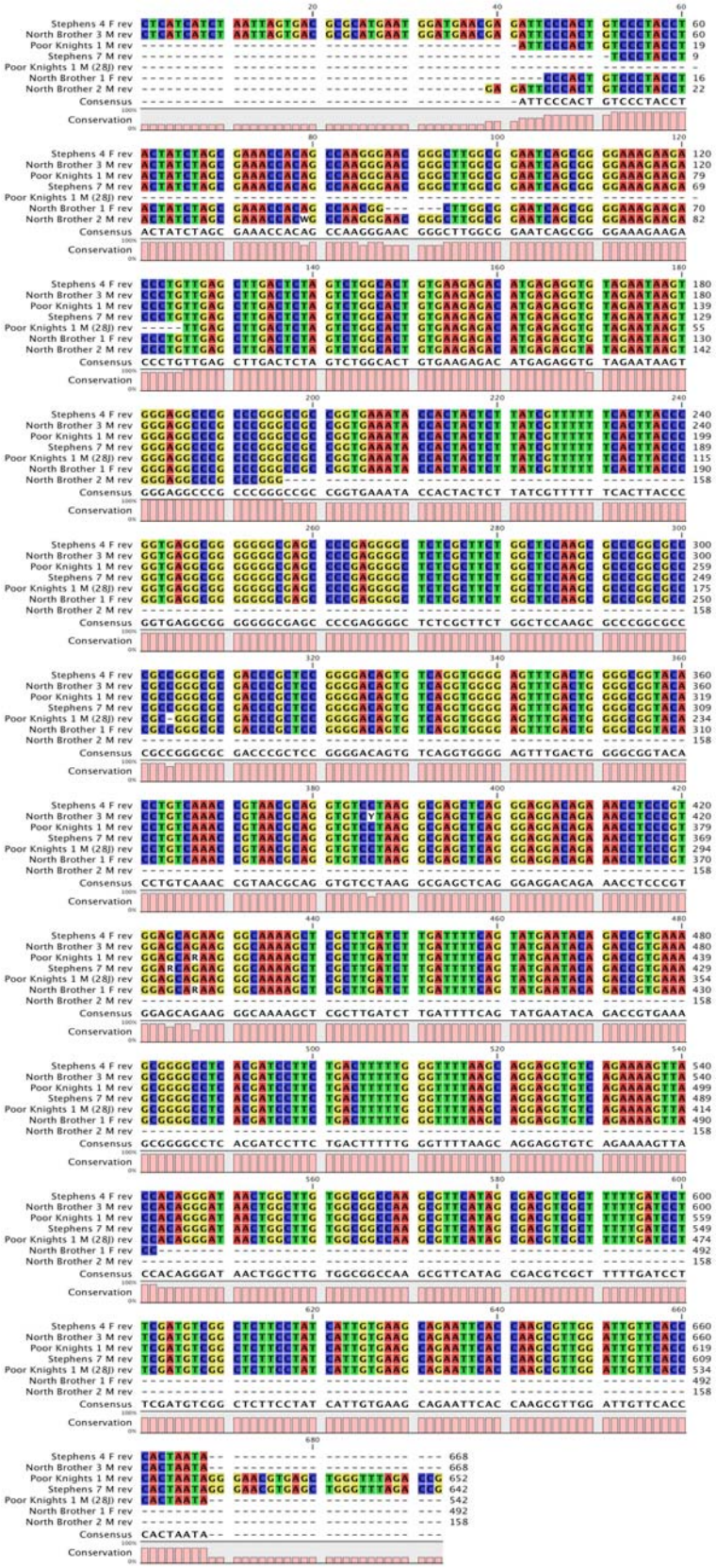
DMRT1 alternative Sphenodon *sequence*



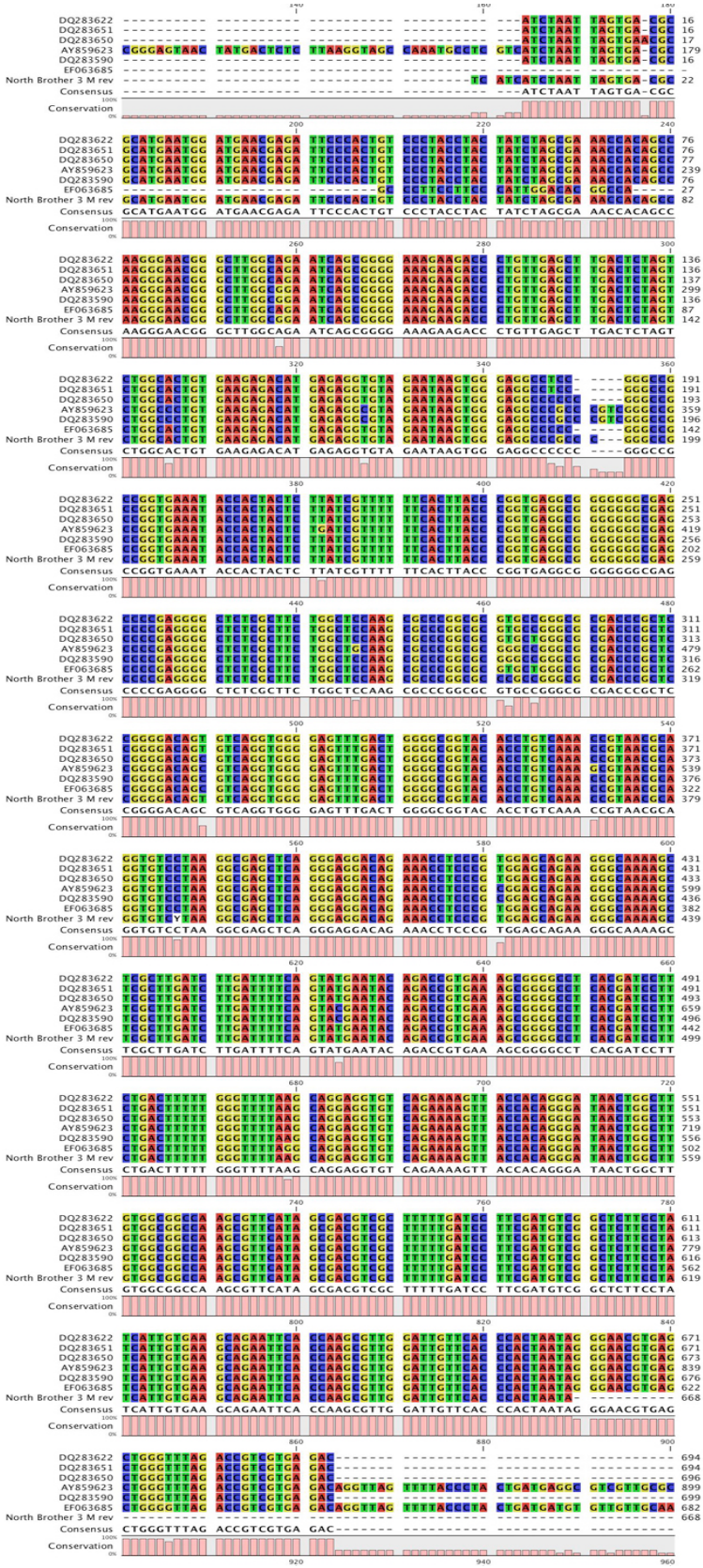
FoxG1 Sphenodon *sequence*



28S Sphenodon *sequence*



28S Reptile sequence alignment



Appendix 4 : Publications

T.B. Norris, G.K. Rickards, and C.H. Daugherty. Chromosomes of tuatara, *Sphenodon*, a chromosome heteromorphism and an archaic reptilian karyotype. *Cytogenetics and Genome Research* 105: 93-99 (2004).

Running title:Chromosomes of Sphenodon

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Abstract
We examined karyotypes of the endemic New Zealand reptile genus Sphenodon (tuatara) from four populations, finding a karyotype unchanged for at least 8000 years. Animals were karyotyped from four geographically distinct populations, representing three groups, namely S. guntheri, S. punctatus (Cook Strait group), and S. punctatus (northeastern North Island group). All four populations have a diploid number of 36 chromosomes, consisting of 14 pairs of macrochromosomes and four pairs of microchromosomes. Except for one animal with a structural heteromorphism, chromosomal differences were not found between the four populations nor between female and male animals. Similarity between Sphenodon and Chelonian karyotypes suggests an ancestral karyotype with a macrochromosome complement of 14 pairs and the ability to accumulate variable numbers of microchromosome pairs.

Introduction
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 by 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 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 squamates within the Lepidosauria (Benton 1985; Olmo 1986). 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 Reptilia. Examination of combined nuclear protein-coding gene sequences, totalling 785aa, demonstrated that the morphological grouping

of Sphenodon with squamates is not supported (Hedges and Poling 1999). Rather, the authors grouped Sphenodon with crocodiles, turtles and birds, a conclusion supported by examination of haemoglobin subunits (Brown 1993). Also, examination of Sphenodon and turtle sperm demonstrated a high level of similarity, and did not support a close relationship 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 Chelonia (turtles) is in debate. Traditionally classified as anapsid (Lee 1997; Wilkinson et al. 1997), recent molecular work (Hedges and Poling 1999; Platz and Conlon 1997; Zardoya and Meyer 1998) 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 Chelonia and Crocodilia (crocodiles) and extensive variation in morphology and number within the Squamata (lizards and snakes). Chelonian 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). Karyotypic evolution in turtles has decelerated and is now virtually non-existant (Bickham 1981). Crocodilia have two karyotypic forms, a predominately submeta- and meta-centric form, and a predominately subtelo- and telo-centric form. The only heteromorphism observed in crocodiles consists of variation in the size of secondary constrictions (Cohen and Gans 1970; King et al. 1986; Olmo 1986; Valleley et al. 1994). The Squamata on the other hand, are more speciose than other reptilian orders, and exhibit greater chromosome morphology variability (Olmo 1986). Evidence exists for chromosomal differences within the Squamata leading to speciation (King 1979; Moritz 1986).

Previous reports of tuatara chromosomes are limited, pre-date 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 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 nucleolous organising 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 on 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 35 specimens of known sex of S. punctatus northeastern group, for chromosome analysis. Sphenodon guntheri from North Brother Island (41o07' S, 174o73' E) were bled at night by drawing 0.5 ml of whole blood from the caudal vein using a heparinsed 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 (8-12oC) and returned by helicopter to the New Zealand mainland within 12 hours of sampling. Sphenodon punctatus Cook Strait group was represented by 12 tuatara from Stephens Island (40o40' S, 173o59' E) held in captivity, S. punctatus northeastern group was represented by one female Stanley Island (36o38' S, 175o53' E) tuatara held in captivity, and 34 tuatara from Ruamahua-iti (Aldermen Island group, 36o58' S, 176o5' 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 26oC, at which point 0.3 ml of 0.05% colchicine (BDH) was added to the cultures and samples incubated for a further 50 minutes. Where a second culture from Ruamahua-iti samples was available, colcemid was used as a cell cycle inhibitor (0.5 ml of 10µg/ml). Cells were then harvested using an adaptation of the human lymphocyte protocol (D. Romain pers comm.) as follows. After colchicine pre-treatment, cultures were spun for 10 minutes at 1000 rpm, the supernatant removed and cells re-suspended in 6 ml of hypotonic solution (1:1 0.4% KCl : 0.4% NaCl) for 20 minutes at 26oC. After hypotonic treatment, cultures were centrifuged for 10 minutes at 1000 rpm and the supernatant removed to 0.5 ml. The cell button 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 to the total volume to 6 ml. After 20 minutes incubation at room temperature, cells were centrifuged at 1000 rpm for 10 minutes, supernatant removed to 1 ml, and the cell button resuspended in 5 ml of fresh fixative. Centrifuging, supernatant removal and cell button resuspension was repeated 3-5 times until the supernatant became clear. Finally, 1-2 ml of fixative was added and the suspension stored at -20oC.

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 (KH2PO4, Na2HPO4, pH6.8) for 10 minutes. Good quality spreads were photographed on Kodak Tmax100 film using an Olympus AX70 photomicroscope. Karyotyping was done by aligning putative pairs in descending order by length. 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 structural 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 normalise 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 chromosome x/TCL of all 36 chromosomes)*100] minimises bias and was used to prepare Figure 2.

For C-banding, slides were placed in 0.02N HCl for one hour, rinsed, then treated with saturated Ba(OH)2 at 60o C for 30 seconds. After further rinses in distilled H2O, slides were placed in 2 x SSC at 60o C for one hour, 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 x 50 mm) coverslip over the liquid. Slides were then heated on a heating block until the mixture turned a 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 TCL and CI. Data for the range of each chromosomes TCL and CI (Table 1) has been used to prepare an idiogram (Fig. 3). Chromosome pairs one to five can be defined on the basis of TCL and CI. Chromosome pairs six and seven are very similar in TCL, but can be distinguished using CI as subtelocentric and telocentric chromosomes respectively. Further separation of the two pairs is possible as the telocentric pair always contains the Ag-NOR (Fig. 1b). Chromosome pair eight is, in all spreads, a submetacentric chromosome and can be defined using CI, although it is of similar TCL to pairs nine and ten. Chromosomes nine and ten are metacentric and comprise about 10% TCL (each 5%). They cannot be consistently paired as unequivocal chromologues due to similar TCL and CI. Chromosome 11 can be defined on the basis of CI and TCL as telocentric. Chromosome 12 is metacentric. Chromosomes 13 and 14 are sub-metacentric and comprise 7.5% TCL (about 3.5-4% TCL respectively). The difference in TCL is too small to separate the two chromosome pairs consistently. The remaining four pairs of micro-chromosomes comprise around 6.5% TCL. Their morphology cannot be accurately defined.

C- banding in the Sphenodon karyotype is shown as 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 seven of the Sphenodon karyotype (Fig. 1b).

We noted incidences of both heteromorphic and homomorphic sized pairs of Ag-NORs (Fig. 4) which varied within islands, although within the same animal Ag-NOR morphology was consistent.

Twenty-six spreads were examined from a Ruamahua-iti female tuatara, 19 with colchicine and 7 with colcemid as the division inhibitor. In all spreads one chromosome of pair 3 contained an extended long arm which was more than 40% larger than the other member of the pair (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 growth. The additional material did not stain C-band positive (Fig 5b) and did not contain a silver stained nucleolous organising region (Ag-NOR) (Fig 5c). A distribution diagram of chromosome 3 TCL (mutant and normal) comparing colchicine and colcemid treatment with a normal Sphenodon karyotype is presented as Fig. 6. The 'normal' chromosome 3 from the mutant 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 Chelonia are apparent in the macrochromosome complements. Haploid karyotypes from nine species of turtle, three species of Crocodilia and one species of Sphenodon are presented as Figure 7.

Discussion

The karyotypes of S. guntheri, S. punctatus (Cook Strait group), and S. punctatus (northeastern group) are identical, with a diploid number of 36 consisting of 14 pairs macrochromosomes and four pairs of microchromosomes with the exception of one animal. The chromosomes of males and females from the four populations are not distinguishable. C- and Ag-NOR banding has been demonstrated in the genus Sphenodon. C-bands are located in the centromeric region and all chromosomes within the karyotype have a similar banding pattern, although 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 seven in all populations. The 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, indicating that the tuatara karyotype has remained unchanged since isolation. Research by Hay et al. (accepted for publication Mol Biol Evol) examining allozymes, albumin, mitochondrial DNA, and nuclear DNA indicated unusually low variation, a situation reflected in our chromosomal data. The current study offers no contrary evidence to the division suggested by Hay et al. of S. guntheri, S. punctatus (Cook Strait group), and S. punctatus (northeastern group), 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, Bentzer et al. (1971) suggested for a structural heteromorphism to be unambiguously identified, a difference of 20% within the pair is required. Using this definition, one animal from 84 Sphenodon examined karyotypically did demonstrate a chromosomal heteromorphism. The additional material on one member of chromosome pair 3 is a duplication or interstitial or terminal growth, but is not a translocation 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 mutation 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 pers. obs.) did not appear to differ from other Ruamahua-iti animals.

Absence of C-banding suggests a recent origin for the additional material, although the limited distribution of C-bands in Sphenodon, and 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 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 will not be able to produce balanced meiotic products.

The karyotype of Sphenodon is signficantly different to all other squamates (Olmo 1986). Comparison of the Sphenodon karyotype with other reptilian orders allows consideration of karyotype evolution. Crocodilian karyotypes consist of two forms, a predominantly meta- and submeta- centric chromosome form and a subtelo- and telo- centric form (Cohen and Gans 1970; King et al. 1986; Olmo 1986; Valleley et al. 1994). The crocodilian diploid number ranges from 30 to 42, and the chromosomes are significantly different in morphology to Sphenodon (Fig. 7). The Chelonia have a diploid number between 50 and 70, however 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 Caretochelys insculpta (Family: Caretochelyidae), exhibit high chromosomal divergence compared to other Chelonians 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). We suggest, given the long period of divergent evolution between the Chelonia and Sphenodontia and high level of karyotypic conservancy, a karyotype for a hypothesised common ancestor is likely to have had a low diploid number, similar to Sphenodon, and a predisposition towards accumulating variable numbers of microchromosomes

through either macrochromosome breakages or de novo formation. The first 14 chromosome pairs of the hypothesised ancestor would have been similar in morphology to the chromosomes seen currently in Sphenodon and most Chelonian karyotypes. Evidence of chromosomal rearrangements are seen in NOR position between turtles and Sphenodon. No examples of a telomeric NOR on a large telocentric chromosome, as seen in Sphenodon, are seen in turtles (Bickham and Rogers 1985). A recent review of avian microchromosomes by Burt (2002) suggested the primitive avian karyotype had around 20 microchromosomes. A situation similar to Chelonia, suggesting 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.

The Chelonian order split from a reptilian common ancestor around 280 mya (million years ago) (Benton 1990) and the Sphenodontidae are thought to have split from the Lepidosauria line during the Triassic (210-230 mya) (Benton 1990). 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; Hedges and Poling 1999; Zardoya and Meyer 1998) 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.

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

Figure 1: Prepared karyotypes of Sphenodon, (a) giemsa stained karyotype of male S. guntheri, (b) Ag-NOR stained karyotype of female S. punctatus (northeastern group, Stanley Island).
Figure 2: In situ C banded chromosomes of Sphenodon.
Figure 3: Idiogram of Sphenodon indicating TCL, CI and Ag-NOR position.
Figure 4: Variation in Ag-NORs in Sphenodon, (a) Stanley Island (homomorphic), (b) Stephens Island (heteromorphic).
Figure 5: Partial karyotypes of a Ruamahua-iti animal with additional material on chromosome 3, (a) giemsa stained first 7 chromosome pairs; (b) C-banded first 7 chromosome pairs; (c) Ag-NOR stained first 7 chromosome pairs.

Figure 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 materal inhibited with colchicine; (o) other member of chromosome 3 pair inhibited with colchicine; (△) chromosome 3 with additional materal inhibited with colcemid; (▲) other member of chromosome 3 pair inhibited with colcemid.

Figure 7: Haploid karyotypes of: (a) Sphenodon punctatus; (b) Mauremys caspica (Emydidae); (c) Chelydra serpentina (Chelydridae); (d) Geochelone denticulata (Testudinoidea); (e) Platysternon megacephalum (Chelydridae); (f) Caretochelys insculpta (Carettochelyidae); (g) Trionyx spiniferus (Trionychidae); (h) Kinosternon scorpiodes (Kinosternidae); (i) Chelonia mydras (Chelonioidea); (j) Crocodylus palustris; (k) Caiman latirostris; (l) Alligator mississippiensis. This figure has been constructed with material from the current study; Bickham and Baker 1976a; Bickham and Baker 1976b; Bickham et al. 1980; Bickham et al. 1983; Cohen and Gans 1970; Haiduk and Bickham 1982; Valleley et al. 1994. Chromosomes have been resized to allow comparison.

Table 1: Quantitative description of Sphenodon chromosomes. The range of TCL and AR is listed.

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