Is There Sex-Specific DNA In The Tuatara, A Reptile With Temperature-Dependent Sex Determination?

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Abstract

It is widely viewed that there is a dichotomy of sex-determining mechanisms within the reptiles: species either exhibit genotypic sex determination or temperature-dependent sex determination (TSD). However, very few species have been examined for both modes. Although it is often considered that the two mechanisms are mutually exclusive, there is evidence that there may be a weak genetic sex-determining mechanism in species in which the primary sex-determining mode is temperature-dependent sex determination. This infers that some TSD individuals may be sex-reversed; that is, their sexual genotype is discordant with their sexual phenotype. This hypothesis of an underlying genotypic system may also be linked to the question of the evolution of sex-determination within the reptiles. The discovery of sex-specific DNA within a TSD reptile could suggest that genotypic sex determination is ancestral and TSD has evolved many times over within independent reptile lineages.

This study tested the hypothesis that there is a genetic component to sex determination in TSD species. This was accomplished by searching for sex-specific DNA in the tuatara, a reptile with temperature-dependent sex determination, using two different molecular genetic techniques.

The major undertaking of the experimental programme was the completion of a comprehensive minisatellite DNA profiling survey. This incorporated 14 restriction enzymes and five different polycore DNA probes; in total, 66 different probe/enzyme combinations were tested for tuatara genomic DNA. None of the DNA profiles revealed sex-specific fragments. Furthermore, a significant difference in mean fragment numbers for males and females was not detected for any of the probe/enzyme combinations.

In addition, a RAPD analysis was conducted in a search for a molecular sex marker in the tuatara. A total of 27 random-sequence oligonucleotide primers were used to successfully amplify anonymous products from the genomic DNA of male and female tuatara. Again, no sex-specific fragments were detected.

Thus, evidence of sex-specific genetic differences in the tuatara was not found. This result fails to refute the null hypothesis that there are underlying sexual genotypes in the tuatara. This finding may reflect the absence of genetic sex differences in the tuatara. Alternatively, it might also be the result of accidental inclusion of sex-reversed individuals within the analyses, a situation which could have obscured the sex-specific nature of any sex-linked fragments. It would appear that the key to solving the question of sex-specific DNA within TSD reptiles such as the tuatara lies with the problem of ensuring sex-reversed individuals are excluded from molecular analysis.

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Thesis Structure And Format

I have arranged this thesis in the following manner. **Chapter 1** gives a general introduction to the tuatara, describing its evolutionary position, taxonomic status, distribution, and general biology. The aspect of tuatara ecology that is the focus of this study, sex determination, is introduced. Finally, the aim of the thesis is explained, in terms of its relevance to scientific study and also its application to the conservation of this rare species.

In **Chapter 2** I provide background material and theory relevant to this research. The phenomenon of temperature-dependent sex determination (TSD) is described in some detail, including a general review of its occurrence within the four major living groups of reptiles (including the tuatara). The concept of genetic sex differences in a species that exhibits temperature-dependent sex determination is examined. Current insights into the possible molecular mechanisms of TSD are discussed, particularly with respect to reconciling the concept of an underlying genotypic mode of sex determination interacting with the TSD mechanism. This discussion is used to give a context to the methodological approach of the study.

Chapter 3 presents a detailed account of the major experimental undertaking of the investigation; a comprehensive minisatellite DNA profiling survey aimed at testing the hypothesis that tuatara have sex-specific DNA. This survey is divided into three distinct phases, consistent with three different sets of tuatara blood samples. The results of a large number of probe/enzyme combinations are presented and discussed.

Chapter 4 is an account of a brief investigation employing RAPD (Randomly Amplified Polymorphic DNA) assays as a further attempt to detect a molecular marker for gender in this species. In this study, a large number of random-sequence oligonucleotide primers were used to amplify anonymous PCR products from male and female tuatara. Results are presented and discussed.

Chapter 5, the final chapter, presents a summary of the findings of the research. Following a general conclusion, there is a discussion of potential avenues of investigation for future research into the question of sex determination in the tuatara (and TSD reptiles in general).

Contents

List of Figures

List of Tables

1

Introduction: The Tuatara, *Sphenodon* spp.

1.0 EVOLUTIONARY POSITION

Tuatara (Spenodon spp.) are rare, medium-sized , reptiles that are endemic to New Zealand. Once believed to be lizards, they are now known to be the only surviving representatives of the order Sphenodontida (also Rhynchocephalia) (Crook, 1975; Fraser, 1988). Sphenodontida is a relict group of reptiles whose immediate ancestry extends back over 200 million years to before the age of the Dinosaurs. The order has equal taxonomic status to the three other living reptile groups: Chelonia (turtles and tortoises), Squamata (lizards, snakes, and amphisbaenians), and Crocodilia (crocodiles, alligators, caimans, and the one surviving species of gharial). Hence tuatara are of exceptional significance to the evolutionary history of reptiles.

Fossil sphenodontids were small to medium-sized reptiles that lived approximately 120-225 million years ago. The skeletal features of the tuatara have changed very little since this time, and its structure is almost identical in form to the ancient reptiles that were in Europe 140 million years ago. The ancient sphenodontids were at their most abundant during the lower Triassic period (200mybp), and fossils indicate their presence in Europe, Africa, Asia and the Americas, suggesting they were once widespread across the supercontinent of Pangaea (Dawbin, 1982a; Cree & Butler, 1993). The sphenodontid lineage continued throughout the Jurassic and into the lower Cretaceous, but began to decline about 100 million years ago. The lineage from which tuatara evolved was probably isolated when the New Zealand land mass broke away from the southern supercontinent of Gondwanaland about 80 million years ago (Crook, 1975; Lockley, 1980; Robb, 1980). By about 60 million years ago, the Sphenodontida

had become extinct everywhere with the exception of the direct ancestors of the tuatara. It is not known why the tuatara lineage survived while all other sphenodontids died out, but it is thought that the absence of endemic land mammals in New Zealand may have been significant (Cree & Daugherty, 1990; Cree and Butler, 1993).

The tuatara is morphologically very similar to lizards, and these similarities in external structure lead to its initial classification as an agamid lizard. (There are in fact remarkable similarities between male tuatara and eastern water dragons (*Physignathus lesueruii*) of Australia, both in terms of size and external morphology [Dawbin, 1982a]). There are several important distinctions in the primitive characteristics of the tuatara, however. First, the tuatara has a completely diapsid skull with two temporal fossae per side, and the lower temporal bar is rigid and complete (Günther, 1867; Dawbin, 1962). The skull is remarkably similar to Permian eosuchians not far removed from stem reptiles (Dawbin, 1982a). Second, the teeth of tuatara and other sphenodontids are acrodont i.e. fused to the jawbone instead of being implanted in sockets, and occur as a single row of teeth in the lower jaw which fits between two upper jaw rows (Dawbin, 1982a). Third, the caruncle (egg-breaker) is a horny epidermal projection which is unrelated to teeth and is also found in the Chelonia and Crocodilia, but differs from the calcified egg tooth of the Squamata (Dawbin, 1982a). Fourth, the ribs of the tuatara are single-headed and bear uncinate processes like those present in birds and crocodiles but absent in lizards (Günther, 1867; Dawbin, 1962). Fifth, unlike all other reptiles, the male tuatara lacks a copulatory organ (Günther, 1867, Dawbin, 1962). Sixth, while the external ear is reduced in some Squamates and Chelonians, it is entirely absent in the tuatara, as is the tympanic cavity (Baird, 1970). Finally, the structure of both the heart and lungs in tuatara have been described as the most simple of all the living reptile species (Dawbin, 1982a).

In 1867 Albert Günther realised the error of the classification of the tuatara as a lizard, finding more similarity in its structure to that of Mesozoic fossil stem reptiles (Thecodontia), from which dinosaurs, birds and mammals evolved. Günther therefore created a separate reptile order, Rhynchocephalia, to recognise the significant differences between the tuatara and lizards (Günther, 1867).

Although it seems to be almost universally accepted that the tuatara is quite distinct from lizards, there is still some contention over the exact evolutionary relationship of sphenodontids to the other extant reptile lineages: Squamata, Chelonia, and Crocodilia. It is widely accepted that Sphenodontida is the close sister-taxon of Squamata, and in most taxonomies the two orders are united as the Lepidosauria (Evans, 1984; 1988, Fraser, 1986; Gauthier *et al*, 1988; Zug, 1993; Pough *et al*, 1998). Given the striking external similarities of tuatara and some agamid lizards, a sister-group relationship would appear to make sense. Pough *et al* (1990) go further and suggest that separation at the ordinal level based upon anatomical differences is not warranted. They claim there are derived characters of the tuatara and its fossil relatives that link these animals to lizards, and furthermore, that 'differences between the sphenodontids and the other [fossil] diapsids grouped with them as rhynchosaurs have become apparent and have weaked the basis for regarding rhynchocephalians as a natural group' (p 494).

On the other hand, there are at least two phylogenetic studies which not only support the generally accepted view that Sphenodontids represent a truly ancient lineage (thus deserving separate ordinal status), but actually dispute the equally accepted view that the Squamata and the Sphenodontida form a natural sister-taxon.

The first of these studies examined the ultrastructure of tuatara spermatozoa in relation to other major amniote groups (Healy and Jamieson, 1992). A cladistic analysis of apomorphic and plesiomorphic characters suggested that a close, sister-group relationship between Sphenodontida and Squamata is not warranted, although it did confirm the very ancient status of the tuatara (Jamieson and Healy, 1992). For example, the only spermatozoal features shared by tuatara and the Squamata also occur in turtles, *Caiman*, some birds and monotremes, and moreover, all of the apomorphic characters occurring in Squamata are absent from the tuatara. In this analysis, the closest reptile taxon to the Squamata was shown to be the Crocodilia, with the Sphenodontida (represented by the tuatara) forming the next outgroup, and the Chelonia forming the most anciently divergent order amongst the extant reptiles.

A more recent study analysed the sequence data from mitochondrial DNA and several nuclear genes of representatives of each of the major living reptile groups: crocodilians, turtles, tuatara, squamates, and birds (with mammals as the outgroup)

(Hedges and Poling, 1999). A molecular analysis of four nuclear protein-coding genes also supported a dismantling of the Lepidosauria, suggesting that turtles, crocodiles, and birds formed a natural group, with tuatara as the next sister-taxon. Squamates were positioned as the basal reptile group. While the findings of this study agreed with the conclusion of Jamieson and Healy (1992) that Sphenodontida and Squamata are not closely-linked taxa, the two studies did disagree over the evolutionary position of turtles amongst the reptile lineages. The earlier study supported the classical morphological phylogeny which positions the anapsid turtles as the most anciently diverged reptile group, while the recent molecular analysis suggested that they are most closely related to the crocodilians, with birds as the next sister-taxon. It would appear that the evolutionary relationships amongst the major extant reptile groups, including the Sphenodontida, are far from resolved.

1.1 PAST AND PRESENT DISTRIBUTION

It is believed the tuatara, or its immediate ancestors, arrived in New Zealand whilst the country was still a part of Gondwanaland, from South America via Antarctica. It is also thought that the sphenodontid lineage survived here due to the absence of predatory land mammals (Cree and Butler, 1993), while the group became extinct everywhere else in the world by about 60 million years ago.

Fossil records indicate that as recently as 1000 years ago the tuatara was widely distributed over the North and South Islands of New Zealand, but by 1900 was extinct on the two mainland islands (Newman, 1878; Buller, 1879). The major causes of this large-scale extinction on the mainland over the past 1000 years were probably predation by early Polynesian settlers and introduced mammals such as rats, cats, pigs and dogs, and major changes in habitat due to fires and land clearing (Daugherty and Cree, 1990). Within the last century, tuatara have become extinct on at least ten offshore islands, a figure which is probably conservative due to the fact that tuatara are likely to have been isolated on numerous offshore islands when the last ice-age ended about 10,000 years ago, and may have died out on many such islands for which no historic records exist (Daugherty *et al*, 1990). Currently, the tuatara is restricted to around 30 coastal islands in the Cook Strait and the East Cape of the North (Fig. 1.1) (Daugherty and Cree, 1990). These islands range in size from 0.4 to 3083 ha. Five of

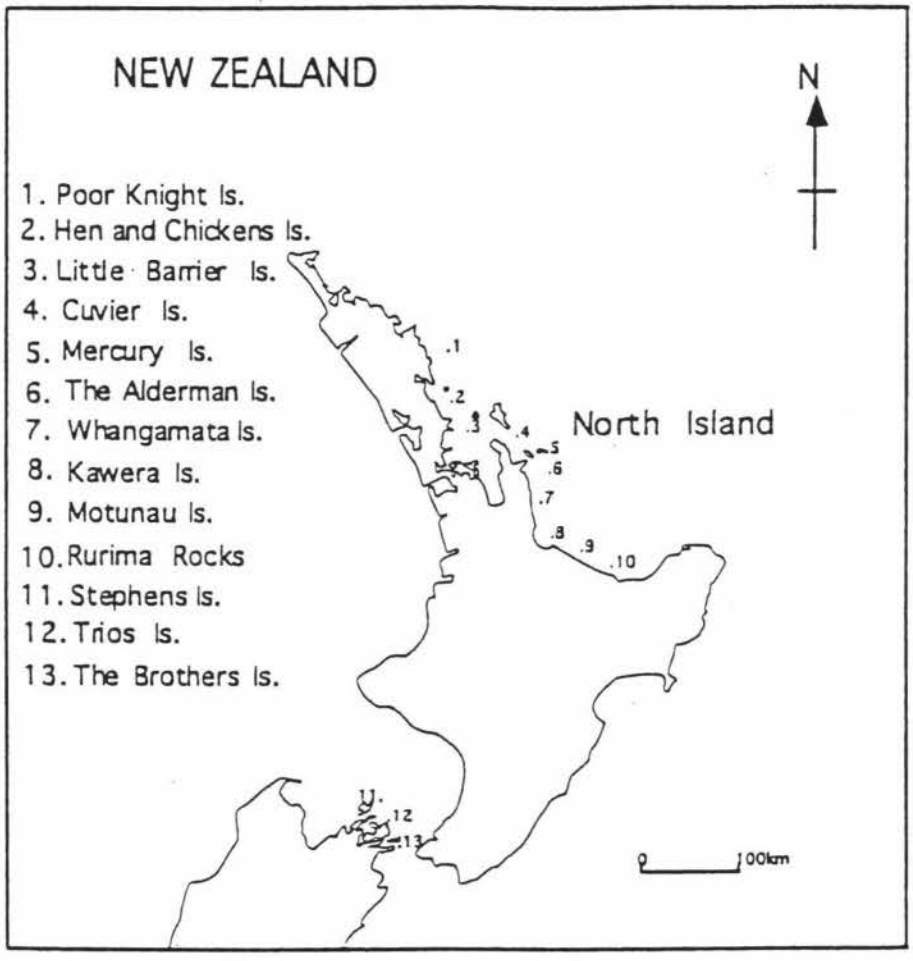

Figure 1.1

Map of New Zealand, showing the locations of the 30 offshore islands that have resident tuatara. The islands are predominantly located off the east coast of the North Island and in Cook Strait.

these islands are in the Cook Strait, and the remaining 25 are off the north-eastern coast of the North Island in the Bay of Plenty and Hauraki Gulf (Cree and Butler, 1993). The survival of tuatara was confirmed on 27 of the islands in surveys between 1988-1991, whereas three small islands of the Aldermen group (Bay of Plenty) have not been surveyed since the early 1970s, when tuatara or their faeces were seen (Crook, 1973). There has also been several releases of captively-reared juveniles onto predator-free islands, such as Somes Island and Titi island.

1.2 TAXONOMY AND CONSERVATION STATUS

Tuatara are classified as rare, and are recognised within New Zealand, and even on the international scene, as a species at risk of extinction. Since 1895, adult tuatara have been legally protected from unauthorised collection, and their eggs since 1898 (Cree and Butler, 1993). Described as 'rare' in the International Union for the Conservation of Nature Red List (IUCN, 1988) and as 'regionally threatened' by Bell (1986), they are also listed in Appendix 1 of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora).

Despite protection by both New Zealand and international law, tuatara numbers continue to decline (Cree and Butler, 1993). The amount of habitat occupied by tuatara when humans first arrived in New Zealand has decreased to less than 0.5% of its original range. For the last century, tuatara have been confined to offshore islands, and during that time at least ten of 40 known island populations have become extinct (Daugherty *et al*, 1990). It is estimated that without conservation efforts to slow or reverse this trend, at least four, and maybe up to seven of the 30 remaining populations will become extinct within 50 years (Daugherty *et al*, 1990; Cree and Butler, 1993). The major threat to tuatara populations is thought to be active predation by introduced rat species, including kiore, the Polynesian rat, which is believed to prey upon tuatara eggs and juveniles (Daugherty and Cree, 1990; Cree and Butler, 1993). Of the 30 islands on which tuatara live (27 confirmed in recent years), 22 are believed to be rat-free, but all face the continual threat of accidental rat introduction. About half of the total tuatara population is found upon Stephens Island in the Cook Strait, where there is believed to be at least 30,000 adults.

Following the publication of Günther's 1867 paper recognising the tuatara's distinctiveness from lizards, a number of papers describing aspects of life history, appearance and habitats of tuatara appeared (Knox, 1870; Buller, 1877, 1878, 1879; Newman, 1878; Reischek, 1882, 1886; Colenso, 1886). Within these papers there were also attempts to distinguish three separate subspecies of tuatara (*S. punctatus*, *S. guntheri*, and *S. diversum*) based on such criteria as colour variation amongst populations (Dawbin, 1982a). Authors such as Dawbin (1982a) dismissed these criteria as invalid: '...in the case of colour variation, the full range can be found in a single population such as that on Stephens Island' (p150). Consequently, up until the early part of this decade, the tuatara was officially recognised for management and legislative purposes as consisting of one single species, *Sphenodon punctatus*.

In 1990, however, a genetic study by Daugherty *et al* recommended reinstatement of an older taxonomy of tuatara, which recognised a second living species, *S. guntheri*. *S. guntheri* consists of a single population of about 300 adults on North Brother Island in the Cook Strait. The study involved a survey of both allozyme and morphological variation in 24 of the 30 island populations of tuatara, but it was the allozyme variation alone which suggested genetic divergence between some of the populations. Whether allozyme variation in itself is sufficient to warrant separate species status for the North Brother Island population is perhaps a little contentious, but the official recognition of that status has undoubtedly been beneficial in procuring more conservation resources for the protection of this endangered, and clearly genetically-different, population. The Department of Conservation (DoC) Recovery Plan for the tuatara (Cree and Butler, 1993), recognises three distinct 'genetic types' of tuatara, based upon the findings of Daugherty *et al* (1990): Northern tuatara (*Sphenodon punctatus punctatus*), composed of the populations on the islands in the Bay of Plenty and Hauraki Gulf; Cook Strait tuatara (*Sphenodon punctatus* – an unnamed subspecies); and the Brothers Island tuatara (*Sphenodon guntheri* – existing only on North Brother Island). The classification of two distinct species now appears to be widely accepted (Daugherty *et al*, 1994).[1]

[1] Occasionally throughout this thesis, tuatara may be referred to as simply, "this species" or "the species", for the sake of simplicity. This is not to advocate single species status; where such an expression is used, it is intended to include all the genetic types of tuatara. The species issue is essentially irrelevant to this study, as it would seem reasonable to assume that these obviously very

A thorough description of the conservation efforts being undertaken to ensure the long-term survival of this unique and endangered animal is presented in the Department of Conservation's Threatened Species Recovery Plan No.9 (Cree and Butler, 1993).

1.3 BIOLOGY AND ECOLOGY OF THE TUATARA

Sexual dimorphism in adult tuatara is amongst the most marked of all reptiles (Dawbin, 1982b). Males are significantly larger in size than females, weighing on average 800gm (occasionally over 1kg), and measuring up to 600mm in length (mean snout-vent length of 265mm). Fully grown females generally weigh about half as much, and may reach 450mm in length (mean snout-vent length of 214mm) (Dawbin, 1982a; 1982b). Adult males typically have a prominent nuchal crest and another crest down the mid-line of the back, both with prominent soft spines. The crest and spines of the female are not as conspicuous. The male also has a proportionately larger head and thinner abdomen than the female (Dawbin, 1982b).

The colour of tuatara changes throughout their lifetime. Hatchlings tend to be uniformly fawn or brown with different colourations on their head and throat. In *S. punctatus*, adult colour varies in background from shades of grey, olive, or even brick-red, and there are spots with varying degrees of distinctiveness (Dawbin, 1982a). *S. guntheri* adults tend to have very white or green spots on a light green background colour (Thompson *et al*, 1992).

Nearly every aspect of the life-history of the tuatara seems to occur at a slow pace, and growth is no exception. Sexual maturity is reached at approximately 13-15 years of age (Cree and Daugherty, 1990), but possibly as late as 20 years (Dawbin, 1982b). Growth continues for a long time after sexual maturation, however, to about 30 years of age (Castanet *et al*, 1988) or maybe even 60 years of age (Dawbins, 1982b), when an asymptotic size limit is approached. Based on the presence of epiphyses in their bone structure, it is thought that the tuatara has determinant growth (Dawbins, 1982a).

closely related taxa have an extremely similar, if not identical, mechanism of sex determination. Moreover, only one species, *S. punctatus*, was examined in this investigation (which included two populations: Lady Alice Island and Stephens Island).

Potential longevity may easily exceed 100 years (Lockley, 1980; Robb, 1980), but it is generally believed that the life span is likely to average around 60 years. Even so, this life span exceeds that of most, if not all, lizard species (Cree and Daugherty, 1990).

Tuatara live in a variety of open scrub and coastal forest habitats on about 30 offshore islands, which they share with breeding colonies of sea birds such as prions, petrels, and shearwaters. The islands are typically cool, rugged, and windswept, and their inaccessibility is undoubtedly one of the reasons why they remain relatively free from predators such as rats. Although juveniles appear to show more diurnal behaviour, adult tuatara are predominantly active at night, particulary in mist or rain (Cree and Daugherty, 1990), which perhaps reduces the risk of dehydration. When not out in the open foraging for food, they are inside burrows which may extend as far into the ground as five metres. Most activity occurs within a three metre radius of the burrow, although they forage up to 25 metres away from an entrance (Newman, 1978). While tuatara may sometimes excavate their own burrows, they typically commandeer one of the numerous forest-floor burrows of the prions, petrels, or shearwaters with whom they share their habitat. During daylight, individuals often bask in areas of sunlight, but usually within close proximity to a burrow entrance (Daugherty and Cree, 1990). Tuatara sit at the top of the food chain within their island communities, and their diet consists primarily of invertebrates (74%), small lizards (4%) and plant material (14%), but they will eat small sea birds or their eggs when available (4%), and adults have been known to eat juvenile tuatara (Walls, 1981). The two upper rows of acrodont teeth in tuatara fit around a single row in the lower jaw, and the jaws work together in a specialised shearing motion which can easily decapitate prey as large as fairy prions.

One of the most remarkable features of tuatara biology is their 'cold-adapted' poikilothermic metabolism in comparison with other reptile species. Like most reptiles, their metabolic rate is affected by ambient temperature, but the optimal activity range is considerably lower in the tuatara than in other reptile species. The optimum temperature for activity is approximately 17-20°C, and activity is possible at air temperatures down to as low as 7°C (the critical minimum is about 3-4°C) (Newman, 1978). With the possible exception of some New Zealand lizards, all other reptile species exhibit no activity at the temperatures at the lower end of the activity range of the tuatara (Dawbin, 1982a). On Stephens Island, home to the largest

remaining population, average night-time temperatures are approximately 16°C in summer, and 9°C in winter months. While tuatara are most active on warmer nights, large males have been known to remain guarding burrow entrances at temperatures as low as 6°C (Daugherty and Cree, 1990). The metabolism of tuatara is so slow that over winter, an adult can survive for up to six months without feeding. In fact, a temperature of 30°C for more than a few hours is lethal to the tuatara, whereas it would probably be within the optimum activity range for many temperate and tropical reptile species.

Another consequence of their low metabolic rate is slow reproduction. In addition to taking 10-15 years to reach sexual maturity, females are only able to reproduce once every four years, although males are able mate annually (Daugherty and Cree, 1990). In contrast, most oviparous lizards lay at least one clutch of eggs a year. Mating takes place in February-March, immediately prior to which males establish territories (about 25 square metres in size) which they defend vigorously against trespassing males. Courtship and mating behaviour is complex (Cree and Daugherty, 1990). Fertilisation of the eggs, which have taken three years to develop within the ovary, occurs a month after mating, and shell deposition takes a further seven months. In November, the females migrate up to a few hundred metres to preferred nesting sites, and eggs are laid approximately 150mm deep in loose soil or within a burrow. The nests are often congregated in groups, or rookeries, usually in exposed areas where soil temperatures are warmer than in native forest. Hatching takes place during summer, 11-16 months later, and the average clutch size is 8-15 eggs (Lockley, 1980; Robb, 1980). Hatching occurs sooner in those nests which experience a warmer average temperature during the incubation period, and embryo development actually pauses during winter months. Sex of the embryo is determined by the temperature experienced during the incubation period (Cree *et al*, 1995) (but see section 1.4). There is no parental care after hatching, juveniles having to feed and fend for themselves. It is thought that the diurnal activity of young juveniles may be a strategy to avoid cannibalism by adult tuatara (Daugherty and Cree, 1990).

Adult tuatara seem to have no natural predators, with the possible exception of some extinct predatory bird species (Holdaway, 1989). However, the decline, and/or extinction, of many island populations of tuatara within the last hundred years appears

to be directly linked to the presence of introduced rat species on those islands. Tuatara have disappeared from all those islands with Norway (*Rattus norvegicus*) or ship rats (*Rattus rattus*), as these species are believed to prey upon both adult and juvenile tuatara. Kiore (*Rattus exulans*) prey only upon tuatara juveniles and eggs (Case and Bolger, 1991; Newman, 1988), thus on those islands on which kiore and tuatara co-exist, there is no juvenile recruitment into the aging, adult populations (Cree and Butler, 1993). In addition, kiore may deplete the invertebrate and small vertebrate fauna on these islands, thereby reducing available food resources for tuatara. Rabbits are also thought to have the same effect in tuatara habitats. Tuatara conservation is thus largely dependent on ensuring their habitats remain free of rats and other introduced mammal species.

1.4 SEX DETERMINATION IN THE TUATARA

There are two types of sex-determining mechanisms in vertebrates (Bull, 1980). In most vertebrate species, sex is determined genetically (genotypic sex determination, or GSD) but in many species, sex is strongly influenced by the effect of environmental factors upon the incubating egg (environmental sex determination, or ESD). Within reptile species with ESD, the crucial environmental factor is the temperature at which eggs are incubated. More specifically, it is the ambient temperature during critical periods of egg incubation that influences the eventual sex of the embryo (Bull, 1983; Lance, 1997). This is known as temperature-dependent sex determination, or TSD.

Recently, Cree *et al* (1995) provided evidence to show that the tuatara, like many other species of reptiles, exhibits TSD. TSD is present to a varying extent in all three of the other orders of living reptiles (Viets *et al*, 1994; Lang and Andrews, 1994; Ewert and Nelson, 1991), and the results of Cree *et al* (1995) showed it was also the mechanism of sex determination in both species of tuatara, the only surviving members of the fourth reptile order, Sphenodontida. Even before this finding, it was suspected this was the case for tuatara (Cree and Thompson, 1988), as total sex biases among locatable offspring had been observed in three zoo-incubated clutches. Furthermore, it was known that the species lacks heteromorphic sex chromosomes (Wylie *et al*, 1968), a necessary precondition for TSD (Bull, 1980; Janzen and Paukstis, 1991). Cree *et al* (1995) presented data on the sex ratios of *S. punctatus* individuals from experiments

conducted in 1986, in which wild eggs were incubated under controlled conditions (Thompson, 1989; 1990). Since juvenile tuatara can not be reliably sexed by their morphology, the experimenters waited several years until the sex of the tuatara could be reliably determined by laparoscopy. Dead individuals were sexed by dissection and/or histological examination. Similar data were obtained for *S. guntheri* individuals, but sex ratios were determined from those individuals that died, as at the time the juveniles were too young for laparoscopy (Cree *et al*, 1994). Their data are summarised in Table 1.1. Despite the small samples sizes within the data, the ratios do deviate significantly from a 1:1 ratio of males to females (expected of a genotypic sex determination mechanism), inferring that the tuatara exhibits TSD. The data indicate that at lower incubation temperatures, there is a bias towards the production of female hatchlings, and a bias towards males at higher temperatures[2]. There is also a suggestion that there may be a subtle difference the two tuatara taxa in their response to incubation temperature, but it would be premature to make any inferences on this matter before additional data on hatchling sex ratios become available.

Thus, TSD has been reasonably established as the mechanism of sex determination in this animal, but there are reasons to suspect that genetic factors may also play a role in tuatara sex determination, and indeed, the sex determination of all reptiles with TSD. These reasons are discussed in Chapter 2.

1.5 AIM AND DESCRIPTION OF THESIS

1.5.1 Sex-specific DNA in the tuatara

The aim of this thesis was straightforward: to test the hypothesis that there is sex-specific DNA in the tuatara. It was anticipated that if genetic sex differences were discovered, this finding could then be developed into a diagnostic molecular marker for sex.

[2] On the basis of this data, tuatara would appear to exhibit Pattern Ib temperature-dependent sex determination (Ewert and Nelson, 1991). However, further investigation is required to rule out Pattern II TSD, in which females are produced at high and low temperatures, and males at intermediate temperatures. This point is discussed in more detail in Chapter 2.

TABLE 1.1 Data of Cree *et al* (1995). Numbers of male and female tuatara hatchlings for different constant temperature regimes for *S. punctatus* and *S. guntheri*, including one variable temperature regime for *S. guntheri*. The general pattern of TSD exhibited appears to be a female biased sex-ratio at lower temperatures and a male bias at higher temperatures. The exclusive bias towards males of *S. guntheri* for the variable incubation temperature regime is thought to be the result of temperatures remaining above 22°C during the middle third of the incubation period (Cree *et al*, 1995).

species incubation temperature	number of female hatchlings	number of male hatchlings
Sphenodon punctatus		
constant 18°C	9	0
constant 20°C	31	3
constant 22°C	4	13
Sphenodon guntheri		
constant 18°C	17	0
constant 22°C	3	0
variable regime (18-23-18°C)	0	7

Although in many vertebrate species, heteromorphic sex chromosomes can be seen in the karyotype, this is not the case for the tuatara (Wylie *et al*, 1968). The next logical step, after cytogenetic examination, was to look at DNA organisation at the genomic level. As part of a wider study into the minisatellite organisation of the tuatara genome, Finch (1994) briefly investigated whether or not sex differences in minisatellite organisation could be found. She observed that for some enzyme/probe combinations female tuatara showed a statistically higher mean number of bands than males, within a specific size range of fragments. This finding lead Finch to conclude that 'subsequent investigations, using additional probes, may separate sex-specific fragments in the genome of the female tuatara' (Finch, 1994).

The objective of this study was to conduct a comprehensive analysis using minisatellite DNA profiling. This involved using a large number of enzymes in conjunction with a variety of probes. The major undertaking of the laboratory work was therefore the completion of a comprehensive survey of probe/restriction enzyme combinations (described in Chapter 3). In addition, a number of RAPD (Randomly Amplified Polymorphic DNA) primers were used to amplify anonymous sequences from genomic DNA, creating band profiles that could potentially reveal sex-specific fragments (described in Chapter 4).

Since the tuatara, like many reptile species, exhibits temperature-dependent sex determination (TSD), the aim of detecting a genetic marker for sex is far from simple. To date, only one other study has revealed a molecular genetic difference between the sexes of a reptile with TSD (Demas *et al*, 1990). It would therefore be of significant scientific interest if sex-specific markers were located for the tuatara, and such a discovery could be an important contribution to the study of molecular mechanisms of sex determination in TSD species.

1.5.2 Application to conservation

Aside from inherent scientific interest in such a finding, it would also have considerable potential application to the conservation of this rare species of reptile (this is, in fact, one of the driving forces behind the research). Tuatara males lack a copulatory organ, unlike all other reptile species, and sexing of tuatara is thus very

difficult prior to the age at which they reach sexual maturity (10-15 years), when sexual dimorphism becomes pronounced (Daugherty *et al*, 1990). A diagnostic molecular sex marker would therefore be an invaluable tool to sex the morphologically indistinguishable juveniles.

Integral to the conservation programme for the tuatara are such measures as establishment of new tuatara populations on predator-free islands, including islands where tuatara have been historically present (that have since undergone successful predator eradication programmes) and islands for which there is no record of their previous existence (Cree and Butler, 1993). The ability to sex captively-reared juveniles by means of a molecular marker would be of enormous use, as it could be ensured that new colonies started from these juveniles have appropriate sex ratios to assure population growth. Furthermore, threatened populations could be augmented with individuals of known sex to maintain or establish appropriate sex ratios.
In this respect, the discovery of a molecular marker for sex in the tuatara could have important implications for the conservation of other endangered reptile species which exhibit TSD. If a genetic marker was found in this species, heterologous probes developed from this marker could potentially establish sex markers in other species. The finding alone would raise hopes enormously that similar methods would be successful in other TSD species. In some rare sea turtles, for example, incubation practices in head-starting programmes have resulted in male-biased sex ratios that were of limited benefit to the conservation of those species (Cree *et al*, 1995). Molecular markers for sex in such cases could be used to establish at an early stage the appropriateness of particular incubation temperature regimes. At some point, understanding of the mechanism of TSD may become sufficiently advanced to allow conservation biologists to manipulate the sex of hatchlings, which could potentially enhance their efforts to save endangered species (Spotila *et al*, 1994).

2

Temperature-Dependent Sex Determination In Reptiles: An Overview

Temperature-dependent sex determination (TSD) is one of the two sex-determining mechanisms found in reptiles. This chapter presents an overview of the phenomenon of TSD. General aspects of the sex-determining mechanism are discussed, and the distribution of TSD within the four orders of extant reptile species is summarised. Evidence supporting the hypothesis of an underlying genetic component to sex determination in TSD species is examined in detail. Current knowledge of the molecular mechanism of TSD is discussed with respect to the possibility of genetic involvement in primary sex determination. The general methodology of the research is explained in terms of the underlying theoretical issues.

2.1 INTRODUCTION

Like many other species of reptile, the tuatara exhibits temperature-dependent sex determination (Cree *et al*, 1995), in which the sex of the developing embryo is influenced by incubation temperature. The objective of this thesis is to test the hypothesis that there is sex-specific DNA in the tuatara. The fact that the tuatara has TSD is thus an important consideration, since it may infer that sexual genotypes do not exist in this animal (see section 2.4.1). For this research to be considered in context, it is necessary that a clear account be given of the phenomenon of TSD. This chapter therefore presents a summary of the current state of knowledge of TSD. It is not intended to be a comprehensive review, however, as the discussion focuses upon only those aspects of TSD which are most likely to have relevance to the hypothesis that there is an underlying genetic component to this sex-determining mechanism. Areas of TSD investigation such as laboratory and field studies, particularly where they are concerned with the ecology,

biogeography, and adaptive significance of TSD, are largely excluded from the discussion (for reviews, see Bull, 1980; Janzen and Paukstis, 1991).

A general introduction to temperature-dependent sex determination is first presented, followed by a description of various aspects of the mechanism, including the general patterns of different TSD thermal regimes (particularly with respect to the tuatara). This is followed by a review of the occurrence and distribution of TSD within the extant reptile taxa.[1] The hypothesis upon which this research is based, that there is a genetic component to TSD in reptiles, is discussed in detail. The case for genetic involvement in TSD systems is presented, including an examination of studies which have suggested the co-occurrence of genetic and temperature-dependent influences in TSD species are examined. This is followed by a summary of the current view of the molecular mechanism of temperature-dependent sex determination, which is discussed in relation to the hypothesis of genetic involvement in TSD. Finally, the experimental methodology of the research is discussed in the context of the theoretical issues raised in this chapter.

2.1.1 Sex determination and sexual differentiation

The term 'sex-determining mechanism' used within this thesis is based upon the definition given by Bull (1983), who referred to the *classical* mechanism as 'the earliest elements in ontogeny common to one sex, including environmental and genetic effects acting in parents or zygotes, that differentially influence the probability of producing or becoming male/female' (p8). This distinguishes sex determination from the separate, but related, phenomenon of sex differentiation. Sex differentiation, or alternatively sex development, is a cascade of programmed genetic, hormonal, and physiological events in which the 'indifferent' gonad develops as a testis or an ovary in association with the corresponding urogenital and secondary sex characteristics (Lance, 1997). This cascade in set in motion by the mechanism of sex determination.

Within the amniotic vertebrates (mammals, birds, and reptiles), there is a variety of sex-determining mechanisms. All mammal and bird species exhibit genotypic sex

[1] Although Aves are a part of the reptile clade (Pough *et al*, 1998), the term 'reptile' used throughout this thesis excludes birds.

determination (GSD), where the sex of the organism is irreversibly fixed by the genome. Most GSD mechanisms involve a genetic switch predetermined at fertilisation (Coriat *et al*, 1994). This sex determination mechanism is sometimes also referred to as *chromosomal* sex determination, since in most GSD species, a genetic mechanism of sex determination is often associated with heteromorphic sex chromosomes. All mammalian species exhibit male heterogamety (XY males, XX females), in which males have heteromorphic sex chromosomes, whereas birds possess female heterogamety (ZZ males, ZW females), in which the females have heteromorphic sex chromosomes.[2]

2.2 TEMPERATURE-DEPENDENT SEX-DETERMINATION IN REPTILES

Reptiles exhibit various sex-determining mechanisms[3], which can be divided into four major types: (1) XX/XY GSD systems, (2) ZZ/ZW GSD systems, (3) GSD systems in which heteromorphic chromosomes have not been identified, and (4) temperature-dependent sex determination systems (Wibbels *et al*, 1994). Temperature-dependent sex determination (TSD) is one particular type of *environmental* sex determination. In environmental sex determination, the earliest ontogenetic distinction between the sex classes is caused by an environmental factor, according to the definition of the classical sex determining mechanism (Bull, 1983). In TSD, the critical environmental factor influencing sex determination is the incubation temperature during embryogenesis. The result is that egg incubation temperature can markedly affect the sex-ratio of an egg clutch, often producing quite dramatic (often exclusive) biases towards one sex or the other (Bull, 1980). In all TSD reptiles, clutches are buried in soil, or laid in burrows or constructed nests (Bull, 1983). Consequently, the ambient environmental temperature at the oviposition site is likely to have an important impact upon sex determination. In TSD species, population sex ratio (at hatching) would therefore appear to be largely dependent on two factors: maternal choice of oviposition sites, and ambient temperature variation at

[2] The only exception might be the ratite species, in which there is little morphological differentiation between the sex chromosomes (although the female still appears to be the heterogametic sex) (Ansari *et al*, 1988).

[3] The large majority of reptile species are dioecious, but there are several species that are known to exhibit parthenogenesis (Bull, 1983). Since the topic of this chapter is the mechanism of sex determination in those species with separate sexes, the parthenogenetic species are not considered.

those sites (which is itself is dependent on several factors, such as soil type, moisture content, depth of the burrow, climate, and so on).

The phenomenon of TSD was first discovered in an agamid lizard by Charnier (1966), and a series of subsequent papers established its occurrence in many other reptiles and brought it recognition (e.g. Pieau, 1971; 1972; 1974; Yntema, 1976; 1981; Ynetma and Mrosovsky, 1980; 1982; Bull and Vogt, 1979; Wagner, 1980). TSD has been demonstrated in laboratory experiments using both constant and fluctuating incubation temperature regimes, and has also been demonstrated to occur in natural nests (reviewed by Bull, 1980; Janzen and Paukstis, 1991). Bull and Vogt (1979) demonstrated for several reptile species that the sex ratio biases are not caused by differential mortality of males and females at specific temperatures, which indicates that temperature is indeed influencing sex determination.

2.2.1 Sex ratio biases and pivotal incubation temperatures

In species with TSD, sex ratios of hatchling clutches change with respect to a gradient of incubation temperature (Fig. 2.1). Along this temperature gradient, the sex ratio switches from exclusive production of one sex to exclusive production of the other (naturally, there are upper and lower limits to this temperature range at which embryonic development ceases, or death occurs). In many cases, the bias towards one sex does not reach fixation. In most turtle and crocodile species, for example, it is rare for more than 80% of hatchlings to be male, at any constant incubation temperature, although exclusive female biases do occur (Ewert and Nelson, 1991; Lang and Andrews, 1994).

In general, sex ratios are heavily or exclusively biased for *most* of the viable temperature range in TSD species. However, there is a transitional range of temperature at which individuals of both sexes are produced (Fig. 2.1). The transitional range may be relatively narrow (<1°C in some species) or broad (4°C in other species) (Pieau, 1996). Within this transitional range, there is an even narrower range at which both males and females are produced in roughly equal numbers (i.e. sex ratio ~1:1). This narrow temperature range occurs at the point where the sex ratio switches from a bias towards one sex to a bias towards the other, and is thus known as the *pivotal incubation*

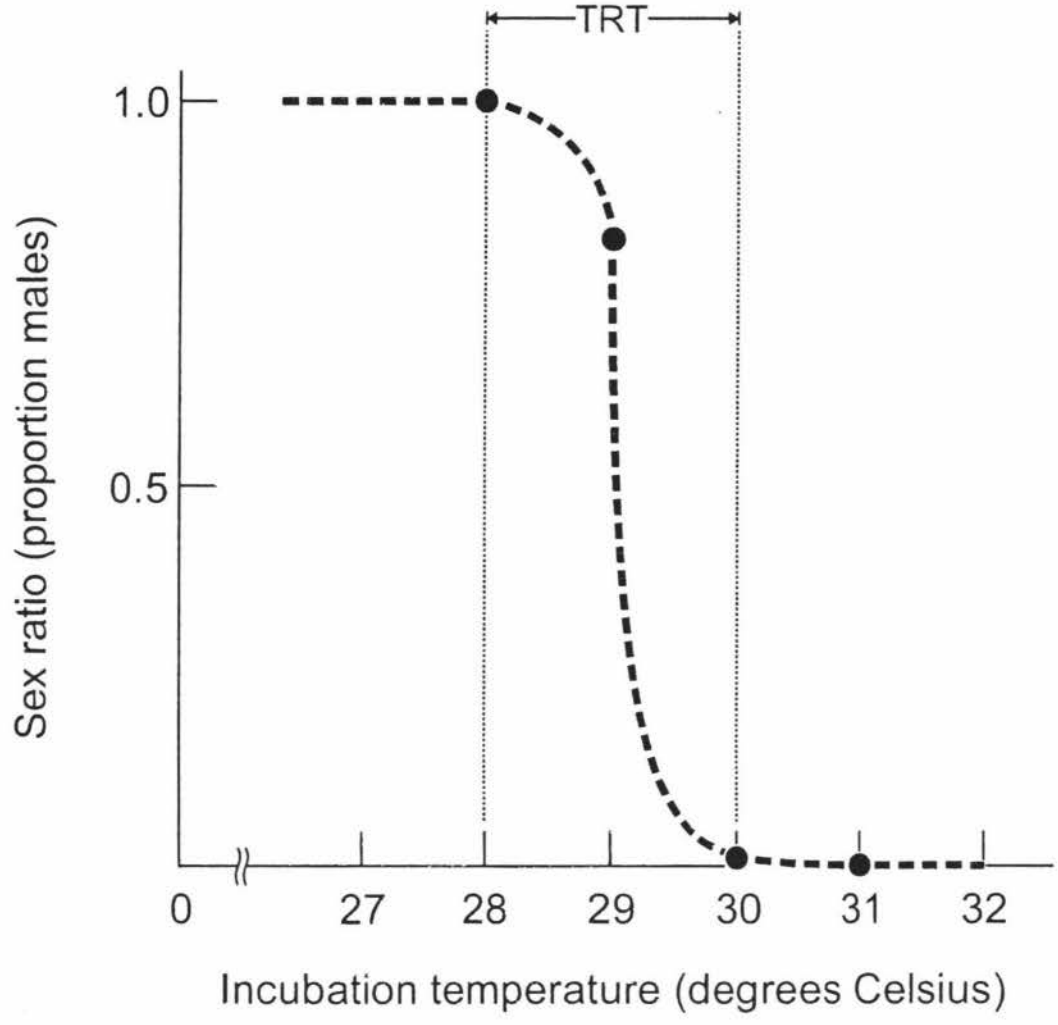

Figure 2.1. Hatchling sex ratio versus constant incubation temperature in the Ouachita Map Turtle, *Graptemys ouachitensis* (Bull and Vogt, 1979; Bull, Vogt, and McCoy, 1982), as an example of a typical pattern of temperature-dependent sex determination in turtles. TRT indicates the *transitional range of temperature* over which the hatchling sex ratio switches from a bias (often exclusive) towards one sex to a bias towards the other (in this particular species, there is an abrupt change from all males to all females over the temperature range 28-30°C). The *pivotal temperature* is the constant incubation temperature at which the bias switches from one sex to the other, hence there is a 1:1 sex-ratio amongst hatchlings incubated at this temperature. (Modified from Bull, 1983).

temperature (Yntema and Mrosovsky, 1980) (sometimes also referred to as the *threshold temperature* [Bull, 1983]). It would appear that male and female determining factors are just balanced at the pivotal temperature, resulting in the observed sex ratio of 1:1 (Mrosovsky and Pieau, 1991).

2.2.2 Patterns of temperature-dependent sex determination in reptiles

Three general patterns of TSD thermal regime have been described for reptile species, (Fig. 2.2). In the first pattern, males are predominant at lower temperatures, and females predominant at higher temperatures (=MF). In the second pattern, the situation is reversed: females are predominant at lower temperatures, and males at higher temperatures (=FM). These first two patterns have a single pivotal temperature. In the third pattern, which has two pivotal temperatures, females are predominant at lower temperatures, males predominant at intermediate temperatures, and females again predominant at higher temperatures (=FMF). These three patterns of temperature-dependent sex determination, MF, FM, and FMF, have been described, respectively, as Patterns (or Types) 1, 2, and 3, or A, B, and C (Bull, 1980, 1983; Bull and Charnov, 1988). In some cases, the first two patterns have been reversed (i.e. FM, MF, FMF) and listed as I, II, and III (Bull and Charnov, 1989; Deeming and Ferguson, 1991).

The descriptions used here are those of Ewert and Nelson (1991), which are different yet again. Since the FMF pattern occurs in all groups of reptiles in which TSD has been extensively studied (turtles, crocodilians, and squamates), they consider that this pattern may be ancestral (Deeming and Ferguson, 1988), and that the other patterns, FM and MF, may be derived (Ewert *et al*, 1994). Therefore, they designate the hypothetically ancestral pattern (FMF) as Pattern II, and the two derived patterns as Pattern Ia (MF) and Pattern Ib (FM) (Ewert and Nelson, 1991; Ewert *et al*, 1994).

These designations are preferable to the others found in the literature because it has become apparent that Pattern Ib may not in fact exist at all within reptiles, as the few species for which this TSD pattern has been reported may actually have Pattern II after all (Ewert *et al*, 1994) (Appendix II examines these cases). If true, this would mean there are only two general patterns: Ia and II, so it would be simpler to refer to Pattern Ia as Pattern I.

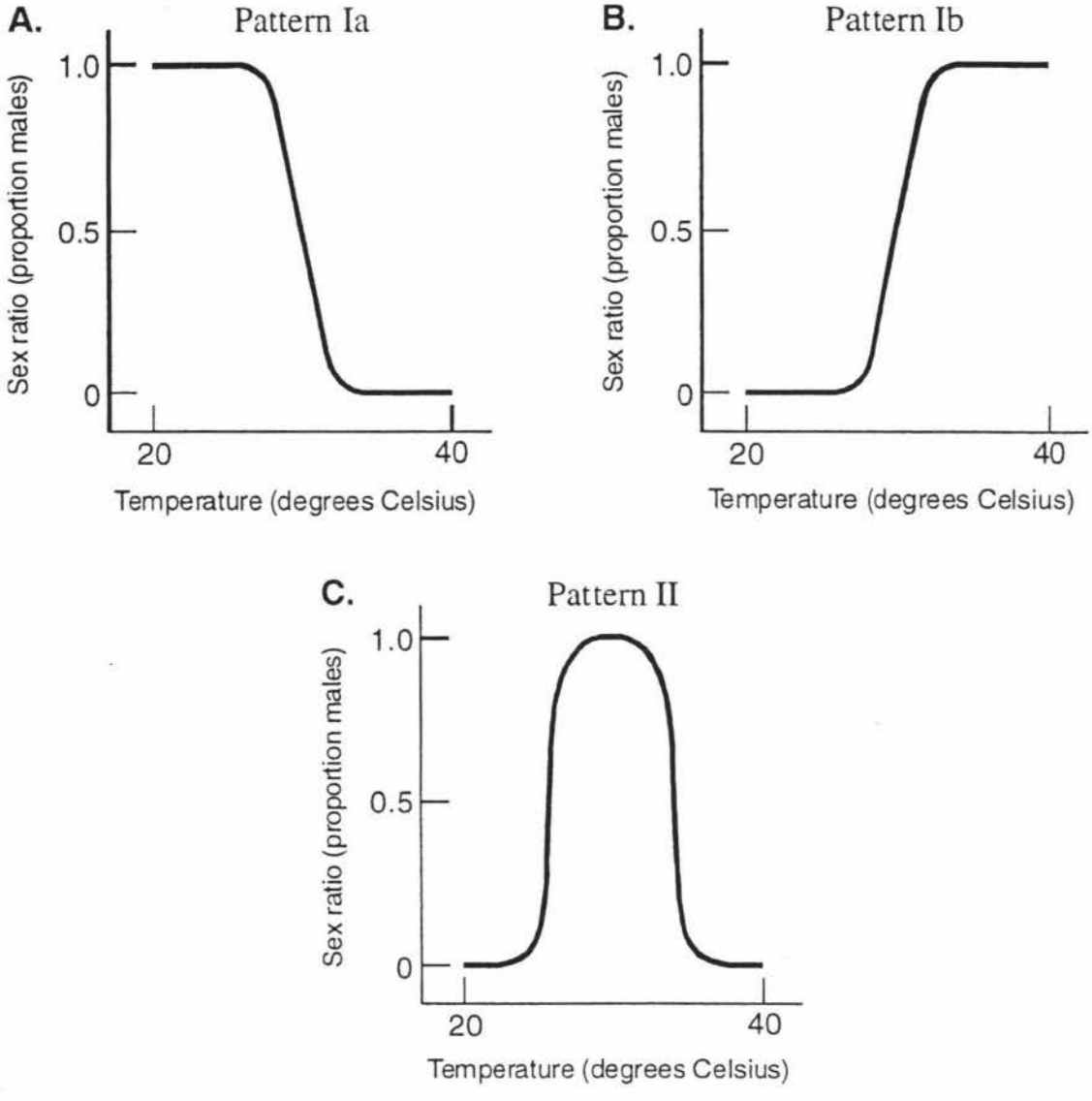

Figure 2.2. Representative plots of hatchling sex ratio as a function of constant incubation temperature, showing the three general patterns of temperature-dependent sex determination in reptiles. **A.** Pattern Ia, in which male hatchlings are produced at lower incubation temperatures, and females are produced at higher incubation temperatures (exhibited by all turtle species with TSD). **B.** Pattern Ib, in which female hatchlings are produced at lower temperatures, and males at higher temperatures (possibly only exhibited by the tuatara). **C.** Pattern II, in which male hatchlings are produced at intermediate incubation temperatures, and female hatchlings are produced at both low and high incubation temperatures (exhibited by all crocodilian species, and probably all squamates with TSD). The classifications are those of Ewert and Nelson (1991). (Redrawn from Bull, 1983).

2.2.3 The effect of incubation temperature upon sex determination

Studies of reptiles with TSD have suggested that temperature only has an effect upon their sex determination during a critical period of incubation (Bull, 1983). This period has been determined from the effect upon sex ratio in temperature-shift experiments, in which eggs are shifted from one incubation temperature to another, at different stages of incubation (Janzen and Paukstis, 1991). The temperature-sensitive period correlates with the period during incubation when sexual differentiation of the gonads occurs (Dorizzi *et al*, 1996; Pieau, 1996). Laboratory experiments have deduced that, in most reptiles, this corresponds approximately to the middle third of embryonic development (reviewed by Janzen and Paukstis, 1991), although in at least some crocodilians, it may be better described as the third quarter of development (Lang and Andrews, 1994). In turtles, for example, the temperature-sensitive period generally correlates with developmental stages 14-20, which corresponds to the middle trimester of egg incubation. In lizards and many crocodilians examined, the temperature-sensitive period commences shortly after oviposition, and lasts for the first half of incubation (unlike turtles). Lizard and crocodilian embryos are at a more advanced stage of development at oviposition than are turtle embryos, which explains the difference in timing of the temperature-sensitive period with respect to the incubation duration (Janzen and Paukstis, 1991). In general, the pattern in all reptiles appears to be that the temperature-sensitive period coincides roughly with the timing of gonadogenesis (Bull, 1987a; Dorizzi *et al*, 1996).

The fact that there are transitional ranges of incubation temperature, where both male and female hatchlings may result from a single temperature, is difficult to reconcile with the notion that the primary event in TSD is a single temperature-dependent 'switch' (Johnston *et al*, 1995). A number of factors may be affected by temperature, and the relative timing of the temperature effect may be critical. One implication of this is that the temperature effect upon sex determination may in fact be cumulative over a period of time, and is not a switch event occurring at a precise developmental stage. Since developmental rates are greater at higher temperatures, it has been proposed that the daily proportion of development occurring at a temperature is the important variable in TSD, rather than the duration of exposure to that temperature (Bull and Vogt, 1981; Pieau,

1982; Georges, 1989). Georges (1989) developed a mathematical model based upon this assumption, which proved to be a good predictor of sex ratio for fluctuating incubation temperature regimes, when tested upon a marine turtle species (Georges *et al*, 1994). This finding also explained the disagreement between sex ratios when mean daily temperatures in natural nests were compared to constant incubation temperatures in laboratory experiments. The model allowed the calculation of a 'constant temperature equivalent' for use in the prediction of sex ratios resulting from cyclical temperature regimes occurring in natural nests (Georges *et al*, 1994).

The precise molecular or cellular mechanism by which temperature actually acts to influence sex determination in TSD species is still unknown, but recent studies have brought to light some of the details of the mechanism (Section 2.5)

2.3 TAXONOMIC DISTRIBUTION OF SEX-DETERMINING MECHANISMS IN REPTILES

2.3.1 General distribution

Temperature-dependent sex determination is widespread amongst the reptiles, but its distribution is best described as patchy. It is present to some extent in all four of the extant major reptile groups; Chelonia (turtles), Crocodilia (crocodilians), Squamata (lizards, amphisbaenians, and snakes) and Sphenodontida (tuatara). Within the Squamata, the most speciose of the reptile orders, TSD is present within many, but not all, lizards, and is seemingly absent from snake species (Janzen and Paukstis, 1991; Viets *et al*, 1994). Genotypic sex determination, on the other hand, appears to be present to a varying degree in turtles, lizards, amphisbaenians, and snakes, and is often associated with cytologically visible heteromorphic sex chromosomes. Heteromorphic sex chromosomes have not been found in the tuatara, or in any crocodilian species. They are rare in amphisbaenians (worm lizards) and turtles.

At present, only a limited picture is available of the relative frequency of the two sex-determining mechanisms with the reptile taxa. There are over 6500 living reptile species (95% of which are squamates [Pough *et al*, 1998]), and of these, around 1300 species

have been examined for sex chromosome heteromorphy (Janzen and Paukstis, 1991). However, of those species, only 100 or so species have been examined for the occurrence of temperature-dependent sex determination. Approximately 27% of the karyotyped species were reported to show heteromorphic sex chromosomes (~330 species), whereas about 75% of the reptile species examined for the presence of TSD have been shown to exhibit this sex-determining mechanism (Janzen and Paukstis, 1991). Of all the species known to have heteromorphic sex chromosomes, only three lizard species, *Lacerta viridis, Sceloporus jarrovi*, and *Gekko japonicus*, and two turtle species, *Staurotypus salvinii* and *S. triporcatus*, have been examined for TSD (Olmo *et al*, 1986; Gorman, 1973; Tokunaga, 1985; Ewert and Nelson, 1991; Bull *et al*, 1974; respectively). Evidence of TSD was found only for *G. japonicus* and *S. salvinii*, but there are some doubts over the validity of these two cases as examples of the co-occurrence of TSD and sex chromosome heteromorphism within the same species (section 2.4.3.2).

2.3.1.1 Turtles

Of the 13 recognised turtle families, TSD is ubiquitous in eight; the Testudinae, Chelydridae, Carettochelyidae, Pelomedusidae, Kinosternidae, Dermatomydidae, Cheloniidae, and the Dermochelyidae. With the exception of the last four, these groups are distantly related. One family, the Platysternidae, which consists of a single species, has not been investigated. In the Staurotypidae, Trionychidae, and Chelidae, GSD is the mechanism in all those species examined, but only a few species have been examined for either mechanism. Lastly, in the Emydidae, TSD predominates, but this family also includes species with different GSD mechanisms, including male heterogamety, female heterogamety, and homomorphic sex chromosomes (which presumably are differentiated at some level) (Janzen and Paukstis, 1991). Most turtles have large diploid numbers, over half of which are microchromosomes, so there is the potential for heteromorphism in the small chromosomes to be overlooked (Ewert *et al*, 1994). Nonetheless, TSD appears to be the predominant sex-determining mechanism amongst turtles, which are the second largest reptile order in terms of species number, after squamates. Most TSD turtle species exhibit Pattern Ia, although several species exhibit pattern II (Ewert *et al*, 1994). It now appears that Chelonia is the only reptile order in which Pattern Ia occurs.

2.3.1.2 Crocodilians

Crocodilia includes 13 species of crocodile (Crocodylidae), seven species of alligator (Alligatoridae), and two species of gharial (Gavialidae). All 22 species have been karyotyped, and in the majority of these both sexes have been examined (Cohen and Gans, 1970). Heteromorphic sex chromosomes have not been found in any of those species. Unlike the karyotypes of other reptiles, crocodiles do not have small microchromosomes, so the overlooking of a sex heteromorphism is less likely than for other reptiles. TSD has been documented in all 11 of the 22 species thus examined, and within those species are representatives of each of the three families (Lang and Andrews, 1994). On the basis of this information it is likely that TSD is ubiquitous in the Crocodilia. This contrasts with the Squamata and Chelonia, in which a diversity of sex-determining mechanisms are present. Pattern II TSD appears to be ubiquitous amongst the crocodilians (Lang and Andrews, 1994), on the basis on five species (two families) examined thoroughly, and another four species incompletely described.

2.3.1.3 Squamates

Lizards, like turtles, show considerable variation in their sex-determining mechanisms. On the basis of the presently available information, it appears that both TSD and GSD are present in the Agamidae, Eublepharidae, and Gekkonidae, and possibly also in the Iguanidae, Lacertidae, and Varanidae (Viets *et al*, 1994). Within the Gekkonidae and Lacertidae at least, there appear to be cases of all four reptilian sex-determining modes: male heterogamety, female heterogamety, GSD with homomorphic sex chromosomes, and TSD (Janzen and Paukstis, 1991). In the Scincidae and Teiidae, however, GSD appears to predominate, as this is the only sex-determining mechanism yet reported (Viets *et al*, 1994). The few reported cases of Pattern Ib TSD in lizards are disputed (Viets *et al*, 1994), so it seems that Pattern II TSD is the only pattern found with TSD squamates.

Little is known about sex-determining mechanisms in amphisbaenians (worm lizards). Only one of 30 karyotyped species has heteromorphic sex chromosomes. No species

have been investigated for temperature-dependent sex determination (Janzen and Paukstis, 1991).

Snakes exhibit more uniformity in their sex-determining mechanisms. While only around 15% of species have been karyotyped, the large majority of these species show female heterogamety (ZZ/ZW chromosomes), with varying degrees of chromosome differentiation between families. The remaining species have morphologically undifferentiated sex chromosomes (Janzen and Paukstis, 1991). Perhaps as a result of the predominance of heteromorphic sex chromosomes, only three snake species have been examined for the occurrence of TSD, which none exhibited. It would appear that GSD is ubiquitous amongst the snakes (Viets *et al*, 1994).

2.3.1.4 Temperature-dependent sex determination in the tuatara

The data upon which the classification of the tuatara as a TSD species is based are clearly limited (Table 1.1 [Cree *et al*, 1995]). Only three constant temperatures were used for *Sphenodon punctatus*, and two constant temperatures and one varied temperature regime were used for *S. guntheri*. Both species produced 100% females at 18°C, and *S. punctatus* produced 91% females at 20°C. At 22°C, *S. punctatus* showed a strong bias towards males (76%). On the other hand, *S. guntheri* showed a complete bias still towards females at 22°C, but the sample size of only three hatchlings in this instance invalidated a statistical analysis. More importantly, under a variable temperature regime ranging from 18-23°C, *S. guntheri* produced exclusively males. Cree *et al* (1995) postulated that this male bias may have resulted from temperatures in the variable regime remaining above 22°C for a period of time during the middle stages of incubation, which corresponds to the temperature-sensitive period of embryogenesis in other species (section 2.2.3).

This limited information would suggest Pattern Ib, but as already noted, confirmation of this pattern requires more rigorous experimental investigation. Given the very ancient separation of the sphenodontid lineage from the other reptile groups, it may be that the tuatara again proves to be remarkably different from other living reptiles, and is in fact

the only reptile with a true Ib pattern, particularly in light of the colder environmental temperature range at which it is active, in comparison to other reptiles (Dawbin, 1982a).

2.4 GENETIC INVOLVEMENT IN TEMPERATURE-DEPENDENT SEX DETERMINATION

2.4.1 The concept of sex-specific genetic differences in reptiles with temperature-dependent sex determination

The hypothesis which this research aims to test, that there is an underlying genetic component to temperature-dependent sex determination, is not restricted to the tuatara, but pertains to all reptiles which exhibit TSD. The concept of genetic distinctions between the two sexes of a species with proven temperature-dependent sex determination appears contradictory. Considering that an environmental variable determines the sex of the embryo, it could well be argued that there should not be any genetic differences between the sexes. To date, all reptile species studied for sex-determination have been classified as exhibiting either TSD or GSD, so it might indeed appear that the two sex-determining modes are mutually exclusive. Sex-specific DNA is obviously associated with genotypic sex determination, but since egg incubation temperature is the critical factor in determining sex in TSD reptiles, it might seem unnecessary to postulate a genetic difference between the two sexes.

Intuitively, the simplest scenario is that prior to the temperature-sensitive period, an embryo is genetically 'indifferent'. That is, every embryo possesses the genetic potential for full phenotypic development as either a male or a female. The effect of temperature would be to 'switch on' one of the two alternate developmental cascades, perhaps via a temperature-sensitive protein or regulatory element. An alternative scenario is that all embryos develop as a 'default' sex, be it male or female, unless the incubation temperature is such that the temperature-sensitive regulatory element is activated and initiates the genetic cascade for producing the opposite sexual phenotype. In some respects, this would be analogous to eutherian mammals, in which embryos develop by default as female in the absence of the *genetic* effect of a testis-determining factor (Lance, 1997).

There is, however, considerable evidence to indicate that the mechanism of TSD is not as simple as a single heat-sensitive protein determining sex, and it has been proposed that there is an underlying genetic component to TSD (Bull, 1980; Bull *et al*, 1982). The first experimental data in support of this proposal was the heritability analysis of the zygotic character of sex ratios at pivotal temperature, in the turtle *Graptemys ouachitensis* (Bull *et al* (1982). They considered that the heritability value of 0.82 was high enough to implicate the involvement of a major gene in the sex determination at pivotal temperature. A similar heritability value was determined for the turtle *Chelydra serpentina* by Janzen (1992), and the finding of Ewert and Nelson (1991) of a broad band of transitional temperature in some species of freshwater turtles, also supports the hypothesis that there is a genetic effect responsible for the mixed sex ratios at these intermediate temperatures. Standora and Spotila (1985) suggested that temperature may act as a modifying agent affecting gene action in the sex determination of turtles. They hypothesised that there are sex-specific DNA sequences scattered throughout the genome, which are controlled by a temperature-sensitive effector molecule.

This hypothesis of a genetic component to sex determination in TSD species raises the question of the ancestry of sex determining mechanisms within the Reptilia. There are currently two alternative hypotheses concerning the evolution of TSD and GSD within this group (Bull, 1980; 1983; Janzen and Paukstis, 1991). The prevailing view is that TSD is the ancestral sex-determining mechanism within the vertebrates, inferring that GSD has evolved independently in many lineages, including the mammals, birds, and reptiles. If this hypothesis is correct, then it is quite possible that no sex-specific genetic differences exist in TSD reptiles. Presumably, sex-specific genetic differences only become established when a genetic mode of sex determination evolves within a lineage.

The alternative hypothesis is that GSD is the ancestral mechanism of sex determination within vertebrate species, and TSD is a derived state. According to this hypothesis, in lineages where TSD has evolved from GSD, the ancestors with GSD had an inherited tendency to develop as one of two alternate sexes. It seems reasonable to postulate that inherited genetic factors predisposing ancestral individuals towards one of two alternate sexual development pathways during embryonic development, would not have been lost from the genomes of the TSD descendants. However, in those descendant individuals,

incubation temperature now activates a mechanism of sex-determination that can override the 'underlying' genetic predisposition of the embryo to develop as one sex or the other. Thus, the primary temperature-dependent sex-determining mechanism is *superimposed* upon the underlying, ancestral genotypic mode of sex determination. This hypothesis also presumes that all embryos have the genetic potential for full phenotypic development as a male or female. Otherwise, a genetically-predisposed male embryo would not be able to fully develop as a female as a result of incubation at a female-producing temperature, or vice versa, as critical genes involved in the development of secondary sexual characteristics would be absent (see Appendix II for a fuller discussion of the ancestry of sex determination in the reptiles).

It is conceivable under such a scenario that there is a wide range of varying degrees of interaction between underlying genetic influences and the effects of incubation temperature in TSD species. However, the most immediate inference of the ancestral GSD hypothesis is the existence of 'sex-reversed' individuals. That is, 'genetic' males that develop with a female sexual phenotype (due to incubation at a female-producing temperature), and 'genetic' females that develop with a male sexual phenotype (due to incubation at a male-producing temperature). This would mean that (at minimum) there are four distinct classes of 'sex': males and females that are *concordant* in their sexual genotype and phenotype, and males and females that are *discordant* in their sexual genotype and phenotype (Table 2.1). The consequences of these (hypothetical) distinctions are a recurring theme throughout this investigation. Consequently, the terms 'concordant' and 'discordant' are used regularly, although 'sex-reversed' and 'discordant' are often used interchangeably.

TABLE 2.1 Theoretical classes of sex if TSD is superimposed upon GSD

	male phenotype (male-inducing temperature)	*female phenotype* (female-inducing temperature)
male genotype	**concordant** male	discordant female
female genotype	discordant male	**concordant** female

The implications of these theoretical classes of sex require careful consideration. If temperature *completely* overrides sexual genotype in a TSD reptile species, then that

species will have, on average, a hatchling sex ratio of 50% concordant individuals and 50% discordant individuals (regardless of the phenotypic sex ratio, which is a function of the environmental temperature variation, nest locations, and so on). The sexual genotype is essentially meaningless in such a scenario, since it is completely independent of the phenotype. Any sex-determining genetic element would simply be a region of DNA found randomly distributed amongst males and females. For this reason, the concept of sex-specific DNA in a TSD species seems contradictory: sex-specific DNA is not in fact *specific* to anything, *unless* the influence of incubation temperature is removed, thereby allowing the sex-specific, sex-determining genetic elements to determine the sexual phenotype. It has been proposed that embryo sex determination is not influenced by temperature at the pivotal temperatures of incubation (Zaborski, 1988), so at this particular point, the underlying genotypic mechanism determines sex. This implies that at pivotal temperatures, individuals will be concordant. It follows that in such cases, the ratio of males to females will be 1:1 (Girondot *et al*, 1994).

This hypothetical scenario that concordant individuals are likely to be in the majority within natural TSD populations, since genotype would determine phenotype in those individuals incubated at or close to the pivotal temperature (which potentially might be a large proportion within natural environments), in addition to at least half (on average) of the remaining population (which would be, at most, 50% discordant). Thus, sex-reversed individuals would be expected to be in the minority.

2.4.2 Examples of the apparent co-occurrence of genotypic and temperature-dependent sex determination within a species

The case for the hypothesis that there is an ancestry of GSD in TSD reptiles is essentially indirect evidence for the hypothesis that there is an underlying sexual genotype in TSD species (Appendix II). The discussion now turns to investigations of TSD species which suggest there are components of both GSD and TSD in operation. These examples are believed to provide more direct evidence for a genetic component underlying TSD.

2.4.2.1 Genotypic and temperature-dependent sex determination responses at different incubation temperature extremes in *Kinosternon baurii*

Ewert *et al* (1990) reported finding a TSD and a GSD-type response at different ends of the incubation temperature range for the kinosternid turtle *Kinosternon baurii*. At the warmer end of the incubation temperature range, there was an exclusive bias towards female hatchlings. At the intermediate temperature of 26°C, only males were produced. However, rather than finding a bias towards females again at cool incubation temperatures (which would fit TSD pattern II), at each of three different cool incubation temperatures tested, there was an approximately 1:1 ratio of males and females produced, suggesting genotypic sex determination.

These results were interpreted by Ewert *et al* (1990) as evidence of a GSD response at lower temperatures having evolved from a more fully expressed TSD pattern across the entire incubation temperature range. However, it is also conceivable that the TSD response at warmer temperatures in *K. baurii* is in fact the derived state. Either way, this apparent co-occurrence of both modes of sex determination within the one species suggests that there are significant similarities in the underlying mechanism of the two modes.

2.4.2.2 Heteromorphic sex chromosomes and TSD in the lizards *Gekko japonicus* and *Staurotypus salvinii*

One species of particular interest is the gekkonid lizard, *Gekko japonicus*, for which Yoshida and Itoh (1974) reported finding distinct heteromorphic sex chromosomes. The lizard was reported to have an XX/XY system (male heterogamety), in which the sex chromosomes differed by both structural rearrangements and in heterochromatin content. However, a later study of the same species, in which the author seemed unaware of the previous report of chromosome heteromorphy, demonstrated conclusively that the species exhibits Pattern II TSD (Tokunaga, 1985). Since it is generally believed that homomorphic sex chromosomes are a necessary prerequisite for TSD systems (Bull, 1980; Janzen and Paukstis, 1991), these surprising findings seem somewhat contradictory, but there are several possible explanations.

First, these separate results bring into question the taxonomic status of the two populations studied (Viets *et al*, 1990); Perhaps unknowingly, two distinct species were studied (one of which had not, or perhaps still has not, been described). Heteromorphic sex chromosomes have been reported in only two other species (of 54) within the Gekkonidae family, but in contrast to *G. japonicus*, both cases are female heterogamety (Bull, 1980). Interestingly, in each of these two cases, the heteromorphism was not observed throughout the species range (King and Rofe, 1976; King, 1977). Thus, the two studies of *G. japonicus* may very well have involved two distinct populations, with different sex-determining modes. If so, this would appear to be the most evolutionarily 'shallow' instance of both GSD and TSD occurrence in sister reptile taxa yet found.[4]

Second, it could be that there is an underlying mode of genetic sex determination in this species that is overridden by temperature, even though the GSD system has evolved to the point of sex chromosome heterogamety. Probably of crucial importance is the fact that in their cytological examination for sex chromosomes, Yoshida and Itoh (1974) only examined a *single* individual of each (phenotypic) sex. It is entirely possible that they fortuitously sampled a concordant member of each sex, or conversely, a discordant member of each sex. In other words, if it can be assumed for a moment that TSD totally overrides sexual genotype, there would be an approximately 1:1 ratio of heterogametic to homogametic individuals amongst phenotypic females (produced at high and low temperatures) and amongst phenotypic males (produced at intermediate temperatures). If the two sampled animals were concordant in their sexual genotype and phenotype, then the investigators would have been correct in assigning male heterogamety (XX/XY chromosomes) to the species. However, if they were both discordant individuals, then the reverse would be true: the phenotypic male shown to have heteromorphic chromosomes was in fact a genetic female, and the species has female heterogamety (ZZ/ZW chromosomes) (which is the system found in the only other two gekkonid lizards with heteromorphic sex chromosomes [Bull, 1980]). Examination of a larger number of animals might therefore be expected to show heterogametic and homogametic individuals of both phenotypic sexes. The problem with this interpretation is that is

[4] There is at least one instance of GSD and TSD reptiles within the same genus; the Emydid turtles, *Clemmys guttata* (TSD) and *Clemmys insculpta* (GSD) (Ewert and Nelson, 1991).

presents the opportunity for YY individuals to be formed from XY male (concordant) and XY female (discordant) crosses, which may be inviable. This would result in selection against sex-reversed individuals, as pointed out by Bull (1980) (section 2.4.1).

Third, it might be that both studies are correct: *G. japonicus* does have both TSD and GSD, but the two modes of sex determination are connected in some unexplained manner which results in complete concordance between genotype and phenotype in individuals. This would infer that temperature can not cause sex-reversal in nature. If this is correct, the species could potentially be an important key to solving questions about the common elements in the two sex-determination systems.

These proposed explanations are speculation at best. Whatever the case, the example of *G. japonicus* does seem to lend some support to the hypothesis that there is an underlying genetic component in TSD systems, because there is undoubtedly some close evolutionary connection in *G. japonicus* between the two mechanisms of sex determination, whether these separate studies investigated two distinct species, two distinct populations, or one and the same population. Clearly, there is a need for a cytogenetic examination of a much larger sample size of individuals from the proven-TSD population, before this example can be claimed as solid evidence for the co-occurrence of GSD and TSD within the same species of reptile.

Another potential example of the apparent co-occurrence of TSD and heteromorphic sex chromosomes within a reptile, is the turtle *Staurotypus salvinii*, but the data in this case are less convincing. Sites *et al* (1979) demonstrated male heteromorphy in the sex chromosomes of this species. Studies on incubation temperature response by Ewert and Nelson (1991) indicated a GSD-like response (~1:1 ratios of male to female hatchlings) for the progeny of one group of breeding pairs, but in another group there was a strong bias toward males at several temperatures. The investigators suggested one of the male parents in this second group could have been the result of a maternal sex reversal (i.e. had a viable YY genotype). The mating of this male with a normal (XX) female would have resulted in male offspring only, which could have biased the group results. Alternatively, it is possible that there were individuals from different populations of *S. salvinii*, with different mechanisms of sex determination, included in the study (as

suggested for *G. japonicus*) (Girondot *et al*, 1994). Further data is obviously needed for *S. salvinii* to determine if TSD is indeed present. On the available data, Ewert and Nelson (1991) concluded that the weight of evidence argued for GSD in this species.

2.4.2.3 Sex-specific DNA fragments in the turtles *Chelonia mydas* and *Lepidochelys kempi*

Another study which lends support to the hypothesis that there is sex-specific DNA in TSD reptiles is the investigation upon which the methodology of this thesis is largely based. Demas *et al* (1990) investigated the possibility of sex-specific DNA in two species of sea turtle, the green turtle (*Chelonia mydas*) and Kemp's ridley sea turtle (*Lepidochelys kempi*). *C. mydas* exhibits Pattern Ia TSD, where females are produced at higher temperatures, and males at lower temperatures (Demas *et al*, 1990). The type of sex determination in *L. kempi* has not been established, although there are indications that it is also a TSD species (Shaver *et al*, 1988). Using minisatellite DNA profiling (fingerprinting), Demas *et al* (1990) revealed sex-specific fragments in phenotypic males of both species, and in phenotypic females of *C. mydas*. Their methodology was quite simple: genomic DNA was digested with a wide range of different restriction enzymes, and Southern-blots of the size-separated fragments were hybridised with the satellite DNA probe Bkm (Singh *et al*, 1980).

This result provides strong evidence of sexual genotypes in these TSD species. If this can be taken to mean that both GSD and TSD are in operation in these turtles, then presumably it infers that there are sex-reversed individuals within the populations. This then poses a question as to why the methodology was successful. If the populations are composed of sex-reversed and concordant individuals, and the investigators sampled the populations randomly and sexed individuals upon the basis of their phenotype, then detection of sex-specific fragments should presumably not have been so straightforward. The inclusion of even one sex-reversed individuals within the DNA profiles would have masked the sex-specificity of a particular genetic fragment, since it would preclude a 100% correlation between the fragment and the sexual phenotype of the sample individuals. However, Demas *et al* (1990) did find 100% correlation between the sex-specific fragments and the sexual phenotype of the individuals. This infers that all of

their sample individuals were concordant. There are at least three possible explanations why no sex-reversed individuals were sampled.

First, even though these species have been shown to exhibit a TSD response under laboratory conditions, in the wild the TSD mechanism is not as predominant for these species, and therefore an underlying system of GSD comes into effect for incubating egg clutches. Possibly there is a large pivotal temperature range for these species, and only very extreme temperatures override genotype. Either way, this would infer that the majority of wild individuals are concordant, and the investigators were simply fortunate enough to avoid sampling any sex-reversed individuals. The sample sizes involved in the investigation were not particularly large, so this idea does seem plausible.

Second, TSD does predominate in the wild, so there is a sizable proportion of sex-reversed individuals within the populations. However, there might be some unknown difference between concordant and discordant animals, that caused a biased sampling. Perhaps discordant individuals exhibit quite different behaviour patterns which for some reason excluded them from being sampled. Alternatively, discordant individuals might have a more ambiguous morphological sexual phenotype, and the investigators declined to sample these individuals, preferring to select the most sexually dimorphic adults (perhaps in an effort to avoid errors in morphological sex assignment). A further possibility is that discordant individuals are less fit, and they have higher mortality than concordant animals.

Third, sex-reversed individuals are non-existent. There is complete concordance between genotype (as defined by the sex markers found) and sexual phenotype. This seems difficult to reconcile how there could be sex-specific DNA in every individual of a particular sex, when gender is supposedly determined by incubation temperature. Demas *et al* (1990) confronted this problem by invoking and modifying a model for sex determination that was first proposed by Singh *et al* (1981). The model proposes the existence of a temperature-sensitive factor that causes a structural modification of Bkm-related DNA in the genome of the developing embryo. This DNA modification, which only occurs at a specific incubation temperature, results in the sex-specific Bkm-related fragments appearing in the DNA profiles for that sex only. In other words, prior to the

temperature-sensitive period, there is no genetic difference between individual embryos; that is, sexual genotypes do not exist. Incubation temperature, by determining the activity of the heat-sensitive protein, in effect *creates* the 'genotype' of an individual during the temperature-sensitive period of embryogenesis. Essentially, the 'genotype' in this sense, is no more than a molecular marker for phenotype, since there was no pre-existing genetic component that had an influence in the sex determination of the embryo.

The model of sex detemination advanced by Demas *et al* (1990) arose from the findings of a number of studies into the nature of Bkm DNA. Bkm is a DNA fraction that was originally isolated from female DNA of an elapid snake, *Bungarus fasciatus* (Indian banded krait) (Singh *et al*, 1980). This Banded krait minor (Bkm) satellite, which contains highly repetitive GATA sequences, was shown to be quantitatively associated with the sex-determining W chromosome (Jones and Singh, 1981). Sex-specific differences in the hybridisation patterns of Bkm-related sequences have also been found for a number of other vertebrate species (Singh and Jones, 1982; 1984; 1986; Kent *et al*, 1988; Sarre, pers.comm). Interestingly, Bkm-probed female-specific DNA fragments were found for both human and mice DNA, an unexpected result since females are the homogametic sex in mammals (Jones and Singh, 1981). Investigations of Bkm hybridisation patterns in sex-reversed male mice lead Singh *et al* (1981) and Jones *et al* (1981) to propose the possibility of modification of Bkm-related DNA as an explanation for the female specific bands found in mice and human DNA. Jones and Singh (1981) suggested that there may be a non-classical mechanism involved in the sex determination of vertebrates, involving DNA splicing of testis or ovary specifying gene transcripts.

Demas *et al* (1990) recognised that their findings were not consistent with the idea of sex-reversed individuals, and thus suggested the possibility of a 'constitutive, temperature-regulated mode of Bkm expression' (p323). They invoked the general sex-determination model of Singh and his colleagues, which postulated the structural modification of DNA by the sex-specific excision of Bkm-related introns in close association with sex-determining genes. However, in the case of the turtles, these splicing events are under the control of incubation temperature, and not the Y chromosome as in mammals (Demas *et al*, 1990). If this model is correct, then there may be sex-specific DNA sequences in these turtles, but it would not be due to an ancestry of

GSD. Furthermore, it would suggest that there is the potential for molecular sex assignment of any TSD reptile, since there would be no sex-reversed individuals to confuse the issue.

There are, however, potential difficulties with this proposed model for the mechanism of TSD. It would require a concerted DNA modification to take place in all cells of the developing embryo during the temperature-sensitive period of incubation (or at least in the blood and gonadal tissue), and a mechanism to explain how this might occur has not been proposed. If there was some degree of thermal gradient across the embryo, this might result in the formation of a sexually chimaeric hatchling. Furthermore, if these genetic alterations are occurring within individuals, then it is uncertain whether the alterations would be inherited by the following generation, or whether the chromosome(s) in question revert to their sexually 'indifferent' state in gamete cells. The original discovery of the apparent association of Bkm-related sequences with the testis determining factor in sex-reversed mice has since been dismissed by many authors as fortuitous, and unrelated to sex determination (Spotila *et al*, 1994). Although the proposed model may therefore be unlikely, the detection of sex-specific fragments in these TSD turtles remains a significant discovery. However, the authors were unable to find sex-specific Bkm fragments in *Alligator mississippiensis* or in a preliminary investigation of freshwater turtles (Demas *et al*, 1990). In any respect, the findings of this study certainly provide further justification for searching for sex-specific DNA in the tuatara using a similar methodology.

2.4.2.4 H-Y antigen expression in the European pond turtle, *Emys orbicularis*

Studies of H-Y antigen expression in the European pond turtle (*Emys orbicularis*) have provided what is perhaps the strongest evidence yet for a TSD mechanism superimposed upon an underlying genotypic mode of sex determination. H-Y antigen is a minor histocompatibility antigen that has remained very conserved throughout vertebrate evolution, found only in males of species with male heterogamety (XX/XY), in mammals, some amphibians, and some fish species (Wachtel, 1983; Nakamura *et al*, 1987b; Spotila *et al*, 1994). In vertebrates with female heterogamety (ZZ/ZW), such as birds, some reptiles, and some amphibians, the antigen is usually specific to females (the

antigen is sometimes referred to as H-W in such cases). H-Y antigen[5] was once proposed as the primary testis-determining factor in vertebrates (Wachtel *et al*, 1975), but later experiments with sex-reversed mice strains demonstrated this is not the case (Mardon *et al*, 1989; Goldberg *et al*, 1991). However, H-Y is certainly a male-specific factor in mammals, and in other vertebrates the antigen shows higher expression levels in the heterogametic sex, for which it can be used as a marker (Girondot *et al*, 1994).[6] Zaborski (1985) demonstrated that there is a dual regulation of H-Y expression in non-mammal species: (i) in gonadal tissue, H-Y expression is under the control of sex hormones, and (ii) in non-gonadal cells, H-Y is constitutively expressed according to a non-inducible genetic mechanism.

Apparent genetic sex differences were revealed in *E. orbicularis* (a species which has TSD and lacks heteromorphic sex chromosomes) by H-Ys typing. Zaborski *et al* (1982; 1988) investigated the H-Y expression levels in gonadal and non-gonadal tissue of both embryos and juveniles obtained from eggs incubated at different temperatures. In gonadal tissue, the H-Y antigen expression phenotype always matched the sexual phenotype; females showed a high expression level of the antigen ($H\text{-}Y^+$), and males showed a low expression ($H\text{-}Y^-$), suggesting female heterogamety. At the pivotal incubation temperature (28.5°C), where both male and female hatchlings were produced, there was nearly complete concordance between sexual phenotype and the H-Y expression phenotype in non-gonadal tissue (i.e. blood) (Zaborski *et al*, 1988). However, at the cool incubation temperature of 25°C, 100% phenotypic males were produced as expected, but approximately half of these males were $H\text{-}Y^+$ and half $H\text{-}Y^-$, in non-gonadal tissue. Similarly, at the warm incubation temperature of 30°C, which produced 100% phenotypic females, roughly half of the females were $H\text{-}Y^+$ and the other rest $H\text{-}Y^-$ (Zaborski *et al*, 1982). These results were interpreted as the effects of temperature being superimposed upon a genotypic sex determination that correlated with the H-Y

[5] According to the immunological technique used for detection, there are three defined H-Y antigen types (Girondot *et al*, 1994): (i) H-Yt, detected by transplantation experiments; (ii) H-Yc, detected by cytotoxic T-lymphocytes, and (iii) H-Ys, detected serologically with anti-H-Y antibodies, which is the antigen type in this discussion. It appears that the genes for these antigens are encoded at different genomic loci, although all have some association with the Y chromosome.

[6] One exception has been found: the turtle *Siebenrockiella crassicolis*. It was reported to have male heterogamety, but the female expresses high H-Ys levels, so it is possible that there is recessive H-Ys expression in this species (Girondot *et al*, 1994).

phenotype in non-gonadal cells. Extreme incubation temperature had the effect of determining the gonadal phenotype, during the temperature-sensitive period of incubation, which in turn determined the sexual phenotype of the animal. At the pivotal temperature, however, when temperature did not have an important influence, the gonads developed according to the sexual genotype, hence the concordance between gonadal and non-gonadal antigen phenotype in these individuals. This interpretation fits exactly with the model of GSD and TSD interaction, under which concordant and discordant individuals are the expected outcome.

2.4.2.5 Non-reptile species: *Pleurodeles waltlii, Menidia menidia, Oreochromis niloticus*, and *Poeciliopsis lucida*

Various forms of environmental sex determination other than temperature-dependent sex determination have been reported to exist within some amphibians and invertebrates (reviewed by Bull, 1983). Most reported cases of sensitivity of sex determination to incubation temperature have been for reptiles, but there are a number of fish and amphibian species for which this phenomenon has also been demonstrated. In general, there appears to be a genetic mode of primary sex determination in these non-reptile species, which is overridden by the effect of extreme temperatures. This is in contrast to reptilian TSD, where temperature seems to be the dominant influence on sex determination over a most of the range of incubation temperature, with the exception of the narrow pivotal range. These examples therefore provide further evidence that genetic and temperature effects may both play a role in sex determination.

Nearly all amphibian species exhibit genotypic sex determination (Janzen and Paukstis, 1991), although it has been demonstrated for many species that the genetic influence is weak, and is susceptible to temperature extremes (Bull, 1983). The most convincing example is the newt *Pleurodeles waltlii*, in which female heterogamety GSD has been demonstrated through immunological, enzymatic, and genetic studies (although heteromorphic chromosomes are not cytologically distinguishable) (Zaborski *et al*, 1988). However, high temperature treatment of larvae causes the production of some sex-reversed ZW males, which has been demonstrated by breeding experiments at normal temperatures (Dournon and Houillon, 1984; Zaborski, 1986). H-Y antigen phenotype is negative in ZZ normal males, and positive in ZW normal females, in both

gonadal and non-gonadal cells of this newt. In sex-reversed ZW males, however, gonadal tissue is H-Y negative, and blood cells are H-Y positive (Zaborski, 1986). This suggests gonad structure, and the corresponding H-Y phenotype of this tisse, is determined by the high temperature, but the blood cell H-Y phenotype is a reflection of the inherited sexual genotype. The H-Y antigen expression pattern therefore emulates that found for *Emys orbicularis* (Zaborksi, 1982; 1988), and provides support for the interpretation that the antigen pattern reflects underlying sexual genotypes in that species of turtle.

Several species of fish are known to exhibit some degree of temperature-dependent sex determination (Bull, 1983). One interesting example is the viviparous fish *Poeciliopsis lucida.* Sullivan and Schultz (1986) tested two different strains of this fish by holding pregnant females at different water temperatures. In one strain, there was an almost exclusive male-bias at high temperature in the offspring, which decreased in correlation to decreasing water temperature, whereas the other strain demonstrated a 1:1 sex ratio of offspring at all water temperatures. The authors suggested there is a genetic polymorphism for temperature-influenced sex determination in this species.

The most well documented example of TSD in a fish, is *Menidia menidia*, the Atlantic Silverside. This species has been demonstrated to exhibit both genetic and temperature-dependent effects in sex determination (Conover and Kynard, 1981; Conover, 1984; Conover and Heins, 1987). Furthermore, Lagomarsino and Conover (1993) found that there is considerable variation in the degree of thermosensitivity in sex determination across a latitudinal gradient of *Menidia* populations. Although this contrasts with the TSD of reptiles, where the dominant effect of temperature is constant for a species over its geographical range (Mrosovsky, 1988), it is further evidence for the contention that genetic and temperature-dependent influences may both operate in the sex determination of a species. Thermosensitivity in sex determination was also demonstrated recently for a second species of teleost, the tilapia mouth brooder, *Oreochromis niloticus* (Baroiller *et al*, 1996).

2.5 THE MOLECULAR MECHANISM OF TEMPERATURE-DEPENDENT SEX DETERMINATION

The exact mechanism by which temperature ultimately acts to determine sex in TSD reptiles is still unknown, but recent research has begun to illuminate some details. In this section, the concept of pre-existing genotype is discussed in relation to the emerging picture of the TSD mechanism.

2.5.1 Estrogen: the critical factor in reptilian sex determination

It has become clear that the process of sex differentiation is very similar in mammals and reptiles, as many of the same genes and hormones are involved in the developmental pathway (Lance, 1997). For example, SRY is a gene located on the Y chromosome which initiates testis formation in placental mammals (Mittwoch, 1996). Tiersch *et al* (1991) detected the presence of SRY-like genes in reptiles, but detected no sex-specific hybridisation patterns using a conserved SRY sequence probe. SRY-like genes have been cloned in two TSD reptiles: *Alligator mississippiensis* (Coriat *et al*, 1994), and the snapping turtle, *Chelydra serpentina* (Spotila *et al*, 1994), but sex-specific expression remains to be demonstrated. One member of a SRY-related gene family, Sox9, has been shown to be expressed in the embryonic testes of all amniote species studied, including male turtle embryos (Lance, 1997). Other genes which appear to be common to both the mammalian and reptilian sex-determining cascade include SF-1 (a key regulator of steroidogenic enzyme gene expression), MIH, and possibly DAX-1 (thought to be involved in ovarian differentiation) (Lance, 1997).

Despite the apparent similarities in sexual differentiation between mammals and reptiles, the mechanism of sex determination, which sets the genetic cascade of sexual differentiation in motion, appears to be quite different. One major distinction is that the 'default' sex in mammals is female, but in reptiles it is male. In mammals, sex is determined genetically by the presence of the SRY gene, which is located on the Y chromosome. The SRY gene is required for the initiation of testis development, and in the absence of this gene, an ovary develops. Thus, in placental mammals the default sex is female.

The most important factor in reptilian sex determination is apparently not a gene, but the hormone estrogen. Sex determination in reptiles, and birds, depends upon the initiation of estrogen synthesis in the gonad. Estrogen inhibits male differentiation and stimulates ovarian development. In the absence of this hormone (or at low concentrations), a testis develops (Lance, 1997). Hence, male is the default sex in reptiles, upon which 'femaleness' is imposed. A number of investigations within the past decade have convincingly demonstrated that estrogen is the critical factor in reptilian sex determination. Typically, these experiments have involved the application of hormones and hormone-related factors to eggs being incubated at a variety of incubation temperatures, for a number of different species of reptile with TSD. It has been discovered that estrogen can in fact override the effect of temperature in TSD reptiles, as the application of estrogens (or estrogenic xenobiotics) to embryos causes female development, irrespective of the incubation temperature (Crews, 1994; 1996; Crews *et al*, 1994; Lance and Bogart, 1992; 1994; Pieau, 1996; Wibbels and Crews, 1992; Wibbels *et al*, 1994). Furthermore, it would appear that incubation temperature and the level of estrogen have a physiologically equivalent effect (Crews, 1996). The closer incubation temperature is to the pivotal temperature, the less estrogen is required to cause all of the embryos to develop as female (Wibbels *et al*, 1991).

In addition to treatment with estrogen, incubating eggs have also been treated with a non-aromatizable androgen, dihydrotestosterone (which, unlike testosterone, can not be converted to estrogen). At an incubation temperature leading to 100% female hatchling production, dihydrotestosterone had no effect on the usual sex ratio, for both turtles and alligators. However, treatment of turtle eggs at pivotal temperature produced a strong bias towards males, rather than the usual 1:1 ratios of sexes, which may suggest that the non-aromatizable substrate is interfering with estrogen production (Wibbels and Crews, 1995). However, the effects of antiestrogen application (tamoxifen) show considerable disagreement between studies, which is yet to be explained (Lance, 1997).

2.5.2 The role of aromatase in temperature-dependent sex determination

The focus of current research into TSD in reptiles is upon the gene that encodes the enzyme necessary for estrogen synthesis, aromatase. The aromatization of androgens

such as androstenedione and testosterone to form estrogens (estrone or estradiol) is catalysed by aromatase (Pieau, 1996). Aromatase inhibitors prevent aromatase from synthesising estrogen from these sex steroids. Thus, it is suspected that embryonic regulation of the aromatase gene may play a key role in sex determination of both TSD and GSD reptiles.

Several studies have found that application of an aromatase inhibitor prevented estrogen synthesis in turtle embryos incubated at a female-producing temperature, resulting in phenotypically male hatchlings (Dorizzi *et al*, 1994; Rhen and Lang, 1994; Richard-Mercier *et al*, 1995). This implies that the genetic potential for male or female development is present in all embryos, and it is simply the primary event of sex determination that decides the sex of the hatchling. It is clear that incubation temperature and estrogen synthesis within the developing embryo are closely linked in temperature-dependent sex determination. On the basis of findings from research into the role of estrogen in TSD, a general model has been proposed for the mechanism of sex determination (Pieau, 1996). In short, this model proposes that aromatase activity or expression is initiated by a female-inducing temperature during the temperature-sensitive period of embryogenesis (Pieau *et al*, 1994; Pieau, 1996). Increased aromatase activity increases estrogen amounts in the embryo which leads to ovarian differentiation. Some studies have suggested that the effect of temperature does not act directly on the activity of the aromatase enzyme, but probably acts upon its synthesis, either directly or indirectly (Desvages and Pieau, 1992a; 1992b). However, it remains to be determined whether it is the activation of the aromatase gene, or gene(s) acting upstream from aromatase, that is the initial temperature-induced trigger (Lance, 1997). There are also unresolved questions about the source of sex steroids, the substrate from which estrogen is synthesised. Studies have focused upon gonadal and kidney-adrenal tissues, but there are also large decreases in the high concentrations of sex steroids in the yolk of alligator (Conley *et al*, 1997) and turtle eggs (Lance, 1997) during the period of gonadogenesis.

2.5.3 Reconciling the hypothesis of an underlying genotypic sex determination with the aromatase model of TSD

For the hypothesis of pre-existing sexual genetic predisposition in TSD species to be plausible, the concept needs to be reconciled with this current model proposed for the TSD mechanism. The emerging picture of the TSD mechanism certainly gives no reason to postulate the existence of underlying genotypes in TSD species. However, the hypothesis of a weak GSD mechanism underlying the primary TSD mode is not inconsistent with the recent findings. If temperature is indeed acting directly, or indirectly, upon aromatase activity, and thus upon estrogen levels within the developing embryo, this does not necessarily preclude the presence of inherited genetic factors which may have a weak influence upon sex determination. For example, at pivotal temperatures, aromatase activity may be at a genetically-default level (male genotype: low expression, female genotype: high expression) and thus both male and female hatchlings are produced. Female-inducing temperature, in appropriately timed doses of exposure, may activate genetic elements (not in operation at pivotal temperature) which directly or indirectly promote aromatase expression sufficiently to induce female development, in genetic males and genetic females. Similarly, appropriate exposure to male inducing temperatures might cause the activation of other genetic elements (also not in operation at the pivotal temperature) which repress aromatase expression, in which case, all embryos (genetic males and genetic females) develop as the default sex (male) due to the low levels of estrogen. In this scenario, it is still not explained how temperature acts to regulate aromatase activity, or expression, by activation or repression of its associated regulatory elements. The point remains that there is still the potential for pre-existing genotypes to be realised at pivotal incubation temperatures. It will probably not be until the mechanism of TSD is fully elucidated that the hypothesis of underlying genotypic sex determination can be confirmed or refuted.

2.6 AN EXPLANATION OF THE METHODOLOGY

In designing the methodology for this study, there was one overwhelming consideration that had to be taken into account. It was believed that a critical issue was successful identification of individuals who are concordant with respect to their sexual phenotype

and genotype. Assuming that the hypothesis being tested is correct, it is conceivable that wild populations are comprised of 50% concordant individuals and 50% discordant individuals. If up to 50% of a sampled population may be sex-reversed, then attempting to find a molecular marker for sex using phenotypically sexed individuals would be is a nearly impossible task, unless there is some way in which concordant individuals can be preferentially selected for analysis.

One possibility is that concordant individuals (whose sexual phenotype is equivalent to their sexual genotype) will have a strong dimorphic sexual phenotype, whereas discordant individuals will have a more ambiguous sexual phenotype. In other words, it might be expected that a phenotypic male with an underlying male genotype, will be a 'better' male than a sex-reversed (discordant) male with an underlying female genotype. This could be due to a reinforcing effect of its male genetic predisposition upon its growth and development.[7] A similar situation might then be expected to be true of concordant versus discordant females.

There is no direct evidence for this notion of two classes of stronger and weaker phenotypes for each sex, but some anecdotal evidence does exist. Daugherty (pers. comm.) has noted the existence of a certain proportion of 'ambiguous' sexual phenotypes amongst the Stephens Island population of tuatara, whose morphology appears indeterminate, or intermediate between female and male. It is possible that these animals have 'intersex' phenotypes that have arisen through the aberrant development of secondary sexual characteristics, caused by factors unrelated to primary sex determination, but it is also possible that these ambiguous phenotypes are the result of antagonistic incubation temperature and genetic sex-determining factors (i.e. sex-reversal).

[7] A hypothetical scenario is that all embryos start to develop as their genotypic sex soon after fertilisation. The incubation temperature may cause this genotypic development to be reversed (discordance), or reinforced (concordance). When the sex is reversed, it could "re-start" the physiological sexual development, so this development is lagging behind the sexual development of a concordant individual at the same point in embryonic development. At sexual maturity there might therefore be two distinct classes of phenotype for each sex: weaker (discordant) and stronger (concordant) sexual phenotype.

The possibility that concordant individuals are more distinguishable as males or females was used as the basis of selection criteria for tuatara samples for this research. As the investigation progressed, care was taken to minimise the proportion of discordant individuals that could potentially have been included within the sample sets. This was believed to be achievable by only including samples of the most phenotypically unambiguous individuals available. Consequently, the research consisted of three progressive phases, each of which incorporated the most recently available tuatara blood samples.

The initial test subjects were tuatara that were sexed according to their morphological phenotype, several years prior to this study. These tuatara were resident at the Auckland Zoological Park, but it is believed they originated from Stephens Island in the Cook Strait. At the time that these individuals were blood sampled (1992), no consideration was given to the possibility that some of them might have discordant phenotypes. Since each of the animals had to be sexed, it is a real possibility that many of the individuals were designated as one or the other sex on the basis of a somewhat less than clear, unambiguous sexual phenotype. If any of the blood samples collected were from sex-reversed animals, this would obviously have a confounding effect upon the search for a sex-specific DNA marker. However, these were the only tuatara blood samples available for analysis at that time. The analysis of this particular sample set was designated as Phase 1 of the minisatellite DNA profiling survey (Chapter 3).

New tuatara samples from Lady Alice Island (Chickens Islands) became available at a later stage of the study. These blood samples were collected in conjunction with morphological measurement data of the sample animals. Thus, specific individuals were able to be selected for minisatellite DNA profiling analysis on the basis of the animals' morphological measurements (Appendix 1). Only the very largest males were included, all of which were well above the maximum size and weight limit of phenotypic females (Fig. 2.5). Similarly, only the very largest females were included. The reason for this selection criterion was the idea that concordant individuals, particularly males, might exhibit the strongest, most vigorous sexual phenotypes, and hence the most growth. At the time these blood samples, with their corresponding morphological data, were the

most appropriate samples available for study. The DNA profiling analysis of this sample set was designated Phase 2 (Chapter 3).

During an advanced stage of the investigation, yet more new samples became available for testing. These blood samples were collected in December 1998 from the tuatara population on Stephens Island in the Cook Strait, and were selected with several criteria in mind. Males were required to exhibit clear male behaviour patterns, and show strong male features, such as large body size, a proportionately large head, a prominent nuchal crest, and conspicuous spines. Females were similarly chosen based upon the strength of their physical phenotype, with the added criterion that they had to be gravid, which was determined by palpation. The analysis of these selected samples constituted the third and final phase of the minisatellite DNA profiling experiments (Chapter 3). This set of samples was also used for the RAPD analysis (Chapter 4).

Thus, it is believed that the chances of identifying a sex-specific marker improved as the research progressed from one phase to the next. The theory that sex-reversed animals have a more ambiguous sexual phenotype may be incorrect, however. There was at least one other factor which potentially assisted in limiting the inclusion of discordant individuals, however. The proportion of sex-reversed animals within a wild population of tuatara may be low. It has been proposed that the influence of temperature may not be as predominant in the sex determination of wild clutches of reptiles, as it is in the sex determination of artificially incubated clutches (Janzen and Paukstis, 1991). This would infer that the majority of hatchlings from natural nests are concordant, due to the operation of a genetic mode of sex determination. Random sampling of the population might therefore be expected to include only a proportionately small number of discordant animals. The lower this proportion, the more chance there is that molecular analyses will detect a genetic marker for sex. Girondot *et al* (1994) employed H-Y antigen expression as a marker for genetic sex for a wild population of the turtle *Emys orbicularis*, and determined that approximately 91% of the population were concordant between their genotype and sexual phenotype. Thus, only a small fraction of (adult) individuals were sex-reversed. A similar population ratio might explain why the investigation of Demas *et al* (1990) was successful in detecting sex-specific fragments in two different species of marine turtles which exhibit TSD. On the basis of these two studies alone, there is

reason to believe that the DNA profiling methodology employed by Demas *et al* (1990) might also be successful in detecting sex-specific DNA in the tuatara.

3

Minisatellite DNA Profiling: Does It Detect Sex-Specific Variation In The Tuatara?

A comprehensive minisatellite DNA profiling survey was conducted to test the hypothesis that there is sex-specific DNA in the tuatara. Five DNA probes were used in combination with 14 restriction enzymes; 66 different probe/enzyme combinations were tested in total. The DNA profiles were examined for both sex-specific fragments and sex biases in fragment numbers. The survey do not reveal any genetic sex differences in the tuatara.

3.1 INTRODUCTION

3.1.1 Sex assignment in the tuatara: previous investigation and current objectives

Prior to this study, there has been no comprehensive attempt at molecular sex assignment of the tuatara, although some studies have made superficial examinations for sex-specific DNA in this species. Like many reptiles, the tuatara exhibits temperature-dependent sex determination (TSD) (Cree *et al*, 1995), and in accordance with this, it also lacks distinct heteromorphic sex chromosomes (Wylie *et al*, 1968; Bruce Norris, pers. comm.). Since cytogenetic examination has failed to detect a difference between the sexes, the next logical step is to examine DNA organisation at the genomic level. An appropriate approach is to investigate minisatellite DNA variation, a method which has been successful in the sexing of many organisms of indistinguishable sexual morphology (e.g. Longmire *et al*, 1991; Millar *et al*, 1992; Graves *et al*, 1993).

This research constitutes only the second investigation of minisatellite DNA variation in the tuatara. Finch (1994) detailed the first such study, in which several genetic issues were investigated using multilocus DNA profiling. These included assessment of genetic relatedness within and between island sub-populations of tuatara, the assessment of inter-island genetic variability in relation to the history of geographical isolation of those islands, and finally, a brief investigation into the possibility of sex-specific differences in minisatellite organisation. In this last part of the study, the polycore probes 33.6 and 33.15 were hybridised to genomic DNA restricted with the enzymes *Alu*I and *Hae*III. While no sex-specific DNA fragments were detected, a sex-bias in the number of fragments was reported for the probe 33.15, in profiles for both of the restriction enzymes. Female tuatara were shown to have significantly more bands than males on average (Mann-Whitney *U*-test), over the fragment size range 3-23+ kilobases (kb). This finding lead Finch to conclude that 'subsequent investigations, using additional probes, may separate sex-specific fragments in the genome of the female tuatara' (Finch, 1994).

Therefore, the principal objective of this research was the completion of a comprehensive DNA profiling survey of the tuatara, involving the use of a large number of restriction enzymes in association with several different polycore DNA probes. It was also intended that other methods of molecular sex assignment would be attempted in the search for evidence of sex-specific DNA variation.

3.1.2 Molecular sex assignment and minisatellite DNA profiling

Correct identification of the sex of an individual is critical to many evolutionary, ethological, and ecological studies, particularly for the purposes of sex ratio estimation, and the management of matings amongst captive animals. In many cases, however, ascertaining the sex of an individual is problematic. Examples include species that are monomorphic in secondary sexual characteristics, species with internal gonads (e.g. birds), and the early life history stages of many species.

The discovery of molecular markers linked to sex chromosomes or sex-determining genes provides one means of sex assignment that has proven to be useful in many cases. There are several different molecular genetic techniques which have been

successful in assigning sex, in a variety of species. Genetic markers for sex that have so far been discovered include restricted genomic DNA fragments probed with unique sequences (Quinn *et al*, 1990; Dvorak *et al*, 1992; Millar *et al*, 1996), or with minisatellite (Longmire *et al*, 1991; Millar *et al*, 1992; Graves *et al*, 1993) or microsatellite DNA sequences (Longmire *et al*, 1993), as well as fragments amplified using specific PCR primers (Griffiths *et al*, 1992, 1996; Sabo *et al*, 1994; Griffiths and Tiwari, 1995; Ellegren, 1996; Nishiumi *et al*, 1996) or short, random sequence PCR primers (Levin *et al*, 1993; Griffiths and Tiwari, 1993; Wardell *et al*, 1993; Antoniou *et al*, 1994; Cushwa and Medrano, 1994; Lessells and Mateman, 1998; Bello and Sánchez, in press).

Genetic markers can be detected using minisatellite sequence probes by the process of *DNA profiling* (also known as DNA fingerprinting). Minisatellite loci are hypervariable regions of DNA, comprised of arrays of short, tandemly-repeated units of DNA sequence (Jeffreys *et al*, 1985a). These repeated units, or minisatellites, are highly variable in number between loci (Jeffreys *et al*, 1985a; Burke, 1991), hence minisatellite regions are also known as variable number of tandem repeats (VNTR) loci (Nakamura, 1987). Each unit contains a short, highly-conserved core sequence of 9-64 bp in length, which is typically G-C rich in content (Jeffreys *et al*, 1985a; Nakamura, 1987; Avise, 1994). The more or less constant core sequence of a repeat unit is flanked by a sequence which varies between the different minisatellite loci in the genome; at some of these loci the repeat units are very similar within that region, at other loci, there is a variety of different forms of each repeat unit (Jeffreys *et al*, 1985a). Minisatellite regions formed by these arrays of repeat sequences may be anywhere from 0.1-20+ kilobases (kb) in length, comprised of up to several hundred repeat units.

Wyman and White (1980) made the first discovery of a hypervariable polymorphic locus, which was characterised by a number of variable length restriction fragments. Their chance discovery suggested the existence of many other DNA regions demonstrating such restriction fragment length polymorphisms (RFLPs). Many additional polymorphic loci were soon found in the human genome, several of which were associated with known genes (Spritz, 1981; Bell *et al*, 1982; Capon *et al*, 1983; Weller *et al*, 1984; Jeffreys *et al*, 1985a; Jarman *et al*, 1986; Thein *et al*, 1986). The restriction fragments were found to contain tandem repeats of short sequences, and it

was realised that the high degree of polymorphism between loci was due to variation in the number of repeat units for each allele. Restriction fragment length differences thus occurred when restriction enzymes with cutting sites outside the repeat unit were used to digest the genomic DNA.

Jeffreys *et al* (1985a) were the first to demonstrate that 'families' of these repeat regions exist within the human genome, meaning that DNA probes based on the tandem repeat core sequence will hybridise to a large number of different genomic loci. They showed that a 33 bp tandem repeat from an intron of the human myoglobin gene hybridised to other loci in the human genome, producing a complex RFLP pattern. A number of regions with the repeat unit were isolated from a human genomic library, each region generally consisting of repeat units varying from 16-62 bp in length. The repeat units were shown to contain a highly conserved core of 10-15 bp, which for each unit was flanked by a sequence which varied between the different regions. The original region was named 33.15.

The complex RFLP patterns produced by hybridisation of the first discovered minisatellite sequences to human genomic DNA were termed multilocus DNA 'fingerprints' by Jeffreys *et al* (1985a). This was because the hypervariability of the minisatellite loci detected produced restriction fragment patterns that were essentially individual-specific. The process of DNA 'fingerprinting', is thus a molecular approach that involves Southern blot assays (Southern, 1975) of hypervariable DNA regions that reveal individually-specific gel banding patterns (the only exception is the DNA fingerprints of monozygotic twins [Jeffreys *et al*, 1987]). The term 'DNA profiling' is now often used in place of the term 'fingerprinting'.

It was also discovered that minisatellite probes differing in core sequence and length could detect new minisatellite families in the genome (Jeffreys *et al*, 1985b), an example of which is the probe 33.6, a shortened derivative of the core sequence of the originally described human minisatellite region, 33.15 (Jeffreys *et al*, 1985a). Following these initial discoveries of Jeffreys and his coworkers, several other human multilocus DNA profiling probes were subsequently developed, many of which have core sequence similarity to 33.15 (Ali *et al*, 1986; Vassart *et al*, 1987; Fowler *et al*,

1988; Longmire *et al*, 1990; Pena *et al*; 1990). Not surprisingly, in some cases these new probes hybridise to the same restriction fragments as the 33.15 probe

Minisatellite regions are not exclusive to humans. They have been identified in a diverse range of other species, including mammals (Hill, 1987; Jeffreys and Morton, 1987; Jeffreys *et al*, 1987; Hoezel *et al*, 1991), birds (Burke and Bruford, 1987; Brock and White, 1991; Hanotte *et al*, 1992; Meng *et al*, 1990; Millar *et al*, 1992), reptiles (Demas *et al*, 1990; Finch, 1994), fish (Baker *et al*, 1992), invertebrates (Coffroth *et al*, 1992; Jarne *et al*, 1990, 1992; Zeh *et al*, 1992), plants (Dallas, 1988; Rogstad *et al*, 1988; 1991), and even micro-organisms (Ryskov *et al*, 1988; Rogstad *et al*, 1989). Furthermore, the original Jeffreys' probes have been demonstrated to cross-hybridise to many species, sometimes producing DNA profiles of similar complexity to humans (Avise, 1994).

Several properties of DNA profiles make them reliable genetic markers (Jeffreys *et al*, 1985a; 1985b). First, they show somatic stability, which means that profiles derived from different tissues of the same individual are identical. Second, segregation analysis has shown detected fragments are independent of one another. Third, segregation analysis has also demonstrated that the fragments segregate in a Mendelian manner; that is, on average, an individual receives approximately half of its restriction fragments from each of its two parents (Jeffreys *et al*, 1986).

This last property of DNA profiling has lead to one of the most widely-used applications of the technique: determination of parentage. Any particular fragment can be directly ascribed to one or the other parent (barring spontaneous de novo mutation [Jeffreys *et al*, 1988]). Generally, unrelated individuals share very few bands, for example, human individuals share approximately 20% of their bands with non-relatives. DNA profiling has also been of use in segregation analysis for detecting fragments in linkage with disease loci or tumours, and in forensic science (Wong *et al*, 1986; McElfresh, 1993).

Paternity/maternity analysis by DNA profiling has had invaluable application in numerous studies of animal species as well (e.g. Gilbert *et al*, 1991; Hoezel *et al*, 1991; Tegelström *et al*, 1991). Determination of parentage and kinship is essential for

deciphering genetic relationships in animal social groups and populations, as well as the measurement of reproductive success in studies of mating systems. DNA profiling studies have been enormously successful in increasing understanding of population structures and behavioural patterns, by clarifying mating strategies and cooperative behaviour between relatives and non-relatives (e.g. Quinn *et al*, 1987; Burke *et al*, 1989; Rabenold *et al*, 1990; Amos *et al*, 1991; Ribble, 1991; Marinelli *et al*, 1992; Haig *et al*, 1994; Watt and Fenton, 1995). DNA profiling has also found application in ecological studies requiring the identification of potential species, population, and strain specific genetic markers (e.g. Nybom *et al*, 1989; Gilbert *et al*, 1990; Meyer *et al*, 1991; Zeh *et al*, 1992).

The application of profiling that is of most relevance to the current study is molecular sex assignment. Despite some of the initial studies of hypervariable repeat regions suggesting that the regions are located predominantly on autosomal chromosomes (Jeffreys *et al*, 1985a; Jeffreys, 1987), several studies have demonstrated the association of these regions with sex chromosomes or sex-determining genes. The technique of multilocus DNA profiling has thus been successful in assigning sex in a range of organisms, using several different probes.

For example, the original human minisatellite probe 33.15 (Jeffreys *et al*, 1985a) has been found to reveal sex-specific fragments in mice (Jeffreys *et al*, 1987), the hooded seal (McRae and Kovacs, 1991), the striped-back wren (Rabenold *et al*, 1990; 1991), and *Aratinga* parrots (Miyaiki *et al*, 1992). Another multilocus probe, pV47-2, which was originally isolated from the human chromosome 16 (Longmire *et al*, 1990), detected female-specific restriction fragments in the brown skua (Millar *et al*, 1992). In this particular study, it was found that 33.15 also hybridised to some of the female-specific fragments to which pV47-2 strongly hybridised, which is not surprising given that there is similarity between the sequences of the probes.

Bkm is another multilocus DNA probe that has been found to hybridise preferentially to one sex or the other, in a range of species. Banded krait minor satellite DNA (Bkm) was first isolated from the W chromosome of an elapid snake, *Bungarus fasciatus* (the Indian Banded krait) (Singh *et al*, 1980). This satellite DNA sequence has been shown to consist of highly repetitive sequences largely comprised of the tetranucleotide repeat

GATA (Singh *et al*, 1984). The Bkm probe is a clone of the whole Bkm sequence, so essentially it is a unique sequence DNA probe. Bkm was in fact the first sex-associated DNA identified in a species, when the sequences were confirmed as being on the W chromosome of *Bungarus fasciatus*, using *in situ* hybridisation (Jones and Singh, 1981). Sex-specific differences in Bkm hybridisation, or sex-specific fragments, have also been found in quail (Jones and Singh, 1981), mice (Singh and Jones, 1982; 1984), horses (Kent *et al*, 1988), humans (Singh and Jones, 1986), possums (Sarre, pers.comm), and, of course, two species of marine turtles (Demas *et al*, 1990). However, Bkm has not been found to hybridise sex-specifically to the DNA of all species thus examined (e.g. Lloyd *et al*, 1989).

All three of these DNA probes, 33.15, pV47-2, and Bkm, were used in this investigation, in addition to two other available probes: 33.6 and *per*. 33.6 (Jeffreys *et al*, 1985a) is a shortened derivative of the 33.15 core sequence, and as such, sometimes hybridises to the same restriction fragments, but in general, it produces quite distinct profiles. *Per* is a sequence originally isolated from the *per* locus of *Drosophila* (Shin *et al*, 1985), and within the sequence is a large number of tandemly-repeated hexamer sequences. It has been demonstrated to hybridise to the genomic DNA of a range of species, including humans, chickens, and mice (Shin *et al*, 1985), although without any sex-specific pattern. Despite this, there is probably no reason to think that *per*, and 33.6, might not have an equal potential to reveal a sex-specific fragment in the tuatara, as do 33.15, pV47-2, and Bkm. These five probes, and their core sequences, are shown in Table 3.1.

3.2 METHODS AND MATERIALS

3.2.1 Strategies for detecting sex-specific DNA using minisatellite DNA profiling

The experimental programme was divided into three distinct stages, designated Phases 1,2, and 3. Each of these phases corresponded to a different set of tuatara samples. The first two sets of samples were replaced by different samples during the course of the investigation, in an effort to minimise the potential for accidental inclusion of sex-reversed individuals in the DNA profiles (Chapter 2). The initial Phase 1 tuatara blood

TABLE 3.1 DNA probes used in all three Phases of the DNA profiling survey, their core sequences, and the organisms from which they originate. Of these probes, only Bkm originates from a reptile species; the Indian banded krait (*Bungarus fasciatus*).

Probe	Core sequence	Source	Reference
33.6	$(AGGGCTGGAGG)_3$	human myoglobin gene	Jeffreys *et al*, 1985a
33.15	AGAGGTGGGC AGGTGG	human myoglobin gene	Jeffreys *et al*, 1985a
Bkm	$(GATA)_n$	banded krait minor satellite	Singh *et al*, 1980
per	ACNGGN	*Drosophila per* locus	Shin *et al*, 1985
pV47-2	GAGGGTGGXG GTCT	human chromosome 16	Longmire *et al*, 1990

samples were replaced by a new set of samples (Phase 2) for which it was believed there was less chance of the inclusion of discordant animals. Similarly, the Phase 2 samples were replaced at an advanced stage of the investigation by newly-available Phase 3 samples, for which it was considered there was even less chance of the inclusion of sex-reversed individuals. The progression from one set of samples to the next reflected the increasing use of specific selection criteria that were believed to assist in the identification of sexually concordant tuatara. These selection criteria were based upon the theory that concordant individuals may have less ambiguous sexual phenotypes than sex-reversed animals (discussed in Chapter 2).

The Phase 1 blood samples were collected in 1992 at Auckland Zoo, and at that time no consideration was given to the possibility of sex-reversed individuals being sampled. It is a distinct possibility that some of the individuals were designated as one or the other sex on the basis of a somewhat less than clear, unambiguous sexual phenotype. This could mean that there were discordant animals amongst the Phase 1 samples. Unfortunately, these samples were all that were available at the beginning of the investigation.

At a later stage, new samples (from the Lady Alice Island population) became available for analysis (Phase 2). The sexing of these animals is considered to have been more reliable than the sexing of the Phase 1 animals. Furthermore, these blood samples were collected in conjunction with morphological data measurements (Appendix I). Only the largest males and females were used for the Phase 2 DNA profiling survey (the reasons for which, were discussed within Chapter 2). Again, these were the most appropriate tuatara samples available at that time.

The third and final Phase of DNA profiling began when another new set of samples became available at a late stage of the investigation. These were collected from the Stephens Island population, and the animals bled were selected upon the basis of several criteria aimed at excluding individuals with sexually ambiguous phenotypes. Males were required to exhibit clear male behaviour patterns, and show strong male features, such as large body size, a proportionately large head, and a prominent nuchal crest and spines. Females were similarly chosen based upon the strength of their

physical phenotype, with the added criterion that they were gravid, which was determined by palpation.

Therefore, the DNA profiling survey was divided into three separate phases, and consequently, many of the probe-enzyme combinations were repeated for each distinct sample set. It is believed that the chances of detecting a sex-specific fragment improved from one phase to the next.

The strategy employed to detect sex-specific DNA by the method of minisatellite DNA fingerprinting consisted of two distinct steps. The initial step was pilot surveys of each probe/enzyme combination, involving nine tuatara of each sex. When a potential sex-specific fragment was detected in these initial surveys, a second step was performed, in which the particular probe/enzyme combination was repeated for a considerably larger sample number of animals (24 of each sex), in order to verify the sex-specificity of the fragment in question. Moreover, the increased sample size experiments were conducted upon an independent set of individuals, to avoid the bias arising from the inclusion of the animals in which the potential sex-specific fragment had first been detected.

3.2.2 Sites and collection of blood samples

Blood samples were obtained from tuatara at three different sites, corresponding to the three phases of DNA profiling conducted. Blood samples from the Phase 1 tuatara were collected in 1992 by Michelle Finch from the Auckland Zoological Park, but these individuals are believed to be originally from the Stephens Island population. Blood samples from the Phase 2 animals were collected from Lady Alice Island, of the Hen and Chickens Island group (off the north-eastern coast of the North Island), in 1997 and 1998 by Graham Ussher of Auckland University. Phase 3 samples were collected by Victoria and Massey University staff from Stephens Island in the Cook Strait in December 1998. The tuatara were caught by hand and restrained while blood was taken ventrally from the caudal vein in the tail. During the collection of the Phase 1 samples, heparinised syringes were used, but during the Phase 2 and 3 collections, the needles were treated with 0.5M dipotassium EDTA to prevent the blood clotting. The animals were held until blood clotting of the wound had occurred and then

released. Blood samples were frozen upon the completion of sampling. All blood samples were kept at –80°C until the time of DNA extraction.

3.2.3 Extraction of genomic DNA

Total DNA was extracted from the whole blood. 15-20µl of blood was added to 500 µl lysing solution (131mM NH_4Cl, 0.9mM NH_4HCO_3), and rocked gently for 10 minutes. The lysate was centrifuged at 13000 rpm for 10 minutes and the supernatant discarded. The pellet was resuspended in 400µl of SET buffer, to which 10µl of Proteinase K (20mg/ml) and 20µl of sodium dodecyl sulphate (10% SDS) were added to final concentrations of 0.5mg/ml and 0.5% respectively. Samples were then incubated overnight at 55-65°C while rotating inside a Hybaid oven. DNA was isolated by phenol/chloroform extraction: an equal volume (430 µl) of phenol was first added, followed by two steps of addition of an equal volume of phenol/chloroform/isoamyl alcohol (PCI, 25:24:1). The DNA was then cleaned by addition of an equal volume of chloroform/isoamyl alcohol (CI, 24:1). At each step, after the addition of phenol, PCI, or CI, the samples were rocked for 30 minutes and centrifuged at 5000 rpm for 10 minutes, then the organic phase was removed, leaving the aqueous phase. DNA was precipitated after the CI wash by addition of 1/10 volume (43µl) 3M sodium acetate (pH 5.2) then 2x volume (860µl) cold 100% ethanol. Samples were rocked for 15 minutes and centrifuged at 13000 rpm for 30 minutes. The DNA was then washed twice with 1ml 70% ethanol. After each wash the DNA was centrifuged at 13000 rpm for 10 minutes, and the ethanol removed. The DNA pellet was allowed to air-dry for a few minutes. Finally, 40-60µl of nano-pure H_2O was added to the pellet, and the DNA was left for 1-3 days to allow it to fully resuspend. Resuspension was tested for by checking the viscosity of the DNA with a micropipette.

3.2.4 Digestion of genomic DNA

Resuspended DNA was digested with one of 14 restriction enzymes (GibcoBRL, Boehringer Mannheim, New England Biolabs) (Table 3.2). 10 µl samples of resuspended DNA were digested at 37°C for an initial period of at least six hours (but typically overnight) in the enzyme manufacturer's supplied buffer. The digestions

TABLE 3.2 Restriction enzymes used in the DNA profiling survey. The quality of the DNA profiles in Phase 1 varied considerably from one enzyme to another, hence in Phase 2, only those enzymes that produced profiles of good band resolution were used. Phase 3 only incorporated the six most effective enzymes. Interestingly, all of the enzymes that were abandoned after Phase 1 have cutting site sequences of 6 base-pairs.

Restriction enzyme	Cutting site sequence 5' → 3'	length of sequence (bp)	quality of profiles	Phase 1	Phase 2	Phase 3
*Alu*I	AG↓CT	4	excellent	•	•	•
*BamH*I	G↓GATCC	6	poor	•		
*BstN*I	CC↓(A,T)GG	5	good	•	•	
*EcoR*I	G↓AATTC	6	poor	•		
*Hae*III	GG↓CC	4	excellent	•	•	•
*Hind*III	A↓AGCTT	6	poor	•		
*Hinf*I	G↓ANTC	5	good	•	•	•
*Mbo*I	↓GATC	4	good	•	•	•
*Mbo*II	GAAGA$(N)_8$↓	5 (+8)	good	•	•	
*Msp*I	C↓CGG	4	good	•	•	
*Pvu*II	CAG↓CTG	6	poor	•		
*Rsa*I	GT↓AC	4	good	•	•	
*Sst*I	GAGCT↓C	6	poor	•		
*Xho*I	C↓TCGAG	6	poor	•		

were in an initial total volume of 19.5µl which included 5 units of enzyme, 4mM spermidine trihydrochloride and 100µg/ml of bovine serum albumin (BSA). Following the initial period of incubation, a further 5 units of enzyme (0.5µl) was added to each sample, and incubation at 37°C continued for 3-4 hours. 1µl samples of the digested DNA samples were run out in 0.8% agarose minigels, which were stained in ethidium bromide solution and visualised under UV light to confirm complete digestion had occurred. The concentrations of the digested DNA samples were determined with a Hoefer DyNA Quant 200 fluorometer, calibrated with 100µg/µl calf thymus DNA (Sigma Chemical Co.). The digested DNA samples were diluted accordingly with nano-pure H_2O to a standard concentration of 5µg of DNA in each sample, in preparation for loading into the profiling gels.

3.2.5 Gel electrophoresis and Southern blotting

DNA fragments were size-separated by electrophoresis in 0.8% agarose gels measuring 195mm wide x 300mm long. The gels were run in 1xTBE buffer (134mM Tris, 74.9mM Boric Acid, 2.55mM EDTA) at 55 volts (0.183 $V.cm^{-1}$). The running time for each of the 35 gels ranged from 29-48 hours (dependent upon the restriction enzyme). Molecular size markers were run on outside lanes to allow each gel to be standardised with respect to the distance run. During Phase 1, the size marker was λ DNA/*Hind*III cut fragments, whereas in Phase 2 and Phase 3 gels the size marker was a mixture of the λ DNA/*Hind*III cut fragments and 1kb DNA ladder. Following electrophoresis the gel was removed from the running buffer, flipped over and the top right corner was excised to orientate the gel. The top section of the gel, measuring about 37mm deep and including the loading wells, was also removed. The gel was then immersed and rocked gently in 500ml depurination wash (0.2M HCl) for 15 minutes, 500ml denaturation wash (1.5M NaCl, 0.5M NaOH) for 45 minutes, and 500ml neutralisation wash (1.5M NaCl, 0.5M Tris-HCl pH 7.2, 2mM EDTA) twice for 15 minutes. Next, the DNA was transferred in 6xSSC (0.9M NaCl, 90mM sodium citrate) from the gel by Southern blotting to a nylon membrane (GeneScreen Plus, NEN™ Life Science Products). Paper towels in the blotting 'stack' were replaced at intervals during the blotting time to expedite transfer of the DNA. After blotting for a minimum period of 12 hours, the membrane was rinsed briefly in 6xSSC then allowed to air-dry at room

temperature. It was then sandwiched between 4 sheets of Whatman chromatography paper and wrapped in metal foil. DNA was bound to the nylon membrane by baking the wrapped membrane at 80°C in a Contherm oven for 2-3 hours.

3.2.6 DNA hybridisation and autoradiography

Membranes were prehybridised in 0.25M Na_2HPO_4 pH 7.2, 1mM EDTA pH 8.0, 7% SDS solution at a set temperature within the range 55-65°C for 2-3 hours before addition of the probe. The various combinations of probe and hybridisation temperature are shown in tables 3.3 (a),(b), and (c). The probes 33.6, 33.15, Bkm, *per*, and pV47-2 (for references see table 3.1) were labelled with α-^{32}P by random priming (GibcoBRL RTS RadPrime DNA Labeling System), and were spun through sephadex columns to remove unincorporated isotope before being added to the prehybridisation solution. Hybridisation duration was for a minimum of 12 hours. Following hybridisation, membranes were twice washed at the hybridisation temperature with 5xSSC, 0.1% SDS solution for 30 minutes to remove excess unincorporated probe. For a few hybridisations where greater stringency was required, the membrane was then also washed for 30 minutes once in 3xSSC, 0.1% SDS, once in 1xSSC, 0.1% SDS, and on a very few occasions, once or even twice with 0.5xSSC, 0.1% SDS. Membranes were then wrapped in Saran plastic wrap and exposed to autoradiography film at -80°C with one intensifying screen for typically 1-21 days. Exposures were developed for three minutes in a developing solution (Kodak), rinsed for two minutes in running water, placed in a fixing solution (Kodak GBX) for two minutes, and then rinsed again in water for several minutes. The exposures, or 'fingerprints', were then allowed to air-dry.

Following hybridisation and exposure to autoradiography film, the hybridised probe was 'stripped' from the membrane by washing in 0.4M NaOH solution for 45 minutes at 45°C, followed by neutralisation in 0.1xSSC, 0.1% SDS, 0.2M Tris-HCl pH 7.5 solution for 30 minutes at 45°C. The stripped membrane was allowed to dry, and stored at room temperature until required for re-probing.

TABLE 3.3 (a) Restriction enzyme and probe combinations tested during PHASE 1 of the DNA profiling survey. Phase 1 included samples from the Auckland Zoological Park. Numbers refer to the hybridisation temperatures tested for each combination of restriction enzyme and probe (degrees Celsius). Some of the specific combinations were not tested during Phase 1, but were included in subsequent phases of the study, e.g. *Mbo*II restriction fragments probed with pV47-2.

PHASE 1

Restriction enzyme	DNA probe				
	Bkm	*per*	pV47-2	33.6	33.15
*Alu*I	55, 61, 63	55, 61	55, 57, 58	55, 58	58, 59, 61
*BamH*I	59	55	-	55	61
*Bst*NI	55, 61	55	55, 58	55, 58	55, 58
*Eco*RI	55	55	58	55, 58	55, 58
*Hae*III	55, 58, 61	55	55, 58	55, 58, 61	58, 59, 61
*Hind*III	55, 61	55	55	55, 58	55, 58
*Hinf*I	55	55	55, 58	55, 58	55, 58
*Mbo*I	55, 61	55	55, 58	55, 58	55, 58
*Mbo*II	59	55	phase 2	55	phase 2
*Msp*I	59	55	phase 2	55	-
*Pvu*II	55, 61	55	55, 58	55, 58	55, 58
*Rsa*I	59	55	phase 2	55	61
*Sst*I	59	55	-	55	-
*Xho*I	55	55	58	55, 58	55, 58

TABLE 3.3 (b) Restriction enzyme and probe combinations tested during PHASE 2 of the DNA profiling survey. Phase 2 included tuatara from Lady Alice Island in the Hen and Chickens Island group. Numbers refer to the hybridisation temperatures tested for each probe and enzyme combination (degrees Celsius).

PHASE 2

Restriction enzyme	DNA probe				
	Bkm	*per*	pV47-2	33.6	33.15
*Alu*I	61	57	57	57	57, 60
*BstN*I	61	57	57	59	60
*Hae*III	61	57	57	57	57, 60
*Hinf*I	61	57	57	57	57, 59, 60
*Mbo*I	55	phase 3	57	phase 3	phase 3
*Mbo*II	61	57	57	57	57, 60
*Msp*I	55	phase 1	57	phase 1	-
*Rsa*I	61	57	57	59	60

TABLE 3.3 (c) Restriction enzyme and probe combinations tested during PHASE 3 of the DNA profiling survey. Phase 3 included tuatara from Stephens Island. Numbers refer to the hybridisation temperatures tested for each probe and enzyme combination (degrees Celsius).

PHASE 3

Restriction enzyme	DNA probe				
	Bkm	*per*	pV47-2	33.6	33.15
*Alu*I	63, 65	63	57, 59	63	59, 63
*BstN*I	63, 65	63	57, 59	63	63
*Hae*III	63, 65	63	57, 59	65	59, 63
*Hinf*I	63, 65	63	57, 59	65	63
*Mbo*I	65	63	57, 59	63	63

3.2.7 Analysis of DNA profiles: checking for sex-specificity and band scoring

3.2.7.1 Examination of the DNA profiles for sex-specific fragments

Each of the DNA profiles (autoradiography exposures) was examined thoroughly for potential sex-specific fragments. First, all profiles were inspected for the presence of obvious sex-specific bands. An 'obvious' band meant any band present in every individual of one sex, and absent from all members of the opposite sex. Each gel run (with a few exceptions) consisted of nine individuals of each phenotypic sex. The probability of a DNA fragment appearing in nine of nine of sex A, and zero of nine of sex B (designated as a ratio of 9:0), but not being sex-linked, is extremely low (1 in 24310). Thus, a distribution of 9:0 would have been statistically strong evidence of a genetic marker.

It was also possible that sex-specific fragments were present, but were not obvious (not appearing with a 9:0 distribution). As already noted, there was the potential for inclusion of discordant individuals within the profiles, which would confound the expected ratio of 9:0 for a true genetic marker. The inclusion of one discordant individual within a gel, for instance, would alter this ratio to 8:0, or 9:1, depending on whether the marker was for the genotype of the discordant individual, or for the genotype of the opposite sex. Inclusion of two discordant individuals would have an even greater masking effect, giving possible marker ratios of 9:2, 8:1, or 7:0. The probabilities of these ratios occurring by chance for a non-sex-linked marker are considerably higher than for a ratio of 9:0, but they are still very slim.

If there were three or more discordant animals amongst the 18 individuals in a gel, then it would have been even more difficult to distinguish a true genetic marker for sex from a non-sex-linked fragment with an unlikely distribution. A fragment that has no connection to the sexual genotype would be expected to appear with the same frequency in males as it would in females. For example, a specific fragment occurring in five of nine females and four of nine males (5:4) would seem very unlikely to be a genetic marker for sex (assuming no discordance amongst the sample individuals), but this would be a fairly likely ratio if that fragment was not linked to sex, but was found

amongst all individuals at a frequency of 0.5. That same fragment would be unlikely to appear in the skewed distribution of eight of nine females and one of nine males (8:1), unless it was linked to sex. Given this 8:1 ratio, for that fragment to be a marker for sex requires that one of the phenotypic males, and one of the phenotypic females, are sex-reversed.

The question, then, was at what point should a ratio biased towards one sex or the other be taken as indicative of a genetic marker (confounded by the inclusion of a small number of discordant individuals amongst the samples), instead of being considered as simply a statistically unlikely skew of the distribution of a fragment that is in fact present with the same frequency in males and females? Appendix IV explores some of the possible scenarios of fragment distributions close to the ideal expected for a true genetic marker for sex (i.e. the fragment is present in nine of nine of sex A and zero of nine of sex B). In using nine of either phenotypic sex, it might be reasonably expected that on average there are nine of either genotypic sex amongst that group (i.e. 50%), regardless of the degree of correlation between sexual phenotype and genotype within those individuals. Hence the table in Appendix IV centres around the possible variations on the distribution of a fragment present in nine of the eighteen individuals within a profiling gel. The possible distributions for seven, eight, ten, and eleven individuals possessing the fragment in question are also examined. The figures within this table are based upon the assumption that that there are no discordant individuals within the sample set. The probabilities given are the likelihood of occurrence of each biased distribution, assuming there is no connection between that fragment and the sexual phenotype. Where a distribution is very unlikely to occur, there is a high probability that the fragment is in fact a sex-specific marker (which would infer that the slight deviation from 9:0 is a result of one or more sex-reversed individuals being included amongst the samples). For example, if a band appeared in seven of nine females and one of nine males, the probability of that distribution occurring by chance would be 0.0148 or about 1.5%. Such a low probability would tend to suggest that there is a link between the fragment in question and females phenotype. It might therefore be reasonably hypothesised that the fragment is a genetic marker for an underlying sexual genotype, and that amongst the phenotypic males there was one sex-reversed individual, and two amongst the phenotypic females (hence the 7:1 ratio).

Due to this potential for the inclusion of sex-reversed samples, the DNA profiles were therefore inspected not only for a fragment in the ratio 9:0, but were also examined for instances of specific fragments appearing as slight variations upon this ratio, such as 9:1, 9:2, 8:1, 8:0, or 7:0. When suspicious ratios of fragment distribution arose, they were tested for sex-linkage by repeating the probe-enzyme combination on a increased sample size of independent samples.

3.2.7.2 Band scoring to test for sex-biases in fragment numbers

A total of 50 DNA profiles were scored to determine the number of DNA fragments revealed for each individual animal.[1] Each scored autoradiography exposure represented a unique probe-enzyme combination, for a given hybridisation temperature, for a specific phase of the DNA profiling. In many cases, for a specific probe-enzyme combination, several exposures of varying intensity were compared to aid in the accurate scoring of light and dark profile bands.

For every profile scored, the mean number of fragments for each phenotypic sex was calculated, and the distributions of the ranked observations were compared using a Mann-Whitney *U*-test to detect any significant bias in fragment number towards one sex or the other. The fragments were only scored over the region of the profiles for which scoring could be done accurately and reliably. This restricted the counts to a particular size range of fragments, typically 4.4 kilobases (kb) up to 23+ kb. This range varied slightly for many profiles, however, due to differences between the probe-enzyme combinations in their distribution of clearly resolvable fragments.

To avoid potential bias due to subjective judgement, no prior assumption was made as to which of the two sexes might be heteromorphic and hence display the higher average number of bands. Although Finch (1994) reported that females had a higher number of bands on average (suggesting a ZZ/ZW chromosome system), it was always presumed there was a possibility this result was a very unlikely statistical deviation (or simply

[1] This is somewhat less than the total number of possible combinations, since the remaining number of exposures were not scored due to poor band resolution of the profiles. Even though the majority of these unscored profiles did show sufficient band resolution to allow satisfactory checking for sex-specific fragments, it was felt that sufficiently accurate band scoring to generate reliable band counts was not always possible.

incorrect), and that males might in fact have a larger average number (suggesting an XX/XY chromosome system).

3.3 RESULTS

3.3.1 Probe-enzyme combinations

Each of the probe-enzyme combinations tested produced a unique 'profile' pattern of DNA fragments. However, the quality of these profiles varied considerably from one restriction enzyme to the next. Generally, the 4- and 5-base pair (bp) cutting site restriction enzymes produced DNA profiles of high quality, but the 6-bp cutting site enzymes did not (Table 3.2). An example of the obvious differences between restriction enzymes can be seen in Figure 3.1, which shows the DNA of the same six individuals restricted with three different enzymes, *EcoR*I, *Msp*I, and *Rsa*I. In this profile, the *EcoR*I restriction pattern produced a high-molecular weight DNA smear, suggesting it is not a good enzyme for profiling tuatara. *Msp*I gave a markedly better result, showing several easily distinguishable fragments in the high molecular weight size range. *Rsa*I is clearly the most suitable profiling enzyme of the three, however, producing a good resolution of fragments over the widest range of fragment size.

The profiles for the enzyme *Msp*I actually improved with further attempts, producing a better resolution of bands over a wider size range than shown for Figure 3.1. *EcoR*I, on the other hand, did not improve with subsequent attempts, continuing to produce high molecular weight DNA smears in additional gels. This pattern was typical of all the 6-bp cutting site enzymes, each of which were used for at least two separate profiling gels. The reason for the failure of this group of restriction enzymes to give profiles of good resolution is not known. It might be due to an inherent property of the tuatara genome: the appropriate cutting site sequences could be so few and far between that a large excess of high-molecular weight fragments is invariably the result of the digestion. Alternatively, it may reflect a technical deficiency in the digestion procedure, but considering that profiles for these enzymes were repeated without further success, this would not seem to be the case.

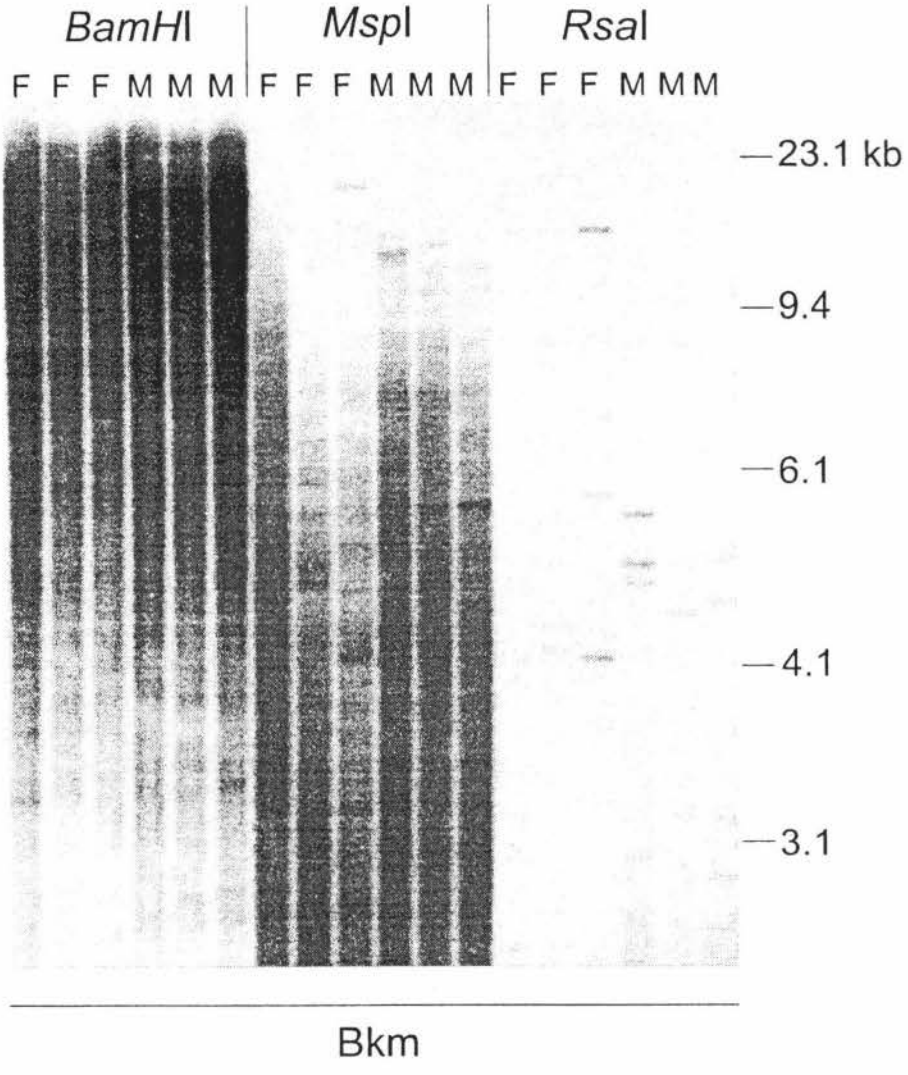

Figure 3.1 DNA profile showing tuatara DNA digested with three different restriction enzymes and hybridised with Bkm at 59°C. This demonstrates the variation in quality of tuatara DNA profiles for different restriction enzymes. Each of the three enzymes was used to restrict the genomic DNA of the same three males and females. *BamH*I, a 6-bp cutting site enzyme, resulted in poor resolution of profile bands, which appear as a high-molecular weight smear. This was typical of the 6-bp cutting site enzymes used in the survey. *Msp*I, a 4-bp cutter, is markedly better, although there is good band resolution only in the high molecular weight range. *Rsa*I proved to be the best profiling enzyme of these three for tuatara DNA, showing distinct bands over a wide fragment size range. *BamH*I was not persisted with beyond Phase 1 of the DNA profiling survey. (N.B. subsequent profiles of *Msp*I-digested DNA showed a considerable improvement in band resolution). Numbers show molecular weight markers. M = male, F = female.

Regardless of the reason, the 6-bp cutting site enzymes were abandoned at the completion of Phase 1 of the profiling survey. Phase 1 constituted the most comprehensive survey of probes-enzyme combinations, involving 14 enzymes and six DNA probes. Unfortunately, since six of those enzymes failed to produce quality profiles, many of those combinations could not be properly analysed, and were certainly unable to be scored for fragment numbers. While this undoubtedly impacted upon the comprehensiveness of the survey, it seemed that these particular enzymes were unlikely candidates for revealing sex-specific fragments. This was because in the cases where bands could be resolved, they were few in number, of high molecular weight, and often showed very little variation.

Phases 2 and 3 were thus carried out using only those restriction enzymes that were proven in Phase 1 to produce high band resolution in tuatara DNA profiles (Table 3.2). Eight enzymes were used for Phase 2, and the five best profiling enzymes used for Phase 3, in an effort to maximise the chances of discovering a sex-specific marker.

One general result of interest arising from the DNA profiling survey was that the DNA probes Bkm (Figs. 3.2 and 3.3) and pV47-2 (Figs. 3.4 and 3.5) both hybridised extremely well to tuatara DNA, indicating strong homology, particularly in the case of Bkm. This finding is contrary to the previous study of tuatara minisatellite variation conducted by Finch (1994), in which no homology was found between these two probes and tuatara DNA.

Each of the five DNA probes produced a distinctive DNA profile pattern, which was generally consistent for that probe for all of the different restriction enzymes (for Phases 2 and 3). Typical DNA profiles for each of the probes are shown in Figures 3.2 and 3.3 (Bkm); Figures 3.4 and 3.5 (pV47-2); Figure 3.6 (33.6); Figure 3.7 (33.15); and Figures 3.8 and 3.9 (*per*).

3.3.2 Examination for sex-specific fragments

No sex-specific fragments were detected in the minisatellite DNA profiling survey. On no occasion did a fragment appear with a distribution of 9:0, or even a distribution close to this (e.g. 8:2, 7:1). Only on one occasion, was it considered justified to

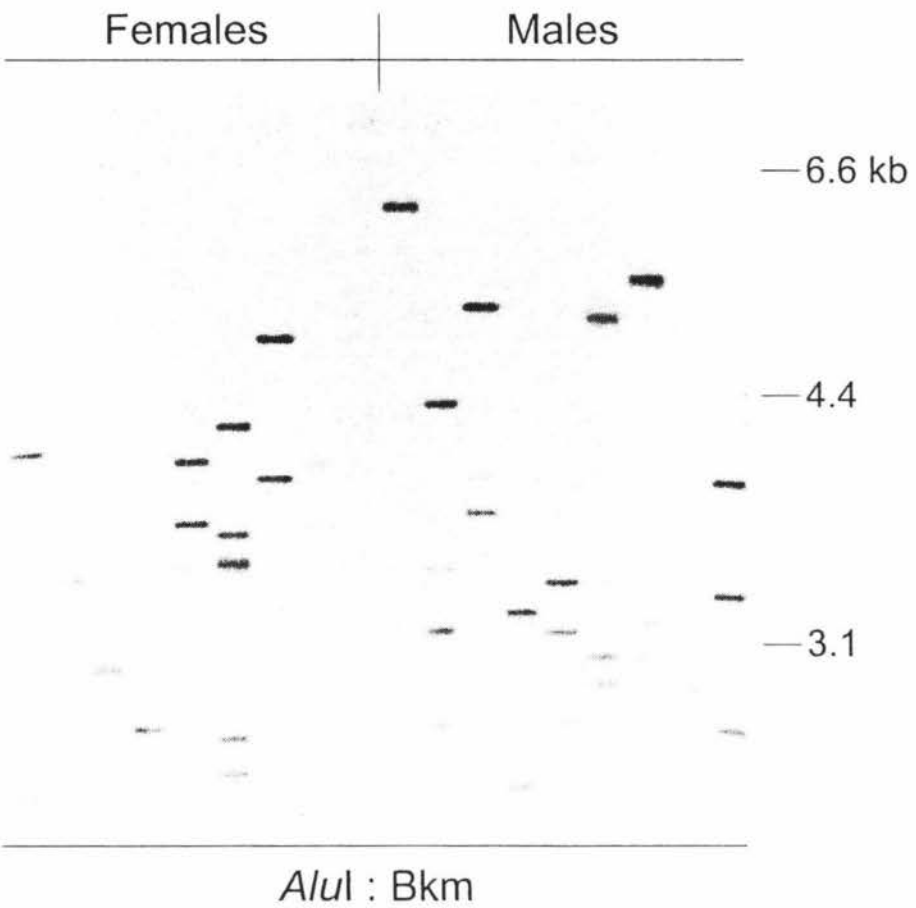

Figure 3.2 DNA profile of *Alu*I-digested DNA hybridised with Bkm (63°C). This is a typical DNA profile for Bkm-probed tuatara DNA. Only a small number of fragments are generated, which with the odd exception are all of small molecular weight, typically below 6.6 kilobases (kb). Despite earlier reports to the contrary (see text), Bkm showed strong homology to tuatara DNA, even at hybridisation temperatures as high as 65°C. Numbers show molecular weight markers.

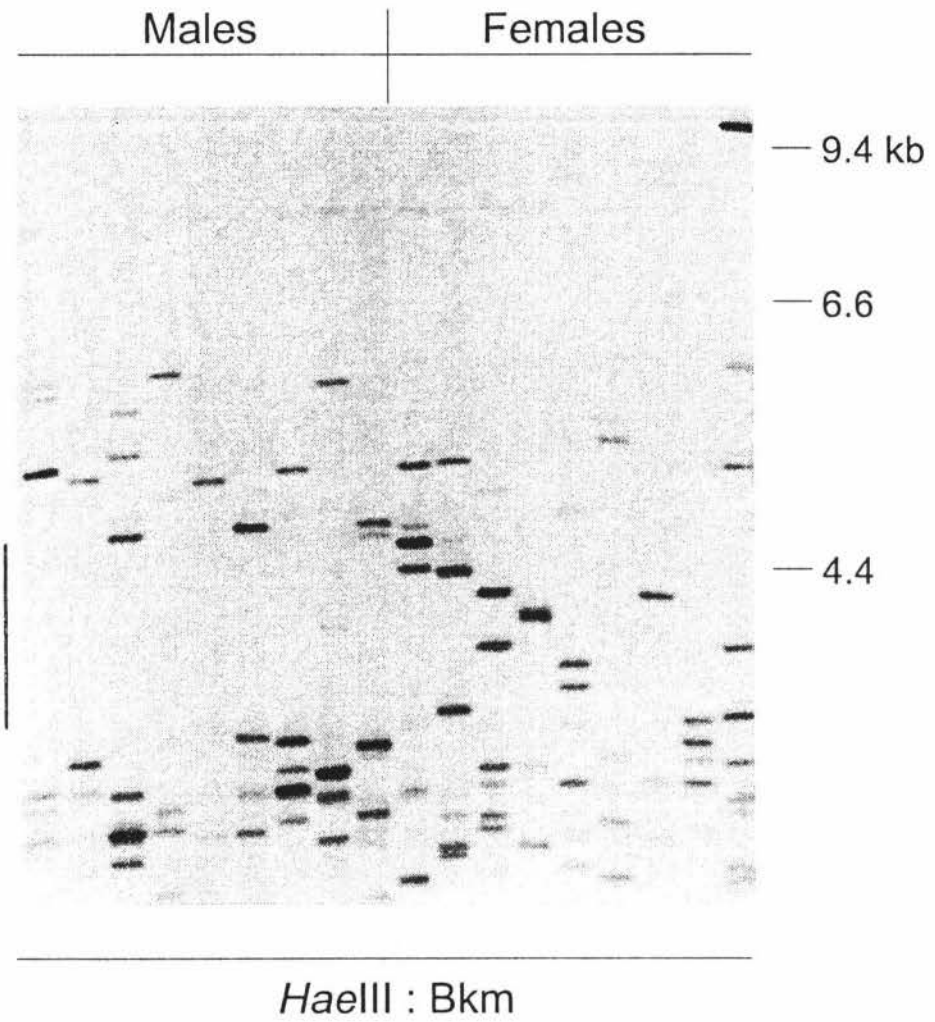

Figure 3.3 DNA profile of *Hae*III-digested DNA probed with Bkm (65°C). Another example of a typical Bkm hybridisation pattern for tuatara DNA, in which there are only a few, small molecular-weight bands of strong homology. This profile is interesting in that there appears to be a region of fragment size (indicated by the bar) in which there is an apparent absence of fragments in all males, but where seven of the nine females show fragments. None of these female fragments suggest a pattern of sex-specificity, however. Furthermore, this pattern was not revealed in other DNA profiles for this probe/enzyme combination. Numbers show molecular weight markers.

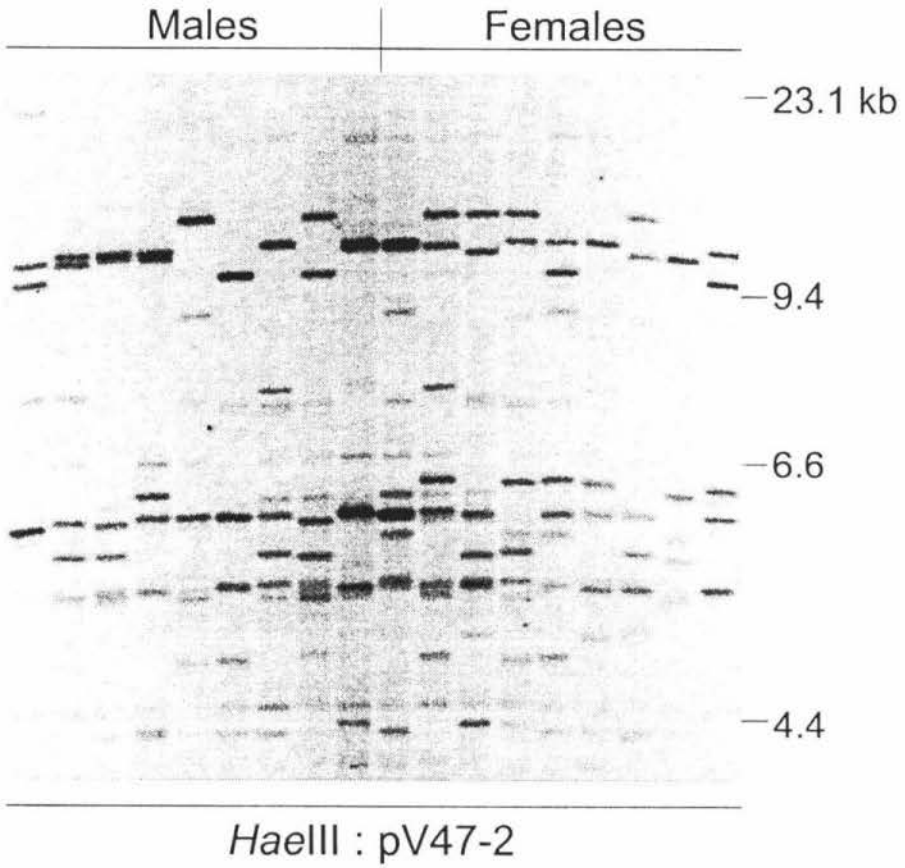

Figure 3.4 DNA profile of *Hae*III-digested DNA hybridised with pV47-2 (59°C). This is a typical pV47-2 hybridisation pattern for tuatara DNA. More fragments are found than for Bkm. There are concentrations of bands showing stronger homology, within particular fragment size regions, just above 9.4 kb and just below 6.6 kb. Numbers show molecular weight markers.

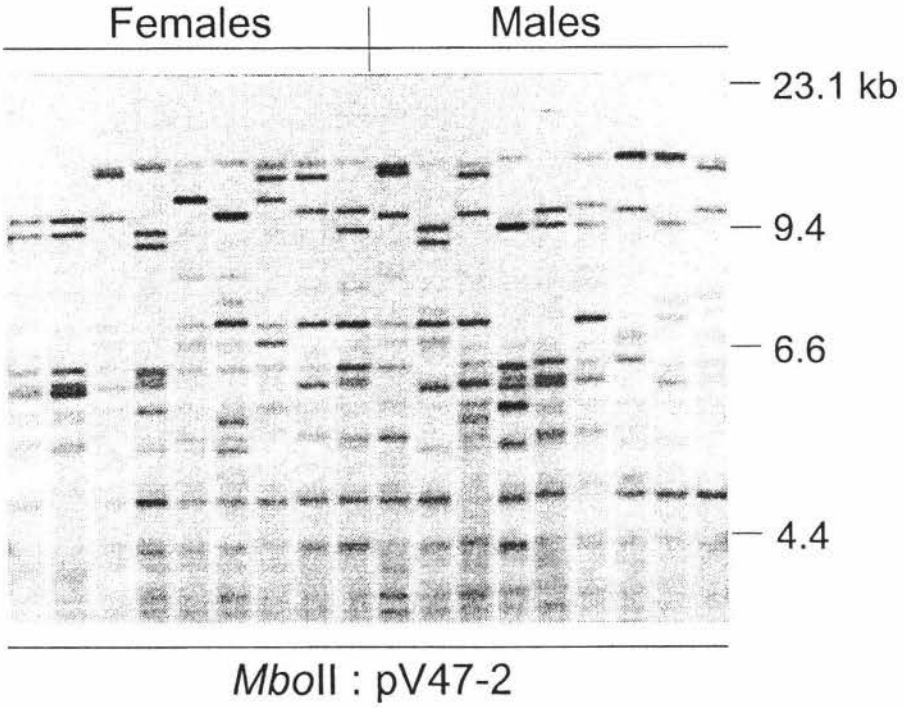

Figure 3.5 DNA profile of *Mbo*II-digested DNA hybridised with pV47-2 (57°C). Another example of the typical pV47-2 hybridisation pattern, exhibiting a marked contrast between weaker bands and fragments of stronger homology. It had been previously reported that pV47-2 does not exhibit homology to tuatara DNA (see text). Numbers show molecular weight markers.

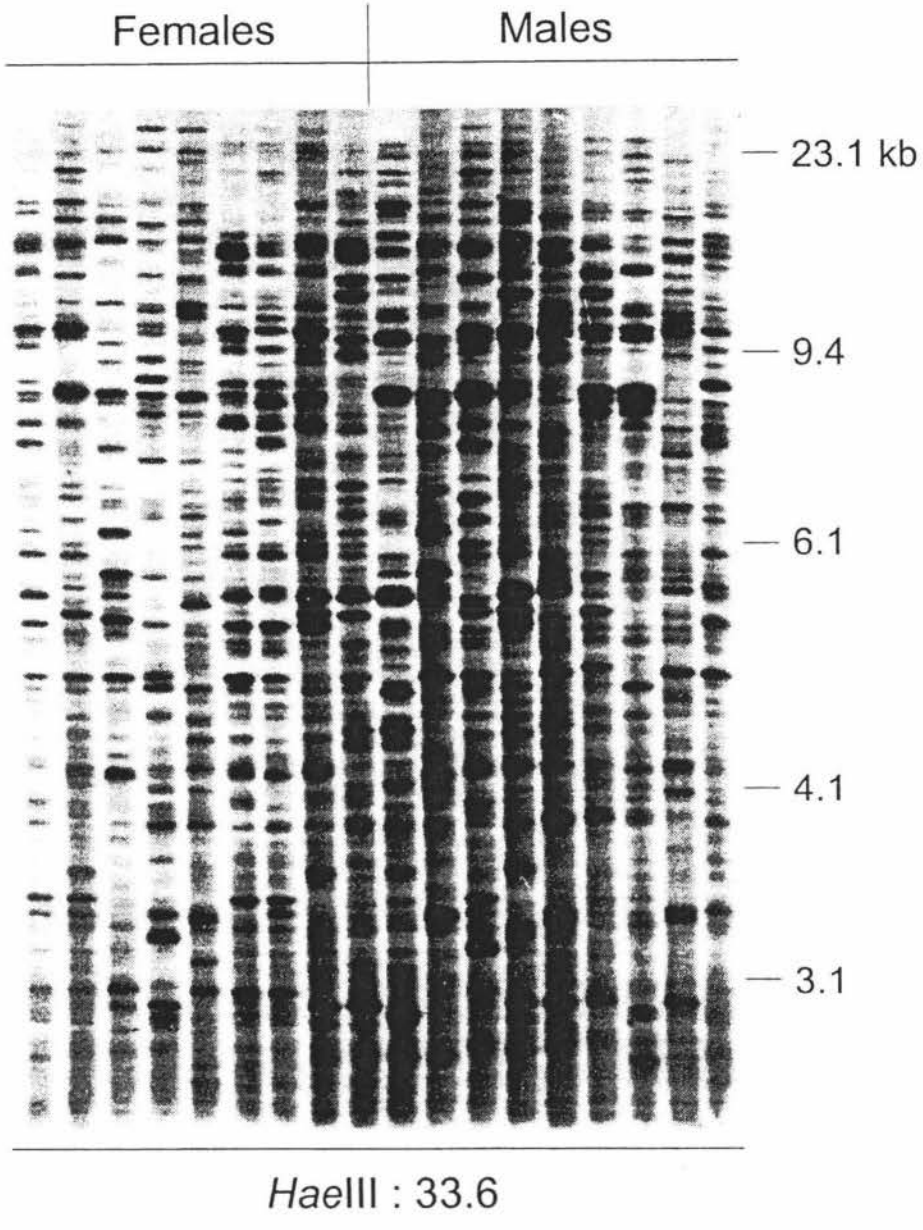

Figure 3.6 DNA profile of *Hae*III-digested DNA probed with 33.6 (61°C). An example of the typical hybridisation pattern for 33.6. A very large number of fragments are generated, which show an even distribution over a large fragment size range, in contrast to Bkm and pV47-2. Bands are difficult to resolve below 3.1 kb. Numbers show molecular weight markers.

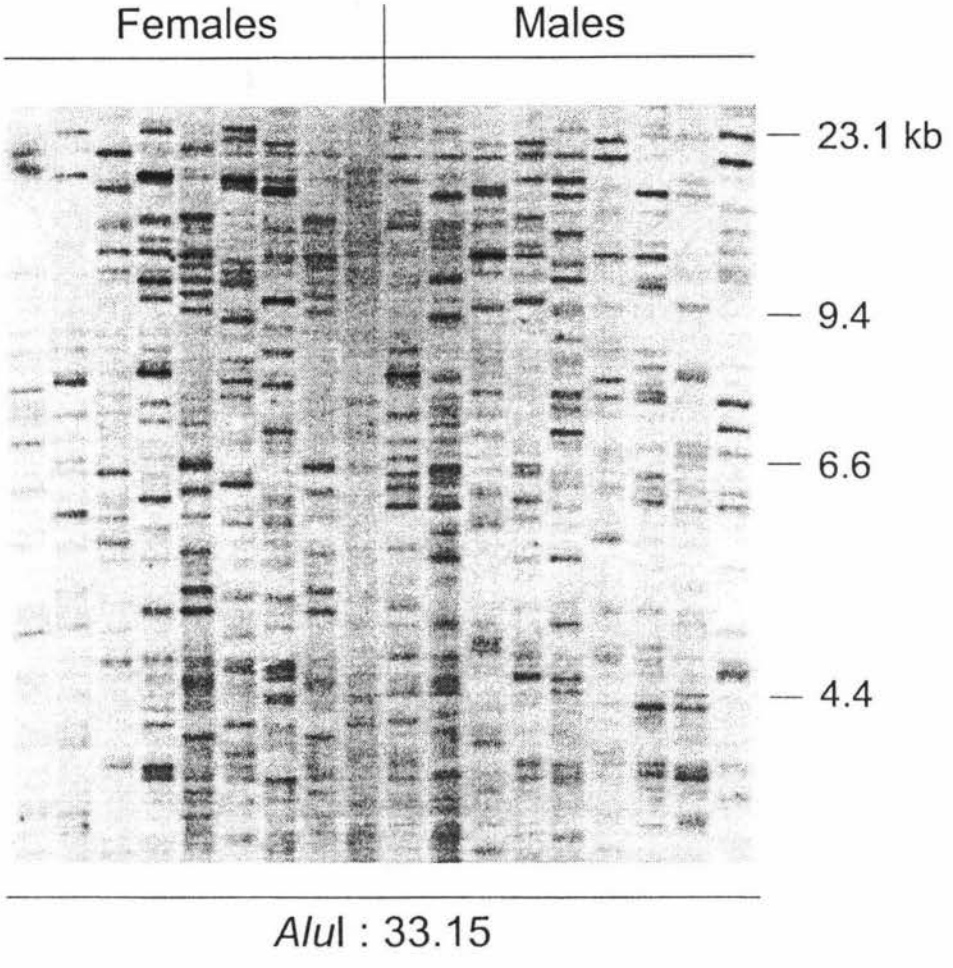

Figure 3.7 DNA profile of *Alu*I-digested DNA hybridised with 33.15 (59°C). This typical DNA profile for 33.15 show similarity to the hybridisation patterns shown for 33.6: there is an even distribution of a large number of fragments. Like all the probes, band resolution is poor for fragments less than 3.1 kb in size. Numbers show molecular weight markers.

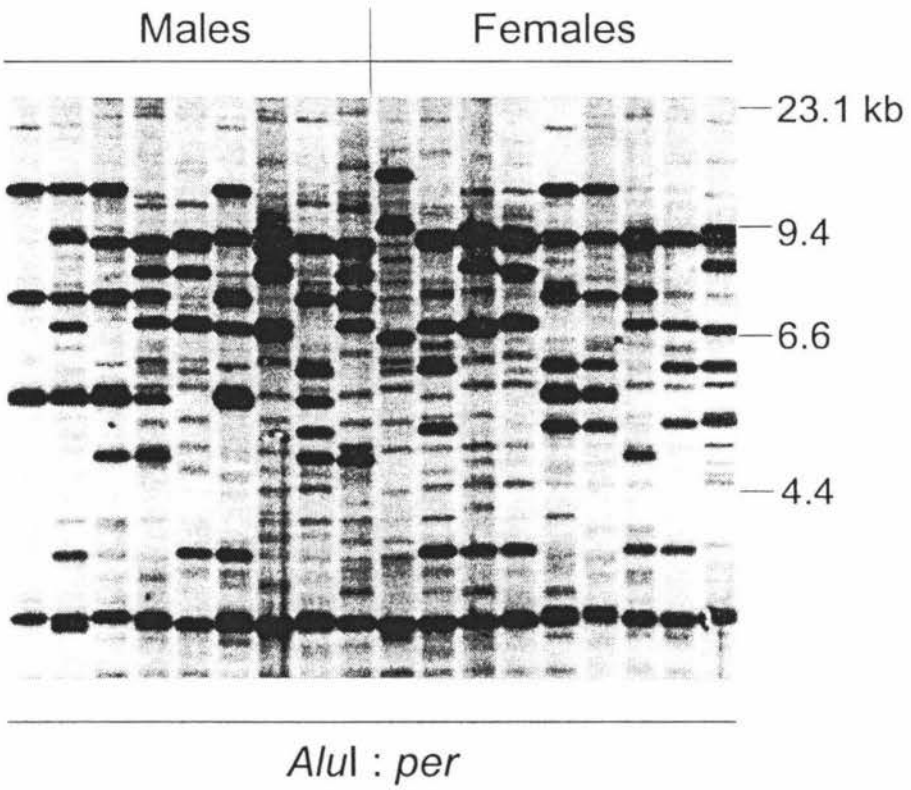

Figure 3.8 DNA profile of *Alu*I-digested DNA hybridised with *per* (63°C). *Per* showed some variation in hybridisation pattern, but as this DNA profile demonstrates, there were often two distinct classes of fragments generated: weak bands (low homology), which showed an even distribution across the size range of fragments, and dark bands (strong homology), which were often concentrated within particular size ranges or at single loci. Numbers show molecular weight markers.

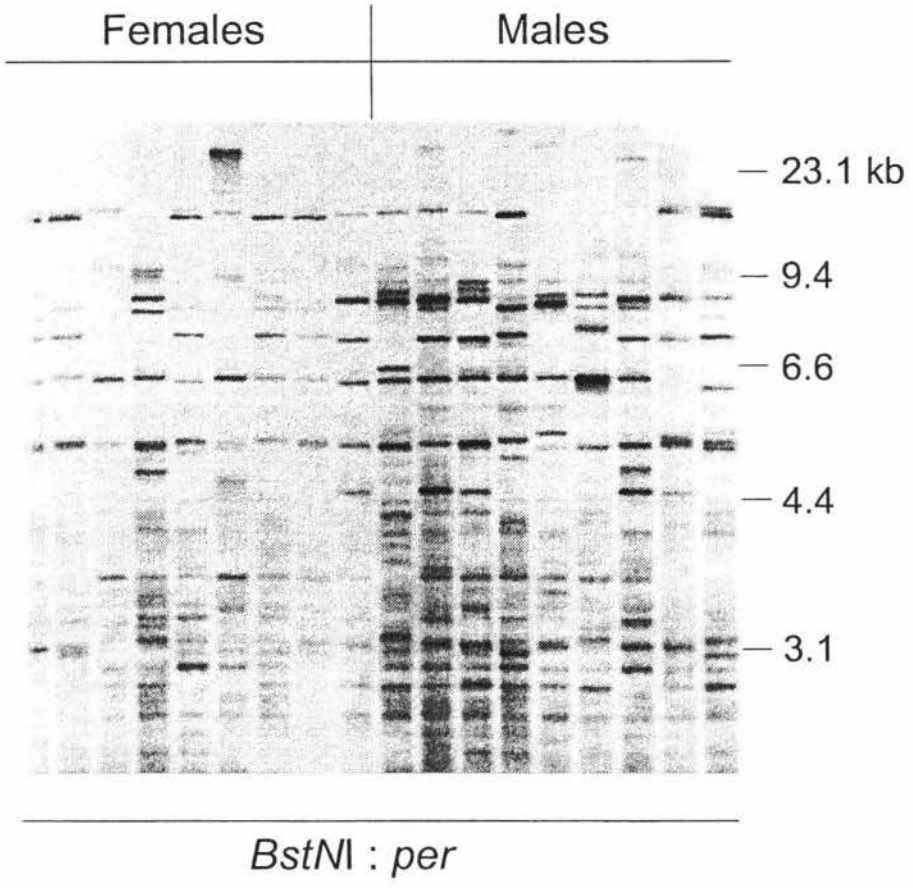

Figure 3.9 DNA profile of *BstN*I-digested DNA probed with *per* (57°C). A less typical pattern for *per*-probed tuatara DNA. The interesting aspect of this profile is the apparent difference in band intensity between male and female individuals. No sex-specific bands are apparent, however. This difference in profile intensity between the sexes was presumed to be a technical artifact, since the male and female DNA samples were quantified at separate times, and subsequent *BstNI/per* DNA profiles did not exhibit this effect for the sexes. Numbers show molecular weight markers.

investigate a particular fragment further by expanding the sample size to 24 individuals of each sex, and repeating the probe/enzyme combination. A potential female-specific fragment was revealed in a Phase 3 profile of *Hae*III-restricted DNA probed with 33.15, but only when that profile was washed at high stringency. This band appeared to be present in nine of nine phenotypic females, and four of nine phenotypic males (Fig. 3.10A). In itself, this ratio is not entirely convincing; the probability of such a ratio occurring by chance for a non-sex-linked fragment is approximately 0.029 (Appendix IV). This infers that such a split between males and females (biased towards either sex), would be expected to arise once in every 34 fragments (for a fragment appearing in any 13 of 18 individuals).

However, there was a second band of a considerably lower molecular weight that was present in eight of those nine females and in exactly the *same* four males. This lead to the suspicion that the four males with the band were sex-reversed, whereas all nine of the females (all of whom were known to be gravid when their blood was collected) were concordant. The fragment was tested for sex-specificity by expanding the sample size from nine of each phenotypic sex to 24 previously untested individuals of each phenotypic sex. That is, the 48 new samples were independent of the initial set of 18 individuals, an important consideration for the statistical validation of the test. Three new *Hae*III-cut DNA profiles were produced, and probed with 33.15 under identical hybridisation conditions. However, these revealed a lack of correlation between the female phenotype and the presence of the fragment (Fig. 3.10B,C, and D). On this basis, it was concluded that the initial profile result was a simply an unlikely statistical occurrence, and the fragment was not a marker for genetic sex.

3.3.3 Band-scoring as a test for sex-bias in fragment numbers

The means of the band counts for male and female tuatara are shown in Table 3.4. The differences between the male and female counts were tested for significance using a Mann-Whitney *U*-test. This non-parametric technique is appropriate for these data since it does not require that the data are normally distributed (Fowler and Cohen, 1990). The test makes a comparison between the medians (not the means) of the data by comparing the distributions of the ranked observations for the males and females. Thus, there is no link between the mean data shown in Table 3.4 and the significance

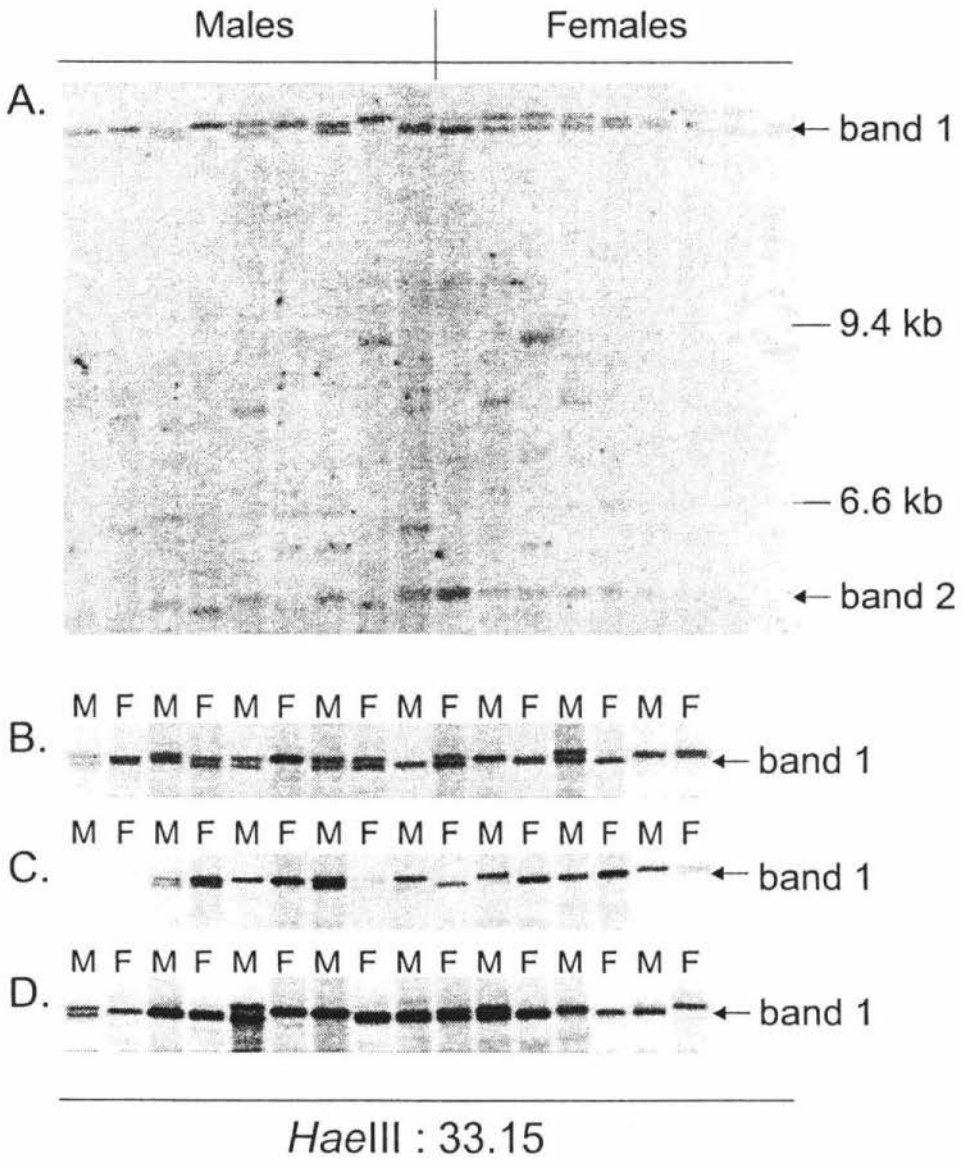

Figure 3.10 DNA profiles of *Hae*III-digested DNA probed with 33.15 (59°C). **A.** An old membrane was hybridised with 33.15 at high stringency, and revealed a high molecular weight fragment (~23 kb) that appeared in nine out of nine females, and four of the nine males (band 1). Furthermore, a second band of lower molecular weight appeared in eight of those females, and in the *same* four males (band 2). This lead to the suspicion that those four males might be sex-reversed, and the fragment(s) in question were female-specific. The probe/enzyme profiling combination was thus repeated for 24 new individuals of each sex, to test this (**B, C, and D**). This revealed no correlation between band 1 and female phenotype. Band 2 was similarly randomly distributed amongst males and females (not shown). It was concluded that the originally skewed distribution between males and females was simply an unlikely occurrence. M = male, F = female. Number show molecular weight markers.

TABLE 3.4 (a) Mean numbers (standard deviations) of DNA profile bands for phenotypic males and phenotypic females, for PHASE 1 probe-enzyme combinations. Counts were made over the reliably scorable size range of the fragments. All means were calculated from a sample size of nine individuals, except in cases where the sample size is indicated by a superscript number. The difference between male and female data was tested for significance using a non-parametric Mann-Whitney *U*-test, which compares the sample distributions of the ranked observations. Note that only a few of the total probe-enzyme combinations tested in Phase 1 were able to be accurately scored for band numbers.

PHASE 1

probe	enzyme	temperature	mean number of male bands	mean number of female bands	difference
33.6	*Hae*III	61°C	44.77 (3.73)	46.33 (3.67)	not significant
33.15	*Alu*I	59°C	55.33 (2.50)	55.00 (3.40)	not significant
	Hae*III*	59°C	56.00 (3.42)	57.44 (3.25)	not significant
Bkm	*Alu*I	63°C	3.22 (1.09)	3.11 (1.69)	not significant
	*Hae*III	55°C	2.22 (0.67)	2.89 (1.45)	not significant
per	*Alu*I	55°C	28.11 (3.30)	29.00 (3.61)[7]	not significant
		61°C	40.11 (3.59)	40.29 (1.98)[7]	not significant

TABLE 3.4 (b) Mean numbers (standard deviations) of DNA profile bands for phenotypic males and phenotypic females, for PHASE 2 probe-enzyme combinations. Counts were made over the reliably scorable size range of the fragments. All means were calculated from a sample size of nine individuals, except in cases where the sample size is indicated by a superscript number. The difference between male and female data was tested for significance using a non-parametric Mann-Whitney *U*-test, which compares the sample distributions of the ranked observations.

PHASE 2

probe	enzyme	temperature	mean number of male bands	mean number of female bands	difference
33.6	*Alu*I	57°C	40.90 (4.70)[10]	41.14 (2.48)[7]	not significant
	*BstN*I	59°C	25.44 (2.46)	26.56 (3.78)	not significant
	*Hae*III	57°C	30.44 (1.74)	30.63 (1.30)[8]	not significant
	*Hinf*I	57°C	35.78 (2.82)	37.22 (1.39)	not significant
	*Mbo*II	57°C	22.67 (2.60)	24.22 (2.17)	not significant
	*Rsa*I	59°C	27.22 (2.68)	25.38 (2.67)[8]	not significant
Bkm	*Alu*I	61°C	5.10 (1.66)[10]	5.00 (1.41)[7]	not significant
	*BstN*I	61°C	4.22 (1.39)	4.33 (1.80)	not significant
	*Hae*III	61°C	4.50 (1.69)[8]	4.56 (0.73)	not significant
	*Hinf*I	61°C	7.00 (2.20)[8]	7.89 (2.20)	not significant
	*Mbo*II	61°C	3.67 (1.11)	4.00 (0.71)	not significant
	*Rsa*I	61°C	12.78 (3.87)	10.88 (1.54)[8]	not significant
per	*Hae*III	57°C	27.63 (2.00)[8]	29.22 (2.59)	not significant
pV47-2	*Alu*I	57°C	20.00 (1.94)[10]	20.71 (3.25)[7]	not significant
	*Hae*III	57°C	8.75 (1.28)[8]	8.67 (1.00)	not significant
	*Mbo*II	57°C	12.78 (1.99)	13.78 (1.56)	not significant

TABLE 3.4 (c) Mean numbers (standard deviations) of DNA profile bands for phenotypic males and phenotypic females, for PHASE 3 probe-enzyme combinations. Counts were made over the reliably scorable size range of the fragments. All means were calculated from a sample size of nine individuals, except in cases where the sample size is indicated by a superscript number. The difference between male and female data was tested for significance using a non-parametric Mann-Whitney *U*-test, which compares the sample distributions of the ranked observations.

PHASE 3

probe	enzyme	temperature	mean number of male bands	mean number of female bands	difference
33.6	*Alu*I	63°C	26.89 (3.55)	27.22 (3.53)	not significant
	*BstN*I	63°C	19.50 (2.72)[8]	19.11 (3.37)	not significant
	*Hae*III	65°C	28.00 (3.58)[8]	29.00 (3.93)	not significant
	*Hinf*I	65°C	20.56 (4.09)	19.56 (4.09)	not significant
	*Mbo*I	63°C	20.00 (3.00)	18.44 (3.68)	not significant
33.15	*Alu*I	63°C	32.44 (2.60)	30.78 (3.08)	not significant
	*BstN*I	63°C	33.00 (2.00)	32.56 (4.34)	not significant
	*Hae*III	59°C	34.67 (1.80)	35.11 (4.14)	not significant
	*Mbo*I	63°C	21.11 (3.88)	18.89 (2.98)	not significant
Bkm	*Alu*I	63°C	5.56 (2.18)	6.22 (2.05)	not significant
		65°C	8.78 (2.11)	8.11 (1.83)	not significant
	*BstN*I	63°C	7.89 (1.83)	7.56 (2.13)	not significant
		65°C	4.88 (1.55)[8]	4.44 (1.13)	not significant
	*Hae*III	63°C	15.67 (1.50)	16.00 (1.87)	not significant
		65°C	7.22 (1.86)	7.78 (2.78)	not significant
	*Hinf*I	65°C	16.67 (1.80)	15.11 (1.96)	not significant
	*Mbo*I	65°C	11.89 (1.76)	12.67 (3.47)	not significant

TABLE 3.4 (c) ***continued***

			PHASE 3		
probe	enzyme	temperature	mean number of male bands	mean number of female bands	difference
per	*Alu*I	63°C	28.33 (2.54)	25.78 (1.98)	not significant
	*BstN*I	63°C	18.50 (5.45)[8]	17.22 (3.38)	not significant
	*Hae*III	63°C	29.89 (4.93)	28.44 (6.27)	not significant
	*Hinf*I	63°C	19.33 (1.65)	19.11 (2.47)	not significant
pV47-2	*Alu*I	59°C	10.00 (2.12)	10.11 (2.85)	not significant
	*BstN*I	59°C	14.67 (2.34)	15.00 (3.00)	not significant
	*Hae*III	57°C	26.33 (3.39)	25.67 (3.53)	not significant
		59°C	14.11 (2.47)	13.78 (3.08)	not significant

TABLE 3.5 A comparison of the band counts from the current study with the data of Finch 1994), in which a significant excess in fragment numbers was found in female tuatara DNA for the probe-enzyme combination of 33.15 and *Hae*III. The current study, however, was unable to replicate this finding. Differences between the band count data for males and females were testing using the Mann-Whitney *U*-test, a non-parametric technique which compares the distributions of the ranked observations (the means are simply presented here as a convenient representation of the data).

	FINCH (1994)			**CURRENT STUDY**		
Probe-enzyme combination (59°C)	Mean number of fragments (standard deviation)			Mean number of fragments (standard deviation)		
	Males	Females	Difference	Males	Females	Difference
33.15 / *Alu*I	24.5 (3.0)	30.8 (4.5)	significant	55.3 (2.4)	55.0 (3.2)	not significant
33.15 / *Hae*III	15.7 (3.3)	19.0 (4.4)	significant	27.6 (1.9)	27.7 (2.3)	not significant

tests; the means are simply the most convenient way to represent the full band count data.

No significant differences between male and female band counts were detected, for any combination of probe and restriction enzyme. In fact, the data in Table 3.4 reveal very little difference between males and females for mean band counts. Of particular interest is that *Hae*III-digested DNA and *Alu*I-digested DNA probed with 33.15 did not reveal a significant sex-bias in fragment numbers, as it was these two probe-enzyme combinations reported by Finch (1994) to reveal a significantly higher number of bands in female tuatara. This was despite the use of nearly identical hybridisation conditions in the two separate studies, for these particular probe-enzyme combinations. Table 3.5 shows a comparison of the new data with the corresponding data of Finch (1994). The actual size of the counts is not important, since there could be a number of reasons why there would be variation in these numbers when making a comparison between two independent profiling analyses (such as the intensity of exposures, for example), however, the relative degree of difference between the two sexes for the two studies is puzzling. It could be argued that it would be logical to put more faith in the new data, since the current study examined a far larger number of different probe-enzymes combinations, none of which revealed a significant difference between male and female band counts.

3.4 DISCUSSION

This study failed to detect sex-specific DNA in the tuatara using the method of minisatellite DNA profiling. A total of 14 different restriction enzymes were used in conjunction with five DNA probes; in total, 66 different probe-enzymes combinations were tested. DNA profiles were examined for the presence of sex-specific fragments, and many of them were scored to calculate band numbers for individual males and females. Mean band counts revealed no significant differences between the DNA of male and female tuatara, despite a previous report (Finch, 1994) that the probe/enzyme combinations of 33.15/*Alu*I and 33.15/*Hae*III revealed a bias in the number of female fragments. This particular result could not be repeated, despite adherence to the methodology of the previous study. Also in contradiction to the study of Finch (1994),

was the discovery that the DNA probes Bkm (Singh *et al*, 1980) and pV47-2 (Longmire *et al*, 1990) exhibit strong homology to tuatara DNA.

The inability of this investigation to detect a genetic difference between the two sexes of tuatara fails to refute the null hypothesis that there is no genetic mode of sex determination in the tuatara. However, the result does not constitute a refutation of the alternative hypothesis that there is sex-specific DNA in the tuatara. It can be seen to be consistent with both hypotheses.

In accordance with the null hypothesis, it is entirely possible that genetic sex differences simply do not exist in this species, and the negative result of this study is a reflection of this fact. This could mean that sex determination in the tuatara (and by extrapolation, potentially all TSD reptiles) is wholly determined by the effect of incubation temperature, and there is a complete lack of genetic interplay with this environmental 'switch' (other than the gene activity associated with sexual development after the event of sex determination).

Alternatively, it could be that there is indeed a genetic distinction between the sexes, but this method was unable to reveal any difference. There are a number of reasons why this may be so. First, the DNA profiling survey may not have been as comprehensive as necessary to detect a genetic difference. That is, a much larger number of combinations of common probes and restriction enzymes (of which there is a very large possible number) might eventually reveal the 'magic' combination that produces a sex-specific fragment. There is no way of knowing what that combination might be until it is discovered, but it still seems likely that Bkm is a prime contender for the appropriate DNA probe, given that it exhibits sex-specific hybridisation patterns in other species (section 3.1.2).

Second, it may be that there is only a very subtle genetic difference between the two sexes, one that is too slight for this method of minisatellite DNA profiling to have revealed. It might remain undetected even if a far greater number of common probe and restriction enzyme combinations were attempted. This would also explain why band counts were also unsuccessful in demonstrating a genetic difference between males and females. A noticeable difference in fragment numbers would not be

expected unless there was a significant difference between the hypothetical 'sex chromosomes' of the tuatara.

Third, and perhaps most likely, is the possibility that the proportion of discordant individuals amongst the samples was too high to make detection of a sex-specific fragment possible. That is, if any true sex-specific fragments were revealed, they may have not been apparent due to the masking effect of sex-reversed individuals amongst the phenotypic males and phenotypic females. A sex-specific fragment would have been distributed amongst the two sets of phenotypic sexes in a ratio similar to that assumed for a fragment unlinked to sex, for example, 4:5 or 6:4. This would infer that the theory that concordant individuals have a more robust sexual phenotype is flawed. In other words, despite deliberate selection based upon the strength of their sexual phenotypes, the Phase 2 and Phase 3 tuatara might well have had a significant number of sex-reversed individuals amongst them. Of course, it could still be that there are in fact subtle phenotypic differences between concordant and discordant sexual phenotypes, but the physical criteria used for selection of the Phase 2 and Phase 3 samples were not appropriate indicators of these differences.

It should perhaps be pointed out that this investigation was not based entirely upon the critical assumption of phenotypic differences being a reasonable basis for judging concordance. Another assisting factor, which has been discussed in Chapter Two, is that there is reason to believe that the effect of TSD might not be as prevalent in nature as it has been demonstrated to be for incubations under laboratory conditions. Genetic factors are hypothesised to play a more influential role in sex determination in natural nests, due to temperature fluctuations supposedly diminishing the effect of TSD. According to this theory, there will only be a small proportion of sex-reversed individuals within a wild population of TSD reptiles. For this viewpoint, it was reasonable to expect that the approach of profiling might reveal a sex-specific fragment, due to the potential inclusion of only a very few discordant individuals because of their low frequency in the sampled population.

It is interesting, then, that sex-specific fragments were revealed for the marine turtles *Lepidochelys kempi* and *Chelonia mydas*, using DNA profiling (Demas *et al*, 1990). It is possible the limited number of samples in that study were, by sheer fortune alone, all

concordant in their sexual phenotype and genotype. No mention is made within the paper of any efforts to select concordant, or strong phenotype individuals; or indeed, of the very possibility that such sex classes as 'concordant' and 'discordant' even exist. Perhaps these are examples of natural populations in which sex-reversed individuals are found at a very low frequency. By this logic, the tuatara populations which were sampled for the current study, are not. Alternatively, the more radical hypothesis proposed by Demas *et al* (1990) to explain their results, that TSD involves physical alteration of the genome, might be correct. This would infer that discordant, or sex-reversed, phenotypes do not exist at all. However, this does not explain why this investigation, which was identical in methodology, was not successful for the tuatara.

4

Randomly Amplified Polymorphic DNA (RAPD) Assays For Sex-Specific DNA In The Tuatara

RAPD assays were performed to test the hypothesis that there are sex-specific genetic differences in the tuatara. This involved PCR amplification of fragments from anonymous genomic DNA sequences of phenotypic male and female individuals, using a number of short, random-sequence primers. The amplifications revealed no sex-specific products.

4.1 INTRODUCTION

4.1.1 Molecular sex assignment in the tuatara: previous investigations

The tuatara exhibits temperature-dependent sex determination (Cree *et al*, 1995), which may mean that sex-specific genetic differences do not exist in this animal. However, several studies have suggested that there may be a genetic component to sex determination in reptiles with temperature-dependent sex determination (Chapter 2). Three previous genetic studies of the tuatara have addressed the question of sex-specific differences in its genome. Wylie *et al* (1968) examined the karyotype of the tuatara, and reported that it lacks distinct heteromorphic sex chromosomes, like many other reptiles (Bull, 1983). As part of a wider study into the minisatellite DNA variation of the tuatara, Finch (1994) briefly investigated whether or not sex-specific fragments were revealed by minisatellite DNA profiling, using a combination of two restriction enzymes and two human DNA probes, 33.6 and 33.15. No sex-specific fragments were found. The third, and first comprehensive study, was the minisatellite DNA profiling survey described in the previous chapter, in which a large number of different probe/enzyme combinations were tested in an effort to detect a sex-specific marker for this species. Again, no such fragments were revealed.

The investigation described in this chapter tested the hypothesis that there are sex-specific differences in the tuatara genome using a previously unattempted approach of molecular sex assignment in the tuatara: RAPD markers (Williams *et al*, 1990).

4.1.2 RAPDs: a useful tool for molecular sex assignment

A number of different molecular genetic methods have been successful in assigning sex in species for which sex is difficult to determine from morphology alone. Genetic markers for sex that have been discovered include: restricted genomic DNA fragments probed with unique sequences (Quinn *et al*, 1990; Dvorak *et al*, 1992; Millar *et al*, 1996), or with minisatellite (Longmire *et al*, 1991; Millar *et al*, 1992; Graves *et al*, 1993) or microsatellite DNA sequences (Longmire *et al*, 1993), and fragments amplified using specific PCR primers (Griffiths *et al*, 1992, 1996; Sabo *et al*, 1994; Griffiths and Tiwari, 1995; Ellegren, 1996; Nishiumi *et al*, 1996).

One of the newer molecular approaches applied to the problem of sex assignment is RAPD analysis (randomly amplified polymorphic DNA [Williams *et al*, 1990]). RAPD markers (or RAPDs) are produced by PCR amplification (Saiki *et al*, 1985) with short, usually 10 bp, oligonucleotide primers of arbitrary or random sequence. Amplification occurs at random from a few anonymous genomic sequences (Welsh *et al*, 1991). This method typically yields a small number of polymorphic fragments which can be electrophoretically size-separated and visualised as a band profile.

Sex-specific amplified fragments may be revealed in the band profiles of species with genetically-distinct species. Many recent studies have successfully employed RAPD markers as a means of assigning sex. Hadry *et al* (1992) and Griffiths and Tiwari (1993) first proposed RAPD markers as a means of sexing bird species, in which newly-hatched juveniles are invariably unable to be sexed on the basis of their morphology. Examples of its successful application in birds include the chicken (Levin *et al*, 1993), the ostrich (Bello and Sánchez, in press), ratite species (Huijnen, pers.comm.), and the jacana, oystercatcher, European bee-eater, European robin, Seychelles magpie robin, Seychelles warbler, and the Great tit all within one study (Lessells and Mateman, 1998). RAPD assays have also been used to detect sex-

specific markers in mammals (Wardell *et al*, 1993), particularly in domestic livestock species (Antoniou *et al*, 1994; Cushwa and Medrano, 1994).

Hence RAPD assays presumably also have the potential to detect sex-specific markers in the tuatara, assuming that specific genetic differences between the sexes exist. In this investigation, 43 RAPD primers were used to amplify anonymous fragments from the genomic DNA of phenotypic male and female tuatara. One study identified RAPD markers for gender in seven different bird species after screening, on average, only 30 different RAPD primers for each species (Lessells and Mateman, 1998). For some of those species, considerably less than 30 primers were required. This number of primers might therefore be reasonably expected to reveal any sex-specific differences in the tuatara.

4.2 METHODS AND MATERIALS

4.2.1 Strategies for detecting sex-specific fragments by RAPD analysis

A total of 43 different RAPD primers (Appendix III) were used to amplify anonymous sequences (sequences of unknown location within the genome) by PCR from the genomic DNA of both phenotypic male and female tuatara. The resulting band profiles were thoroughly inspected for the presence of sex-specific fragments.

As discussed in Chapter 2, a complication of this approach was that the tuatara exhibits temperature-dependent sex determination (TSD) (Cree et al, 1995). It is therefore possible that sex-reversed individuals exist within natural populations, due to incubation temperatures 'overriding' the (hypothetical) underlying sexual genotype. These *discordant* individuals may be phenotypic males with a female genotype, or phenotypic females with a male genotype. Inclusion of such sex-reversed individuals amongst the samples could obviously have confounded this analysis, by masking the detection of a potential sex marker.

Individuals that are *concordant* in their underlying sexual genotype and temperature-determined sexual phenotype may exhibit stronger, or less ambiguous, sexual phenotypes than discordant, or sex-reversed, individuals (Chapter 2). With this in

mind, certain criteria were employed for the selection of individuals that were used in this analysis. Males were required to exhibit clear male behaviour patterns, and show strong male morphology, such as large body size, a proportionately large head, and a prominent nuchal crest and conspicuous spines. Females were similarly chosen based upon the strength of their physical phenotype, with the added criterion that they were gravid, which was determined by palpation, or the inducement of egg-laying.

There was, however, no guarantee that these selection criteria were sufficient to avoid the sampling of sex-reversed individuals (especially if the theory that concordant animals have stronger sexual phenotypes is incorrect). Therefore it was necessary to consider the consequences of accidental inclusion of sex-reversed individuals within the RAPD assay samples.

4.2.1.1 Amplification from DNA cocktails

The initial methodological approach for the RAPD analysis was amplification of products from 'cocktails' of male and female DNA. Two DNA cocktails were prepared: one consisting of equal concentrations of DNA from eight phenotypic males, and the other consisting of equal concentrations of DNA from eight phenotypic females. For each primer, RAPD products were amplified from both of the cocktails and compared. An amplified fragment specific to a particular sex would have appeared in the band profile for that cocktail only, assuming all the individuals were concordant.

Inclusion of just one sex-reversed individual amongst the samples contributing DNA to one of the cocktails could potentially have obscured a sex-specific fragment, giving a false-negative result. For example, if a phenotypic male, that was actually genotypically female, was included within the all-male cocktail, the specificity of a sex-specific female product within the profile of the all-female cocktail would not have been apparent due to the appearance of that same product within the supposedly all-male cocktail. This would also have been true in the reverse instance: if the DNA of a genetically male, but phenotypically female individual was included by accident in the cocktail of all-females. The cocktail method was therefore only foolproof if it could be guaranteed that the individuals included within each single-sex cocktail all had the same *genetic* sex. This is clearly difficult without a phenotypic marker for genetic sex.

There was one way in which this difficulty could presumably be overcome. If there was one, or maybe even two, discordant individuals within a cocktail, a RAPD product specific to the genetic sex of the discordant individual(s) would, in theory, be far more intense in the band profile of the *other* cocktail than it was for that cocktail, as a result of the greater number of individuals of that particular genetic sex contributing amplified DNA products in the other profile.

Therefore, when a band appeared weakly-amplified in one profile, but much stronger in the other, it was tested for sex-specificity by repeating the amplification with that primer using several individual DNA samples of each sex (i.e. not in cocktail form). It was presumed that this would expose any discordant individuals. In theory, this method of detecting and screening such instances of variable intensity of equivalent bands was reasonably straightforward. In practice, however, it was found to be problematic. It became apparent that the sensitivity of PCR amplification to slight technical variations made it unrealistic to expect the band profiles to clearly depict such a scenario. Interpreting occurrences of equivalent bands varying in intensity as possible instances of sex-specific products (masked by discordant individuals) proved to be a difficult undertaking, as varying degrees of intensity for equivalent bands invariably appeared in the profiles of all the primers tested. Each of these instances probably could be explained by experimental inconsistencies such as differential amplification of the cocktail DNA samples or variation in the relative concentrations of the DNA of the different individuals within the cocktails (which were difficult to equalise with great accuracy). In short, the DNA cocktail approach made interpretation of the banding profiles difficult, and it was therefore abandoned due to its unreliability.

4.2.1.2 Amplification from genomic DNA of individuals

This prompted a change to a new method: amplification from the DNA of individuals only, rather than from cocktails of the DNA of several individuals. For each RAPD primer, the separately amplified products of two males were run out on minigels alongside those of two females. In any instance where a band appeared unique to one or the other sex, that primer was then used for an increased sample size of eight males and eight females, to determine if the band was truly sex-specific, merely a technical artifact, or simply a result of the small sample size of only two of each sex.

Again, the implications of a discordant sex individual being assayed had to be taken into consideration. With two of each sex constituting the four samples, it required that *two* discordant individuals were selected for one phenotypic sex only, for this method to fail to reveal any potential sex-specific bands (i.e. a false-negative result). In other words, for a sex-specific band to be obscured, all four individuals had to be the same genetic sex, e.g. the two male samples could have been discordant (genetically female, phenotypically male) and the two females concordant (both genetically and phenotypically female).

If no discordant individuals were included (or in the presumably unlikely case that all four were sex-reversed), a sex-specific fragment would appear in the band profiles of two individuals of one phenotypic sex, and neither of the two individuals of the opposite phenotypic sex. However, if one, or three, of the four individuals were sex-reversed, a sex-specific fragment would appear in three of the four band profiles, or one of the four. It was possible that such fragments were not sex-specific, but were simply a result of the small sample size of two of each phenotypic sex.

For this reason, instances of these fragment distributions were checked for sex-specificity either by repeating the PCR reactions for the same four individuals (to first eliminate the possibility of technical error), or by increasing the sample size to eight individuals of each phenotypic sex, and repeating the amplification with the same primer. PCR products of these larger sample-size experiments were run out in minigel lanes alternating with respect to sex, so that a true sex-specific fragment would be apparent in an obvious on/off pattern.

4.2.2 Site and collection of blood samples

The blood samples were collected by Victoria and Massey University staff from the *Sphenodon punctatus* population resident on Stephens Island in the Cook Strait, which is the largest existing tuatara population. The sampled tuatara were specifically selected according to the criteria outlined in section 4.2.1. The animals were caught by hand and restrained while blood was taken ventrally from the caudal vein in the tail. Needles were treated with 0.5M dipotassium EDTA to prevent the blood clotting. The animals were held until blood clotting of the wound had occurred and then released.

The samples were frozen at -80°C upon the completion of sampling, tthen transferred in liquid nitrogen to the Molecular Ecology Laboratory at Massey University, Palmerston North. The blood samples were kept at -80°C until DNA extraction.

4.2.3 DNA extraction

Total DNA was extracted from the whole blood. 15-20µl of blood was added to 500 µl lysing solution (131mM NH_4Cl, 0.9mM NH_4HCO_3), and rocked gently for 10 minutes. The lysate was centrifuged at 13000 rpm for 10 minutes and the supernatant discarded. The pellet was resuspended in 400µl of SET buffer, to which 10µl of Proteinase K (20mg/ml) and 20µl of sodium dodecyl sulphate (10% SDS) were added to final concentrations of 0.5mg/ml and 0.5% respectively. Samples were then incubated overnight at 55-65°C while rotating inside a Hybaid oven. DNA was isolated by phenol/chloroform extraction: an equal volume (430 µl) of phenol was first added, followed by two steps of addition of an equal volume of phenol/chloroform/isoamyl alcohol (PCI, 25:24:1). The DNA was then cleaned by addition of an equal volume of chloroform/isoamyl alcohol (CI, 24:1). At each step, after the addition of phenol, PCI, or CI, the samples were rocked for 30 minutes and centrifuged at 5000 rpm for 10 minutes, then the organic phase was removed, leaving the aqueous phase. DNA was precipitated after the CI wash by addition of 1/10 volume (43µl 3M sodiuma acetate (pH 5.2) then 2x volume (860µl) cold 100% ethanol. Samples were rocked for 15 minutes and centrifuged at 13000 rpm for 30 minutes. The DNA was then washed twice with 1ml 70% ethanol. After each wash the DNA was centrifuged at 13000 rpm for 10 minutes, and the ethanol removed. The DNA pellet was allowed to air-dry for a few minutes. Finally, 40-60µl of nano-pure H_2O was added to the pellet, and the DNA was left for 1-3 days to allow it to fully resuspend.

4.2.4 PCR amplification of RAPD products with 10-mer primers

Genomic DNA samples were diluted 100-fold with nano-pure H_2O, to a concentration range appropriate for the PCR reactions. This was done in two 10-fold dilution steps, before each of which the approximate concentrations of the samples were determined on a Hoefer DyNA Quant 200 fluorometer. The concentrations of the individual DNA

samples of 10 males and 10 females ranged from approximately 3-14 ng/µl. In both the DNA cocktail and individual DNA PCRs, samples with concentrations of ~6-7 ng/µl were used. Each PCR reaction contained: 5-10 ng of genomic DNA, 25µM of each dNTP, 67mM Tris (pH8.8), 16.6mM $(NH_4)_2SO_4$, 5-10ng of primer DNA, 2.5mM $MgCl_2$, and 0.2 units of *AmpliTaq*® DNA polymerase (Perkin-Elmer), in a final volume of 10 µl (made up with nano-pure H_2O). In negative control reactions, absent template DNA was substituted with an equivalent volume of nano-pure H_2O. PCR reactions were carried out in a Hybaid OmniGene thermal cycler using the following temperature programme: 94°C/2 min, followed by five cycles of 94°C/15 sec, 42°C/30 sec, 72°C/30 sec, followed by 30 cycles of 94°C/15 sec, 38°C/30 sec, and 72°C/30 sec. After PCR amplification, the RAPD products were size-separated on 2% agarose/agarose-1000 (GibcoBRL) minigels run for approximately 25 minutes at 12.5 $V.cm^{-1}$. Gels were stained in ethidium bromide solution for a minimum of 30 minutes, then the resulting band profiles were visualised with UV light and photographed using a Biorad InstaDoc™ system.

Primer screening was with Primers #51 to #66 of a 50 primer set of unknown origin (labelled RAPD Oligo Project 10-mers Set 50/1[2]), the entire Operon Technologies RAPD-primer kit A (numbered OPA-01 to OPA-20), and seven other miscellaneous primers from various Operon Technologies primer kits: OPB-03, OPF-15, OPS-11, OPX-08, and OPY-06, -07, and -11. The sequences of these primers are given in Appendix III.

4.3 RESULTS

Products were successfully amplified from tuatara genomic DNA with 27 different RAPD primers. Sixteen other primers from a set of 50 (RAPD Oligo Project 10-mers Set 50/1[2]) failed to amplify any products. These primers were the first sixteen within the set of 50, so it is reasonable to assume that the remainder would also have failed to amplify products. Moreover, the stocks of this particular set of 50 primers were over two years old, and it is assumed that age-related primer degradation caused this failure.

The primers that did amplify RAPD products were Operon Technologies Primer Set A (numbered OPA-01 to OPA-20), and miscellaneous Operon Technologies primers OPB-03, OPF-15, OPS-11, OPX-08, OPY-06, OPY-07, and OPY-11. No sex-specific fragments were detected for any of the primers. Figure 4.1 shows examples of typical RAPD fragment profiles for male and female DNA cocktails, amplifed with the 10-bp primers OPA-02, and OPA-05. Examples of typical RAPD band profiles for individual male and female tuatara samples, amplified with the primers OPA-02, 04, 10, and 11 are shown in Figure 4.2.

There were numerous instances where a RAPD fragment appeared to be potentially sex-specific. This could have been when it was present in two individuals of the same phenotypic sex, or less convincingly, when it appeared in one of the four or three of the four individuals (which would be expected for a sex-specific fragment if just one sex-reversed individual was amongst the four samples). However, upon repeating the reactions for these primers, they were invariably found to be caused by experimental inconsistencies (meaning that amplifications in some reactions were more successful than others, resulting in pronounced differences in band intensity between profiles). Figure 4.3 depicts an example of a potential female-specific band which appeared in the RAPD band profiles for the primer OPF-15 (Fig. 4.3A), but upon further examination with an enlarged sample size of eight of each phenotypic sex, turned out to have been a result of unsuccessful amplification in the initial two male samples (Fig. 4.3B).

In general, amplifications with each of the 27 successful RAPD primers needed to be repeated at least twice, and sometimes three times, before it could be satisfactorily established that they did not produce sex-specific fragments (as frequently the success of amplication differed amongst the four individual samples). The number of individual fragments amplified by any one primer ranged from 1-12, but typically there were 5-10 separate fragments. For almost all of the primers, the band profiles produced were not in fact individual specific (as DNA fingerprints are), as they invariably showed few or no polymorphic fragments, which is perhaps a little surprising. However, this may have potentially aided detection of possible sex-specific markers anyway, which would presumably have been more apparent had they been revealed.

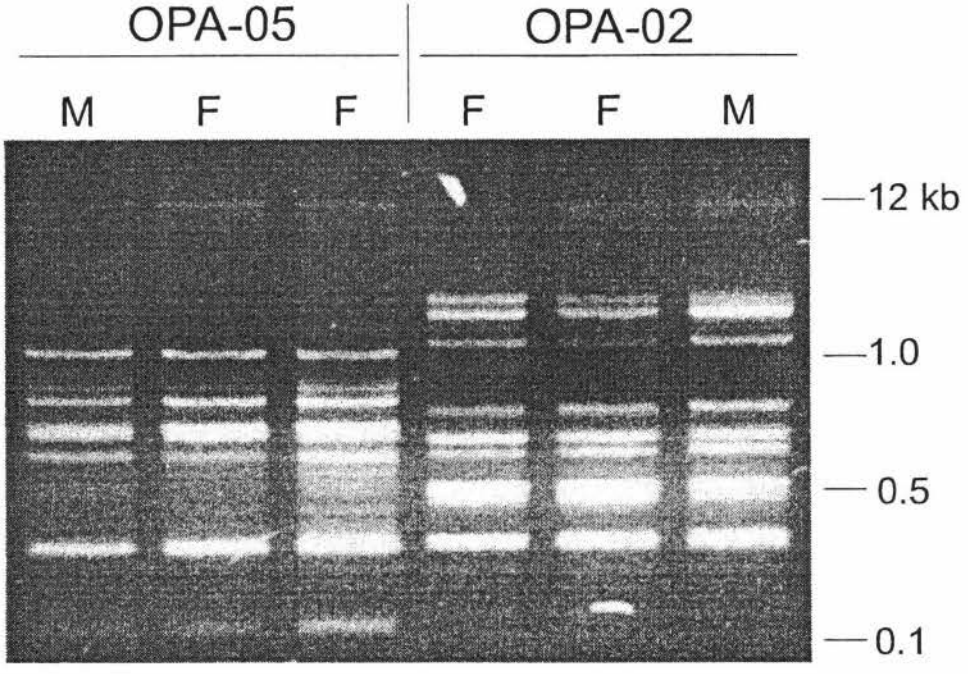

Figure 4.1 Typical RAPD band profiles for cocktails of male and female tuatara DNA. These products were amplified with the 10-mer primers OPA-05 and OPA-02 (Operon Technologies). There was the DNA of eight females in the female cocktails (F), and seven males in the male cocktail (M). Any sex-specific band should have appeared in the cocktail for one sex only. Number should molecular size markers.

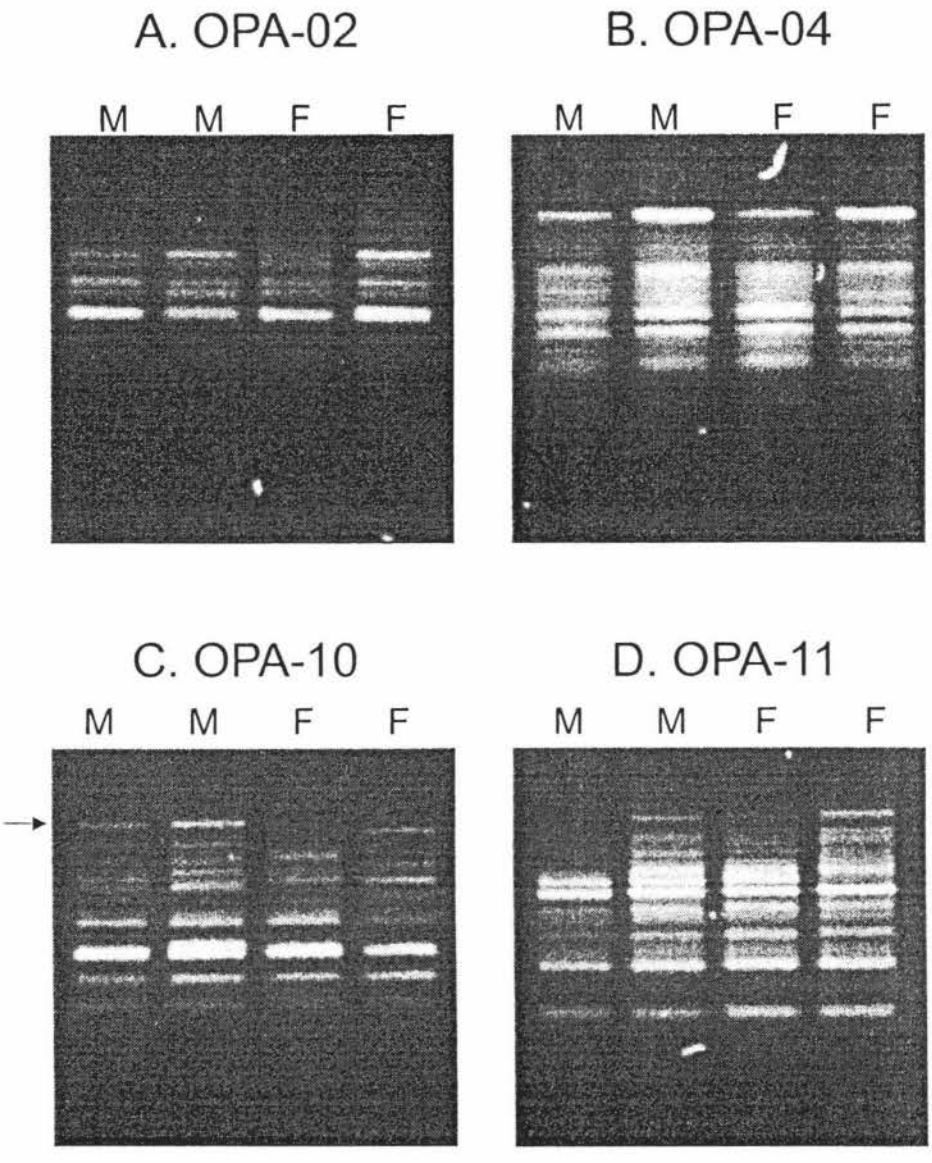

Figure 4.2 Typical RAPD band profiles for products amplified from individual tuatara DNA. These products were amplified with OPA-02 (**A**), OPA-04 (**B**), OPA-10 (**C**), and OPA-11 (**D**). Note the potential male-specific fragment indicated in **C**. This turned out to not be sex-specific upon repeating the amplification for these individuals, indicating that in this instance there was differential amplification between the four samples. This occurred for many primers, and often amplifications needed to be repeated two or three times to ensure that particular bands were present in all four individuals.

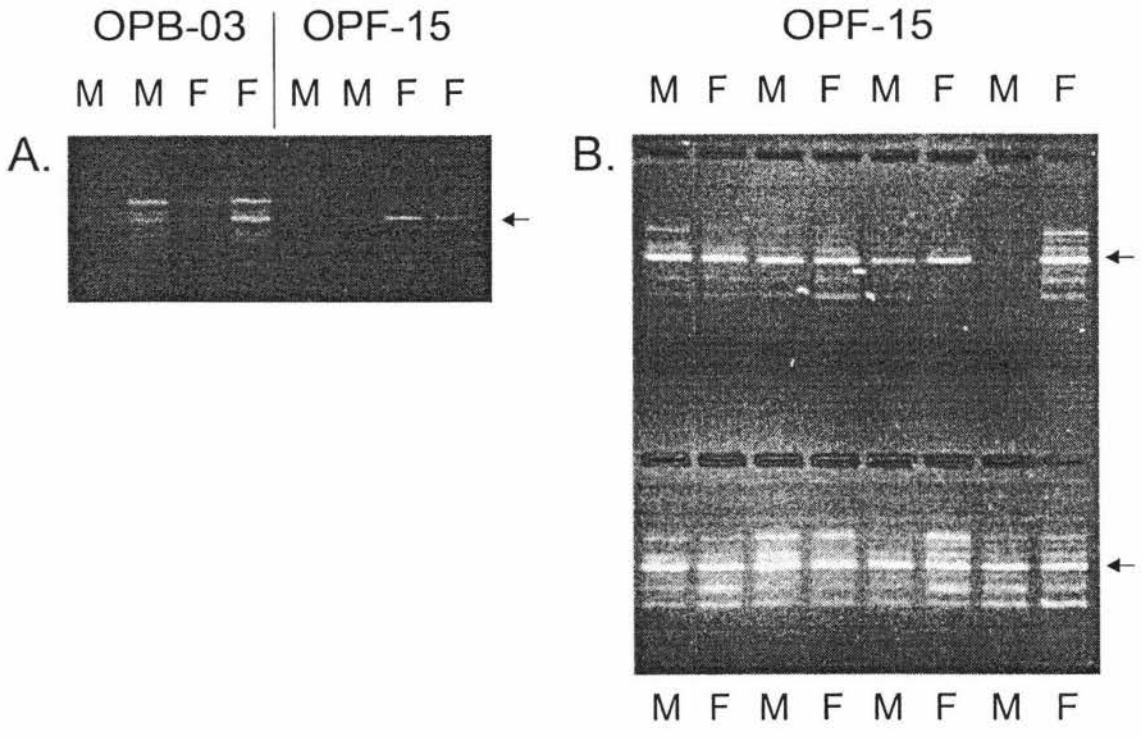

Figure 4.3 **A.** A RAPD profile indicating a potential female-specific band (indicated by an arrow) for the primer OPF-15. **B.** Amplification with OPF-15 repeated for eight individuals of each sex. In this assay it is clear that the fragment in question is not sex-specific, as it appears in almost every single individual. Thus, the original assay result was probably due to poor amplification within the two male reactions.

4.4 DISCUSSION

The inability of this RAPD analysis to detect sex-specific DNA in the tuatara fails to refute the null hypothesis that there are no sex-specific differences in the the tuatara genome. If the null hypothesis is correct, it would infer that the effect of incubation temperature is the only factor[1] influencing the determination of sex of the embryo, and there is no genetic predisposition within an embryo to become one sex or the other. Alternatively, the embryo might develop as a 'default' sex, unless the temperature effect is such that the alternative sexual development pathway is initiated. Such a scenario is consistent with the theory that TSD is the ancestral sex-determining mechanism within reptile groups (which infers that genotypic sex determination is a derived mechanism that has evolved independently in many lineages). According to this theory, genetic differences between the sexes presumably do not become established *until* a genetic mode of sex determination has evolved within a lineage.

On the other hand, while the result fails to support the hypothesis that genetic differences between male and female tuatara exist, it does not necessarily refute the hypothesis either. An alternative theory to the status of TSD as the ancestral sex-determining mechanism within reptiles contends that genotypic sex determination is the ancestral state. If genotypic sex determination is ancestral, then in lineages where TSD has evolved, there are presumably underlying sexual genotypes within individuals, which are no longer the primary sex determining factor due to the over-riding effect of incubation temperature (by an unknown molecular mechanism). If we assume for a moment that this theory is correct, at least two possible explanations can be postulated as to why the RAPD analysis was unsuccessful in detecting this underlying genetic difference between male and female tuatara.

First, it is very possible that an insufficient number of RAPD primers were screened. Only 27 primers successfully amplified RAPD fragments. In a comparable analysis of ten different bird species, Lessells and Mateman (1998) found female-specific RAPD fragments in seven of those species (birds have female heterogamety, hence markers

[1] This is not necessarily true. It could be that other effects such as hormone levels within the egg, which is a maternally-dependent factor, also play a role in sex determination of the embryo. See Chapter 2 for a discussion of this point.

would be expected to be found for females). On average, it required the testing of 30 or so primers to dectect these fragments. However, for only three of those seven species were *less than* 30 primers required. The other four of the seven species required 32-63 primers to be screened, and in the three species for which sex markers were not detected, 25, 48, and 67 primers were tested respectively. Moreover, Bello and Sánchez (in press) found that for the ostrich, only one of 200 different primers detected a RAPD marker specific to female DNA. Taking into account these previous results, it is perhaps not surprising that a sex-specific fragment was not revealed in the tuatara after screening only 27 primers. Moreover, bird species (with the exception of ratites) have very distinct heteromorphic sex chromosomes (ZZ males, ZW females), so it is reasonable to expect that RAPD analysis would be far quicker to detect sex-specific differences in avian DNA than it would in the genome of a species which lacks distinct heteromorphic sex chromosomes, and presumably has only minor genetic distinctions between the sexes. Conceivably, it might require the screening of a very large number of RAPD primers (possibly several hundred) before one is discovered that amplifies a sex-specific fragment in the tuatara.

Second, despite the efforts made to avoid the possible confounding effect of potential inclusion of a sex-reversed individual within the samples, this may still have occurred. There could have been a sex-specific fragment amplified within the RAPD assays, that was not apparent due to the two phenotypic males and two phenotypic females all being of the same genetic sex. While this does not seem likely, it can not be discounted.

Thus, it would appear that the method of RAPD analysis is far from exhausted as a potential approach to test for sex-specific DNA in the tuatara. Essentially, this survey constituted only a brief initial investigation of RAPD markers in this species. There is no reason to think that a considerably more comprehensive RAPD survey might not succeed in finding a molecular sex marker, if genetic sex differences exist. Such an investigation would first require the availability of a much larger number of RAPD primers for screening. In addition, the chances of success would be improved if many individuals of each phenotypic sex were tested, rather than only two of each, as this would reduce the potential confounding influence of a small number of sex-reversed individuals within the samples. Finally, the most important advance upon this study

would be to use individuals for which concordance between sexual phenotype and genotype is as certain as possible. Perhaps the most simple way of achieving this would be to test individuals that were incubated at the pivotal temperature(s) of the TSD thermal gradient, as at this temperature, it is thought that sexual phenotype is a manifestation of the underlying sexual genotype (Chapter 2).

5

Summary, Conclusion, and Future Directions

This final chapter reviews the research objectives and the experimental results, and presents the general conclusion arising from the research. The implications of the research are discussed. Potential avenues for future research into the question of a genetic component to TSD in the tuatara, and other TSD reptiles, are examined.

5.1 REVIEW OF THE RESEARCH AIMS AND OBJECTIVES

The fundamental aim of this research was to test the hypothesis that there is a genetic sex determining mechanism to temperature-dependent sex determination (TSD) in the tuatara, *Sphenodon punctatus*. The null hypothesis was that sexual genotypes do *not* exist in this species, as TSD is the sole mechanism of sex-determination. This was approached by employing molecular genetic techniques in a search for sex-specific DNA in the tuatara genome. It was hoped that this analysis would result in the discovery of a molecular marker specific to either male or female tuatara.

The motives for this research were two-fold. First, the discovery of sex-specific DNA in the tuatara would have significant implications for theories concerning the interaction of genetic factors and environmental influences in sex determination. It could contribute to an increased understanding of the mechanism and ecology of TSD within reptiles, as further research initiatives might potentially be enhanced by the availability of a diagnostic molecular sex marker. A discovery of this nature would also have implications for the unresolved evolutionary debate over the ancestry of sex-determining mechanisms within the Reptilia. There are two hypotheses regarding this ancestry: the first holds that TSD is the ancestral state, whereas the alternative hypothesis postulates that GSD is the ancestral mechanism. A derived TSD mechanism would presumably be superimposed upon underlying, ancestral genotypes within individuals. The discovery of genetic sex differences in the tuatara would therefore suggest that GSD is ancestral.

Second, a molecular marker for gender in the tuatara could potentially be of considerable benefit to the conservation programme for this endangered reptile.

Juvenile tuatara are unable to be reliably sexed from their morphology until they are at least ten years of age, and sometimes older. A diagnostic genetic marker for sex would allow the molecular assignment of sex in captively-reared juveniles, which could assist in establishing appropriate sex ratios in new island colonies, and in maintaining appropriate sex ratios when augmenting existing populations with new juveniles.

5.2 SUMMARY OF THE EXPERIMENTAL PROGRAMME AND RESULTS

Two different molecular genetic techniques were employed in a search for sex-specific genetic markers in the tuatara, or evidence of a statistically significant difference between the genomes of males and females. A minisatellite DNA profiling survey constituted the major undertaking of the experimental programme (Chapter 3). A secondary analysis involving RAPD assays of tuatara DNA was also conducted (Chapter 4).

In the minisatellite DNA profiling survey, 66 different pairings of DNA probe and restriction enzyme combinations were tested, using Southern blotting and radioactively-labelled probe hybridisation. This comprehensive survey employed five probes and 14 different enzymes, including 4-, 5-, and 6-bp cutters. The survey was divided into three distinct phases, correlating to different sample tuatara populations, and the reliability of the sexing of those samples. Sixty probe/enzyme pairings were involved in Phase 1, 36 in Phase 2, and 30 in Phase 3. DNA profiles (multilocus fingerprints) were examined for the presence of sex-specific fragments, and a considerable number of the probe/enzyme combinations were scored to obtain band counts. Mean fragment numbers for male and female tuatara were compared to determine if a statistically significant difference between the sexes was present. No sex-specific fragments were detected in this survey, and not one of 50 band counts revealed a significant distinction in male and female fragment numbers.

The RAPD analysis involved low-stringency PCR amplification of anonymous genomic fragments from male and female tuatara DNA using 10-mer oligonucleotide primers of random sequence. A total of 27 primers successfully amplified RAPD products. No sex-specific amplified fragments were revealed within the RAPD products, for any of the tested primers.

5.3 CONCLUSION

The fundamental aim to test the hypothesis of genetic involvement in the sex determination of tuatara, using techniques of molecular genetic analysis, was

accomplished. The experiments conducted did not reveal sex-specific DNA in the tuatara.

This finding fails to refute the null hypothesis that underlying sexual genotypes are not present in the tuatara. The result certainly lends some degree of support to the null hypothesis, but by no means is it a conclusive validation of the notion that sexual genotypes are non-existent. It is possible that sexual genotypes do exist in this reptile, but the assays were unable to detect any of the associated sex-specific genetic differences, for any one of a number of potential reasons (5.3.1). In theory, it is not possible to refute an alternative hypothesis, but there must come a point when the weight of evidence in support of the null hypothesis is so substantial that it is practical to assume that the alternative hypothesis is incorrect. The result of this research constitutes only limited support for the null hypothesis, so the alternative hypothesis should not yet be disregarded.

5.3.1 General implications

In general terms, the inability of this study to detect any sex-specific genetic markers is consistent with either of two scenarios. First, there is not genotypic gender in the tuatara, in which case, any further efforts to detect a sex-specific genetic difference will be equally unsuccessful. Second, such gender differences do exist, but the methodological approach employed to detect such differences was lacking in some respect. At least two potential explanations are apparent.

The first is that, despite every effort being made to minimise the risk of inclusion of sex-reversed tuatara in the analyses, a significant number of discordant animals were in fact included. This could have occurred if two conditions were met: (i) the proportion of sex-reversed individuals within the populations sampled was large, and (ii) the theory that concordant individuals could be preferentially selected based upon the physical phenotype criteria applied during sampling was wrong. There is little evidence to suggest that these two conditions were not met. Thus, it is quite possible that sex-specific fragments were present on any number of occasions within either the minisatellite DNA profiles or the RAPD band profiles, but the DNA of sex-reversed animals within the same profiles masked the sex-specific nature of these markers.

An alternative possibility is that the molecular methods employed were simply insufficiently comprehensive, or even inappropriate. Minisatellite DNA profiling may have been too 'shotgun' an approach to detect what could be a sex-specific genetic difference of a very subtle nature (possibly only a minor sequence difference at a single

locus). In this respect, RAPD analysis would supposedly allow greater resolution of slight genetic distinctions between the sexes, but it might require the amplification of products from an extremely high number of 10-mer primers, before an appropriate primer was encountered. As already noted, the RAPD investigation conducted within this investigation was limited to a relatively small number of different primers.

With respect to the evolutionary debate over the ancestry of sex determination in reptiles, the result that no sex-specific bands were detected is consistent with the hypothesis that TSD is the pleisomorphic state. According to this hypothesis, sex-specific genetic differences are unexpected for TSD reptiles. However, the result does not constitute a refutation of the alternative hypothesis that GSD is ancestral, since sex markers may have remained undetected due to these potential shortcomings of the methodology.

5.3.2 Implications for conservation

The concept of sex-reversal, which implies that sexual genotypes are overridden by temperature to produce discordant individuals (Chapter 2), has major implications for the presumed value of a molecular sex marker to the conservation of the tuatara. A marker for sex is required by conservation biologists to determine gender in the morphologically indistinguishable juveniles. However, it needs to be considered exactly which 'type' of gender is relevant for conservation practices, such as the establishment or maintenance of sex-ratios within populations or colonies. Sex ratios in such circumstances are taken to mean the ratio of *phenotypic* males to *phenotypic* females. The ratio of sexual genotypes within a population is irrelevant, since reproduction can presumably only occur as a result of the mating of a phenotypic male and a phenotypic female. Therefore, knowing the sexual genotype of a juvenile may be of limited benefit to conservation managers, if it is revealed at sexual maturity that the individual has developed as the opposite phenotypic sex as a result of the incubation temperature regime it experienced during embryogenesis. Hence, the relevant information for breeding purposes is the incubation temperature regime of an embryo, rather than the genotype of the hatchling. For captively-reared juveniles the incubation temperature regime will presumably already be known.

This potential for sex-reveral would significantly diminish the usefulness of a molecular marker for sex in the conservation of a TSD reptile. There may be particular circumstances in which a genetic marker would be of benefit, however. Concordant individuals might have a fitness advantage over their discordant consexuals, and in some conservation scenarios it could be advantageous to use the fittest individuals

available to establish or augment a population. Knowledge of both genotype (ascertained with a molecular sex marker) and phenotype (predicted through control of incubation temperature) would enable the identification of concordant (more fit) and discordant (less fit) males and females. In fact, if it was established that at pivotal temperatures temperature-influences upon sex are not in operation, then it would be advantageous to incubate all eggs of captive-reared juveniles at the pivotal temperatures, since phenotypes would be a true manifestation of sexual genotypes. All the hatchlings raised would be concordant, and their sex could be determined by the use of the molecular marker. This would eliminate the chance that a significant proportion of these individuals turn out to be the opposite sex when their phenotype becomes apparent at sexual maturity. Presumably, it would be of benefit to the conservation effort if all those released captively-reared individuals possess a high fitness (due to concordance), as this might increase the average individual's chances of survival and reproduction.

It is conceivable that concordant and discordant sex classes do not exist, as sex-reversal due to the effect of incubation temperature may not occur. This would be the case if genetic predispositions for development as one or the other sex do not exist in fertilised embryos. However, the effect of temperature during sex-determination may involve the structural modification of DNA, as proposed by Demas *et al* (1990) (section 2.4.3.3). According to this hypothesis, sex-specific differences might be 'created' in individuals during embryogenesis. This would imply that the detection of sex-specific DNA markers is possible. Furthermore, this scenario implies that diagnostic genetic markers would be of undiminished value in the conservation management of the species, as the potential problem of sex-reversals would be non-existent.

5.7 FUTURE DIRECTIONS FOR RESEARCH

There are a number of possible directions for future research into the question of genetic involvement in the sex determination of TSD reptiles. Some of these measures would be applicable to a species like the tuatara, whilst others would be more applicable to other species of TSD reptiles.

First of all, the preceding discussion raises the interesting point that the methodology employed in this study might succeed in detecting sex-specific markers in the tuatara if it was conducted using sampled individuals for which concordance is certain (or at least, highly probable). Until a genetic sex-marker is found (which was the aim of this study!), this would appear particularly difficult. There may be potential means of

identifying concordant animals, however, which were not available options at the time that this research was undertaken.

For example, at the incubation temperatures of *S. punctatus* eggs which were shown to produce large biases towards one phenotypic sex, there were a small number of individuals of the opposite phenotypic sex produced (Table 1.1 [Cree *et al*, 1995]). At a constant incubation temperature of 20°C, three out of 34 individuals turned out to be males, when sexed at maturity. There is every reason to believe that these three individuals were true genetic males, since they were incubated at a temperature which produced 91% phenotypic females. At a constant temperature of 22°C, four out of 17 individuals were female. It is reasonable to assume that these individuals were genetic females, since this temperature obviously produces a bias towards male development. Hence, individuals which develop as the opposite sexual phenotype to that which is typically induced by a certain incubation temperature, are very likely to be concordant, whereas the remainder of the hatchlings from that incubation temperature will presumably be a mixture of concordant and sex-reversed individuals.

A second potential method of identifying concordant individuals for analysis would be to sample only those individuals that are incubated at the pivotal temperature (which is yet to be established precisely for the tuatara). It has been proposed that the 1:1 sex ratio at this incubation temperature is explained by the absence of temperature influence upon sex determination within this narrow temperature range, which infers that the underlying genotype alone directs the sex determination of the eggs. Thus, all those individuals incubated at the pivotal temperature(s), are presumably concordant. Unfortunately, examples of such individuals were not available for analysis at the time of this investigation

Another possible method of identifying concordant individuals is H-Y antigen testing of gonadal and non-gonadal tissue. Girondot *et al* (1994) identified putative sex-reversed individuals within a wild population of the European pond turtle, as those animals which with opposite H-Y antigen phenotype (positive or negative expression) in their blood cells and gonadal tissue (section 2.4.2.4). This could presumably also be done for tuatara. The most certain way of identifying concordant individuals might in fact be a combination of techniques: the incubation of eggs at the pivotal temperature, testing of the hatchlings for H-Y antigen expression, and the morphological determination of sexual phenotype when those animals reach sexual maturity. In theory, the concurrence of H-Y antigen phenotype with morphological phenotype for animals raised at pivotal temperature would effectively maximise the chances of correct identification of concordant individuals.

The next logical step in the search for sex-specific DNA in this species would then be to repeat the minisatellite DNA profiling and RAPD analyses, using these individuals for which genetic and phenotypic sexual concordance is believed to be very likely. In the case of DNA profiling, it would be advisable to repeat only those probe/enzyme combinations shown by this study to produce profiles of high band resolution for the tuatara, but there are a number of probes and enzymes which are yet to be tested which might also produce high quality minisatellite DNA profiles. Any future RAPD survey would be more likely to detect sex-specific genetic distinctions if a much larger number of 10-mer primers were available for analysis.

Other available approaches for molecular sexing could also be attempted for the tuatara (using probable concordant individuals). Sex-specific sequences isolated from other species, such as the CHD (Ellegren, 1996; Griffiths *et al*, 1996) and SRY sequences (Mittwoch, 1996), could be hybridised to tuatara DNA in an attempt to detect related sex-specific sequences in this reptile. Admittedly, this has been unsuccessful in other TSD reptiles for which it has been attempted (Lance, 1997), but this does not necessarily preclude the chances of success in the tuatara.

Another potential approach for testing the hypothesis of genetic involvement in the sex determination of TSD species, would require two sister reptile taxa in which genotypic sex determination is present within one group, and temperature-dependent sex determination is exhibited by the other (i.e. tuatara are not appropriate). There are many instances of such a scenario within reptiles; it has even been found for two species within the same genus. An example is the turtles *Clemmys guttata* (TSD) and *C. insculpta* (GSD) (Ewert and Nelson, 1991). Such closed-related taxa provide an excellent opportunity for investigating the common elements in the two sex-determining mechanisms. A sex-specific DNA marker detected in the GSD species (supposedly a straightforward exercise) could be developed as a probe to test for related DNA in the sister species with temperature-dependent sex determination. If GSD is ancestral, then the taxon with the derived TSD mechanism will presumably have the ancestral sex-determining genetic elements within its genome, although this DNA may be scattered amongst phenotypic males and females, due to sex-reversal. If such DNA was found within the TSD species, it could be tested for linkage to genetic sex, by determining the distribution of the putative sex-specific sequences amongst males and females incubated at the pivotal temperature. In theory, the occurrence of the putative sex-specific DNA within the TSD species should correlate to the phenotypic sex revealed at sexual maturity (or earlier, if juveniles are sexually dimorphic), if the assumption that sex at pivotal temperature is determined by genotype is correct.

5.5 FINAL REMARKS

It is widely viewed that there is a dichotomy of sex-determination mechanisms within the reptiles: species either exhibit genotypic sex determination or temperature-dependent sex determination. Only a few species have been examined for both modes. Although it is often considered that the two mechanisms are mutually exclusive, there is evidence that there may be a weak genetic sex-determining mechanism in species in which the primary sex-determining mode is TSD. This infers that some TSD individuals may be sex-reversed; that is, their sexual genotype is discordant with their sexual phenotype. This hypothesis of an underlying genotypic system may also be linked to the question of the evolution of sex-determination within the reptiles. The discovery of sex-specific DNA within a TSD reptile would suggest that GSD is ancestral and TSD has evolved many times over within independent reptile lineages. This project tested the hypothesis that there is a genetic component to sex determination in TSD species, by searching for sex-specific DNA in the tuatara, a reptile with temperature-dependent sex determination. Evidence of sex-specific genetic differences was not found. This may reflect the absence of genetic sex differences in the tuatara. Alternatively, it might also be the result of accidental inclusion of sex-reversed individuals within the analyses, a situation which could have obscured the sex-specific nature of any sex-linked fragments. It would appear that the key to solving the question of sex-specific DNA within TSD reptiles such as the tuatara lies with the problem of ensuring sex-reversed individuals are excluded from molecular analysis.

Appendix I

Morphological data of the Lady Alice Island tuatara (PHASE 2 samples: Chapter 3)

sample code	phenotypic sex	snout-vent length (mm)	weight (g)	ranking within sex (weight)	ranking overall (weight)
sp23	M	249	706	1	1
sp12	M	250	678	2	2
sp22	M	244	615	3	3
sp15	M	242	549	4	4
sp18	M	243	528	5	5
sp07	M	241	426	6	6
sp11	M	247	415	7	8
sp09	M	219	395	8	9
sp16	M	211	394	9	10
sp17	F	227	334	1	15
sp24	F	210	423	2	7*
sp06	F	223	379	3	11
sp03	F	213	364	4	12
sp25	F	197	358	5	13
sp10	F	211	335	6	14
sp21	F	209	332	7	16
sp19	F	215	325	8	17
sp05	F	208	321	9	18
sp04	F	204	308	10	19
sp13	F	208	305	11	20
sp20	F	210	291	12	21

* Based upon weight as the ranking parameter, only one female overlapped with the male size range. It was thought this might indicate that all the males were concordant, due to their large size. The largest nine males and nine females were used for Phase 2 of the minisatellite DNA profiling survey.

Appendix II

The patterns of temperature-dependent sex determination: two or three?

It has long been believed that there are three general patterns of temperature-dependent sex determination exhibited by reptiles (section 2.2.2), however, there may be only two general patterns. It appears now that the few species for which Pattern Ib TSD was originally described, may actually exhibit Pattern II. The species in question are two species of lizard and several crocodilian species (Bull, 1983); Gutzke and Crews, 1988; Deeming and Ferguson, 1989, 1991). The lizards are *Agama agama*, the first reptile for which TSD was reported (Charnier, 1966), and *Eublepharis macularius*. The latter species was reported to exhibit a Pattern Ib TSD (Bull, 1987; Wagner, 1980), but it was later shown to be Pattern II (FMF) upon closer investigation of sex ratios across a wider thermal incubation range (Viets *et al*, 1993), i.e. there was a transition back towards a female hatchling bias at higher temperatures. In the case of *Agama agama*, which has been frequently cited as a Pattern Ib species (Bull, 1980, 1983; Deeming and Ferguson, 1988; Ewert and Nelson, 1991), there has been no further study of its sex determination since it was first studied by Charnier (1966), and since only two different incubation temperatures were investigated in that study, it is possible that studies at warmer incubation temperatures would yield a female-bias in hatchling sex ratios. This seems all the more likely when it is considered that *Agama caucasia*, the only closely-related TSD species which has been thoroughly investigated in this respect, exhibits Pattern II TSD.

The crocodilian species *Alligator mississippiensis* and *Caiman crocodilus* were once believed to have Pattern Ib (Deeming and Ferguson, 1991; Lang *et al*, 1989), but were recently shown to exhibit Pattern II TSD when a wider range of incubation temperature was tested (Lang and Andrews, 1994). Several other crocodilian species also appeared on the basis of early preliminary data to exhibit Pattern Ib TSD, but these too were revised to Pattern II upon further investigation (Lang and Andrews, 1994). The FMF pattern is now thought to be ubiquitous in crocodilians for which sex determination has been documented, and since the 11 (of 22) species studied include members of each of the three main lineages of crocodilians (alligators/caimans, crocodiles, and gharials), and heteromorphic sex chromosomes are universally absent from crocodilians, it is

believed that all extant species of Crocodilia exhibit TSD (Lang and Andrews, 1994). Furthermore, the ubiquitous FMF pattern could be taken as further evidence that Pattern II is the ancestral pattern of TSD in reptiles, especially in light of the 'antiquity of phylogenetically distinct lineages (alligators vs crocodiles), diversity of nesting habits (mound vs hole nesting), and the widespread distribution of representatives in wet and dry tropical and warm temperate habitats' found within Crocodilia (Lang and Andrews, 1994). If this is the case, the single transition pattern Ia (MF) must have been derived in certain turtle lineages. All lizards with TSD that have been studied across a reliable range of incubation temperatures show Pattern II (Viets *et al*, 1994). None show pattern Ia, and the dubious nature of the few reports of species with Pattern Ib has already been noted.

Appendix III

THE EVOLUTION OF SEX DETERMINATION IN REPTILES

The evolution of the two modes of sex determination in the reptiles is clearly relevant to the hypothesis that there is an underlying genetic component to primary sex determination in temperature-dependent sex determination. For this reason, the evidence for the ancestry of both modes of sex determination is examined here. The transition from TSD to GSD, or GSD to TSD, must have occurred independently many times within the evolutionary history of the modern reptiles, since there are many instances of TSD and GSD species occurring within a single subfamily, and sometimes even within the same genus.

The case for an ancestry of temperature-dependent sex determination

The prevailing view appears to be that the most remote ancestors of reptiles had some form of environmental sex determination. The earliest reptiles were present some 320 mybp, during the early Carboniferous period (Pough *et al*, 1988). From those early reptiles, many vertebrate lineages arose, including the dinosaurs, birds, modern reptiles, and mammals. Within the modern vertebrate groups, genotypic sex determination is ubiquitous in birds and mammals, whereas both TSD and GSD (in various forms) are found in the reptiles. Nearly all birds and mammals possess heteromorphic sex chromosomes[1], compared to only 27% of reptile species that have been karyotyped (Janzen and Paukstis, 1991). Most invertebrates, fish, and amphibians also lack heteromorphic sex chromosomes, and furthermore, many organisms within these groups show lability in their expression of sex (Janzen and Paukstis, 1991). It is believed that early reptiles evolved from ancient amphibians, and it is known that modern amphibians exhibit components of both GSD and TSD. In a few amphibian

[1] The terms *heteromorphic* and *homomorphic sex chromosomes* are used frequently in this discussion. In vertebrate GSD, the sex chromosomes are a pair of homologous chromosomes, one or both of which contain sequences involved in primary sex determination. In this discussion, heteromorphy is taken to mean sex chromosomes that are morphologically distinguishable at the level of cytogenetic examination. Homomorphy means sex chromosomes that are not sufficiently differentiated to be visibly distinct upon such examination. In the latter case, it is presumed that the sex chromosomes are differentiated at some level, perhaps only at a single locus.

species studied, it appears that sex determination is only weakly controlled by genotype, as incubation of eggs at extreme temperatures results in sex-biases (which are not a result of differential mortality) (Bull, 1980). Moreover, heteromorphic sex chromosomes are rare in amphibians.

It has been argued upon this basis that environmental sex determination is the ancestral state in vertebrates (Ohno, 1967), and therefore in reptiles also (Bull, 1980), which would infer that GSD evolved at a later stage in 'higher' vertebrates. The transition from one sex-determining mode to the other must have occurred independently within a number of separate reptile lineages, implying that there is a straightforward developmental modification involved. There is a considerable amount of evidence to support the hypothesis of TSD ancestry, including the general pattern of sex-determining mechanisms within the modern vertebrates just described.

Current theories of the evolution of sex chromosomes also support the ancestral TSD hypothesis. GSD is thought to progressively evolve from an initial state of chromosome homomorphism through increasing, and probably irreversible, stages of differentiation, until a point of morphologically distinguishable sex chromosome heteromorphism is attained (Bull, 1983; Charlesworth, 1991). There is evidence that heteromorphic sex chromosomes have evolved only recently in the snakes, lizards, and turtles in which they are found (Bull, 1980). For example, the snakes (Squamata) are phylogenetically grouped on the basis of skeletal characters (Romer, 1956), and these families correlate to the varying degrees of sex chromosome differentiation (Bull, 1980). The Boidae (boas and pythons) are the most ancestral in their skeletal form, and they exhibit GSD but have homomorphic sex chromosomes. The Colubridae skeletal morphology is derived from the boids, and they exhibit female heterogamety, where the Z and W chromosomes are equal in size, but differ in the position of the centromere. Finally, the Viperadae have the most derived skeletal characters, which obviously evolved from the colubrid condition. The viperids also exhibit female heterogamety, but have the highest degree of chromosome differentiation, as the Z and W chromosomes are unequal in size (Ohno, 1967). The karyotype is preserved across the families, and it is the same chromosome pair in which heteromorphism is observed each time (Bull, 1980).

Therefore, it would appear that GSD with chromosome homomorphism is the ancestral condition in snakes, since TSD has not been found in any snake species thus examined (Janzen and Paukstis, 1991). Lizards (Squamata) differ from the snakes in that species with heteromorphic sex chromosomes occur infrequently, and the sporadic distribution of these instances indicate multiple origins of sex chromosome heteromorphy, and not a single ancestral type, as in snakes (Bull, 1980). Snakes, in fact, belong within the 'lizard' clade (Pough *et al*, 1998), so it would seem that the snakes evolved from a lizard lineage which had homomorphic chromosome GSD. Finally, there are some lizards, and turtles, in which the heteromorphic sex chromosomes are unique to the species or genus, indicating their recent evolution (Bull, 1980; Janzen and Paukstis, 1991). These facts suggest that the evolutionary trend in reptiles with genotypic sex determination is from an ancestral state of morphologically undifferentiated chromosomes to heteromorphic sex chromosomes, which may reflect a trend towards increased genetic control of sex determination (Bull, 1980).

It is generally considered that once heteromorphic sex chromosomes evolve, there is no returning to a state of homomorphism (Charlesworth, 1991). Homomorphic sex chromosomes are presumed to be a necessary prerequisite for TSD (Bull, 1980; Janzen and Paukstis, 1991). Therefore, it would seem that an evolutionary transition from GSD with heteromorphic sex chromosomes to a TSD mechanism is unlikely. Bull (1980) postulated a theoretical explanation for this, which can be summarised using male heterogamety as an example. If a susceptibility for incubation temperature to override genetic sex arises in a few embryos, then some of those embryos will develop as XY females or XX males (i.e. sex-reversed individuals). The proportionately small number of XY females are more likely to mate with the numerous XY males than with the small number of XX males. The resulting XY-XY crosses would produce 25% YY individuals, which is assumed to be a lethal genotype. This is because differentiation of the X and Y chromosomes is likely to have caused the accumulation of detrimental recessive genes on the Y chromosome. Hence, if YY progeny *are* inviable, then XY females are at a selective disadvantage to normal XX females, since less of their progeny will survive. Thus, there would be selection *against* the evolution of temperature sensitivity in the sex determination of those species with differentiated sex chromosomes (Bull, 1980).

A final argument for the remote ancestry of environmental sex determination arises from the distribution of TSD within the reptile groups. TSD is widespread throughout the Reptilia, and is absent from only one major lineage (snakes), and this has been taken to indicate that the ancestral condition was environmental sex determination. The fact that tuatara have TSD, and are the only living representatives of the ancient sphenodontid lineage, has also been suggested as support for the hypothesis of TSD ancestry in reptiles (Cree *et al*, 1995).

It is the distribution of TSD within turtles, however, that presents the best case in this respect. It is widely considered that the turtles represent the most anciently divergent lineage within the reptiles, based upon the anapsid skull structure which separates them from the living diapsid reptiles (Pough *et al*, 1998), and the early appearance of this group in the fossil record (Janzen and Paukstis, 1991). TSD predominates within the turtles, as it is present in over 80% of species examined, whereas GSD occurs in less than 10% of studied species (Janzen and Paukstis, 1991). These two facts considered together suggest an ancestral state of TSD within the early reptiles prior to the putative Anapsida/Diapsida divergence. In addition, phylogenetic analysis of the turtle taxa has suggested that it is more parsimonious to assume ancestry of TSD rather than GSD (Janzen and Paukstis, 1991). It would require a minimum of seven evolutionary events (transitions within a single lineage from one sex-determining mechanism to the other) to explain the current distribution of TSD within Chelonia, if TSD were ancestral, compared to a minimum of 13 evolutionary events if GSD were ancestral.

The case for an ancestry of genotypic sex determination

The case for a remote ancestry of genotypic sex determination in the reptiles does not so much rely upon direct evidence for this theory, as it does question the validity of the arguments for an ancestry of temperature-dependent sex determination.

First, the argument for TSD ancestry based upon the occurrence of the sex-determining mechanism within the major vertebrate groups can be turned around to some degree. Although environmental sex determination is present in various forms within fish and amphibians, TSD as a single mechanism of sex determination is not. Certainly, there are many cases of fish and amphibian species in which there is a degree of

thermosensitivity in sex determination, but in all such cases found to date, there is an obvious genotypic sex determination system in effect as well (Bull, 1980; 1983; Janzen and Paukstis, 1991; Lagamarsino and Conover, 1993; Baroiller *et al*, 1996); a situation which is not true of reptiles with TSD. For example, the rarity of heteromorphic sex chromosomes in the Amphibia has been suggested as support for the ancestry of TSD in reptiles. Yet genotypic sex determination (albeit often a weak form) is present in most amphibians, despite the absence of morphologically differentiated sex chromosomes (Janzen and Paukstis, 1991). Hence, it is conceivable that GSD (with chromosome homomorphism) existed in the ancient amphibian stock from which the first reptiles are thought to have evolved.

All mammal and bird species have GSD, and these two groups are generally believed to have had common ancestors amongst the early proto-reptiles, prior to the Synapsida/Reptilia divergence in the Permian period (Pough *et al*, 1998). However, this may have little relevance to either of the hypotheses concerning the ancestry of sex determination in reptiles, since all modern representatives of mammals and birds thermoregulate the development of their young. This could quite possibly preclude the operation of TSD, so it may be that it was simply not possible for TSD to persist in these lineages, whether it was present in the early ancestors of mammals and birds, or not (Bull, 1980).

Second, the widespread occurrence of TSD within the reptiles, and the seemingly larger proportion of species that exhibit TSD in relation to GSD, may be misleading. In a thorough review of TSD in the Reptilia, Janzen and Paukstis (1991) note that even though many more of the species examined have TSD, the total number of species known to have *either* TSD or GSD is approximately only 25% of the extant reptile species. It is conceivable that information from the remaining uninvestigated taxa might paint a quite different story of the probable ancestry of sex determination within this group.

This is especially true in the case of the phylogenetic analysis of turtles (Janzen and Paukstis, 1991). Additional data from the large number of unstudied turtle species might change the result that it is more parsimonious to assume TSD is ancestral. Even if it did not, the original finding may not be all that significant. It is thought that a

transition from GSD to TSD would probably be a fairly simple evolutionary step (Bull, 1983), and it may well have occurred on a large number of occasions within different turtle lineages, perhaps many more times than the minimum number established by phylogenetic analysis.

The fact that TSD is predominant within the Chelonia (9 of 13 families, one of which is not studied), may not necessarily be strong evidence for the ancestral TSD hypothesis either. There is some dispute over the assumption that the turtles, as the living representatives of the Anapsida, represent the most ancient divergence amongst the modern reptile groups (Carroll, 1988). For example, at least two recent phylogenetic studies have presented molecular evidence which indicates a close affinity between the Chelonia and the Archosauria (birds and crocodilians). Kumazawa and Nishida (1999) determined the complete mitochondrial sequences of a turtle and a lizard species, and subsequent phylogenetic analyses using amino acid sequences of 12 mitochondrial proteins established the turtles as a sister group of the archosaurs, with the squamates as a distinct clade. Hedges and Poling (1999) analysed the sequence data from mitochondrial DNA and from several nuclear genes of representatives of each of the major living amniote groups: crocodilians, turtles, tuatara, squamates, and birds (with mammals as the outgroup). The results also indicated that birds, crocodilians, and turtles form a natural group. Interestingly, tuatara were the sister taxon to this group, and the Squamata became positioned as the most anciently diverged lineage. Like turtles, the squamates exhibit a wide range of sex determination mechanisms, but amongst lizards at least, it is not nearly so clear whether GSD or TSD is the predominant mechanism (all snakes have GSD it seems) (Viets *et al*, 1994). On this basis, the proposed new phylogeny probably could not be taken as solid support for either of the two hypotheses regarding the ancestry of sex determination in reptiles.

As a final point of the argument against the ancestral TSD hypothesis, it is certainly difficult to counter the observation that TSD has not been conclusively demonstrated to exist in any reptile with heteromorphic sex chromosomes. Only five species known to have heteromorphic sex chromosomes have been examined for TSD (Janzen and Paukstis, 1991), but on the other hand, it has been determined that heteromorphic sex chromosomes are absent in many reptiles with TSD (Janzen and Paukstis, 1991). There is little disagreement to the view that homomorphic sex chromosomes tend to

differentiate into heteromorphic sex chromosomes (probably irreversibly), and that a transition from a genetic sex-determining mechanism involving chromosome heteromorphism to a temperature-dependent mechanism is an unlikely evolutionary step (Bull, 1980). Thus, for TSD to have evolved from an ancestry of GSD, the transition from one mechanism to the other must presumably have occurred in taxa with (morphologically) undifferentiated sex chromosomes. In this context, it is assumed that chromosome heteromorphism is associated with the accumulation of deleterious alleles in one or the other of the sex chromosomes. There does not seem to be any reason why TSD may not operate in a GSD species that has homomorphic sex chromosomes, where this accumulation presumably has not occurred.

If GSD is ancestral, then it has to be questioned why heteromorphic sex chromosomes did not evolve in the early amniotes and stem reptiles (prior to evolution of TSD), thus precluding the possibility of a later transition from GSD to TSD. Bull (1980) suggests a possible solution to this potential dilemma for the ancestral GSD hypothesis: in addition to the obvious requirement that there is a system of genetic sex determination, the evolution of sex chromosome heteromorphism (which typically involves structural gene rearrangements or deletions) might require other unknown factors to be present, or in operation. These additional factors may have been absent from the ancestral lineages, where heteromorphic sex chromosomes failed to evolve despite the presence of a GSD mechanism.

The adaptive significance of temperature-dependent sex determination

The adaptive significance of TSD has been one of the more controversial areas of sex determination research. Several hypotheses have been proposed to give a reason for the occurrence of TSD in reptiles, and there is still dispute over the plausibility of the various alternative explanations (Janzen and Paukstis, 1991; Shine, 1999).[2]

[2] While it is often assumed that there is some adaptive significance to TSD in reptiles, it is quite possible that there is not. If GSD is ancestral, the evolution of TSD from GSD could conceivably have occurred by an evolutionary process without the involvement of any positive selection for this mechanism. Alternatively, if TSD is ancestral, it does not require a selective advantage to explain why TSD has been 'retained' in some lineages, when GSD has evolved in other related taxa. It might simply be that there is

Shine (1999) reviews the hypotheses, which he groups into four categories: phylogenetic inertia; group-adaptation; inbreeding avoidance; and differential fitness. The phylogenetic inertia hypothesis proposes that TSD is the ancestral mode of sex determination, and that TSD may persist in most reptile lineages because the genetic variation which is presumably required to allow the evolution of GSD is lacking. That is, there is no current adaptive significance to TSD; it is simply the status quo.[3] This hypothesis is difficult to test, but Ewert and Nelson (1991) point out that the number of transitions from TSD to GSD required to explain the current taxonomic distribution of turtles with TSD, as well as the evidence for the evolutionary plasticity of TSD species, makes this explanation suspect. They advanced the group-adaptation hypothesis as a possible alternative, which proposes that TSD is advantageous because it facilitates group fitness through the skewing of population sex-ratio. This skew could be towards a female bias at sexual maturity, which might enhance group productivity. Of course, such a hypothesis invokes the troublesome assumptions associated with the theory of group selection (Williams, 1966), and moreover, it is inconsistent with the available data on TSD reptile population structure (Shine, 1999). Ewert and Nelson (1991) also proposed the hypothesis of inbreeding avoidance to explain the advantage of TSD over GSD. This idea contends that TSD results in large proportions of unisexual clutches within a population, which reduces the potential for sibling matings. Thereby inbreeding depression is avoided, and outbreeding is promoted, which may increase genetic variation. This is not entirely consistent with TSD in many crocodilian and turtle species, in which offspring from many annual cohorts interbreed with little risk of a high proportion of sibling matings (Shine, 1999).

Perhaps the most plausible theoretical models proposed, and certainly the first to be advanced (Charnov and Bull, 1977; Bull, 1980; 1983), postulate that TSD would be favoured over GSD in situations where a lifetime fitness advantage would accrue to individuals of a particular sex in a given environment. In other words, incubation

some unknown barrier to this transition to GSD in those TSD species, for example, or that the transition has not occurred due to 'phylogenetic inertia'.

[3] The inverse view is that adaptive significance of TSD might explain *why* this sex-determining mechanism has persisted in many reptilian lineages, when it has been superseded by GSD in many other, often closely-related, taxa (if it is assumed that the ancestral TSD hypothesis is correct).

temperatures might differentially affect the fitness of male and female offspring, making it more advantageous for one sex to develop at a cooler incubation temperature, and the other at a warmer incubation temperature. Various explanations for this have been proposed, many of which emphasize differences in post-hatching growth rate benefits for males and females in given environments (reviewed by Wilbur and Morin, 1988; Janzen and Paukstis, 1991; Shine, 1999). All of these hypotheses invoke a series of assumptions, and at this time they still appear to have little empirical support (Elphick and Shine, 1999).

With respect to the two hypotheses of sex determination ancestry, it is the ancestral GSD hypothesis that would gain the most support from solid evidence for the adaptive significance of TSD, since it could explain why the hypothetical transition from GSD to TSD took place within so many different reptilian lineages. It is in fact quite simple to perceive an evolutionary transition to TSD from GSD, if genotypic sex determination is the ancestral mechanism (it would also appear to be a simpler step than the reverse transition). If there is at least some small degree of thermosensitivity in the sex determination of a GSD species (which is known to be the case for several fish and amphibian species [Bull, 1983; Janzen and Paukstis, 1991]) then the initially low level of temperature-sensitivity could be increased under selection until it becomes the dominant influence upon embryonic sex determination (Bull, 1983). This selection would operate if there was a selective advantage conferred on these individuals by the unknown adaptive significance of TSD. Alternatively, if there is no such temperature-sensitivity to begin with, a (heritable) mutation might cause a protein or genetic factor within the genetic sex-determining pathway to become temperature-sensitive, thereby producing the initial thermosensitivity within the pathway that selection may act upon to commence an evolutionary transition to TSD. There are certainly precedents for this theory, such as temperature-sensitive sex mutations in *Drosophila*, *Caenorhabditis*, and the mosquito *Aedes*, in which the mutant strains have heterogametic sex determination at permissive temperatures, but produce sex-ratio biases at restrictive temperatures (Bull, 1983).

In summary, it is quite apparent that the ancestry of reptilian sex determination is far from satisfactorily resolved. The demonstration of sex-specific DNA in a reptile with TSD would provide more support for the hypothesis that TSD is a derived sex-

determining mechanism superimposed upon an ancestral genetic sex-determining mechanism.

Appendix IV

A statistical analysis of potential sex-specific fragments

Assuming complete concordance between sexual genotype and phenotype of the selected individuals, nine members of each genetic sex were included in a typical fingerprinting gel of 18 lanes (it is certain that there were nine of each phenotypic sex). A genetic marker would therefore appear as a fragment in all nine individuals of one sex, and none of the other. This 'distribution' is here denoted as 9:0; that is, it is given as the ratio of the number of individuals of sex A with the fragment (out of nine) to the number of individuals of sex B with the fragment (out of nine). (Note that sex A could be male or female). However, if there were a few discordant individuals included unwittingly, then this fragment ratio would deviate from 9:0 for a genetic marker for sex. For example, one discordant animal amongst both sets of sexes would produce a fragment distribution for a genetic sex marker of 8:1. The problem then arises as to whether a particular fragment with a distribution biased towards one sex should be ignored as a fairly likely distribution for a fragment not linked to sexual phenotype, or investigated further as a potential genetic marker for sex, that has a distribution deviating from 9:0 *because* of the inclusion of one or more discordant individuals. It has been assumed that there will only be a few, if any, discordant individuals included amongst the 18 samples as a result of the selection strategies employed in the study (section 2.6). Indeed, if there were as few as only five or six discordant individuals amongst the 18 sampled animals, then the detection of a genetic marker for sex would be very difficult.

This analysis therefore investigates the probabilities of particular fragment distributions occurring by chance, that is, the probability that the fragment in question is *not* linked to sexual phenotype. The table below gives these probabilities for the possible distributions for a particular fragment with a frequency close to 0.5 (the frequency expected on average for a genetic marker for sex). For example, a fragment may occur with a frequency of 0.44 in the sample population, so might therefore be found in eight of the 18 individuals sampled within the fingerprinting gel (on average). (If that fragment is linked to genetic sex, this infers there is a 44:56 sex ratio in the sample population). If the fragment is *not* sex-linked, then it should occur with the same

frequency in phenotypic males as it does in phenotypic females. For a fragment with a 0.44 frequency in the population, it should (on average) be present in four of the nine phenotypic males within the gel, and four of the phenotypic females, that is, a distribution of 4:4 in the table below. However, if there is some linkage between that particular fragment and phenotypic sex, then it would be expected to occur in a more biased ratio, for example, 6:2, or even 7:1. The table gives the probabilities of possible distributions occurring by chance, assuming there is no correlation between the fragment and phenotypic sex. When the probability of a particular distribution is highly unlikely (e.g. p(8:0) = 0.00042), it is suggestive of a correlation between that fragment and the phenotypic sex for which is nearly always present, indicating that the fragment in question deserves to be investigated further as a potential genetic marker for sex.

Probabilities of particular fragment distributions occurring by chance when the fragment is not linked to phenotypic sex

Number of individuals with fragment (out of 18: nine phenotypic females plus nine phenotypic males)	Distribution of the number of phenotypic males with the fragment to the number of phenotypic females with the fragment (given as ratio **a:b**)	Probability of the distribution occurring by chance
7 individuals (fragment frequency = 0.39)	7:0 or 0:7	0.00226
	6:1 or 1:6	0.04751
	5:2 or 2:5	0.28507
	4:3 or 3:4	0.66516
8 individuals (fragment frequency = 0.44)	8:0 or 0:8	0.00042
	7:1 or 1:7	0.01480
	6:2 or 2:6	0.13822
	5:3 or 3:5	0.48376
	4:4	0.36281
9 individuals (fragment frequency = 0.50)	9:0 or 0:9	0.00004
	8:1 or 1:8	0.00334
	7:2 or 2:7	0.05332
	6:3 or 3:6	0.29026
	5:4 or 4:5	0.65306
10 individuals (fragment frequency = 0.56)	9:1 or 1:9	0.00042
	8:2 or 2:8	0.01480
	7:3 or 3:7	0.13822
	6:4 or 4:6	0.48376
	5:5	0.36281
11 individuals (fragment frequency = 0.61)	9:2 or 2:9	0.00226
	8:3 or 3:8	0.04752
	7:4 or 4:7	0.28506
	6:5 or 5:6	0.66516

Calculation of probabilities:

(i) For **x** number of individuals with the fragment, there are **n** number of ways in which that number of fragments can be distributed amongst the total number of individuals (**y**) in the gel:

x = 7, 8, 9, 10, or 11 (fragment frequencies close to 0.5, that is, 9 individuals)
y = 18

$$\mathbf{n} = {}^{x}C_{y} = \frac{x!}{y!(x-y!)}.$$

(ii) For each distribution **a:b**, that is, the number of individuals of sex A with the fragment (**a**) to the number of individuals of sex B with the fragment (**b**), (where a+b=x), there are **r** number of different ways in which those numbers of fragments can be distributed amongst the *specific* individuals of each phenotypic sex. A fragment present for two out of nine females, for example, might be present for the first two individuals, last two, first and last individual, or any other specific combination of two individuals amongst those nine females.

$$\mathbf{r} = \frac{9!}{a!(9-a!)} \times \frac{9!}{b!(9-b!)}.$$

(iii) The *probability* of a particular distribution **a:b** occurring is the number of possible ways in which those numbers of fragments can be distributed amongst the two sexes (**r**), divided by the number of possible ways (**n**) in which the number of fragments (**x**) can be distributed amongst the total number of individuals (**y**)

r / n = probability of a particular distribution occurring by chance

Note: In the table, the probabilities have been doubled, to represent the probability of the distribution occurring in favour of *either* phenotypic sex (e.g. a bias of 7:1 might be for males or females).

Example calculation (for section 3.3.2):

A specific fragment is present in nine out of nine females, and four out of nine males (i.e. 13 fragments in total). The distribution is denoted 9:4.

(i) number of possible different distributions of 13 fragments amongst 18 individuals:

$$n = \frac{18!}{13!5!} = 8{,}568$$

(ii) number of possible ways of the distribution 9:4 occurring:

$$r = \frac{9!}{9!0!} \times \frac{9!}{4!5!} = 1 \times 126 = 126$$

(iii) probability of distribution 9:4 occurring by chance (i.e. if there is no link between the fragment and phenotypic sex):

$$r / n = 126 / 8{,}568 = 0.0147$$

Since there is an equal chance of the distribution 4:9 occurring if there is no linkage between the fragment and phenotypic sex, then the probability of this fragment distribution arising by chance is twice this value, i.e. 0.0294. Such a distribution might therefore be expected to arise at random once in every 34 fragments which occur with a frequency within the sample set of approximately 0.72 (that is, in 13 out of every 18 individuals).

Appendix V

Sequences of the 10-bp primers used in the RAPD analysis (Chapter 4).

Operon Technologies Primer Set A

OPA-01	5'-CAGGCCCTTC-3'
OPA-02	5'-TGCCGAGCTG-3'
OPA-03	5'-AGTCAGCCAC-3'
OPA-04	5'-AATCGGGCTG-3'
OPA-05	5'-AGGGGTCTTG-3'
OPA-06	5'-GGTCCCTGAC-3'
OPA-07	5'-GAAACGGGTG-3'
OPA-08	5'-GTGACGTAGG-3'
OPA-09	5'-GGGTAACGCC-3'
OPA-10	5'-GTGATCGCAG-3'
OPA-11	5'-CAATCGCCGT-3'
OPA-12	5'-TCGGCGATAG-3'
OPA-13	5'-CAGCACCCAC-3'
OPA-14	5'-TCTGTGCTGG-3'
OPA-15	5'-TTCCGAACCC-3'
OPA-16	5'-AGCCAGCGAA-3'
OPA-17	5'-GACCGCTTGT-3'
OPA-18	5'-AGGTGACCGT-3'
OPA-19	5'-CAAACGTCGG-3'
OPA-20	5'-GTTGCGATCC-3'

Miscellaneous primers (from various Operon Technologies Primer Sets)

OPY-07	5'-AGAGCCGTCA-3'
OPY-06	5'-AAGGCTCACC-3'
OPY-11	5'-AGACGATGGG-3'
OPB-03	5'-CATCCCCCTG-3'
OPF-15	5'-CCAGTACTCC-3'
OPS-11	5'-AGTCGGGTGG-3'
OPX-08	5'-CAGGGGTGGA-3'

RAPD Oligo Project 10-Mers Set 50/1(2)

#51	5'-CTACCCGTGC-3'
#52	5'-TTCCCGGAGC-3'
#53	5'-CTCCCTGAGC-3'
#54	5'-GTCCCAGAGC-3'
#55	5'-TCCCTCGTGC-3'
#56	5'-TGCCCCGAGC-3'
#57	5'-TTCCCCGAGG-3'
#58	5'-TTCCCGGAGC-3'
#59	5'-TTCCGGGTGC-3'
#60	5'-TTGGCCGAGC-3'
#61	5'-TTCCCCGACC-3'
#62	5'-TTCCCCGTCG-3'
#63	5'-TTCCCCGCCC-3'
#64	5'-GAGGGCGGGA-3'
#65	5'-AGGGGCGGGA-3'
#66	5'-GAGGGCGTGA-3'

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