EVOLUTION OF SEX-DETERMINING MECHANISMS IN REPTILES

by

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Male Central bearded dragon (*Pogona vitticeps*) at twilight, southwest Queensland.

"The three evolutionary questions – what, how, and why – may be applied separately to the study of sex determination, but a perspective combining all three levels can lead to a particularly enriched paradigm of study."

J.J. Bull (1985)

Statement of Originality

This thesis is my original work and has not been submitted, in whole or in part, for a degree at this or any other university. Nor does it contain, to the best of my knowledge and belief, any material published or written by another person, except as acknowledged in the text.

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Abstract

Reptiles exhibit marked diversity in sex-determining mechanisms. Many species exhibit genotypic sex determination (GSD) with male heterogamety (XX females/XY males), others have GSD with female heterogamety (ZW females/ZZ males), and still others exhibit temperature-dependent sex determination (TSD). The distribution of these mechanisms throughout the reptile phylogeny implies evolutionary lability in sex determination, and in some lineages there has been a number of transitions between GSD and TSD. Despite this diversity, GSD and TSD have traditionally been viewed as mutually-exclusive mechanisms of sex determination in reptiles, since there is little evidence for their co-occurrence. Considerable empirical and theoretical effort has been directed towards understanding the adaptive significance of TSD in reptiles. In comparison, there has been little focus on understanding how evolutionary transitions between GSD and TSD occur at a genetic and mechanistic level. I addressed this question by applying both empirical and theoretical approaches to investigate interaction of genotypic and temperature influences in the sex determination of two endemic species of Australian lizards.

The three-lined skink, *Bassiana duperreyi*, has XX/XY chromosomal sex determination, yet a previous investigation reported a significant male bias in the sex ratio of eggs incubated at low temperatures. To enable an explicit test for temperature-induced sex reversal in this species, a 185 bp Y chromosome marker was isolated by Amplified Fragment Length Polymorphism (AFLP) analysis. The marker was subsequently converted into a duplex PCR assay that co-amplified a 185 bp (or 92 bp) Y chromosome fragment and a 356 bp fragment of the single-copy nuclear gene C-*mos* (from both sexes) as a positive control. The accuracy of the PCR sex assay was tested on 78 individuals for which sex reversal was not expected. PCR genotype and sex phenotype were concordant for 96% of the animals. This is one of the very few sex tests developed for a reptile, and the first report of Y chromosome sequence from a reptile. The PCR assay was subsequently applied to genotype hatchlings from both cool (16±7.5°C) and warm (22±7.5°C) cyclical incubation temperature treatments, and identified sex reversal in 15% of genotypically female (XX) embryos (n=26) from the cool treatment, but no sex reversal in eggs from the warmer treatment (n=35). Thus, low incubation temperatures can over–ride genotypic sex determination in *B. duperreyi*, indicating that GSD and TSD co-occur in this species.

The Central bearded dragon, *Pogona vitticeps* (Agamidae), has ZZ/ZW chromosomal sex determination, and is a member of a lizard family in which GSD and TSD are both widespread, indicating evolutionary lability in sex determination. AFLP analysis was applied to isolate homologous Z and W chromosome-linked markers (71 bp and 72 bp, respectively) from this species. The AFLP sequences were subsequently extended into larger genomic fragments by a reiterated genome walking procedure, producing three non-overlapping contigs of 1.7 kb, 2.2 kb and 4.5 kb. The latter two fragments were verified as distinct, homologous Z/W chromosome fragments by PCR analyses. An amplified 3 kb fragment of the 4.5 kb contig was physically mapped to metaphase spreads, identifying the W microchromosome, and for the first time in this species, the Z microchromosome. PCR analyses indicated the presence of homologous sequences in other Australian agamid species, including both GSD and TSD species. The isolated sequences should therefore prove useful as a comparative genomic tool for investigating the genomic changes that have occurred in evolutionary transitions between sex-determining mechanisms in agamids, by enabling the identification of chromosomes in TSD species that are homologous to the sex chromosomes of *P. vitticeps.* The isolated sequences were further converted into a duplex DNA sex assay that co-amplified a 224 bp W chromosome fragment and a 963 bp positive control fragment in both sexes. This PCR assay diagnosed chromosomal sex in three *Pogona* species, but was not effective outside the genus.

Incubation treatment of *P. vitticeps* eggs revealed a strong and increasing female bias at high constant temperatures (34-36°C), but an unbiased sex ratio between 22-32°C. Hatchlings from three clutches split between 28°C and 34 or 36°C incubation treatments were genotyped with the W chromosome AFLP marker. At 28°C, the sex ratio was 1:1 but the high temperature treatments produced 2 males and 33 females. All but one of the 30 lizards (97%) incubated at 28°C had concordant sex phenotype and genotype, but only 18 of 35 animals (51%) from the high temperature treatment were concordant. All discordant animals were genotypic males (ZZ) that developed as females. Thus, temperature and genotypic influences can interact to determine sex in *P. vitticeps*.

These empirical findings for *B. duperreyi* and *P. vitticeps* were extended into a novel theory for the evolution of sex-determining mechanisms in reptiles, working within the framework that species with temperature-induced reversal of chromosomal sex

determination are a window to transitional stages of evolution between GSD and TSD. A model was derived from the observation that in both lizards, an extreme of incubation temperature causes sex reversal of the homogametic genotype. In this model, the strength of a genetic regulatory signal for sex determination must exceed a threshold for development of the homogametic sex to occur (male in *Pogona*, female in *Bassiana*). The strength of this signal is also temperature-sensitive, so diminishes at extremes of temperature. Simulation modelling demonstrated that increasing the relative magnitude of the threshold for sexual development can cause evolutionary transitions between GSD and TSD. Even more remarkably, decreasing the relative magnitude of the threshold value causes an evolutionary transition between female and male heterogametic GSD. Quantitative adjustment of a single model parameter (the threshold value) thus charts a continuous evolutionary pathway between the three principal mechanisms of sex determination in reptiles (XX/XY—ZZ/ZW—TSD), which were previously considered to be qualitatively distinct mechanisms.

The experimental demonstration of temperature-induced reversal of chromosomal sex determination in both *B. duperreyi* and *P. vitticeps* presents a challenge to the traditional view that reptilian sex determination is strictly dichotomous (GSD *or* TSD), and suggests instead that sex determination in reptiles consists of a continuum of systems of interaction between genotypic and temperature influences. Simulation modelling provided solid theoretical support for this proposition, demonstrating that transitions along this continuum are effected simply through shifts in the mean population value for the sex-determining threshold, without requiring substantial genotypic innovation. An important implication of this theory is that transitions between XX/XY and ZZ/ZW modes of GSD may retain the same sex chromosome pair, and the same primary sex-determining gene, in contrast to previous models for heterogametic transitions. A more immediate implication of these findings is that many reptile species believed to have strict TSD (in particular, lizards and crocodilians), may in fact have a sex-determining system of GSD-TSD interaction, where there is an equilibrium between GSD and TSD individuals within the population.

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Statement of Contribution

This thesis includes papers for which I am senior but not sole author. I took the lead in this research in that I designed the research, conceived and developed the novel ideas, undertook the fieldwork, analysed the data and wrote the manuscripts. I was, however, assisted by my co-authors. Manuscripts for which I am senior author have been included as central chapters, and papers for which I am a co-author are included as appendices. My contributions to the papers included as Appendices, were as follows:

Appendix 1 – I contributed as an equal partner in both the generation of the ideas and in the writing of the manuscript.

Appendix 2 – I was involved in the animal and laboratory work leading to the generation of the results secondary only to the senior author, and made a major contribution to the writing of the manuscript.

Appendix 3 – This paper depended on the development and application of the DNA sex test, both of which were my contribution, and I had input into writing the paper.

Alex Quinn

March 2008

Statement of Co-authorship

Core manuscripts:

Chapter 2: Quinn AE, Radder RS, Georges A, Sarre SD, Ezaz T, Shine R.
Isolation and development of a molecular sex marker for *Bassiana duperreyi*, a lizard with XX/XY sex chromosomes and temperature-induced sex reversal. *To be submitted.*

Chapter 3: Quinn AE, Ezaz T, Sarre SD, Georges A, Graves JAM.
From AFLP to Z: Isolation, conversion, and physical mapping of sex chromosome sequence in a dragon lizard. *To be submitted.*

Chapter 4: Quinn AE, Georges A, Sarre SD, Guarino F, Ezaz T, Graves JAM (2007) Temperature sex reversal implies sex gene dosage in a reptile. *Science* 316:411, plus supplementary online material.

Chapter 5: Quinn AE, Georges A, Sarre SD, Ezaz T, Graves JAM.
The evolutionary dynamics of sex as a threshold trait. *To be submitted.*

Appendix 3: Radder RS, Quinn AE, Georges A, Sarre SD, Shine R (2008)
Genetic evidence for co-occurrence of chromosomal and thermal sex-determining systems in a lizard. *Biology Letters* 4: 176-178

Other associated manuscripts:

Appendix 1: Sarre SD, Georges A, Quinn AE (2004)
The ends of a continuum: genetic and temperature-dependent sex determination in reptiles. *Bioessays* 26: 639-645.

Appendix 2: Ezaz T, Quinn AE, Miura I, Sarre SD, Georges A (2005)
The dragon lizard *Pogona vitticeps* has ZZ/ZW micro-sex chromosomes. *Chromosome Research* 13: 763-776.

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Chapter 1

Introduction: The evolution of sex determination in vertebrates, with special reference to reptiles.

Sex determination and sexual differentiation

Sex is a dichotomous phenotypic trait of fundamental importance to all dioecious organisms (those with two distinct sexes) and for the reproductive structures of hermaphroditic organisms. Consequently, it has been the subject of philosophical speculation and empirical investigation since the earliest days of biological enquiry (Mittwoch 2000). The developmental process leading to separate male and female phenotypes is often divided into the processes of *sex determination* and *sexual differentiation*. While the distinction between these terms is generally understood, there appears to be no consensus on precise definitions, and some authors use the terms interchangeably (for examples of various uses, see Mittwoch 1992). I will consider *sexual differentiation* to refer to the series of developmental events in an embryo leading to the structural formation (morphogenesis) of a functional testis or ovary from an undifferentiated gonadal ridge, that is, gonadal differentiation. *Sex determination* describes the process which directs and commits gonadal development to proceed down one of the two alternative pathways of differentiation, male or female. This is clearly a critical developmental decision for the individual (since in most cases the decision is irreversible), but the determination of sex also has wider implications at the level of the population, and for the species.

A variety of mechanisms of sex determination exist in vertebrates, and these can be divided into two broad phenomenological categories: *genotypic sex determination* (GSD) and *environmental sex determination* (ESD) (Figure 1.1) (Bull 1983). In GSD, *sex-determining genes* provide the initial regulatory signal directing sexual differentiation. Although a number of genes may be involved in initially directing and committing an embryo to male or female differentiation, and thus can be considered to be sex-determining in their function, this term typically refers to a *primary* sex-determining gene which operates as a master switch, triggering only one of the two alternative pathways of sexual differentiation. There is a consistent genotypic difference

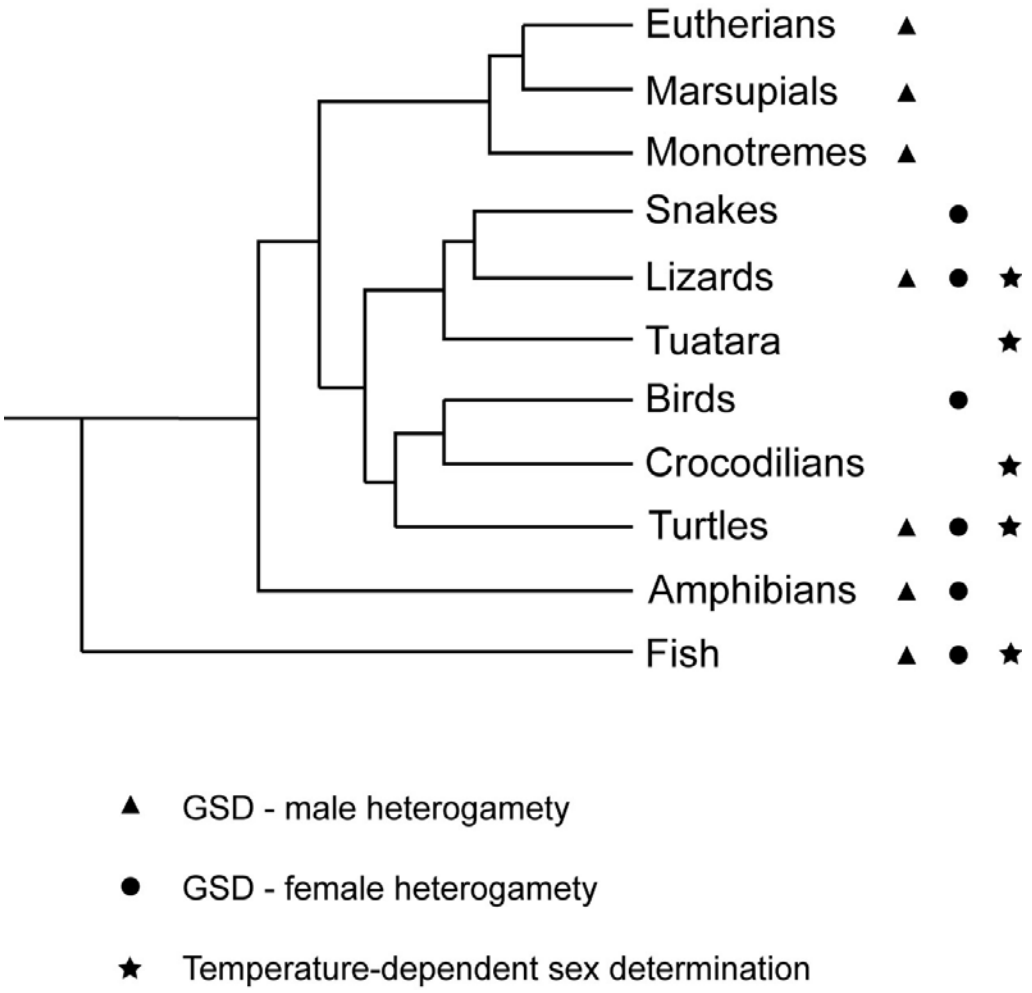

Figure 1.1 Sex-determining mechanisms mapped onto the vertebrate phylogeny. Figure modified from Ezaz et al. 2006a.

between males and females with respect to the primary sex-determining gene; it may be present in one sex only, or found in different copy number in the sexes. In a sense, sex under GSD is determined at fertilization by the segregation and inheritance of the sex-determining gene, even though the gene itself may not operate to influence the direction of gonadal differentiation until later in development.

In ESD, the sex of an individual embryo is determined by the value of an environmental variable encountered during a critical period of the embryo's development. Unlike GSD, there is no consistent genotypic difference between the sexes – embryos are sexually indifferent until the environmental factor determines sex, some time after fertilization. The environmental sex-determining signal is analogous to a sex-determining gene in that it triggers or enables only one of the sexual differentiation pathways, to the exclusion of the alternative pathway. In vertebrates, the most prevalent form of ESD is *temperature-dependent sex determination* (TSD), in which the incubation temperature experienced during embryonic development is the critical environmental factor which determines sex (Figure 1.1) (Bull 1980; 1983). TSD is found in many fish, and is widespread in reptiles. Other environmental influences on sex determination in vertebrates (reported only in fish) include pH (Romer & Beisenherz 1996), social conditions or relative juvenile size (Francis & Barlow 1993; Holmgren & Mosegaard 1996).

In all vertebrates, gonadal differentiation is directed by the co-ordinated action of a complex network of genes and their products. The sex-determining signal, whether genotypic or environmental, initiates a regulatory signal cascade through this genetic network, thereby acting as a switch to push the activity of the network in the direction of either testis or ovary development. Across the vertebrates, there is remarkable conservation in the structural organisation of fully developed testes and ovaries. Gonads arise in both sexes from bilateral genital ridges which are capable of developing as either ovaries or testes. Most knowledge of the molecular and cellular events involved in the formation of distinct testes or ovaries from the bipotential gonadal primordia has come from studies of mammals (particularly the mouse) and the chicken (reviewed by Koopman et al. 2001; Lovell-Badge et al. 2002; Place & Lance 2004; Smith & Sinclair 2004; Yao & Capel 2005). Gonadal differentiation has also been described for a number of reptile species, including several turtle species (with both GSD and TSD) and the

American alligator (TSD) (reviewed by Raynaud and Pieau 1985; Pieau et al. 1999; 2001; Yao & Capel 2005). Despite the diversity in the initial sex-determining mechanism amongst vertebrates, the striking similarities in gonadal structure and function across vertebrates led to an expectation that the downstream molecular pathways and cellular mechanisms involved in gonad morphogenesis would be highly conserved (Yao & Capel 2005). This is supported by a wealth of data indicating that many of the same genes are involved in the sexual differentiation of mammals, birds, fish and both TSD and GSD reptiles (Morrish & Sinclair 2002; Smith & Sinclair 2004; Devlin & Nagahama 2002; Place & Lance 2004). The similarities may not run quite as deep as once suspected, however, since it now appears there are marked differences between divergent vertebrate taxa in the roles and expression profiles (direction and timing of expression) of some of those genes (Morrish & Sinclair 2002; Crews 2003; Place & Lance 2004), as well as important differences in the morphogenetic events involved in gonad development (Yao & Capel 2005).

Genotypic sex determination

Sex chromosomes and sex-determining genes

GSD is apparently ubiquitous in mammals, birds, amphibians, and snakes, and sex is also genotypically-determined in most fish and lizards, as well as a few species of turtles (Figure 1.1) (Bull 1983; Solari 1994; Olmo & Signorino 2005). In most GSD species, the primary sex-determining gene is borne on a single homologous pair of chromosomes, which by definition, are termed the *sex chromosomes*. The simplest form of chromosomal sex determination is a so-called *two-factor* system (Bull 1983), in which two types of sex chromosome are present in the population. The two chromosome types differ in their gene content; the degree of differentiation may vary enormously (depending on the taxon), but the minimum difference, common to all sex chromosome systems, is that one type bears the sex-determining gene and the other lacks this locus. The sex that produces haploid gametes bearing one or the other of the different sex chromosomes is the *heterogametic sex*. Mammals have *male heterogamety*, in which the two distinct chromosome types are designated as the X and Y chromosomes; males have one copy of each sex chromosome (XY), whereas females have two copies of the X chromosome (XX). In the opposite system of *female heterogamety*, found in all birds and snakes, the two sex chromosome types are

designated the Z and W, and thus females are heterogametic (ZW) and males are homogametic (ZZ).

If the heterogametic sex chromosome (Y or W) bears the primary sex-determining gene, sex is determined by the presence (XY or ZW) or absence (XX or ZZ) of that gene. For instance, the primary sex-determining gene in therian mammals is the Y-borne *SRY* gene (Sinclair et al. 1990; Koopman et al. 1991; Foster et al. 1992); it is found only in those embryos destined to become males, since the expression of *SRY* initiates the regulatory cascade of gene interactions leading to the development of a testis from the undifferentiated gonad. In the absence of *SRY*, ovarian development ensues. Alternatively, sex may be determined by the differential dosage of a sex-determining gene borne on the homogametic sex chromosome; expression of the gene in the homogametic sex (XX or ZZ) is double that of the heterogametic sex (XY or ZW). The *DMRT1* gene, which appears to have a conserved role in testis differentiation in metazoans (Erdman & Burtis 1993; Raymond et al. 1998; 1999; 2000), is a candidate for such a role in avian sex determination (Smith & Sinclair 2004), as it is located on the Z, but not the W chromosome of birds (Nanda et al. 1999; 2000). These two genic mechanisms of sex determination are referred to as *dominance* and *dosage* mechanisms, respectively.

In principle, the primary sex-determining gene need not be a testis-determinant, as sex could also be determined by the double dosage of an ovary-determining gene on the X chromosome, or the presence of a dominant ovary-determining gene on the W chromosome. An X gene dosage mechanism has not yet been found in vertebrates, but the *HINTW* gene in carinate birds (Hori et al. 2000; O'Neill et al. 2000; Pace & Brenner 2003; Ceplitis & Ellegren 2004; Moriyama et al. 2006) and the recently discovered *DM-W* gene in the South African clawed frog *Xenopus laevis* (Yoshimoto et al. 2008) are candidates for the role of a dominant sex-determining W chromosome gene. Interestingly, the molecular mechanism of GSD in birds remains unknown, with the double-dosage of a Z-borne testis-determining gene or the presence of an ovary-determining gene on the W chromosome (Ellegren 2001; Smith 2007) as primary alternatives. There is some evidence that both elements play a part in determining avian sex (Arlt et al. 2004; Smith & Sinclair 2004; Nakagawa 2004).

Irrespective of the system of heterogamety, or the genic mechanism of sex determination, simple chromosomal sex determination systems are characterised by a stable 1:1 sex ratio in the population, resulting from the Mendelian segregation of the sex chromosome homologues.

Evolution and differentiation of sex chromosomes

In mammals and most birds, the sequence content of the two types of sex chromosome homologues (X and Y, or Z and W) are differentiated to the extent that the sex chromosomes are *heteromorphic*, meaning they can be visibly distinguished under a light microscope on the basis of their gross morphology by metaphase chromosome staining or banding. The mammalian Y and the avian W are considerably smaller than their homologues, and contain only a small fraction of the number of genes. This is proposed to be the result of an evolutionary process of *sex chromosome degeneration*, in which the genetic content of the heterogametic sex chromosome (Y or W) was progressively eroded (Charlesworth 1991; Rice 1987; Graves 2006). Sex chromosomes are believed to evolve from an ordinary autosomal pair, according to a well-accepted model (Muller 1914; Ohno 1967; Charlesworth 1991; Schartl 2004; Bachtrog 2006). In male heterogamety, for example, this evolutionary process is initiated when an autosomal gene acquires a (primary) male-determining function (e.g. by mutation or duplication), converting the autosome into a nascent Y chromosome. Sexually antagonistic genes (advantageous to males, detrimental to females) or genes simply advantageous to males, are linked or become linked (through recombination or transposition) to this new sex-determining locus. Chromosomal modifications that suppress meiotic recombination within the linked chromosomal region, such as inversions, prevent the advantageous gene combination (male-determining gene and male-advantage genes) being disrupted by X-Y recombination, resulting in positive selection for these modifications. Further accumulation of sexually-antagonistic or specialised male development genes in this region of the Y chromosome, followed by further suppression of recombination, progressively extends the non-recombining region of the Y chromosome. The fraction of the Y chromosome outside this region, still undergoing recombination with the X chromosome, is a *pseudoautosomal region*. With the exception of positively-selected genes with a vital male-specific function, the Y chromosome genes are vulnerable to mutation, loss of function and deletion in the absence of recombination, and so the gene content of the non-recombining region of the

Y chromosome progressively erodes (Rice 1992; 1994). Repetitive sequences also invade and spread throughout the non-recombining Y region (*heterochromatinization*). A parallel process occurs in W chromosome degeneration. If the critical sex-specific genes are transposed to autosomes or their function is usurped by autosomal genes, the Y or W chromosome may even decay to the point where it is lost entirely from a population, producing XX/XO or ZZ/ZO systems (Bull 1983; Solari 1994; Graves 2006).

Heteromorphic sex chromosomes are also present in a small proportion of lower (i.e. non-mammalian and non-avian) vertebrates. However, in the vast majority of fish, amphibians and reptiles with GSD, the sex chromosomes are weakly differentiated, or *homomorphic* (Solari 1994; Schartl 2004). Morphologically differentiated sex chromosomes have been identified in only 4% of the amphibians that have been karyotyped (Hillis & Green 1990; Schmid & Steinlein 2001; Eggert 2004), and in about 10% of karyotyped fish species (Devlin & Nagahama 2004; Ezaz et al. 2006b). Under the accepted model of sex chromosome evolution, homomorphic sex chromosomes represent an early stage of differentiation, which implies they evolved relatively recently. The frequent occurrence of 'young' sex chromosomes in the lower vertebrates may reflect evolutionary lability of sex-determining mechanisms. Transitions between TSD and GSD, between heterogametic systems, or simply switches in the sex chromosome pair, would all restart the process of sex chromosome differentiation anew. Alternatively, unidentified factors may modify the rate of progress of sex chromosome differentiation in these taxa (Graves 2006), and so in many cases, sex chromosomes may be evolutionarily 'older' than their homomorphic appearance suggests.

Sex chromosomes in reptiles

A variety of GSD mechanisms are found in reptiles. These include simple male and female heterogamety, but also more complicated two-factor systems with multiple segregating sex chromosomes, including $X_1X_1X_2X_2$ females:X_1X_2Y males, $Z_1Z_1Z_2Z_2$ males:Z_1Z_2W females and ZZ males:ZW_1W_2 females (Olmo 1986; Solari 1994; Olmo & Signorino 2005). Chromosomal sex determination is present in all snakes, most lizards, and a few turtles. Although reptilian orthologues for a number of mammalian sexual differentiation genes have been identified (Morrish & Sinclair 2002; Place & Lance 2004), no strong candidates for primary sex-determining genes on sex

chromosomes have been identified for reptiles, and there is no indication of the genic mechanism of sex determination (dominance or dosage) for any species (Modi & Crews 2005; Ezaz et al. 2006b). The sex chromosomes of snakes are the best characterised among reptiles. Snakes have a conserved ZZ/ZW system of sex determination with chromosome pair 4 conserved as the sex chromosome pair, but there is striking variation in the degree of W chromosome degeneration between families, ranging from near homomorphy to extreme differentiation of the Z and W pair (Beçak et al. 1964; Ohno 1967; Beçak & Beçak 1969; Singh 1972; Solari 1994; Matsubara et al. 2006). Similarity in size and morphology suggested the snake and avian Z chromosomes could be homologous (Ohno 1967; Ezaz et al. 2006a), but recent comparative gene mapping data indicates the snake and bird sex chromosomes evolved from different pairs of ancestral autosomes (Matsubara et al. 2006; Kawai et al. 2007).

Turtles and lizards show considerable variation in their sex chromosomes, in contrast to snakes, but almost nothing is known about the gene content of their sex chromosomes. The sex chromosome pair has been clearly identified for only seven of almost 160 karyotyped turtle species (Olmo & Signorino 2005), with male heterogamety in five species (Bull et al. 1974; Sites et al. 1979; Carr & Bickham 1981; Ezaz et al. 2006b; T.Ezaz pers. comm) and female heterogamety reported for two species (Sharma et al. 1975; Kawai et al. 2007). However, sex chromosomes have been identified in 172 species of 953 karyotyped lizard species (Olmo & Signorino 2005; Ezaz et al. 2006a). Simple male heterogamety (XX/XY) is clearly established as present in the families Gekkonidae, Teiidae, Gymnophthalmidae, Iguanidae and Scincidae, and species with male heterogamety involving multiple sex chromosomes are also found in the latter three families (Olmo 1986; Solari 1994; Olmo & Signorino 2005). Simple female heterogamety (ZZ/ZW) is clearly established as present in the Agamidae, Gekkonidae, Varanidae and Lacertidae, with female heterogamety involving multiple sex chromosomes also found in some lacertid species (Olmo 1986; Solari 1994; Olmo & Signorino 2005). Standard cytogenetic techniques (chromosome staining and banding) have failed to detect differentiated sex chromosomes in many species of lizards and turtles. Many of those species karyotyped (particularly turtles) are likely to have TSD, and thus heteromorphic sex chromosomes are expected to be absent (Bull 1980). However, in many species (including some for which TSD has been excluded by incubation experiments) the sex chromosomes are evidently homomorphic. For poorly differentiated sex chromosomes, the distinction between sex chromosome heteromorphy

and homomorphy is one of degree, and depends on the resolution of the cytogenetic technique. For instance, where chromosome staining has failed to identify heteromorphism in GSD reptiles on the basis of gross morphology of the chromosomes, chromosome banding has succeeded (e.g. Ezaz et al. 2005; Kawai et al. 2007), and where banding has been insufficient, higher resolution techniques such as comparative genomic hybridisation (CGH) have proven successful (e.g. Ezaz et al. 2006b). Unlike mammals, birds and snakes, the sex chromosomes are a microchromosome pair in at least some lizards and turtles (Makino & Asana 1950; Gorman 1973; Olmo et al. 1987; Ezaz et al. 2005; Ezaz et al. 2006b; Kawai et al. 2007), and consequently they may have been overlooked in some species in which the karyotype has been examined using only basic chromosome staining or banding techniques. Thus, many turtle and lizard species may have cryptic sex chromosomes which are potentially detectable using higher resolution cytogenetic approaches. In others, the degree of differentiation may be so subtle that even the highest-resolution cytogenetic techniques available will be unable to distinguish the sex chromosomes.

Multifactorial and polyfactorial sex determination

Two-factor systems (i.e. XX/XY and ZZ/ZW chromosomal sex determination) are not the only type of GSD found in vertebrates. In *multifactorial sex determination*, three or more *major* sex-determining factors are segregating in the population (Bull 1983; Kallman 1984; Wilkins 2002). In contrast to male and female heterogamety, a diagnostic feature of such systems is that some genetic crosses produce biased sex ratios (Scudo 1967), and there is usually no association with chromosomal heteromorphism (Bull 1983). Multifactorial systems may also be an intermediate stage in the evolution between different types of two-factor systems (Bull & Charnov 1977; Bull 1983). Vertebrates with three sex chromosomal variants are apparently rare, but examples include the platyfish *Xiphophorus maculatus* (Poeciliidae) (Kallman 1984; Volff & Schartl 2001) and several species of lemmings (Cricetidae) (Fredga et al. 1976; 1977; Gileva 1980; Gileva & Chebotar 1979). In *polyfactorial* (or *polygenic*) *sex determination*, sex is determined by the additive effect of many genes of individually minor effect (*minor* sex factors*)*, so no single gene has a major influence on sex determination. The genes combining to determine sex are distributed throughout the autosomes, so sex chromosomes *per se* are not associated with polyfactorial sex determination. The distinction between multifactorial and polyfactorial sex

determination is somewhat arbitrary (Bull 1983), and it can be difficult to distinguish the number of sex factors based on the heritability of variable sex ratios (Scudo 1967), since polyfactorial systems are expected to behave similarly to multifactorial systems with some environmental variance (Bulmer & Bull 1982; Bull 1983; Kallman 1984; Vandeputte et al. 2007). Polyfactorial sex determination is suspected for several fish species (Baroiller et al. 1999; Volff & Schartl 2001; Devlin & Nagahama 2002; Vandeputte et al. 2007), but the best characterised example is the swordtail fish *Xiphophorus helleri* (Poeciliidae) (Kosswig 1964; Price 1984; Volff & Schartl 2001). Evidence for this sex-determining mechanism is scarce for vertebrates, but this may be because the carefully controlled crossing and back-crossing genetic experiments required to detect many independently segregating sex factors are difficult to perform, or unfeasible for most species (aquaculture species being an obvious exception). Evolutionary instability of polyfactorial sex determination has also been proposed to explain its apparent rarity (Rice 1986).

Temperature-dependent sex determination in reptiles

Temperature-dependent sex determination is detected by incubating developing embryos at constant temperatures, under controlled laboratory conditions, and comparing the sex ratios of offspring incubated at different temperatures. Species with TSD show different directions or degrees of sex ratio bias at different temperatures. Sex ratio biases at different temperatures can also result from differential mortality of genotypically-determined sexes, but this can be ruled out in favour of TSD if all embryo mortality during incubation is assumed to belong to the under-represented sex and the bias towards the over-represented sex remains significant. Although sex determination is influenced by temperature in many species of fish (Conover & Kynard 1981; reviewed by Baroiller et al. 1999; Devlin & Nagahama 2002; Conover 2004), it is in reptiles that temperature-dependent sex determination is most widespread and probably best characterised. TSD was first identified in the agamid lizard *Agama agama* (Charnier 1966), and it is now known to be exhibited by all crocodilians (Ferguson & Joanen 1982; reviewed by Lang & Andrews 1994; Deeming 2004), both species of tuatara (*Sphenodon*) (Cree et al. 1995; reviewed by Nelson et al. 2004), most turtles (Pieau 1972; Bull & Vogt 1979; reviewed by Ewert & Nelson 1991; Ewert et al. 2004), and many lizards (reviewed by Viets et al. 1994; Harlow 2004). TSD is apparently

absent in snakes (Bull 1980; Viets et al. 1994), which appear to have a conserved system of female heterogametic GSD (Ohno 1967; Matsubara et al. 2006).

Three generic patterns of TSD are recognised in reptiles (Figure 1.2) (Bull 1980; Valenzuela & Lance 2004). In the *MF* pattern, seen in many turtles (Ewert et al. 2004), low temperatures produce predominantly or exclusively males, and high temperatures produce predominantly or exclusively females. For example, in the pig-nosed turtle (*Carettochelys insculpta*, Carettochelydidae), constant temperatures below 31.5°C produce 100% males, constant temperatures above 32.5°C produce 100% females, and both sexes are produced at 32.0°C (Young et al. 2004). The reverse *FM* pattern, in which low temperatures produce predominantly or exclusively females and high temperatures produce predominantly or exclusively males, is probably restricted to the tuatara *Sphenodon punctatus* and *S. guntheri* (Sphenodontidae) (Cree et al. 1995; Mitchell et al. 2006). In *S. punctatus*, males are produced above 22°C, and females are produced below this temperature (Mitchell et al. 2006). In the *FMF* pattern, both high and low temperatures produce predominantly or exclusively females, whereas an intermediate band of temperatures produces predominantly or exclusively males. This pattern occurs in all crocodilians (Deeming 2004), some turtles (Ewert et al. 2004), and probably all lizards that have TSD (Harlow 2004). An example is the Australian water dragon (*Physignathus lesueurii*, Agamidae); mostly (or exclusively) females are produced below ca. 25°C and above ca. 28°C, whereas the range of temperatures in between produces mostly males (Doody et al. 2006). It was once believed that some crocodilians and lizards exhibited the FM pattern of TSD, but this was because sex ratios were not examined across the full range of viable temperatures for some species; in many such cases, a true FMF pattern was revealed by further examination (Deeming 2004; Harlow 2004).

For any given embryo of a species with TSD, the dichotomous nature of sex determination dictates that the thermal reaction norm for sex is a step function, in which the alternative phenotypic outcomes of sexual differentiation are divided by a single 'switchpoint' (or threshold) temperature (Figure 1.3A). If threshold temperatures vary among embryos within a population (to greater or lesser degree) (Figure 1.3B), this variation could be the basis of the *transitional range* of temperatures for the population, conventionally defined as the range of temperatures in which both sexes are produced (Figure 1.3C). Under this hypothesis, the *pivotal temperature* is the mean

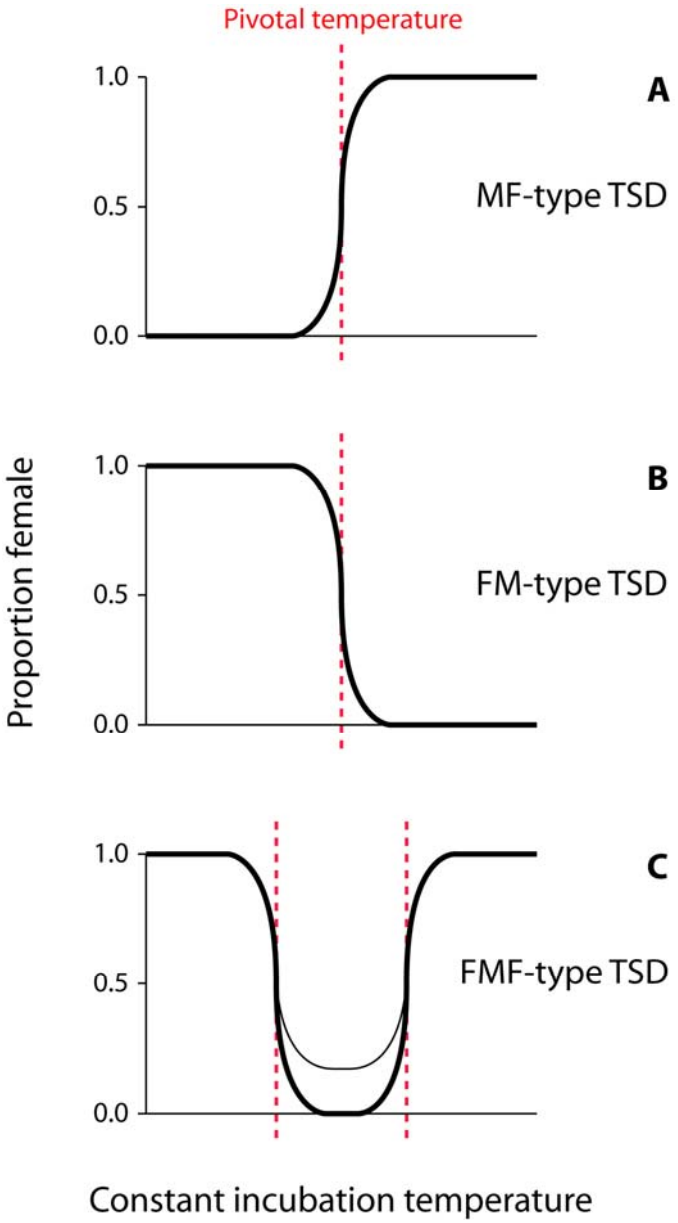

Figure 1.2 Reptiles with TSD show three generic patterns of sex ratio in relation to constant incubation temperature. Panel A: the *male-female* pattern (MF) of TSD is exhibited by some turtles. Panel B: the *female-male* (FM) TSD pattern is exhibited by the tuatara. Panel C: the *female-male-female* (FMF) TSD pattern is exhibited by all crocodilians, some turtles, and some lizards. In FMF-type TSD, intermediate temperatures produce a bias towards males, but for most species, no temperature produces 100% males. Dashed red lines indicated *pivotal temperatures*, at which the interpolated population sex ratio is 1:1. MF and FM patterns have one pivotal temperature, but there are two in the FMF pattern.

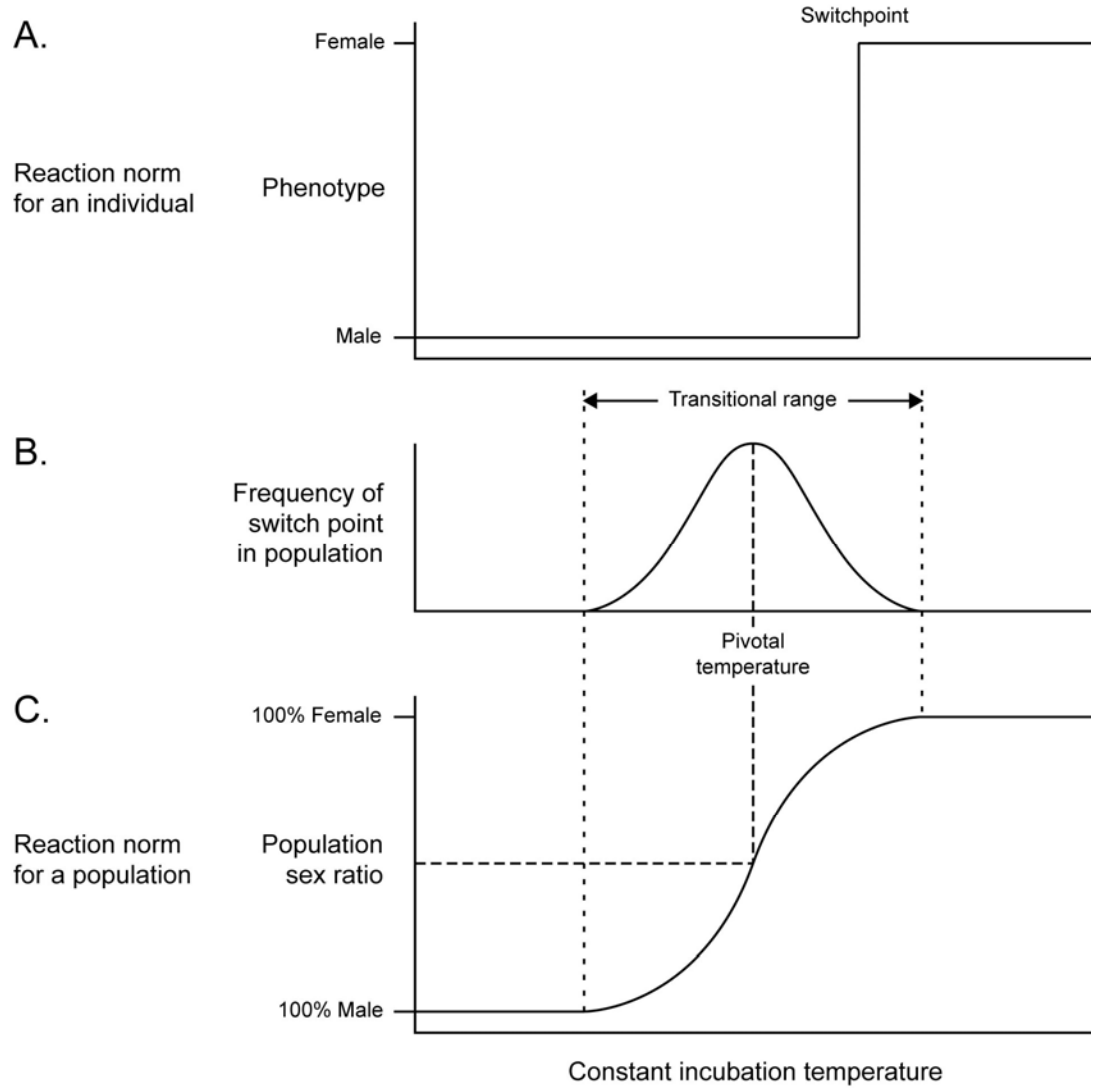

Figure 1.3 Hypothetical relationship between threshold temperatures and transitional range of temperature (shown for MF-type TSD). Panel A: For an individual, the reaction norm for sex in relation to incubation temperature is a step function, where there is a precise threshold temperature. Panel B: Variation between individuals in their threshold temperatures may be the basis of the *transitional range* of temperatures, defined as the range of temperatures producing a mixed sex ratio. Panel C: For the population as a whole, the reaction norm for sex in relation to temperature is a curved function. Under this hypothesis, when threshold temperatures are normally distributed, the pivotal temperature is equivalent to the mean threshold temperature (see also Hazel et al. 1990).

population value for the threshold temperature, conventionally defined as the (interpolated) value producing a 1:1 sex ratio. There is a single pivotal temperature for species with MF or FM patterns of TSD, but two for FMF species (Figure 1.2). Reptile species often vary markedly in pivotal temperature, the width and slope of the transitional range, and indeed, even the range of temperatures that produce viable offspring. Constant temperatures applied in laboratory incubation experiments do not emulate the thermal conditions in natural nests of reptiles, where temperatures fluctuate on a diel and seasonal basis, but temperature has been demonstrated to influence sex ratios in field nests for a number of reptile species (e.g. Bull & Vogt 1979; Morreale et al. 1982; Vogt & Bull 1984; Georges 1992). In many species shown to have FMF-type TSD by laboratory experiments, only one of the two pivotal temperatures may actually be realised under natural incubation conditions. Moreover, sex ratio and development time can vary markedly between a constant temperature regime and a fluctuating temperature regime with an equivalent nominal mean temperature (Georges 1989; Georges et al. 1994).

Temperature shift experiments, in which eggs are strategically shifted between male- and female-producing temperatures (or vice versa) at specific time points during incubation, have identified that temperature influences reptilian sex determination only during a specific window of embryonic development. This *thermosensitive period* encompasses approximately the middle trimester of embryogenesis, which corresponds with the initial stages of gonad differentiation (Yntema 1979; Pieau 1996). The precise mechanism by which temperature acts as a sex-determining signal is unknown. Sexual differentiation in birds, reptiles, amphibians and fish is ultimately dependent on whether the hormonal environment of the developing embryo is androgenic (testis-promoting) or estrogenic (ovary-promoting) (Elbrecht & Smith 1992; Crews et al. 1989; Wallace et al. 1999; Devlin & Nagahama 2002), in contrast to the embryos of placental mammals in which sexual differentiation is insensitive to levels of steroid hormones in the uterus (Greene et al. 1940; Couse et al. 1999; Britt et al. 2000; Cupp et al. 2003). In these egg-laying vertebrates, the steroidogenic enzyme aromatase converts the androgens testosterone and androstenedione into the estrogens estrone and estradiol-17β. In TSD species, application of estrogens to eggs can produce females at masculinizing incubation temperatures, and application of aromatase inhibitors to eggs can produce males at feminizing temperatures (Crews et al. 1991; Crews & Bergeron 1994). Aromatase inhibitors are increasingly more potent as the transitional range of

temperatures is approached, indicating that the sex-determining effects of steroid hormones and incubation temperature are physiologically equivalent (Crews et al. 1991; Crews 1996). Thus, it is thought that temperature exerts its effect by acting on the genes or proteins in the molecular pathways involved in the regulation of *aromatase* expression, or on the production of steroid hormone receptors (Crews 1996; Pieau et al. 2001; Sarre et al. 2004). In this way, incubation temperature ultimately determines sex by altering the proximate influence of the hormonal environment of the differentiating gonads, in either an androgenic or estrogenic direction.

At incubation temperatures close to the pivotal temperature(s), where some embryos develop as males and some as females, intersex phenotypes are rare (Crews et al. 1991). Sex under TSD is evidently a *threshold trait*; a continuous variable (temperature) determines the state of a dichotomous outcome (sexual phenotype), which necessitates the involvement of at least one threshold, above which one outcome occurs and below which the alternative occurs (Falconer 1989; Roff 1996). The decision to be male or female hangs in the balance of competing influences on the regulatory signal cascade involved in sexual differentiation. At some point in gonadal differentiation, the regulatory signal is either above or below a critical threshold, and the gonad becomes committed to its fate as either testis or ovary. Beyond this point-of-no-return, positive feedback mechanisms in the strongly canalised sexual differentiation pathways must act to reinforce the sex-determining decision, and the entire organism is recruited to male or female development (Yao & Capel 2005).

Intermediate modes of sex determination

Co-occurrence of GSD and TSD in amphibians and fish

In some anamniote vertebrates with GSD, sex can also be influenced by temperature during embryonic development, in contrast to birds and mammals which are considered to have strictly genotypically-determined sex. In the simplest pattern of interaction between genotypic and temperature influences on sex determination, a 1:1 sex ratio is produced across a range of incubation temperatures (consistent with the Mendelian segregation of major sex factors), but at one (or both) extremes of the viable temperature range, the sex ratio is skewed. If differential embryonic mortality of the sexes can be excluded as an explanation, it can be inferred that the genotypic signal for

sexual differentiation has been over-ridden by the influence of extreme temperature in some embryos, such that they develop with a phenotypic sex (i.e. their functional, gonadal sex) that is discordant to their genotypic sex. *Temperature-induced sex reversal* has been reported in a number of fishes (Devlin & Nagahama 2002; Conover 2004) and amphibians (Chardard et al. 2004; Eggert 2004).

GSD appears to be ubiquitous in amphibians (Hayes 1998; Schmid & Steinlein 2001). Only 12 of over 4800 species have been examined for temperature effects on sex, but in all these species either the homogametic sex or the heterogametic sex (or both) is susceptible to temperature reversal at extreme temperatures (Chardard et al. 2004; Eggert 2004). For instance, high incubation temperatures induce sex reversed (ZW) males in the salamander *Pleurodeles waltl*, but induce sex reversed (ZZ) females in the congeneric *P. poireti*, and in both cases the sex reversed animals have been demonstrated to be fertile (Dournon & Houillon 1984; 1985; Dournon et al. 1984; 1990). The extreme temperatures applied in laboratory experiments to induce sex reversal are rarely encountered by amphibians in their natural habitat (Chardard et al. 2004). TSD can be considered to occur when undifferentiated offspring have the potential to develop as male or female, depending on environmental conditions naturally encountered during development (Conover 2004), thus the temperature-induced sex reversal exhibited by amphibians is usually not considered to represent TSD. Although it is often difficult in practice to assess the range of incubation temperatures naturally experienced by a species, comparison of experimental sex-reversing temperatures with temperatures encountered in the wild suggests that natural temperature sex reversal could occur in the salamander *Triturus cristatus* (Wallace & Wallace 2000). Considering how few species have been examined for temperature effects on sex, it is too early to conclude if strict TSD, or systems of GSD-TSD interaction, occur in amphibians.

In some atherinid fishes and in cichlid fishes of the genus *Apistogramma*, there is a gradual and linear shift in sex ratio from mostly females at low temperatures to mostly males at high temperatures (Conover & Kynard 1981; Römer & Beisenherz 1996; Strüssmann et al. 1996a;b; 1997; Conover 2004). In the *Apistogramma* genus, and in at least one atherinid species, the influence of genotype on sex ratio appears to be very weak or non-existent, and therefore the primary sex-determining mechanism seems to be TSD (Römer & Beisenherz 1996; Strüssmann et al. 1996a; 1997). In the atherinid

Menidia menidia, however, there is clearly a strong genotypic component to sex determination as well, and this is also the only fish species for which TSD has been demonstrated to occur in the wild (Conover & Kynard 1981; Conover & Heins 1987a;b). *Menidia* exhibits a latitudinal gradient in its sensitivity to temperature, with sex ratios weakly influenced by temperature in a northern population, but strongly influenced in a southern population. Sex determination in this species is thought to be controlled by an interaction between major sex factors (multifactorial sex determination), minor sex factors (polygenic sex determination), and temperature (TSD), with the relative importance of each component changing with latitude (Lagomarsino & Conover 1993).

Co-occurrence of GSD and TSD in reptiles

The co-occurrence of GSD and TSD in reptiles is more contentious. Certainly, reptiles with TSD exhibit genetic variation in thermosensitivity. A number of studies, encompassing a range of reptile species, have reported interclutch and interpopulational variation in pivotal temperatures or sex ratios at constant incubation temperatures (Bull et al. 1982; Janzen 1992; Ewert et al. 1994; Rhen and Lang 1998; Dodd et al. 2006), in some cases leading to high heritability estimates for a presumed underlying polygenic component to sex determination (e.g. Bull et al. 1982; Lang and Andrews 1994; Rhen and Lang 1998). However, excluding the work reported in this thesis, there has been no unequivocal demonstration that incubation temperature can influence sex in reptiles with chromosomal sex determination, as has been done for fish and amphibians.

Indeed, GSD and TSD are often presented as mutually-exclusive sex-determining mechanisms in reptiles (e.g. Bull 1980; Valenzuela et al. 2003). This view stems from theoretical considerations that imply the two mechanisms are incompatible and empirical data that suggest that co-occurrence of GSD and TSD is either extremely rare or non-existent in reptiles (Bull 1980; Olmo 1986; Janzen & Paukstis 1991; Solari 1994; Valenzuela et al. 2003). If temperature can reverse genotypic sex in species with male heterogamety, XY females and XX males can arise. Assuming XY females are fertile, their mating with XY males produces ¼ YY offspring, which are expected to be inviable if the sex chromosomes have differentiated to the extent that X chromosome genes vital for development have been deleted from the Y chromosome (or lost their function). Sex reversed XY females would then have a lower reproductive fitness than

XX females, so there would be selection against a thermal influence in sex determination. In addition, the Y chromosome may have accumulated genes critical for male development or fitness, such that sex reversed XX males are less fit than XY males. These theoretical predictions, which apply beyond reptiles, are supported by empirical data indicating that TSD is not found in reptiles with heteromorphic sex chromosomes (Bull 1980; 1983; Olmo 1986; Janzen & Paukstis 1991; Solari 1994).

There is a slippery slope of inference from the prediction that heteromorphic sex chromosomes and TSD are incompatible, to the notion that sex chromosomes are incompatible with TSD, and from there to the idea that GSD and TSD are incompatible. Some GSD mechanisms (e.g. polyfactorial sex determination) do not involve sex chromosomes, and more often than not in vertebrates, sex chromosomes are homomorphic. Questions regarding semantics and definitions arise. First, the distinction between polyfactorial GSD and major sex factor GSD is not clear-cut, and there is ample evidence for polygenic influences on sex determination in many fish and reptiles with TSD. At what point can minor sex factors be considered as major sex factors, and for that matter, what is the maximum number of major sex factors that still constitutes chromosomal sex determination? Second, in two-factor systems of chromosomal sex determination (heterogametic GSD), the degree of genetic differentiation between the X and Y (or Z and W) chromosomes can range continuously, from heterozygosity at a single sex-determining locus to complete loss of the Y (or W) chromosome. At what point does a sex chromosome become a heteromorphic sex chromosome? For species with weakly differentiated sex chromosomes, any reproductive fitness disadvantage for sex reversed genotypes may be non-existent or inconsequential. In theory, a continuum of neutral equilibrium states, involving varying proportions of XX, XY and YY sex reversal, connects strict GSD (no temperature influence) with strict TSD (no genotypic influence), provided there is equivalent fitness for all possible genotypes within a sex (Bull 1981; 1983; 1985). In the first major review of sex-determining mechanisms in reptiles, Bull (1980) outlined that TSD and GSD can coexist in principle, and that the coexistence is unlikely only when the sex chromosomes are differentiated to the point of heteromorphy. In spite of this, the view that GSD is incompatible with TSD in reptiles has become firmly established in the past three decades (e.g. Janzen & Paukstis 1991; Solari 1994; Valenzuela et al. 2003; Janzen & Krenz 2004; Valenzuela 2008).

An alternative view (and the one to which I adhere) is that sex-determining mechanisms in reptiles are not dichotomous, and that instead, a continuum of systems may exist in reptiles, ranging from strict GSD with heteromorphic sex chromosomes, through systems of varying interaction between genotypic and temperature influences, to strict TSD (Sarre et al. 2004). Some empirical evidence exists to support the proposition of GSD and TSD co-occurrence in reptiles, but it is equivocal. Early evidence came from studies examining expression of the Y-chromosome linked histocompatibility (H-Y) antigen. H-Y antigen was originally identified as a male-specific antigen in mammals (Eichwald et al. 1958; Wachtel & Koo 1981) but it shows sexually dimorphic expression in many vertebrates, and even in species lacking differentiated sex chromosomes, positive H-Y antigen expression is considered to be a marker for the heterogametic sex (Engel & Schmid 1981; Engel et al. 1981). In a series of laboratory experiments involving the European pond turtle (*Emys orbicularis*), a species with MF-type TSD, serologically defined H-Y antigen expression was tested in both blood and gonadal tissue for animals incubated at masculinizing or feminizing temperatures, or within the transitional range of pivotal temperatures (Zaborski et al. 1982; Zaborski 1985; Zaborski et al. 1988). At all temperatures, H-Y antigen expression was positive in ovarian tissue, but negative in testicular tissue. H-Y antigen expression in the blood was highly correlated with gonadal expression in the transitional range of temperatures, but at masculinizing and feminizing temperatures, it was positive in approximately half the males and half the females, and therefore did not correlate with gonadal expression. This was interpreted as evidence for an underlying genotypic system of female heterogamety in a species with TSD, with H-Y antigen expression in non-gonadal tissue serving as a marker for genotypic sex. It was proposed that genotypic sex was manifested at temperatures close to the pivotal temperature (1:1 sex ratio), but feminizing and masculinizing temperatures caused sex reversal of genotypically male and genotypically female embryos, respectively.

In another study, H-Y antigen expression was tested in various non-gonadal tissue types for 14 turtle species, including *E. orbicularis* (Engel et al. 1981). Female heterogamety was indicated in 13 species (positive expression in females) and male heterogamety was indicated for one species (positive expression in males). Although incubation studies have indicated that eight of these species have TSD (Pieau 1972; Ling 1985; Ewert & Nelson 1991; Eendebak 1995; Ewert et al. 2004), non-gonadal H-Y antigen expression gave no indication of any discordance with sex phenotype, as found for *E. orbicularis*

by Zaborkski et al. (1982; 1988). This may reflect that sex reversal is rare in nature in species for which TSD has been assigned on the basis of laboratory experiments (Girondot et al. 1994). More concerning, however, is that sex chromosomes have been identified by karyotyping in only one of the 14 species, *Siebenrockiella crassicolis* (Carr & Bickham 1981), and indicated male heterogamety, contradicting the female heterogamety suggested by H-Y antigen typing. Further investigation is clearly required before H-Y antigen can be considered as a reliable marker for genotypic sex in either GSD or TSD reptiles.

Another indication of GSD and TSD co-occurrence in reptiles was provided by a study which found that the multilocus minisatellite DNA probe Bkm, a sequence originally isolated from the W chromosome of a snake (Singh et al. 1984), hybridised in a male-specific pattern to genomic DNA of two marine turtle species, *Chelonia mydas* and *Lepidochelys kempi* (Demas et al. 1990). Like all marine turtles, these species have demonstrated MF-type TSD (Miller & Limpus 1981; Morreale et al. 1982; Shaver et al. 1988; Wibbels et al. 1989). The sample sizes were small, however, and similar experiments on other TSD species, including the alligator, freshwater turtles (Demas et al. 1990) and the tuatara *Sphenodon punctatus* (A.E. Quinn, unpublished data), did not find sex-specific hybridization of the Bkm sequence. Again, further work is necessary to verify the finding in marine turtles.

More recently, Shine et al. (2002) reported a thermally-induced bias in sex ratio in a GSD lizard. The Australian three-lined skink (*Bassiana duperreyi*) has male heterogamety with morphologically differentiated sex chromosomes (Donnellan 1985). Fluctuating incubation temperatures, designed to mimic the conditions experienced in the coolest nests at the highest elevations of its range, produced over 70% males, whereas warmer incubation temperatures that emulated lower elevation nests produced sex ratios that did not depart significantly from 1:1, consistent with the Mendelian segregation of sex chromosomes (Figure 1.4). Even assuming all embryonic mortalities were XX embryos, the male bias at low temperatures remained significant, and so it was inferred that some of the males from cool incubation temperatures were sex reversed XX males. This study is the strongest evidence yet that chromosomal sex determination and TSD can interact to determine sex in a reptile, but it relied on indirect inference from sex ratios, rather than an explicit demonstration that XX males were in fact induced by incubation temperature.

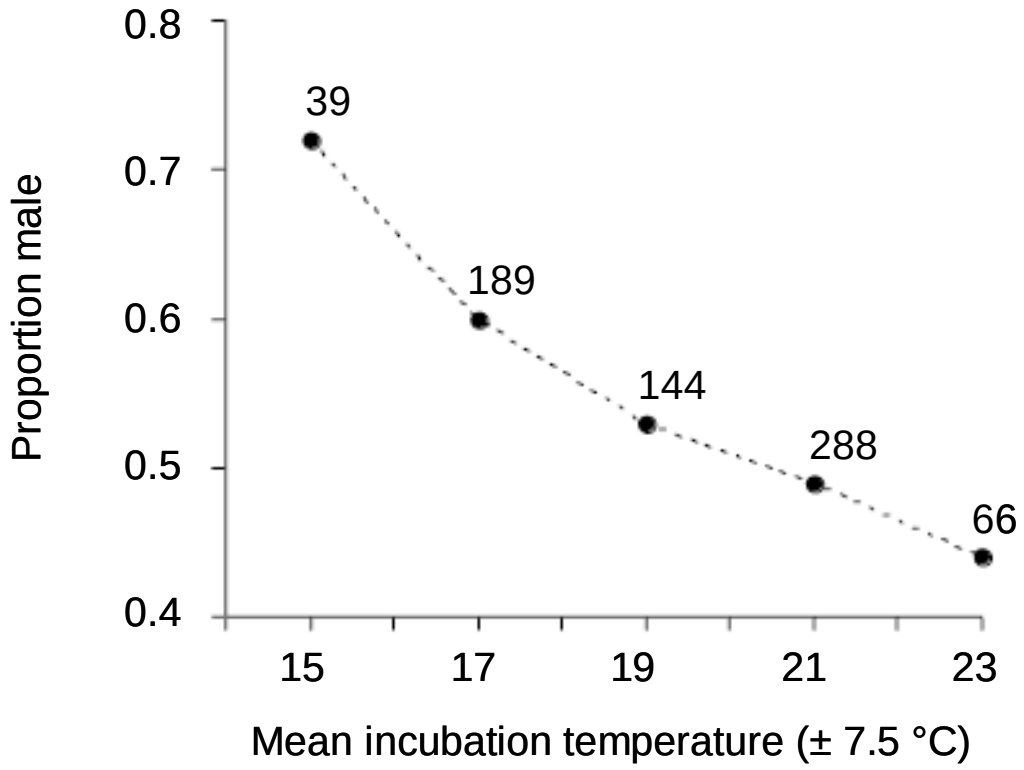

Figure 1.4 Sex ratio in relation to incubation temperature for *Bassiana duperreyi* (Scincidae). Numbers above data points indicate sample size at each temperature. Temperatures are shown as nominal means for a diel fluctuating temperature regime, with 15°C range of fluctuation. Figure modified from Shine et al. 2002.

Sex reversal and molecular sex identification in non-model organisms

In certain cases, it may be possible to demonstrate sex reversal of chromosomal sex determination using classical breeding experiments, by analyzing the sex ratios of progeny from specific crosses. This approach can be unfeasible for non-model organisms for a variety of reasons, e.g. the species may be unamenable to captive breeding, sexual maturity is attained slowly, or the number of progeny is small. Ideally, sex reversal is demonstrated explicitly by identifying that the chromosomal sex of individuals conflicts with their sex phenotype. This may be achieved by cytogenetic approaches if sex chromosomes are sufficiently differentiated to be distinguishable at mitotic metaphase by standard staining or banding, or by higher resolution techniques such as comparative genomic hybridization. For instance, C-banding identified sex reversed XX males in the newts *Triturus carnifex* (Wallace et al. 1999) and sex reversed XY females in the congeneric *T. cristatus* (Wallace & Wallace 2000). Other cytogenetic 'markers' may also identify sex reversed individuals; lampbrush W chromosome loops, observable in the meiotic karyotype of oocytes in the salamander *Pleurodeles poireti*, were used to identify ZZ females sex reversed by high temperatures (Dournon et al. 1984). Unfortunately, cytogenetic approaches are often technically challenging, or simply unfeasible, since cytogenetic markers may not be available, the degree of sex chromosome differentiation may be too subtle, or live cell cultures for metaphase chromosome preparation cannot be established from the organism of interest. Moreover, if it is necessary to screen a large number of individuals to detect rare incidences of sex reversal, cytogenetic approaches are impractical because they are labour-intensive.

Molecular markers (protein or DNA) provide an alternative to cytogenetic identification of chromosomal sex. For instance, the peptidase 1 enzyme in the salamander *Pleurodeles waltl* is encoded by two allelic variants, located on the Z and W chromosomes. A difference in the electrophoretic mobility of the two allozymes was used to identify ZZ, ZW, and WW genotypes (Dournon et al. 1988). These are the only genes encoding enzymes known to localise to urodelan sex chromosomes (Chardard et al. 2004). Several genes encoding enzymes have been mapped to the sex chromosomes of frogs in the genus *Rana* (Sumida & Nishioka 2000; Schmid & Steinlein 2001). For most non-model organisms, however, identified enzyme markers for the sex chromosomes are unlikely to be available, so identifying appropriate DNA sex markers is a more realistic alternative.

Griffiths (2000) suggests three strategies for identifying DNA sex markers: (i) search for and test known sex markers from closely-related species, (ii) test known repetitive sequences (microsatellites and minisatellites) for sex-specific hybridisation, and (iii) screen for novel sex markers in the species of interest. The first strategy may be practical for fish taxa, in which a number of sex markers have been identified (Devlin & Nagahama 2002), but very few sex markers have been identified for amphibians and reptiles, negating this as an approach in these groups. The second strategy is likely to be successful only when the sex chromosomes are well-differentiated, and it is laborious in comparison with PCR-based sex identification. For organisms such as reptiles, the third strategy of developing DNA sex markers *de novo* will probably be necessary.

Developing novel DNA sex markers requires the isolation of sequences from the heterogametic (Y/W) chromosome, using molecular genetic approaches which screen the genome for sequences found only in the heterogametic sex. The size of the sex-specific fraction of the genome largely determines the difficulty of finding markers. In principle, but perhaps not always in practice (e.g. Li et al. 2002), molecular genetic approaches can identify sex-specific sequences even when the degree of sex chromosome differentiation is so subtle that the sex chromosomes cannot be distinguished by high resolution cytogenetic approaches. The most commonly applied molecular techniques include the PCR-based DNA fingerprinting techniques of Randomly Amplified Polymorphic DNA (RAPD) analysis (Welsh & McClelland 1990; Williams et al. 1990) and Amplified Fragment Length Polymorphism (AFLP) analysis (Vos et al. 1995), as well as subtractive hybridisation techniques such as Representational Difference Analysis (RDA) (Lisitsyn et al. 1993). AFLP appears to have overtaken RAPD as the method of choice in recent years, and it has been applied successfully to detect sex markers in a diverse range of organisms, including plants (Reamon-Büttner et al. 1998; Lebel-Hardenack et al. 2002; Peil et al. 2003), birds (Griffiths & Orr 1999) fishes (e.g. Griffiths et al. 2000; Ezaz et al. 2004; Felip et al. 2005) and amphibians (A.E. Quinn, unpublished data). Sex markers identified by RAPD, AFLP, or RDA can be subsequently converted into more reliable single-locus PCR tests for the Y or W chromosome sequence, which co-amplify positive control products of higher molecular weight (in both sexes) to eliminate the possibility of incorrect diagnosis of sex due to amplification failure (Griffiths 2000). In addition,

single-locus PCR sex assays are relatively inexpensive, and amenable to high-throughput genotyping.

Sex chromosome sequences and sex markers in reptiles

Few sex chromosome sequences have been identified for reptiles, but the notable exceptions are in snakes. Repetitive satellite sequences, such as Bkm (Banded krait minor, named for the snake from which it was first isolated), are interspersed throughout the chromosomes of snakes in high copy number (Singh et al. 1976; 1980), but are concentrated in particularly high density on the W chromosome, especially in those taxa where the sex chromosome pair is highly differentiated (Solari 1994). Recently, 11 functional genes, with autosomal homologues in chickens and humans, were mapped to the Z chromosome in three snake species: all 11 genes also mapped to the W chromosome in *Python molurus* (Pythonidae); three mapped to the W chromosome in *Elaphe quadrivirgata* (Colubridae); and none mapped to the W chromosome in *Trimeresurus flavoviridis* (Viperidae) (Matsubara et al. 2006). There have been no reports about the gene content of the sex chromosomes in turtles and lizards. This situation is likely to change rapidly as sequences start to emerge from the genome project for the green anole lizard, *Anolis carolinensis* (Iguanidae), which has male heterogamety (http://www.broad.mit.edu/models/anole).

Only two DNA sex markers have been reported for reptiles. Sequence from the W chromosome of the largest extant species of lizard, the Komodo dragon (*Varanus komodoensis*, Varanidae), was identified by RAPD analysis and converted into a PCR sex test (Halverson & Spelman 2002). This PCR test also identifies sex in the Australian varanid *V. rosenbergi* (W. Smith, pers. comm), but does not identify sex in agamid lizards (A.E. Quinn, unpublished data). It is possible this test could diagnose sex in other varanid species because the family has a conserved sex-determining mechanism of female heterogamety (Olmo & Signorino 2005). In the second example, a polymorphic microsatellite locus on the X chromosome, with no corresponding Y chromosome locus, was identified in the Australian shingleback lizard (*Tiliqua rugosa*, Scincidae) (Cooper et al. 1997), enabling the sex of heterozygotes to be identified as female in this species. The microsatellite locus was also found to be polymorphic, and therefore identify heterozygotes as females, in another skink (*Egernia cunninghami*) (Stow et al. 2001).

The evolutionary lability of sex chromosomes and sex-determining mechanisms in reptiles (Sarre et al. 2004; Modi & Crews 2005) means that DNA sex markers developed for reptiles are unlikely to have broad taxonomic applicability. Sequences within the sex-specific fraction of the heterogametic chromosome, the target of searches for sex markers, are presumably lost, or at least lose their association with genotypic sex, each time that a sex chromosome pair is replaced by another. This could also occur whenever a chromosomal mechanism of sex determination is replaced by TSD. For most turtles and lizards, it seems likely that DNA sex markers will need to be developed *de novo* for species of interest. Snakes have a ubiquitous system of female heterogamety, and it appears likely the Z and W chromosome pair is homologous in all species (Matsubara et al. 2006), albeit with considerable variation in the degree of W chromosome degeneration between families (Ohno 1967). It may be possible to isolate conserved W chromosome markers with universal applicability in snakes. By the same token, conservation of the sex chromosome pair, and the apparent absence of TSD, renders snakes less interesting from the point of view of evolutionary transitions between reptilian sex-determining mechanisms. TSD is ubiquitous in crocodilians (Deeming 2004), so sex markers are not expected to exist in this reptile group.

Diagnostic sex tests and definitions for sex markers

Various terms have been applied to describe DNA sex markers, depending on the chromosomal location of the sequence, and there is inconsistent use of these terms in the literature. To avoid confusion, I will use the following definitions throughout this thesis (using male heterogamety as an example). Markers that are *sex-specific* are located in the non-recombining region (NRR) of the Y chromosome, and they identify male chromosomal sex (presence of the marker) or female chromosomal sex (absence of the marker) correctly in 100% of cases. These are also referred to as *Y-specific* or *male-specific* markers. Markers in the pseudoautosomal region (PAR) of the Y chromosome, but in close proximity to the NRR of the Y chromosome and therefore exchanged only occasionally to the X chromosome PAR by recombination, are *Y-linked* or *male-linked*, because they identify chromosomal sex correctly in most cases. Markers in the X chromosome PAR which rarely cross-over to the Y chromosome PAR are *X-linked* and markers on that part of the X chromosome which does not recombine with the Y chromosome are *X-specific*. These X chromosome markers cannot be used to diagnose chromosomal sex on the basis of simple presence or absence PCR tests. If there is

allelic polymorphism in X chromosome markers, chromosomal sex is identifiable for heterozygotes: if the marker is X-specific, heterozygotes are always female, but if the marker is X-linked, most heterozygotes are female. The chromosomal sex of homozygotes cannot be diagnosed. The terms *female-specific* and *female-linked* do not apply for male heterogamety. In the context of diagnostic sex tests, *sex-linked* markers will refer to both Y- and X-linked markers, in contrast to the classical genetics definition, where sex-linkage is often applied only to sequences from the X chromosome. The term *sex markers* itself can be an umbrella term for all these definitions, but it is usually intended to mean markers that can be used to diagnose chromosomal sex, even if not with total accuracy.

Evolutionary transitions between sex-determining mechanisms

The diversity and distribution of sex-determining mechanisms exhibited by the lower vertebrates indicate remarkable evolutionary lability of sex determination in these groups, in striking contrast to the evolutionary conservatism of heterogametic sex determination systems in mammals (XX/XY) and birds (ZZ/ZW).

Evolutionary lability of sex-determining mechanisms in amphibians and fish

TSD has not been reported as a primary sex-determining mechanism in amphibians, but both male and female heterogametic GSD are widespread, almost always with homomorphic sex chromosomes (Schmid & Steinlein 2001; Chardard et al. 2004; Eggert 2004). ZZ/ZW is thought to be the ancestral mechanism in Amphibia, and it is more frequent than XX/XY (Hillis & Green 1990). Phylogenetic analysis suggests a bias in the direction of these independent heterogametic transitions, in the direction ZZ/ZW to XX/XY (Hillis & Green 1990). Amphibians have also provided the only vertebrate example of a species with has evolved separate ZZ/ZW and XX/XY populations, the Japanese frog *Rana rugosa* (Nishioka et al. 1993; Ogata et al. 2003; Miura et al. 1998). Homology of the XY and ZW pairs indicates that they evolved from the same chromosome pair, which is proposed to have occurred through hybridisation of two ancestral types with male heterogamety involving homomorphic sex chromosomes; it is hypothesised that one pair retained a primary male-determining locus, but the other acquired a primary female-determining locus (reviewed by Miura 2007).

Fish show even greater evolutionary lability, and include species with male or female heterogamety, TSD, GSD-TSD interaction, hermaphroditism and parthenogenesis. Character mapping onto molecular phylogenies of teleosts reveals a highly patchy distribution of the different sex-determining mechanisms, and indicates there have been frequent transitions between mechanisms within several evolutionary lineages (Mank et al. 2006). A few chromosomal sex-determining systems are ancient and highly conserved, but in many lineages sex chromosomes appear to have evolved only recently, because there is minimal differentiation of the sex chromosomes, or because sex chromosome sequences in one species are autosomal in sister species (Ezaz et al. 2006a). An outstanding example of recent sex chromosome evolution in vertebrates is provided by the Japanese medaka, *Oryzias latipes*, where a duplicated copy of the autosomal *DMRT1* gene, named *DMY*, was evidently retroposed to another chromosome, assuming a male-determining function and thereby creating a neo-Y chromosome (Nanda et a. 2002; Matsuda et al. 2002; Matsuda et al. 2007). *DMY* is present and functional in the congeneric *O. curvinotus*, but not in other *Oryzias* species, indicating that the duplication event occurred in a common ancestor of *O. latipes* and *O. curvinotus* only ca. 10 million years ago (Kondo et al. 2003; Matsuda et al. 2003; Zhang 2004; Tanaka et al. 2007). Some closely-related species amongst salmonids and stickleback fish also have different sex chromosome pairs (Woram et al. 2003; Peichel et al. 2004). In the poeciliid *X. maculatus*, sex is determined by the segregation of three chromosomal variants (designated X, Y and W), in which XY and YY are male and XX, XW and YW are female (Kallman 1984). XX/XY with autosomal influences, strict XX/XY, and strict ZZ/ZW sytems are found in congeneric species, so the multifactorial system in *X. maculatus* may be a transitional stage between male and female heterogamety (Kallman 1984; Volff & Schartl 2001).

Evolutionary lability of sex-determining mechanisms in reptiles

Reptiles, like fish, show striking diversity in their sex-determining mechanisms. Snakes have a conserved system of female heterogamety, and all crocodilians and both species of tuatara have TSD. Amongst turtles and lizards, however, are species with male and female heterogamety, parthenogenesis and TSD. In the turtle subfamily Kinosterninae, there is clear diversity in the expression of TSD; species can exhibit the MF or FMF pattern, or an intermediate pattern (Ewert 2004). It is unclear whether TSD or GSD is the ancestral sex-determining mechanism in reptiles, and whether there has been a bias

in the direction of transitions (GSD to TSD, TSD to GSD). In a recent phylogenetic analysis, Janzen and Krenz (2004) attempted to reconstruct the most parsimonious history of transitions between TSD, XX/XY and ZZ/ZW systems that could account for the current distributions of these modes in reptiles. Their analysis suggested that GSD is ancestral in vertebrates, but TSD is ancestral in sauropsids (reptiles, dinosaurs, and birds). Further, it suggested that TSD to GSD transitions have occurred at least six times in turtle lineages, and that at least three GSD to TSD transitions have occurred in lizard lineages. Overall, their analysis supported a case for multiple, independent evolutionary origins of the two modes of sex determination within reptiles (Janzen & Krenz 2004).

Evolutionary transitions between GSD and TSD in reptiles

Two fundamental questions, applicable to most evolutionary phenomena, arise in regard to the evolutionary transitions between GSD and TSD modes in reptiles: how and why? That is, *how* do the transitions occur, with respect to the genetic changes that effect modification of a vital developmental mechanism? And *why* do they occur, with respect to the evolutionary forces (natural selection, genetic drift) that drive genetic changes within populations? The reasons for why TSD might be favoured over GSD in reptiles have been the subject of intensive theoretical and empirical research in the past three decades. Most investigations of the adaptive significance of reptile TSD have focussed on the expectations of a model proposed by Charnov and Bull (1977) for the adaptive significance of environmental sex determination in a general sense. The Charnov-Bull model proposes two key conditions for the adaptive evolution of ESD: (1) the environment encountered by a developing individual is spatially or temporally variable (patchy) in such a way that there are lasting, differential effects on the fitness of the sexes (i.e. some patches are good for males but poor for females, or vice versa); and (2) neither the parents nor the offspring can predict the environment encountered during development. Hence, if incubation temperature differentially affects the fitness of male and female offspring, TSD allows an embryo to develop as the sex that gains the most benefit from the incubation conditions. Under the umbrella of this theory, an array of hypotheses for why reptiles might have differential fitness of the sexes with respect to developmental temperature have been forwarded and tested empirically (reviewed by Shine 1999; Valenzuela 2004). Empirical evidence in support of the various explanations is often equivocal, however, or contradictory for different taxa, and no

satisfactory general explanation for adaptive TSD evolution in reptiles has emerged (Janzen & Phillips 2006).

In comparison, the question of *how* the mechanism of GSD might evolve to (or from) TSD has received less attention. Yet a better understanding of the genetic process underlying switches between the two sex-determining mechanisms might provide important insights as to why the evolutionary transitions occur, since the transitional pathway between the two modes might be genetically or developmentally constrained, or canalised, in important ways. At a theoretical level, Bull and Charnov laid the foundations for the study of evolutionary transitions in sex-determining mechanisms some 30 years ago (Bull & Charnov 1977; Charnov & Bull 1977; Bull 1980; Bull 1981). In particular, Bull noted that both male and female heterogamety have the potential to evolve to ESD (and vice versa), provided there is some degree of environmental sensitivity in the genotypic mechanism of sex determination, selection for a greater (or lesser) environmental sensitivity than presently exists, and equal fitness of genotypes within a sex (which largely depends on the degree of sex chromosome degeneration, as described previously). Thermosensitivity at extremes of incubation temperature normally encountered by GSD reptiles may provide the raw material for selection for a greater influence of temperature in sex determination, provided there is heritable variation in the level of thermosensitivity. With the potential exception of the lizard *Bassiana duperreyi*, however, thermosensitivity of sex determination at extreme temperatures has not been demonstrated for GSD reptiles (prior to this study), in the way it has for fish and amphibians.

More recently, attempts to elucidate the mechanistic basis of GSD-TSD transitions have focussed on identifying molecular differences between the two mechanisms. Commonality of gonad structure and function in vertebrates, and conservation of many sexual differentiation genes, suggests that simple genetic changes, perhaps mutations conferring greater or lesser thermosensitivity in gene expression or activity, may be sufficient to effect the transitions. With the advent and rise of genomic technologies, attention is increasingly being focussed on comparing regulation of gene expression in the sexual differentiation networks of both divergent GSD and TSD taxa (Morrish & Sinclair 2002; Place & Lance 2004; Yao & Capel 2005), as well as closely-related GSD and TSD taxa (Valenzuela et al. 2006; Valenzuela & Shikano 2007; Valenzuela 2008). An ultimate aim of this avenue of research is to identify the molecular mechanism by

which the temperature signal influences sex determination: it remains to be seen if the mechanism operates via the expression of a single gene product, many genes, or whether the temperature signal operates at a higher level of molecular or cellular organisation.

Lizards: Model taxa for investigating the evolution of sex-determining mechanisms in reptiles

Amongst the major reptile groups, lizards (suborder Sauria) stand out as model taxa for the study of sex determination evolution. Available data indicates lizards are particularly diverse with respect to sex-determining mechanisms (Table 1.1). In addition, the early maturation and short-life span of many lizards, in comparison with crocodilians, turtles, and tuatara, facilitate empirical investigations of the potential adaptive significance of TSD, enabling more accurate estimates of lifetime reproductive success (Warner & Shine 2005; Janzen & Phillips 2006; Warner & Shine 2008). For the same reasons, pedigree studies, genetic linkage mapping, and even experimental evolution studies may be viable research avenues for some lizard species. The ancestral sex-determining mechanism in squamates appears to be GSD, in contrast to turtles, which implies there have been multiple, independent, and possibly quite recent origins of TSD in saurian lineages (Janzen & Krenz 2004). Again, this highlights lizards as model taxa for investigating the adaptive and molecular basis of evolutionary transitions between GSD and TSD in reptiles (Janzen & Phillips 2006).

Bassiana duperreyi

As described previously, the most credible evidence for the co-occurrence of GSD and TSD within a reptilian species (prior to this study) is for the three-lined skink *Bassiana duperreyi* (Shine et al. 2002; Figure 1.4), a montane lizard distributed throughout southeastern Australia. Sex determination in *B. duperreyi* may represent an evolutionary intermediate state between GSD and TSD, hence this is a candidate model species for investigating the molecular and evolutionary basis of reptilian sex determination. An important first step towards establishing this status will be to verify the putative interaction between genotype and temperature influences in its sex determination. This necessitates the development and application of molecular sex markers, to demonstrate unequivocally the occurrence of temperature-induced sex reversal.

Table 1.1 Occurrence of GSD and TSD mechanisms within eight lizard families. Both GSD and TSD occur in the Agamidae, Gekkonidae and Scincidae. Mechanisms are assigned to a family only where there is unequivocal empirical evidence for its occurrence. Cytogenetic reports of heteromorphic chromosomes which examined only a single individual of each sex, and reports of TSD based on experiments involving single incubation temperatures, very small sample sizes, insignificant sex ratio biases, or where the reliability of sexing was questionable, are considered insufficient (see Harlow 2004). Hence the diversity presented here is conservative in comparison with other reviews of sex-determining mechanisms in lizards (e.g. Janzen & Paukstis 1991; Viets et al. 1994; Janzen & Krenz 2004). It is likely that further rigorous cytogenetic and incubation studies will establish even greater diversity of the modes of sex determination within lizards (including for several families not listed).

Lizard family	Male heterogamety	Female heterogamety	TSD
Agamidae		•	•
Gekkonidae	•	•	•
Gymnophthalmidae	•		
Iguanidae	•		
Lacertidae		•	
Scincidae	•		•
Teiidae	•		
Varanidae		•	

Agamid lizards

The lizard family Agamidae (dragons) comprises almost 400 species in at least 34 genera worldwide, distributed across central, southern, and southeast Asia, as well as New Guinea, Australia and Africa (Greer 1989; Pianka & Vitt 2003; Uetz 2008). Available data on the occurrence of GSD and TSD mechanisms within the Agamidae are the most robust for any lizard family (Harlow 2004). Four congeneric African-Asian species, *Agama agama*, *A. caucasia*, *A. impalearis* and *A. stellio*, have been reported to have TSD (Charnier 1966; Langerwerf 1983; 1988; El Mouden et al. 2001), and two Asian species, *Calotes versicolor* and *Phrynocephalus vlangalii*, have been reported to have GSD (Ganesh & Raman 1995; Zeng et al. 1997). The monophyletic Australian clade of approximately 70 species (Cogger 2000) is particularly well-characterised, with varying degrees of evidence for either GSD or TSD for about 20 species (Figure 1.5; and references therein). The distribution of GSD and TSD species within the Australian clade indicates remarkable evolutionary lability in the mechanism of sex determination (Harlow 2004; Sarre et al. 2004). It appears that multiple transitions between GSD and TSD have occurred, and possibly quite recently, however the available data are insufficient to infer the directionality of transitions with any confidence. Australian agamids are gaining increasing recognition for the opportunities they present as a model reptile group, for the study of sex determination evolution (Harlow 2004; Sarre et al. 2004; Ezaz et al. 2005; Warner & Shine 2005; Doody et al. 2006; Janzen & Phillips 2006 Quinn et al. 2007; Warner & Shine 2008), and for the related fields of sex ratio evolution and sex allocation (Uller & Olsson 2006; Uller et al. 2006; Warner & Shine 2007; Warner et al. 2007).

Indeed, a recent study of the Australian jacky lizard (*Amphibolurus muricatus*) has provided the strongest empirical evidence yet for the adaptive significance of TSD in a reptile (Warner & Shine 2008). *A. muricatus* has FMF-type TSD (Harlow & Taylor 2000), with females only produced at high and low temperatures (e.g. 33°C and 23°C), and a mixture of the sexes produced at intermediate temperatures (e.g. 27°C). Warner and Shine (2008) measured the lifetime reproductive success (in number of offspring produced) of animals incubated at these three temperatures. This included males produced at feminising temperatures by the application of an aromatase inhibitor to developing eggs, thereby blocking the conversion of androgens to estrogens. Males from intermediate temperatures produced more offspring over their lifetime (3-4 years)

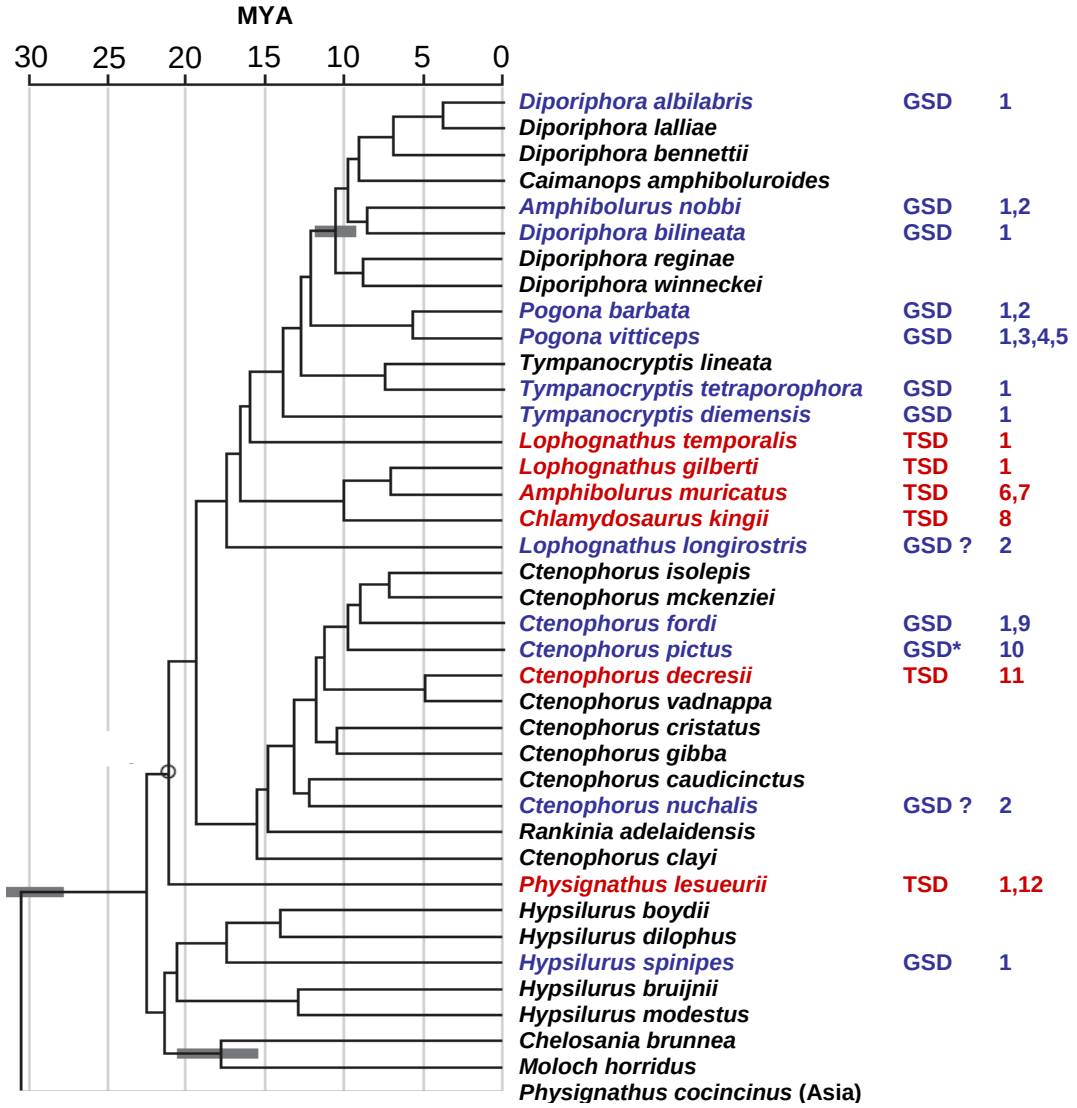

Figure 1.5 TSD and GSD modes of sex determination mapped onto an ultrametric chronogram for 38 representatives of the Australian Agamidae and one Asian species as an outgroup (modified from Hugall et al. 2008; chronogram generated from a Bayesian combined mitochondrial and nuclear gene phylogeny, under penalised likelihood rate smoothing). Numbers denote references for evidence for sex-determining mechanism: 1) Harlow 2004; 2) T. Ezaz & A.E. Quinn, unpublished data (cytogenetic, incubation, or molecular data); 3) Viets et al. 1994; 4) Ezaz et al. 2005; 5) Quinn et al. 2007; 6) Harlow & Taylor 2000; 7) Warner & Shine 2005; 8) Harlow & Shine 1999; 9) Uller & Olsson 2006; 10) Uller et al. 2006; 11) Harlow 2000; 12) Doody et al. 2006. Question marks indicate preliminary cytogenetic indications for heteromorphic sex chromosomes. * *C. pictus* was originally suggested as TSD (Harlow 2004), but incubation experiments with larger sample sizes indicates GSD (Uller et al. 2006), albeit with a consistent female bias across a range of temperatures. MYA, millions of years ago.

than ‘sex reversed’ males from the feminising temperatures. High temperature females produced more offspring than females from intermediate (and low) temperatures. These patterns imply differential fitness of the sexes with respect to incubation temperature, in accordance with the theoretical predictions of the Charnov-Bull model for the adaptive significance of environmental sex determination.

This finding for *A. muricatus* has not necessarily unveiled the reason *why* TSD evolved in this lizard lineage. TSD may have arisen originally for other adaptive or non-adaptive reason(s), followed by subsequent selection for canalisation and optimisation of the male or female developmental programs at the different temperatures producing the two sexes. Thus, the question of *cause* or *effect* arises: are different temperatures optimal for male and female development in this lizard as the result of TSD evolution, or the reason for its evolution? A more conservative interpretation is that differential fitness is the reason why TSD has been *maintained* evolutionarily, to the exclusion of GSD. The Warner and Shine study emphasises that a complete understanding of TSD evolution will require both an understanding of *why* TSD evolves from GSD, and an appreciation of *how* this evolution proceeds at the developmental and genetic level.

Agamid lizards hold considerable promise as a model reptile group for addressing the latter question, and for gaining a better understanding of sex chromosome evolution. Within the Australian clade, there is the potential for different sex chromosome pairs to have arisen in closely-related species, for variation in the degree of sex chromosome differentiation amongst species, and for some species to exhibit transitional forms of sex determination, intermediate to GSD and TSD. An essential first step towards the reconstruction of GSD-TSD transitions in agamids will be the isolation of sex chromosome sequences, for the identification and comparative analysis of sex chromosomes in GSD agamids, and for the identification of their homologues in related TSD agamids. In some agamid species, sex may be determined by an interaction between temperature and genotype, and as for *B. duperreyi*, this question can only be addressed through the development of sex chromosome sequences, or more specifically, sex-linked markers.

Karyotypically, the agamids are a reasonably well-studied group with data available for about one quarter of the almost 400 species worldwide, including 22 of the Australian species (Witten 1983; Janzen & Paukstis 1991; Ezaz et al. 2005; Olmo & Signorino

2005). Heteromorphic sex chromosomes appear to be very rare. Morphologically differentiated Z and W macrochromosomes have been reported for the Asian species *Phrynocephalus vlangalii* (Zeng et al. 1997), and more recently, female heterogamety was reported also for the Australian species *Pogona vitticeps* (Ezaz et al. 2005). *P. vitticeps* has a conserved karyotype typical of Australian agamids, with a diploid chromosome complement of 2n=32, comprising 12 macrochromosomes and 20 microchromosomes (Witten 1983; Ezaz et al. 2005). Comparative genomic hybridisation, GTG- and C-banding detected a highly heterochromatic W microchromosome (Ezaz et al. 2005). The Z chromosome, presumed to be a microchromosome also, is not identifiable even by fluorescent *in situ* hybridisation of the microdissected W chromosome to male (and female) metaphase spreads, suggesting the W and Z chromosomes are highly differentiated. Reports of sex ratios at different constant incubation temperatures for *P. vitticeps* indicate no significant bias from 1:1, consistent with GSD (28, 30, 32°C, Viets et al. 1994; 26, 29, 32°C, Harlow 2004).

P. vitticeps presents itself as a candidate model species for investigating the molecular basis of agamid sex determination, for several reasons. First, the Z and W chromosomes are evidently differentiated, suggesting it should be possible to detect sex-linked DNA markers. It is also a common and widespread species within Australia, so it is relatively straightforward to collect gravid females, and it is particularly amenable to captive husbandry, having become one the most popular reptile pet species in Europe and the USA. Finally, the largest females can produce a remarkably large clutch size for a lizard (up to 40+ eggs, personal observation), which facilitates incubation experiments designed to detect an influence of temperature on sex ratios. For these reasons, it is an ideal species to test the hypothesis that extremes of incubation temperature can over-ride chromosomal sex determination in agamid lizards.

Thesis aim and objectives

My broad aim in this study was to gain some insight into the nature of evolutionary transitions between sex-determining mechanisms in reptiles. I focussed on understanding *how* evolution between GSD and TSD occurs at a genetic and mechanistic level, rather than understanding *why* these transitions occur, in terms of the selective forces which may drive such transitions.

I tested the hypothesis that incubation temperature can over-ride chromosomal sex determination in some reptiles, to challenge the traditional view that TSD and GSD are mutually-exclusive sex-determining mechanisms in this group of vertebrates. I worked within the framework that important insights into transitional stages of evolution between GSD and TSD can be gained by investigating species with sex chromosomes and temperature-induced sex reversal. A specific experimental objective was to develop DNA sex markers for two distantly-related Australian lizards, and to apply those markers to explicitly test for sex reversal at extremes of incubation temperature. The first study species was the three-lined skink *Bassiana duperreyi* (Gray 1838) which has XX/XY sex chromosomes (Donnellan 1985). Incubation experiments have indicated previously that GSD and TSD may co-occur in this species (Shine et al. 2002). The second study species was the Central bearded dragon *Pogona vitticeps* (Ahl 1926), which has ZW/ZZ sex chromosomes (Ezaz et al. 2005). This species was chosen as a representative of the Australian agamids, a group exhibiting marked evolutionary lability in their mechanism of sex determination.

Another experimental objective was to further develop the Australian agamid lizards as a model research system for elucidating the molecular and evolutionary basis of reptile sex determination. A specific objective was to isolate and develop sex chromosome sequences from *P. vitticeps* as a comparative genomic tool for the analysis of sex chromosomes in GSD agamids, and their homologues in TSD agamids.

In addition to these experimental objectives, a final objective was to contribute to the theoretical understanding of the evolution of reptilian sex determination, in the context of viewing reptile sex determination as a continuum of states between strict TSD and strict GSD (*sensu* Sarre et al. 2004).

Thesis structure

With the exception of this Introduction (chapter 1) and the final Synopsis (chapter 6), this thesis is structured as a series of self-contained scientific manuscripts, comprising four chapters and three appendices. Consequently, there is occasional overlap in the content of the manuscripts. This thesis is my own research, but because my study formed part of a wider collaborative investigation, these manuscripts have multiple authors. Manuscripts for which I am first author are included as chapters, and manuscripts I have co-authored are included as appendices. The co-authors are listed on the title page for each chapter.

Chapter 4 has been published, but has been reformatted for the thesis. Appendices 1, 2 and 3 have been published and are presented as they appeared in publication. Chapters 2, 3 and 5 have been written as papers for submission to peer-reviewed journals. Figure 1.6 presents an overview of the thesis structure, indicating the logical flow and links between the chapters and Appendix 3.

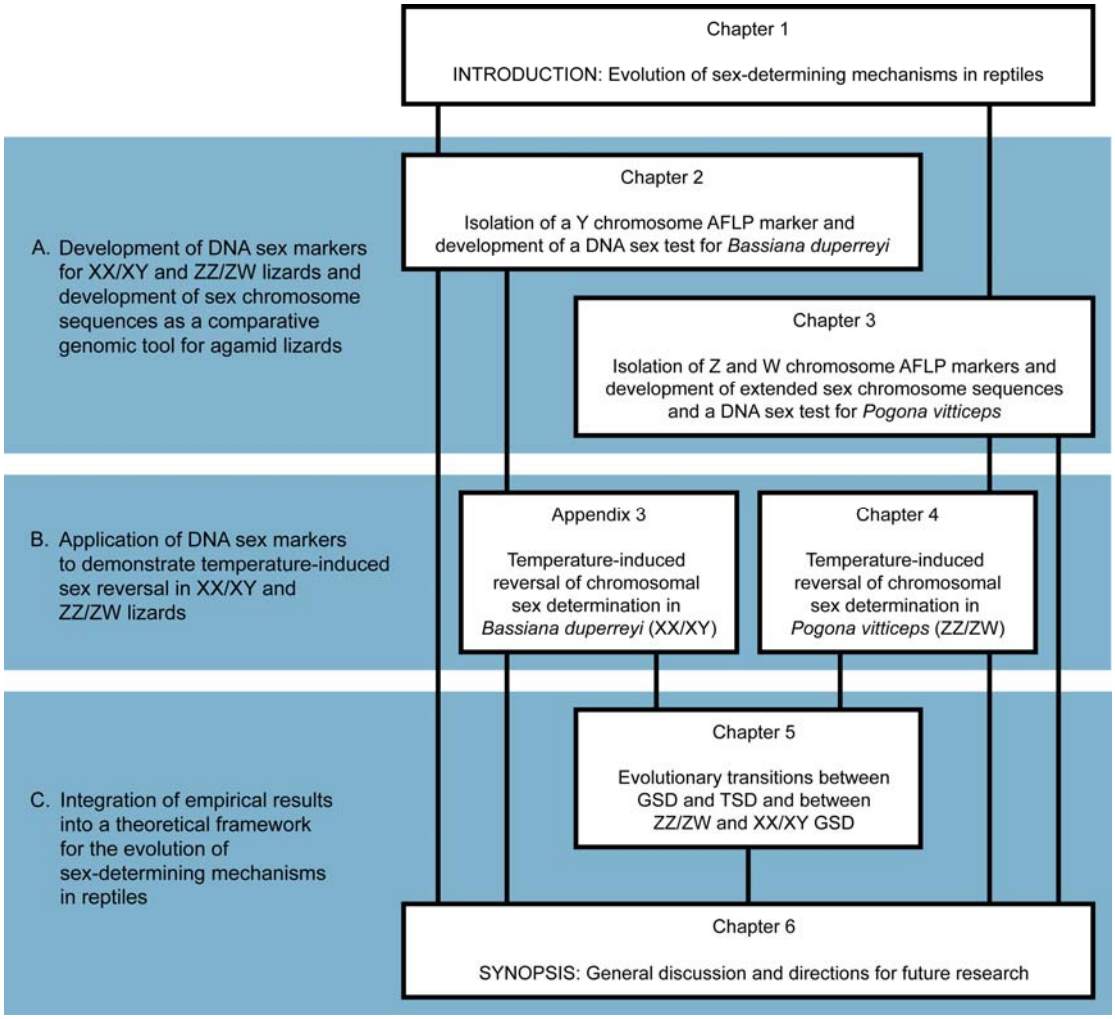

Figure 1.6 Schematic diagram of the thesis structure.

Chapter 2

Isolation and development of a molecular sex marker for *Bassiana duperreyi*, a lizard with XX/XY sex chromosomes and temperature-induced sex reversal

Manuscript to be submitted:

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Abstract

Sex determination in the endemic Australian lizard *Bassiana duperreyi* (Scincidae) is under the influence of both sex chromosomes (XX/XY) and incubation temperature, challenging the traditional dichotomous classification of reptilian sex determination as either genotypic or temperature-dependent. The demonstration, and exploration, of an interaction between sex chromosomes and temperature in sex determination requires the development of molecular markers for chromosomal sex, in order to identify cases of temperature-induced reversal of chromosomal sex. Here, we report on the isolation of Y chromosome DNA sequence from *B. duperreyi* using AFLP-PCR, and the subsequent conversion of the isolated AFLP marker sequence into a single-locus PCR assay for chromosomal sex. We developed a duplex PCR assay that co-amplified a 185 bp (or 92 bp) Y chromosome fragment and a 356 bp fragment of the single-copy nuclear gene *C-mos* (from both sexes) as a positive control. The accuracy of this PCR sex test was tested on an independent sample of individuals with known phenotypic sex, for which temperature-induced sex reversal was not expected. This is one of the very few sex tests developed for a reptile, and the first report of Y chromosome sequence from a reptile.

Introduction

In most vertebrates, sex is determined by the segregation and inheritance of genes borne on sex chromosomes (genotypic sex determination, or GSD). Mammals have male heterogamety, where males are heterozygous with respect to a pair of differentiated sex chromosome homologues (the X and Y) and females are homozygous (two X chromosomes). Birds have an opposite system of female heterogamety, so females are designated as ZW and males as ZZ. Almost all mammals share a homologous pair of X and Y chromosomes (Graves 2006), and similarly, the same Z and W pair is likely to be homologous in all species of birds (Stiglec et al. 2007). It is currently believed the avian and mammalian sex chromosomes evolved independently from different pairs of ancestral vertebrate autosomes (Nanda et al. 2000; Graves & Shetty 2001; Ezaz et al. 2006).

In contrast to the conservatism of mammals and birds, other classes of vertebrates exhibit remarkable diversity both in their fundamental mechanisms of sex determination and in their sex chromosomes. Both male and female heterogametic GSD are found amongst reptiles, amphibians and fish, but the system of heterogamety, and even the sex chromosome pair, varies amongst closely-related taxa (Olmo 1986; Solari 1994). Moreover, many reptiles and some fish exhibit temperature-dependent sex determination (TSD), in which incubation temperature during embryonic development is the dominant influence on the outcome of sexual differentiation (Bull & Vogt 1979; Bull 1980; Conover & Kynard 1981; Valenzuela & Lance 2004). For fish at least, it is accepted that in some species both temperature and sex chromosomal genes interact to determine sex (Conover & Kynard 1981; Devlin & Nagahama 2002; Conover 2004). In reptiles, however, sex determination has been traditionally viewed as either genotypic or temperature-dependent, and so systems of interacting influences were considered non-existent (Bull 1980; Janzen & Paukstis 1991; Valenzuela et al. 2003). Recently, this view of reptilian sex determination has been challenged both theoretically (Sarre et al. 2004) and empirically (Shine et al. 2002; Quinn et al. 2007; Radder et al. 2008).

In species where sex chromosomes are present, but developmental temperature also affects sex determination, some individuals develop with a sex phenotype discordant to their genotypic (chromosomal) sex. Temperature-induced sex reversal is sometimes inferred from significantly skewed sex ratios at temperature extremes (e.g. Shine et al.

2002), but alternative explanations, such as differential mortality of the sexes (e.g. Burger & Zappalorti 1988), are possible. Explicitly demonstrating sex reversal, and ascertaining *which* individuals are sex reversed, requires the unambiguous identification of chromosomal sex. In some cases, this may be achieved by cytogenetic methods (e.g. metaphase chromosome staining or banding, or comparative genomic hybridisation). Such approaches are often laborious and technically challenging, or simply unfeasible, since the level of chromosome differentiation may be too subtle for the resolution of the technique, or live cell cultures (for metaphase chromosome preparation) cannot be established.

DNA sex markers provide an alternative means to identify chromosomal sex, but require the isolation of sequences unique to the heterogametic (Y/W) chromosome. Polymorphic X- or Z-linked markers can also be applied, but are less powerful than Y- or W-linked markers because sex is certain for heterozygotes only. For species with little genomic information available, sex markers are detected by molecular genetic approaches which screen the genome for sequences found only in the heterogametic sex. The size of the sex-specific fraction of the genome largely determines the difficulty of finding markers. Commonly applied methods include Randomly Amplified Polymorphic DNA (RAPD) analysis (Welsh & McClelland 1990; Williams et al. 1990) and Amplified Fragment Length Polymorphism (AFLP) analysis (Vos et al. 1995). AFLP in particular has been successfully applied to detect sex markers in a diverse range of organisms, including plants (e.g. Reamon-Büttner et al. 1998; Lebel-Hardenack et al. 2002; Peil et al. 2003), birds (Griffiths & Orr 1999), fish (e.g. Griffiths et al. 2000; Ezaz et al. 2004; Felip et al. 2005), reptiles (Quinn et al. 2007), and amphibians (A.E. Quinn, unpublished data). RAPD or AFLP sex markers can be subsequently converted into more reliable single-locus PCR tests for the Y or W chromosome sequence, which co-amplify positive control products of higher molecular weight (in both sexes) to eliminate the possibility of incorrect diagnosis of sex due to amplification failure (Griffiths 2000).

The three-lined skink *Bassiana duperreyi* is a montane scincid lizard endemic to southeastern Australia. Chromosome banding has revealed highly differentiated X and Y sex chromosomes (Donnellan 1985). Cyclical incubation temperatures (16.0±7.5°C), designed to mimic the conditions experienced in the coolest nests at the highest elevations of its range, cause sex reversal of some genotypically female (XX) embryos

(Shine et al. 2002; Radder et al. 2008). This lizard is one of only two reptiles (to date) for which temperature-induced reversal of chromosomal sex has been clearly demonstrated through the application of DNA sex markers (Radder et al. 2008; see also Quinn et al. 2007). In this paper, we report the isolation of the Y chromosome AFLP marker, and the development of the single-locus PCR sex test, used to demonstrate sex reversal in *B. duperreyi*.

Materials and Methods

Animals

Adult female *B. duperreyi* were collected from the Brindabella Range (148°50'E, 35°21'S) of southeastern Australia, one week before laying, and allowed to lay in captivity at the University of Sydney (see Radder et al. 2007). Eggs were incubated in moistened vermiculite (water potential -200kPa). Animals used for AFLP sex marker screening were incubated at a diel cycle of 20±7.5°C, and animals used for subsequent PCR analyses were incubated at a diel cycle of 22±7.5°C (see Radder et al. 2007). These thermal regimes emulate the conditions experienced by natural nests at low elevations, and produce no significant bias in sex ratio in laboratory incubation experiments (Shine et al. 2002; Radder et al. 2008).

Phenotypic sex of the hatchlings was assessed by hemipene eversion (Harlow 1996; Shine et al. 2002), and verified by histological examination of gonads at 8-10 weeks post-hatching (n=7) (Radder et al. 2007). Tail-tips (10mm) of hatchlings were removed with a sterile blade and stored in 90% ethanol (at -20°C) prior to DNA extraction and analysis.

DNA extraction

Tail-tip tissue (ca 5mm) was macerated, added to 400µl tissue extraction buffer (40mM Tris, 20mM EDTA, 100mM NaCl, pH 7.2) containing 20µl proteinase K (10mg/ml) and 20µl sodium dodecyl sulphate solution (10% w/v), and incubated overnight at 55°C. Genomic DNA was purified from the digested tissue by one of two methods. For AFLP analysis, DNA was purified using standard phenol-chloroform procedures (Sambrook & Russell 2001). For other PCR analysis, DNA was purified using a modified 'salting-out'

protocol (Miller et al. 1988). Briefly, 150µl of NH_4 acetate was added to the digested tissue, which was then chilled at -80°C for 30min, before pelleting the cellular debris by centrifugation and transferring the supernatant to a new tube. 1ml ice-cold 100% ethanol was added to the supernatant, which was again chilled at -80°C for 30min, to precipitate the DNA, followed by centrifugation to pellet the DNA. In both the phenol-choloroform procedure and the salting-out procedure, the DNA pellet was exposed to two consecutive washes of 600µl 70% ethanol followed by centrifugation, then all traces of ethanol were removed and the pellet allowed to dry at RT, followed by resuspension of the purified DNA in TE buffer (10mM Tris, 0.1mM EDTA disodium, pH 7.5).

A duplicate DNA extraction was performed for some animals, using an alternative extraction method. A small amount of tail-tip tissue (2-3mm) was macerated and added to 300µl of a 10% (w/v) solution of Chelex® 100 beads (Biorad), along with 10µl of proteinase K (10mg/ml), and incubated overnight at 55°C. After digestion, the Chelex-extracted DNA samples were incubated at 99°C for 5min, and then allowed to cool to RT. After any period of storage at 4°C, this 'boiling' step was repeated immediately prior to PCR amplification.

AFLP analysis

AFLP analyses were performed using the AFLP Analysis System I kit (Invitrogen) or according to the original protocol (Vos et al. 1995), with minor modifications. All AFLP-PCRs were performed in a 20µl volume using 1.5U BioTaq™ Red polymerase (Bioline). Selective *EcoR*I +3 and +4 primers were labelled with WellRed fluorophores (Sigma), and the selective PCR products were separated by capillary electrophoresis on a CEQ8000 capillary sequencer (Beckman Coulter), and analyzed using the associated Genetic Analysis System software. Appropriate negative controls were included for all stages of the AFLP analyses.

Sex-linked AFLP marker screening by bulk segregant analysis

Preselective AFLP products for 12 animals were combined into four monosex pools, comprising three males (two pools) or three females (two pools). The pools formed the templates for selective amplification reactions. Forty-four selective primer

combinations were tested on the four pools (Table 2.1). AFLP profiles for the two male pools versus the two female pools were compared visually to detect candidate sex-linked markers, designated as fragments amplifying in one or both of the pools for a single sex only. Selective primer combinations generating candidate sex markers were tested further by repeating selective amplification using the preselective products of the 12 animals as separate templates. This was also done for an independent sample set comprising an additional six males and six females, increasing the total sample size to 24.

Cloning and sequencing of sex-linked AFLP marker

To facilitate the isolation and cloning of AFLP fragments of interest, the final annealing temperature of the selective PCR was increased from 56°C to 60°C, further promoting specific amplification of those fragments. This optimised selective PCR was performed on three males with an increased reaction volume of 60µl, and the PCRs were purified using a High Pure PCR product purification kit (Roche). The purified AFLP products were cloned into a pGEM®-T Easy Vector (Promega) and transformed into chemically competent JM109 *E.coli* cells (Stratagene), according to the manufacturers' instructions. Recombinant clones were identified by blue-white selection and plasmid DNA was isolated from 5ml overnight cultures in LB medium (including 50µg/ml ampicillin) using the mini-prep procedure described in Sambrook and Russell (2001). Recombinant clones containing the AFLP fragment of interest were verified by PCR amplification with universal M13 forward and reverse primers and were sequenced using the Beckman Coulter Quick Sequencing kit. Sequencing reactions were run on a CEQ8000 capillary sequencer (Beckman Coulter) and sequences were analysed with the accompanying Genetic Analysis System software.

Conversion of AFLP marker into single-locus PCR sex assay

PCR primers were designed to amplify the entire genomic sequence of a putative AFLP sex marker (primers BdY-F1/BdY-R2), or a nested fragment approximately half the length of the marker (primers BdY-F1/BdY-R1) (Table 2.2). To provide a positive control for PCR amplification, PCR primers were designed to amplify a fragment of the single-copy nuclear gene C-*mos* (from both sexes) that was larger than the putative sex marker sequence. To do so, a reptile-specific reverse primer (G74; Saint et al. 1998)

Table 2.1 Selective AFLP primer combinations screened in the search for a sex-linked marker in *B. duperreyi*. Asterisk denotes selective primer combination which amplified sex-linked AFLP marker Bd199/207Y.

*Mse*I primer selective nucleotides	*EcoR*I primer selective nucleotides					
	AAC	AACT	AAG	ACG	ACGG	AGG
CAA	●	●	●	●	●	●
CAC	●	●	●	●	●	●
CAG				●	●	
CAT	●	●	●	●	●	●
CTA	●	●	●	●	●	●
CTC	●	●	●	●	✶	●
CTG	●	●	●	●	●	●
CTT	●	●	●	●	●	●

Table 2.2 Primer information for the Y chromosome and C-*mos* primers used in the two variants of the PCR sexing assay. * From Saint et al. (1998).

Primer	Sequence (5'-3')	Product
BdY-F1	GAATTCACGGGGATGTTGCG	92 bp Y chromosome marker (with BdY-R1) 185 bp Y chromosome marker (with BdY-R2)
BdY-R1	CCATGCGTAACCACCACG	92 bp Y chromosome marker
BdY-R2	TTAACTCCCATGGGCATCAAC	185 bp Y chromosome marker
ScCmosF1	CAGAACTRAATGTGGCACGC	356 bp fragment of the single-copy nuclear gene C-*mos*
G74*	TGAGCATCCAAAGTCTCCAATC	

was paired with a forward primer (ScCmosF1) designed to anneal to lygosomine skink *C-mos* sequences retrieved from Genbank (http://www.ncbi.nlm.nih.gov/Genbank).

The primers were combined into two duplex PCRs and optimised to the following conditions. PCR #1 co-amplified the entire AFLP-derived genomic fragment and the C-*mos* fragment, with the following final reaction conditions: 1.5mM $MgCl_2$, 200µM each dNTP, 5pmol of primers BdY-F1 and BdY-R2, 10pmol of primers ScCmosF1 and G74, 0.5U of BioTaq™ Red DNA polymerase (Bioline) and 2µl of the accompanying 10X PCR buffer were added to 20-50ng of genomic DNA template in a reaction volume of 20µl, and thermocycled (94°C for 2min, then 40 cycles of 94°C for 30s, 64.5°C for 30s, and 72°C for 30s, followed by 72°C for 5min). PCR #2 co-amplified the shorter, nested section of the AFLP-derived fragment and the C-*mos* fragment, with the following final reaction conditions: 1.5mM $MgCl_2$, 200µM each dNTP, 5pmol of primers BdY-F1, BdY-R1, ScCmosF1 and G74, 0.5U of BioTaq™ Red DNA polymerase (Bioline) and 2µl of the accompanying 10X PCR buffer were added to 20-50ng of genomic DNA template in a reaction volume of 20µl, and thermocycled in a touchdown PCR (94°C for 2min, then 10 cycles of 94°C for 30s, 66°C for 30s decreasing by 0.5°C per cycle, and 72°C for 1min, then 30 cycles of 94°C for 30s, 61°C for 30s, and 72°C for 1min, followed by 72°C for 5min).

The optimised duplex PCRs were tested on an independent sample set of animals for which sex reversal was not expected. PCR #1 was tested on genomic DNA extracted from 54 animals by the 'salting-out' technique (32 males, 22 females). PCR #2 was tested on 27 of those 54 animals (19 males, 8 females), using genomic DNA extracted by the salting-out method, or Chelex-extracted genomic DNA, as template.

Results

Isolation of Y chromosome AFLP marker

Selective AFLP primer screening by bulk segregant analysis and subsequent amplification from individual templates revealed a single male-linked marker. The combination *EcoR*I-ACGG/*Mse*I-CTC amplified a 207 bp AFLP fragment (designated Bd207Y) in 5 of 6 males, but in none of 6 females in the initial sample set. Although most of the selective primer combinations generated 20-50 intense AFLP peaks, this

particular primer combination was atypical in that it produced only two intense peaks: a 445 bp fragment in both sexes, and the 207 bp fragment in males only. A strong AFLP product of 199 bp amplified in the male lacking Bd207Y (with equal fluorescence intensity to the Bd207Y marker in the other males), so it appeared likely this band represented a homologous fragment with a deletion in the sequence (see below). Testing this primer combination marker on another six of each sex (Figure 2.1) expanded the sample to 24 individuals and revealed that Bd207Y amplified in 11 of 12 males (the 199 bp marker amplified in the 12th male), but in none of 12 females.

Sequence of Y chromosome AFLP marker

The Bd207Y marker (two males) and the 199 bp marker were cloned and sequenced. The low complexity of the AFLP profile (only two intense amplification products) made cloning of the markers straightforward; PCR amplification indicated that all recombinant clones included an insert size of either ~200 bp or ~450 bp. The sequence of the 207 bp fragment was identical for the two males, and the sequence of the 199 bp fragment from the third male was identical to the 207 bp fragment, but with an 8 bp deletion, indicating that it represented the same Y chromosome locus. After accounting for the AFLP adaptor sequences, the Bd207Y marker represented 185 bp of genomic sequence from the Y chromosome (Accession no. EU259191; Figure 2.2). BLAST analysis (blastn/megablast: http://www.ncbi.nlm.nih.gov/blast) of the 185 bp sequence detected no significant similarity to available database sequences.

PCR sex assay testing

PCR #1 (Figure 2.3) amplified a 185 bp fragment in 30 of 32 males (94%), but in only one of 22 females (4%), further confirming the AFLP-derived marker as Y chromosomal sequence (Table 2.3). Thus, 3 of 54 (5.6%) individuals showed discordance between their sex phenotype and their genotype according to PCR #1. When combined with the original 24 animals genotyped by AFLP, the overall level of discordance was 3 of 78 animals (3.8%). PCR #2 (Figure 2.3) amplified a 92 bp fragment in 17 of 19 males and one of eight females, which included the three animals (two males, one female) shown to be discordant by PCR #1 (Table 2.3). The genotype results were therefore identical for the 27 animals tested with both variants of the PCR sex test.

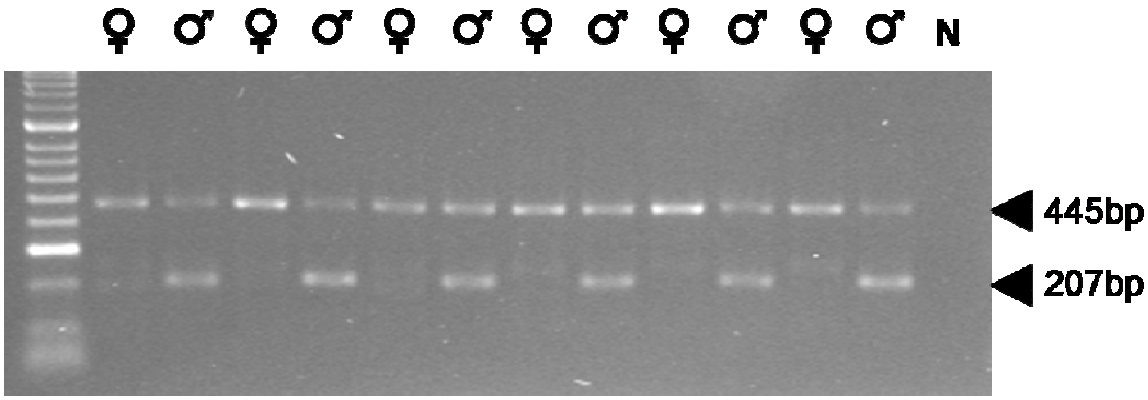

Figure 2.1 Agarose gel showing selective AFLP products for six female and six male *B. duperreyi*. Products were amplified with the primer combination *Eco*RI-ACGG/*Mse*I-CTC. Only two strong products are visible, a monomorphic 445 bp marker, and a 207 bp marker in males only (Bd207Y), representing Y chromosome sequence. N, negative control reaction. Lane 1 shows molecular weight marker.

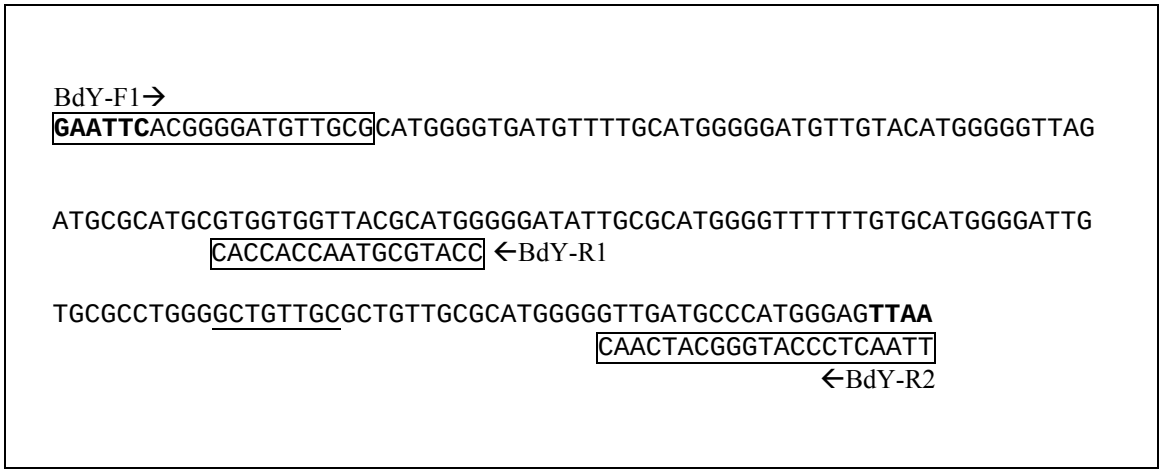

Figure 2.2 Genomic sequence (185 bp) of the AFLP marker Bd207Y (5'-3'). Boxes indicate the sequences and annealing sites for the forward and two alternative reverse primers used to amplify a Y chromosome marker (92 bp or 185 bp) in the PCR sex assay. Nucleotides in bold denote the *Eco*RI and *Mse*I restriction sites for the AFLP marker. Underlined nucleotides denote the 8 bp that were absent from a single male which amplified a 199 bp AFLP marker instead of the 207 bp marker.

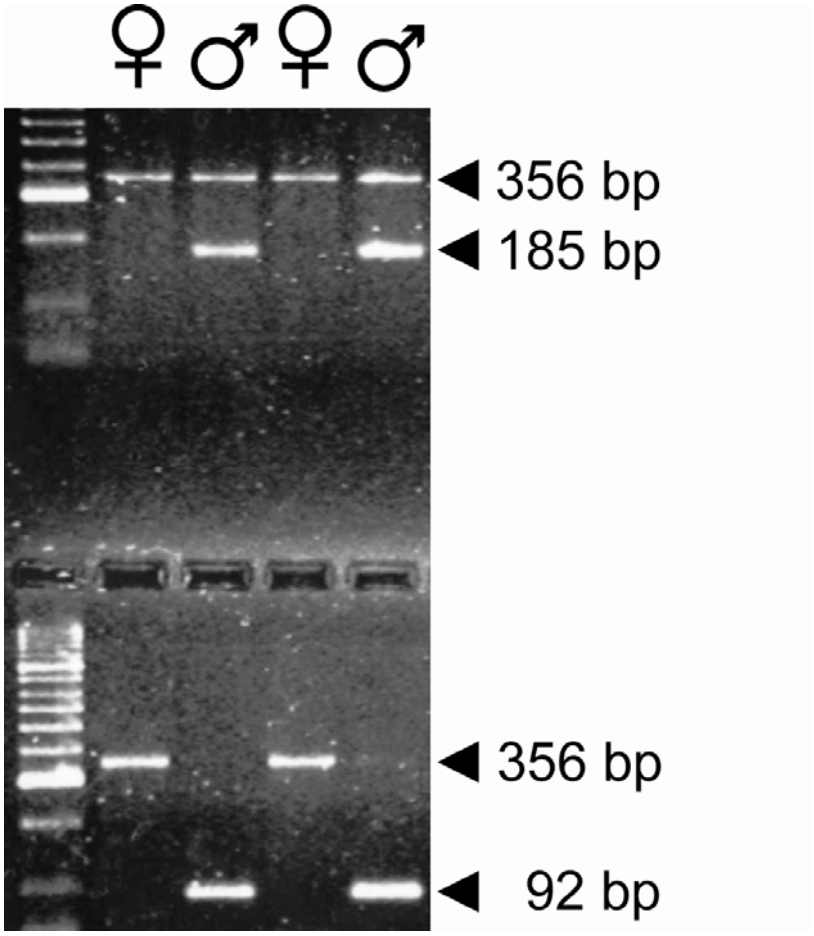

Figure 2.3 Agarose gel showing identification of chromosomal sex for two female and two male *B. duperreyi*. Two variants of a PCR sex assay are shown for the same four individuals. Upper half of gel: Duplex PCR amplification of 356 bp *C-mos* fragment (males and females) and 185 bp Y chromosome fragment (males only) from genomic DNA extracted by salting-out method. Lower half of gel: Duplex PCR amplification of 356 bp *C-mos* fragment (females only) and 92 bp Y chromosome fragment (males only) from genomic DNA extracted by Chelex method. The Y chromosome fragment is amplified preferentially over the positive-control *C-mos* fragment for the Chelex-extracted DNA. Lane 1 shows molecular weight marker.

Table 2.3 Sex genotypes obtained for 78 *B. duperreyi* sexed by hemipene eversion. Putative chromosomal sex was established by amplification (or non-amplification) of the Y chromosome AFLP marker Bd209Y, or by amplification (or non-amplification) of a Y chromosome sequence in the duplex PCR sex assay. *PCR#2 was applied to 27 of the 54 animals genotyped using PCR#1, giving equivalent genotype results.

	Phenotype	n	Male-linked marker	Discordance
AFLP	Male	12	12	
	Female	12	0	
PCR #1 (*PCR #2)	Male	32 (*19)	30 (*17)	2 (6.3%)
	Female	22 (*8)	1 (*1)	1 (4.5%)
Totals	Male	44	42	2 (4.5%)
	Female	34	1	1 (2.9%)
Combined total for both sexes		78	43	3 (3.8%)

Agarose gel electrophoresis indicated that the co-amplifying C-*mos* positive control product, amplified from both sexes, was around 350 bp (Figure 2.3), which approximates the consensus size of 356 bp for this conserved section of the C-*mos* sequence for other species within the same scincid subfamily (Lygosominae: Accession numbers AF039462 to AF039466). The C-*mos* fragment failed to co-amplify, or co-amplified only very weakly, from Chelex-extracted DNA templates when the male-linked product amplified, but this occurred only very occasionally for DNA templates extracted by the salting-out method (Figure 2.3).

Discussion

We succeeded in isolating a male-linked DNA sex marker for the lizard *B. duperreyi*, by screening Amplified Fragment Length Polymorphism (AFLP) markers amplified from pooled monosex templates (i.e. bulk segregant analysis). The heterogametic sex in this species is male (Donnellan 1985), so the isolation of a male sex marker (rather than a female sex marker) was expected. The lack of significant homology to available sequences indicated by BLAST search implies the sex marker represents novel Y chromosome sequence. The AFLP marker was subsequently converted into a single-locus PCR assay to diagnose chromosomal sex. Two variants of this test, one amplifying a 185 bp Y chromosome marker, and the other a nested 92 bp Y chromosome marker, produced equivalent genotype results. PCR genotyping was effective using two different methods of DNA extraction, including Chelex resin–based extraction. This provides a means of performing rapid and reliable DNA sex identification for *B. duperreyi*, as an alternative to the more expensive, time-consuming, and technically-demanding approach of performing AFLP analysis on DNA extracted using commercial kits or by phenol-chloroform methods. The duplex PCR assay favoured amplification of the Y chromosome marker over the co-amplification of the larger C-*mos* positive control fragment. Indeed, for Chelex-extracted DNA templates, the Y chromosome marker out-competed amplification of the C-*mos* product to the extent that the positive control product failed to (visibly) amplify for most XY individuals. C-*mos* still amplified strongly from XX animals, thus serving its purpose as a positive control to prevent misdiagnosis of chromosomal sex in the event of complete PCR amplification failure (Griffiths 2000).

The small proportion of individuals discordant between sex phenotype and genotype observed for the PCR sex assay has four plausible explanations, each of which highlights a specific issue arising in sex marker development. First, it is possible that an error was made in phenotypic sexing of these animals. Given sufficient practice, hemipene eversion is a proven and reliable technique for sexing skinks (Harlow 1996), and this phenotypic sexing method has been shown to be 100% congruent with gonadal histology for *B. duperreyi* (Radder et al. 2007). So we consider this to be an unlikely explanation. Second, the two "XX" males which failed to amplify the male sex marker could be explained by mutation in the primer sites (null amplification). Mutation rates for non-recombining regions of heterogametic sex chromosomes are typically much higher than for pseudoautosomal regions, so primer sites for Y chromosome markers may be particularly susceptible to point mutations, insertions or deletions. Whilst we cannot rule out this possibility definitively for the forward primer, the two variants of our PCR sex test employed distinct reverse primers (BdY-R1 and BdY-R2) and both failed to amplify the male sex marker in the discordant males. This greatly reduces the likelihood that mutations in the reverse primer site caused null amplification. Even if mutations in the forward primer site do cause occasional false identification of XX males, such mutations cannot account for amplification of the the Y chromosome marker in the single discordant female.

A third possible explanation is that all three discordant animals could be the result of meiotic recombination between the X and Y chromosomes at some point in the patrilineal ancestry of these animals, since a recombination event could have exchanged one (or both) of the primer sites on the Y chromosome with homologous sequence on the X chromosome. This would require that the marker is in a pseudoautosomal region of the sex chromosomes. The Y chromosome of *B. duperreyi* is much smaller than the X chromosome, and C-banding of metaphase chromosomes indicates that the Y chromosome is largely heterochromatic (Donnellan 1985; T. Ezaz & A.E. Quinn, unpublished data), implying it is highly differentiated from the X chromosome. It seems unlikely then that the Y chromosome marker was isolated from a pseudoautosomal region, but it is certainly not impossible; for instance, an AFLP-derived sequence isolated from the highly heterochromatic W chromosome of the bearded dragon lizard *Pogona vitticeps* (Agamidae) (Quinn et al. 2007) appears to be located in a homologous region of the Z and W sex chromosomes still undergoing occasional recombination (A.E. Quinn, unpublished data).

The final, and in our view, most likely explanation for the discordance is that the PCR genotype was a true indication of the chromosomal sex of the three animals in question, and they were therefore sex reversed. The eggs of these hatchlings were incubated under a thermal regime that produces sex ratios that do not depart significantly from 1:1 (Shine et al. 2002; Radder et al. 2007; 2008), but this implies only that this temperature regime does not over-ride chromosomal sex determination in the large majority of embryos. The degree of thermosensitivity in sex determination may vary in *B. duperreyi*, such that a small proportion of individuals are sex reversed under these incubation conditions. There is some evidence that it is not only sex chromosomes and incubation temperature that can influence sex in this lizard; for instance, eggs that produce females are significantly larger than eggs that produce males, irrespective of incubation temperature (Shine et al. 2002). The PCR sex assay we have developed will enable investigation of the possibility that egg size can induce sex reversal in *B. duperreyi*. Chromosomal sex identification with this PCR assay has already shown that application of the steroid sex hormone 17β-oestradiol to egg shells can induce male-to-female reversal in XY embryos, suggesting that yolk hormone levels may be able to naturally influence sex determination (Radder et al. 2008). The lability in sex determination clearly evident in this lizard supports the possibility that the discordant animals were rare instances of sex reversal under 'control' incubation conditions.

Reports of sex markers, and indeed sex chromosome sequences, are scarce for reptiles. Sequence from the W chromosome of the largest extant species of lizard, the Komodo dragon (*Varanus komodoensis*, Varanidae), was identified by RAPD analysis and converted into a PCR sex test (Halverson & Spelman 2002), which also identified sex in the Australian varanid *V. rosenbergi* (W. Smith, pers. comm). More recently, a W chromosome AFLP marker sequence was isolated and converted into a single-locus PCR sex assay for the Australian agamid lizard *Pogona vitticeps* (Agamidae) (Quinn et al. 2007) and shown to diagnose chromosomal sex for other species within the *Pogona* genus (A.E. Quinn, unpublished data). The only other sex-linked marker isolated for a skink is an X chromosome microsatellite locus (Tr4.11) in another Australian species, the shingleback lizard *Tiliqua rugosa* (Cooper et al. 1997), and this locus was subsequently shown to also identify heterozygotes as females in the related Cunningham's skink (*Egernia cunninghami*) (Stow et al. 2001). To the best of our knowledge, the present study is the first report of a Y chromosome sequence for a reptile. This sequence will allow comparison with the Y chromosome sequences likely

to emerge from the genome sequence for the green anole (*Anolis carolinensis*, Iguanidae) (http://www.broad.mit.edu/models/anole).

In contrast to mammals and birds, molecular sex markers developed for reptiles, amphibians and fish are unlikely to have broad taxonomic applicability because of the evolutionary lability of sex chromosomes and sex-determining mechanisms within these vertebrate groups. Genes and non-coding sequences within the sex-specific fraction of the heterogametic chromosome, the target of searches for sex markers, are presumably lost (or exchanged) every time that a sex chromosome pair is replaced by another, and this may also occur whenever a chromosomal mechanism of sex determination is replaced by environmental sex determination. For instance, the PCR test for W chromosome sequence for the agamid lizard *P. vitticeps* is ineffective as a diagnostic test for sex beyond the genus (A.E. Quinn, unpublished data), and a PCR test developed for a Y chromosome AFLP marker in the three-spined stickleback fish (*Gasterosteus aculeatus*, Gasterosteidae) is ineffective for congeneric species (Griffiths et al. 2000). For most reptiles, unless markers happen to be available for related species with highly conserved sex chromosomes, sex markers will need to be developed *de novo* for species of interest. An important exception may be snakes; conserved sex chromosome sequences may be universally present in this reptile group because the Z and W chromosome pair is conserved (Matsubara et al. 2006), albeit with considerable variation in the degree of W chromosome degeneration between families (Ohno 1967). This study, in conjunction with the recent isolation of W chromosome sequence from an agamid lizard (Quinn et al. 2007), shows that AFLP is a highly effective approach for detecting sex markers in reptiles, provided there is some differentiation of the sex chromosomes.

The Y chromosome sequence isolated from *B. duperreyi* awaits testing of its homology and sex-linkage in populations covering the geographical distribution of this species in southeastern Australia, and also in related species of skinks. Notably, the heteromorphic sex chromosome pair in *B. duperreyi* (pair 7) is also the heteromorphic sex chromosome pair for the two other species in the genus, and also in a further 28 species spanning nine other genera of skinks within the subfamily Lygosominae (Hardy 1979; Donnellan 1985, 1991; Hutchinson & Donnellan 1992). Given this apparent conservation of the sex chromosome pair, the PCR sexing test developed for *B. duperreyi* could prove to be useful for a number of species.

Chapter 3

From AFLP to Z: Isolation, conversion, and physical mapping of sex chromosome sequence in a dragon lizard

Manuscript to be submitted:
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Abstract

Temperature-dependent sex determination (TSD) and genotypic sex determination (GSD) are widespread in dragon lizards (Agamidae), implying an evolutionary history of transitions between these sex-determining mechanisms. We report on the isolation of sex chromosome sequence from the Australian Central bearded dragon (*Pogona vitticeps*), as a comparative genomic tool for investigating the evolution of sex determination and sex chromosomes in agamid lizards. We isolated homologous Z and W chromosome AFLP markers, and then generated larger genomic fragments by genome walking. PCR experiments confirmed two non-overlapping fragments (2.2 kb and 4.5 kb) as homologous Z/W chromosome sequences. An amplified 3 kb fragment of the 4.5 kb sequence was hybridised onto metaphase chromosome spreads of both sexes of *P. vitticeps* by fluorescent *in situ* hybridisation, identifying the W microchromosome, and for the first time, the Z microchromosome in this species. The isolated sequences were also converted into a high-throughput, single-locus PCR sexing assay for examining the interaction between temperature and genotype in the sex determination of species in the *Pogona* genus. PCR analyses indicate that other Australian agamid species share sequence homology with these *P. vitticeps* sex chromosome sequences. Comparative chromosome painting with these sequences may therefore shed light on the relationship of sex chromosomes in the agamids, and the nature of genomic differences between GSD and TSD species.

Introduction

Sex in vertebrates is determined either by genes on sex chromosomes (genotypic sex determination, GSD) or by an environmental variable during embryonic development (environmental sex determination, ESD). The most common form of ESD in vertebrates is temperature-dependent sex determination (TSD), in which incubation temperature during embryogenesis influences offspring sex (Bull 1983). TSD is exhibited by some fishes (Conover & Kynard 1981; Devlin & Nagahama 2002; Conover 2004), but is most prevalent in reptiles, being found in the tuatara (Cree et al. 1995), all crocodilians (Ferguson & Joanen 1982; Deeming 2004), many turtles (Pieau 1972; Bull & Vogt 1979; Ewert 2004) and a minority of lizards (Charnier 1966; Harlow 2004). GSD is also widespread in reptiles. Chromosomal sex determination with female heterogamety (ZZ males/ZW females) is apparently ubiquitous in snakes (Beçak et al. 1964; Ohno 1967; Matsubara et al. 2006), whereas turtles and lizards with GSD include species with either female or male heterogamety (XY males/XX females), some of which have multiple sex chromosomes (Solari 1994; Janzen & Krenz 2004; Olmo & Signorino 2005), and many species in which the heterogametic sex is yet to be determined because the sex chromosomes are homomorphic, or cryptic (Ezaz et al. 2005; 2006). The diversity of sex-determining mechanisms and their almost haphazard distribution across the reptile phylogeny alludes to a complex evolutionary history of transitions between male and female heterogametic GSD, and between GSD and TSD (Bull 1980; Kraak & Pen 2002; Janzen & Krenz 2004; Sarre et al. 2004).

Dragon lizards (Agamidae) comprise almost 400 species worldwide (Uetz 2008). The 70 endemic Australian species (Wilson & Swan 2003) are increasingly being adopted as a model group for the study of sex determination evolution, sex ratio evolution, and sex allocation (Harlow 2004; Ezaz et al. 2005; Warner & Shine 2005; Doody et al. 2006; Janzen & Phillips 2006; Uller & Olsson 2006; Uller et al. 2006; Quinn et al. 2007; Warner & Shine 2007; Warner et al. 2007; Warner & Shine 2008). The distribution of GSD and TSD species amongst Australian agamids implies evolutionary lability in their sex-determining mechanisms (Harlow 2004; Sarre et al. 2004), presenting an opportunity to better understand the molecular and chromosomal basis of evolutionary transitions between the two fundamental mechanisms of vertebrate sex determination. Within such a group, there is the potential for different sex chromosome pairs to have arisen in closely-related species, for variation in the degree of sex chromosome

differentiation amongst species, and for some species to exhibit transitional forms of sex determination, intermediate to GSD and TSD.

An essential first step towards the reconstruction of GSD-TSD transitions in agamids is the isolation of sex chromosome sequences for the identification and comparative analysis of sex chromosomes in GSD agamids, and for the identification of their homologues in related TSD agamids. One strategy for isolating sex chromosome sequences in non-model organisms is to screen the genome for sequences unique to the heterogametic sex, that is, Y or W chromosome markers (Griffiths 2000). Common molecular genetic approaches include PCR-based DNA fingerprinting techniques such as Randomly Amplified Polymorphic DNA (RAPD) analysis (Welsh & McClelland 1990; Williams et al. 1990) and Amplified Fragment Length Polymorphism (AFLP) analysis (Vos et al. 1995), as well as subtractive hybridisation techniques such as Representational Difference Analysis (RDA) (Lisitsyn et al. 1993). AFLP in particular has been applied successfully to detect sex markers in a diverse range of organisms, including plants (Reamon-Büttner et al. 1998; Lebel-Hardenack et al. 2002; Peil et al. 2003), birds (Griffiths & Orr 1999) fishes (e.g. Griffiths et al. 2000; Ezaz et al. 2004; Felip et al. 2005), reptiles (Quinn et al. 2007) and amphibians (A.E. Quinn, unpublished data). Linkage mapping of sex chromosome markers can assist in pinpointing the chromosomal region containing the sex-determining locus (e.g. Lebel-Hardenack et al. 2002), and Y- or W-chromosome linked markers can be used to diagnose sex when morphological sex identification is unreliable.

In species where there is an interaction between temperature and genotype in the determination of sex, such as might be expected in some agamids (Sarre et al. 2004), DNA sex markers can be used to identify instances of temperature-induced sex reversal, where the chromosomal and phenotypic (gonadal) sex of an individual are in conflict. The endemic Australian lizard *Pogona vitticeps* (Central bearded dragon) provides one such case (Quinn et al. 2007) and represents an excellent model for studies of sex reversal because it has a large clutch size for a lizard and is particularly amenable to captive husbandry. *P. vitticeps* has a conserved karyotype typical of Australian agamids, with a diploid chromosome complement of 2n=32, comprising 12 macrochromosomes and 20 microchromosomes (Witten 1983; Ezaz et al. 2005). Comparative genomic hybridisation, GTG- and C-banding have demonstrated it has female heterogamety, with a highly heterochromatic W microchromosome (Ezaz et al. 2005). The Z

chromosome, presumed to be a microchromosome also, is not identifiable even by fluorescent *in situ* hybridisation of the microdissected W chromosome to male (and female) metaphase spreads, suggesting the W and Z chromosomes are highly differentiated (Ezaz et al. 2005). *P. vitticeps* is not only the first reported instance of sex microchromosomes in an agamid lizard, but it is one of only two reptile species (to date) for which an interaction between incubation temperature and sex chromosomes in sex determination has been demonstrated convincingly through the application of DNA sex markers (see also Radder et al. 2008).

In this paper, we describe the isolation of the W chromosome AFLP marker (and a homologous Z chromosome marker) used to demonstrate temperature-induced sex reversal in *P. vitticeps* (Quinn et al. 2007), and the subsequent isolation of extended sex chromosome sequences by a reiterated genome walking process. We identified two non-overlapping, genome-walked fragments (2.2 kb and 4.5 kb) as homologous Z/W sequences, and physically mapped a 3 kb fragment of the 4.5 kb sequence to the Z and W microchromosomes of *P. vitticeps*. We tested for homologous sequences in other Australian agamid species by PCR amplification, to gauge the potential usefulness of the *P. vitticeps* sequences as hybridisation probes for investigating the relationship of sex chromosomes in this lizard family. We also developed a single-locus PCR sexing assay from the *P. vitticeps* sequences, to enable investigation of the interaction between incubation temperature and genotype in the sex determination of species within the *Pogona* genus.

Materials and Methods

Animals and DNA extraction

Central bearded dragons (*Pogona vitticeps*) were collected from northwest New South Wales and southwest Queensland, Australia, and a captive breeding colony was established at the University of Canberra. Eggs were individually incubated in moist vermiculite in plastic boxes at a high constant but unmeasured humidity (Fordham et al. 2006). Sex of hatchlings was determined by hemipene eversion (Harlow 2001) and confirmed by dissection and examination of gonadal morphology. Adults were sexed on the basis of external morphology on a number of occasions, and sex assignment was consistent. In addition, all adult females used in this study were known to have been

gravid. Animals were killed by intracranial injection of Nembutal (Sigma), prior to dissection for gonadal sexing (hatchlings) or collection of tissue for fibroblast culture (adults). Blood, liver, or tail-tip tissues were collected or obtained for other Australian agamid species from various locations (see Acknowledgements). Sex of these animals was assessed on the basis of external morphology, or by examination of gonadal morphology, when possible.

Genomic DNA was extracted from whole blood (collected from the caudal vein), tail-tip tissues or liver tissues, using standard phenol-chloroform procedures (Sambrook & Russell 2001).

Amplified Fragment Length Polymorphism (AFLP) analysis

AFLP analyses were performed using the AFLP Analysis System I kit (Invitrogen) or according to the original protocol (Vos et al. 1995), with minor modifications. Polymerase Chain Reactions (PCRs) were performed in a 20µl volume using 1.5U BioTaq™ Red polymerase (Bioline). Selective *EcoR*I primers were labelled with WellRed or HEX fluorophores (Sigma). WellRed-labelled selective PCR products were electrophoretically separated on a CEQ8000 capillary sequencer (Beckman Coulter) and analysed using the associated Genetic Analysis System software. HEX-labelled selective PCR products were electrophoretically separated on a GelScan 2000 system (Corbett Research), and analysed using the associated ONE-DSCAN software. Appropriate negative controls were included for all stages of the AFLP analyses.

Screening for sex-linked markers by AFLP: Three-phase strategy

A three-phase strategy was employed to screen selective AFLP primer combinations for candidate sex markers. In phases 1 and 2, we analysed hatchlings from a clutch incubated at a temperature (constant 28°C) which produces an unbiased sex ratio (Quinn et al. 2007), so the hatchlings were expected to have concordant chromosomal and phenotypic (gonadal) sex. In phase 1, preselective PCR products from five males or five females were mixed in equal concentrations to create monosex pools. These formed the template for selective PCR amplification. Ninety-six combinations of selective primers were examined for amplification of candidate sex markers (Table 3.1), designated as products amplifying from one monosex pool only, or exhibiting a

Table 3.1 Selective AFLP primer combinations tested while screening *P. vitticeps* for sex-linked markers. Nucleotide sequences denote the +3 or +4 selective nucleotides of the primer, and numbers indicate the progressive experimental phases (1, 2, or 3) in which the primer combination was tested. Phase 1 - AFLP profiles for one male pool and one female pool were compared (five individuals represented in each monosex pool); Phase 2 - individual AFLP profiles for six males and six females were compared (included all individuals represented in the monosex pools); Phase 3 - individual AFLP profiles for 16 males and 16 females were compared (including the 12 individuals from phase 2, plus another ten of each sex representing an independent sample set). The primer combination which amplified the sex-linked fragments Pv72W and Pv71Z is underlined.

*Mse*I primer	*EcoR*I primer							
	AAC	AACT	AAG	ACC	ACG	ACGG	AGC	AGG
CAA	1	1, 2, 3	1		1	1	1	1
CAC	1	1	1		1, 2, 3	1	1	1
CAG	1	1	1	1	1	1	1, 2	1, 2
CAT		1	1		1	1	1	1
CCA		1	1		1	1	1	1
CCG		1	1	1	1, 2	1	1	1
CCT		1	1		1	1, 2, 3	1, 2	1
CGA		1	1, 2		1	1	1, 2, 3	1
CGC		1	1, 2	1	1	1	1, 2	1, 2
CGG		1	1, 2		1, 2	1	1	1, 2
CGT		1, 2	1, 2		1	1, 2, 3	1	1
CTA		1, 2	1		1	1	1	1
CTC		1	1		1	1, 2, 3	1	1
CTG		1	<u>1, 2, 3</u>		1	1	1, 2	1, 2
CTT		1	1		1, 2, 3	1	1	1, 2

considerably higher relative fluorescence for one sex pool. In Phase 2, products for primer combinations of interest were amplified again, but using the preselective PCR of six males and six females as individual templates (including the five males and five females constituting the monosex pools) (Table 3.1). AFLP products that amplified in one sex only, or with a heavily sex-biased distribution, were then tested in Phase 3, in which selective amplification was performed on individual templates for an increased sample size of 16 of each sex (Table 3.1). This included the 12 individuals from Phase 2 (as positive controls), and 20 new individuals (as an independent sample set), comprising an additional four males and four females from the same clutch (incubated at 28°C) plus six male and six female adults captured in the wild.

Recovery, cloning and sequencing of candidate sex-linked AFLP markers

To reduce the number of amplified products and facilitate isolation of two AFLP fragments of interest, two additional rounds of AFLP selective amplifications were performed using progressively longer selective *Mse*I primers (i.e. *Eco*RI+3/*Mse*I+4 then *Eco*RI+3/*Mse*I+5). Once the refined *Eco*RI+3/*Mse*I+5 primer combination was identified, the AFLP markers of interest for three females and two males (from the 28°C clutch) were isolated from a 5% high-resolution agarose gel and reamplified by 'band-stab' PCR (Sambrook & Russell 2001). Briefly, the desired bands were stabbed precisely with a sterile hypodermic needle while visualising the ethidium bromide-stained gel under UV light. The amplified DNA was then transferred to a sterile PCR tube containing 5µl nano-pure H_2O, by briefly stirring the needle tip in the water. This formed the template for reamplification using the same selective primer combination. The reamplified products were 'band-stabbed' and reamplified a second time, and the new products electrophoretically separated on a capillary sequencer to check for amplification of single products of the desired size. The second reamplification products were diluted 10,000-fold to form templates for a third and final reamplification. Negative control reactions were included at all steps, including 'sham' needle-rinses with clean needles.

The fragments of interest were smaller than 100 bp, precluding purification with commercial gel purification kits, so products were recovered by excision from a 1% low-melting point agarose gel, followed by melting and digestion of the gel slices with

β-agarase I enzyme (New England Biolabs). DNA was precipitated with ammonium acetate and isopropanol, using glycogen as a carrier (Sambrook & Russell 2001).

The purified AFLP fragments from the three males and two females were TA cloned (pGEM®-T Easy Vector, Promega) and recombinant clones identified by blue-white selection. Plasmid DNA was isolated from overnight cultures by the mini-prep procedure described in Sambrook and Russell (2001). Recombinant clones were verified by PCR amplification, and plasmid inserts sequenced, using universal M13 primers. All sequencing (of cloned inserts or PCR products) was performed on a CEQ8000 capillary sequencer (Beckman Coulter), and sequences were analysed and edited using Sequencher™ 4.7 software (Gene Codes).

Extension of AFLP sequences by genome walking

Larger genomic fragments were generated from the sequenced AFLP fragments using reagents supplied in the GenomeWalker™ Universal kit (BD Clontech). Four genome walking 'libraries' were constructed for one male and for one female from the 28°C clutch. Genomic DNA was digested separately with four blunt-end restriction enzymes (*Dra*I, *EcoR*V, *Pvu*II, and *Stu*I) then purified by phenol-chloroform treatment and precipitated with sodium acetate, glycogen, and ethanol (Sambrook & Russell 2001). GenomeWalker™ Adaptors were ligated to the restricted fragments with T4 DNA ligase (16°C for 8-12 h). Ligation reactions were diluted ten-fold to create template for the primary round of PCR amplification. A set of 'gene-specific' primers (GSPs) were designed (in accordance with the kit manufacturer's guidelines) to anchor within the AFLP fragment sequences and to enable amplification in both directions (Table 3.2). Each primary gene-specific primer (GSP1) was designed in combination with a nested secondary gene-specific primer (GSP2) on the same DNA strand.

GSP1 primers were paired with Adaptor Primer 1 (BD Clontech) to amplify primary PCR products from the eight genome walking libraries. Optimisation of the primary PCR yielded the following conditions: 2mmol/l $MgSO_4$, 200µmol/l each dNTP, 5pmol GSP1 and AP1 primers, 1U of Platinum® *Taq* DNA polymerase High-Fidelity (Invitrogen) and 2.5µl of the accompanying 10X PCR buffer were added to 1µl template (genome walking library DNA) in a final volume of 25µl, and amplified by 'touch-down' PCR (8 cycles of 94°C for 30sec, 68°C for 30sec decreasing by 0.5°C/cycle, and

68°C for 4min, and 23 cycles of 94°C for 30sec, 64°C for 30sec, and 68°C for 4min, followed by 68°C for 10min). GSP2 primers were paired with Adaptor Primer 2 (BD Clontech) to amplify secondary PCR products from the primary products. Optimisation of the secondary PCR yielded the following conditions: 1.5mmol/l $MgSO_4$, 200µmol/l each dNTP, 5pmol GSP2 and AP2 primers, 1U of Platinum® *Taq* DNA polymerase High-Fidelity (Invitrogen) and 2µl of the accompanying 10X PCR buffer were added to 1µl template (50,000-fold dilutions of the primary PCR products) in a final volume of 20µl, and amplified (5 cycles of 94°C for 25sec and 72°C for 4min, and 35 cycles of 94°C for 25sec and 68°C for 4min, followed by 68°C for 10min).

Recovery, cloning and sequencing of genome walking products

Products for seven secondary genome walking PCRs were excised from 2% high-resolution agarose gels and purified using the UltraClean™ Gel Cleanup DNA Purification kit (Mo Bio Laboratories). The purified products were TA cloned (TOPO® TA cloning kit, Invitrogen) and plasmid DNA was isolated using the Wizard Plus® SV Plasmid DNA Purification System (Promega). Recombinants were verified by *EcoR*I digestion as well as PCR amplification with universal M13 primers. Plasmid inserts were sequenced using universal M13 primers. Internal primers were designed to anchor within the obtained sequences and were used to continue sequencing through the genome walking fragments. Sequences were assembled into contiguous fragments.

Reiteration of genome walking procedure

Internal primers were also designed to anchor within the genome walking fragments in order to amplify outwards into unknown flanking sequence. To obtain additional flanking sequences for one of the assembled genome walking contigs, these primers were used as primary and secondary 'gene-specific' primers (in conjunction with Adaptor Primers 1 and 2) for a second iteration of the genome walking process, using the eight genome walking libraries constructed previously. The secondary PCR products were purified using the UltraClean™ PCR Clean-up kit (Mo Bio Laboratories) and sequenced directly using GSP2 and AP2 primers, or the internal primers designed from the initial genome walking products. The additional sequences were assembled onto the contig obtained in the first phase of genome walking.

Table 3.2 (facing page) PCR primers for *P. vitticeps* used in this study. Gene-specific primers (GSPs) for phase 1 of genome walking were designed to target the sex-linked AFLP sequences (Pv71Z and Pv72W) in the male and female genome walking libraries, and GSPs for phase 2 of genome walking were designed to target contig C sequences in these libraries (F2, H, I, J, K). Internal primers A1-Q were designed from the genome walked sequences, for amplification of genomic DNA templates, and were used for: (1) verification of contigs A and C as homologous Z/W sequences (Figures 3.4 and 3.5); (2) testing for amplification of products spanning contigs A, B and C (not all primers listed, and data not shown); (3) testing for the presence of homologous sequences in other agamid species (Figure 3.8); and (4) the PCR sexing assay (Figures 3.4 and 3.7). *F* = forward primer. *R* = reverse primer. * Primer F1 was designed with a 5' polyA tail of 20 nucleotides. This was to lengthen amplification products of internal fragments of the 50 bp Pv72W marker sequence to enable them to be visualised on a capillary sequencer (experiment not described in this paper). F1 was subsequently incorporated into the PCR sexing assay, and since it performed as required, the polyA tail was not removed.

Table 3.2

Primer	Sequence (5'-3')	Site	Application
PvW-*Mse*I-5	AACTGCTGAGGATGAGACACACCTTGTCGG	Pv72W	GSP1 (phase 1)
PvW-*Mse*I-4	ACACACCTTGTCGGGGGCGCTTCCTTG	Pv72W	GSP2 (phase 1)
PvW-*Eco*RI-2	GAATTCAAGGAAGCGCCCCCGACAAGG	Pv72W	GSP1 (phase 1)
PvW-*Eco*RI-6	GCCCCCGACAAGGTGTGTCTCATCCTCAGC	Pv72W	GSP2 (phase 1)
PvZ-*Mse*I-7	CTGCTGAGGATGAGACACAGCTTGTCAGGG	Pv71Z	GSP1 (phase 1)
PvZ-*Mse*I-9	GGATGAGACACAGCTTGTCAGGGTCCTTCC	Pv71Z	GSP2 (phase 1)
PvZ-*Eco*RI-7	GGAAGGACCCTGACAAGCTGTGTCTCATCC	Pv71Z	GSP1 (phase 1)
PvZ-*Eco*RI-8	CCCTGACAAGCTGTGTCTCATCCTCAGCAG	Pv71Z	GSP2 (phase 1)
A1	TCCCTCTCTCCAGATTTTCTGCTG	Contig C	3 kb probe amplification, Homology test
C	CGGTAAGCGAAACAGCTTATCGATC	Contig B	Sex assay (control band *F*), Homology test
E	CTCTGAGAAGGGCGGAGAAGAAG	Contig B	Sex assay (control band *R*), Homology test
F	CAGTTCCTTCTACCTGGGAGTGC	Contig C	Homology test
F1	*(A_{20})CTGCTGAGGATGAGACACAC	Pv72W	Sex assay (female band *F*), Homology test
F2	GCACTCCCAGGTAGAAGGAACTG	Contig C	GSP2 (phase 2)
F4	CTTTCCGCCCTTATTGCCTTCTGC	Contig C	Sex assay (female band *R*), Homology test
G	TCACCTCAAGGAAGGGTCTTCTG	Contig C	Z/W assignment test
H	GAGGCCACCATCTGTTAACTTGG	Contig C	GSP1 (phase 2)
H2	GCCCATATCTCACTAGTTCCCCTCC	Contig C	Homology test
I	CATGGTGGGAGCAGCAAACAT	Contig C (specific to fragment 4)	W-specific GSP1 (phase 2)
J	CCCTCTGGCCTCCTTAATGTTTGCTGCTC	Contig C (specific to fragment 4)	Z/W assignment test, W-specific GSP1 (phase 2)
K	TGCATGTGCCTGCCCTTCCGCTAAGAG	Contig C	GSP2 (phase 2)
L	CCTTTGCAGAAAGACTCACTGTGC	Contig C	Z/W assignment test, Homology test
L1	TATGTGTTTGAGCTTCGGCTACAGTG	Contig C	3 kb probe amplification, Homology test
M	GGACAGAATGGCACAACAAGGAC	Contig A	Z/W assignment test
Q	CCTCCAGTTCCGAATCCTCTCG	Contig A	Z/W assignment test

Blood culture and metaphase chromosome preparation

Metaphase chromosome spreads of *P. vitticeps* were prepared from short-term culture of whole blood or peripheral blood leukocytes as described by Ezaz et al. (2005). Briefly, approximately 100 µl blood was used to set up 2 ml culture in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% foetal bovine serum (JRH Biosciences), 1 mg/ml L-Glutamine (Sigma), 10 µg/ml gentamycin (Multicell Technologies), 100 units/ml Penicillin (Multicell), 100µg/ml Streptomycin (Multicell) and 3% phytohaemagglutinin M (PHA M; Sigma). Cultures were incubated at 30°C for 96-120 h in 5% CO_2 incubators. Six and four h prior to harvesting, 35µg/ml 5'-bromo-2'-deoxyuridine (BrdU; Sigma) and 75ng/ml colcemid (Roche) were added to the culture respectively. Metaphase chromosomes were harvested and fixed in 3:1 methanol:acetic acid following the standard protocol (Verma & Babu 1995). Cell suspension was dropped onto glass slides and air-dried. For DAPI (4'-6-diamidino-2-phenylindole) staining, slides were mounted with anti-fade medium Vectashield (Vector Laboratories) containing 1.5 µg/ml DAPI.

Fluorescent in situ hybridisation (FISH) and C-banding

For FISH mapping, a 3 kb fragment was amplified from the genomic DNA of a female *P. vitticeps* (using primers designed from the genome walking sequences), purified using the UltraClean™ PCR Clean-up kit (Mo Bio Laboratories), and labelled with SpectrumRed-dUTP (Vysis) by nick translation. Labelled probe was precipitated and hybridised overnight onto denatured metaphase chromosome spreads. Slides were washed and counter-stained with DAPI and images were captured and analysed using a Zeiss Axioplan epifluorescence microscope equipped with a CCD (charge-coupled device) camera (RT-Spot, Jackson Instruments) as described in Ezaz et al. (2005). The camera was controlled by an Apple Macintosh computer. IPLab scientific imaging software (V.3.9, Scanalytics) was used to capture grey scale images and to superimpose and co-localise the source images into a colour image. Images were captured and coordinates were recorded for later identification of the same metaphase.

The same slides were then used for C-banding to corroborate the FISH results with the C-banding profile. Probes were stripped off the slides following the protocol described by Müller et al. (2002). Briefly, slides were soaked in 4X SSC/0.2% Tween to remove

the coverslip, and antifading solution for 3-5 min at RT, and then transferred to a new Coplin jar containing 50ml 4X SSC/0.2% Tween and incubated for 60min at RT. The slides were then dehydrated through an ethanol series (70%, 90%, 100%), for 3 min at each step, and fixed in 3:1 methanol: acetic acid for 30min at RT. Fixed slides were incubated in a 37ºC oven overnight before C-banding. C-banding was performed as described in Ezaz et al (2005). Images were captured as described previously.

Conversion of extended sequences into a single-locus PCR sex test

A single nucleotide polymorphism distinguishing putative W and Z chromosome AFLP markers was exploited to design a W chromosome-specific primer. This was paired with a primer designed to anneal at a site in one of the genome walking contigs, common to males and females (Table 3.2). A second pair of primers were designed to amplify a larger fragment of another genome walking contig, in both sexes (Table 3.2). The four primers were combined into a duplex PCR, intended to identify chromosomal sex in *P. vitticeps* on the basis of amplification (or non-amplification) of a W-chromosome fragment, with the larger fragment intended to act as a positive control product for successful amplification (Griffiths 2000). Reaction conditions were as follows: 1.5mmol/l $MgCl_2$, 200µmol/l each dNTP, 5pmol of each primer, 0.5U of BioTaq™ Red DNA polymerase (Bioline) and 2µl of the accompanying 10X PCR buffer were added to 20-50ng of genomic DNA template in a reaction volume of 20µl, and cycled in a touch-down PCR (94°C for 2min, then 10 cycles of 94°C for 30s, 67°C for 30s decreasing by 0.5°C per cycle, and 72°C for 1min, then 30 cycles of 94°C for 30s, 62°C for 30s, and 72°C for 1min, followed by 72°C for 5min). This duplex PCR was tested on 112 *P. vitticeps* for which putative chromosomal sex had previously been determined by AFLP genotyping (ZW, n = 56; ZZ, n=56).

Amplification of homologous sequences in related species of agamids

The PCR assay designed to identify chromosomal sex in *P. vitticeps* was also tested on eight related agamid species, including three other *Pogona* species. As a further test for the presence of homologous sequences in other agamids, PCRs were designed to amplify internal fragments of one of the genome walking contigs, and were tested on five agamid species (one of each sex) outside the *Pogona* genus. Reaction conditions were as follows: 1.5mmol/l $MgCl_2$, 200µmol/l each dNTP, 5pmol forward and reverse

primers, 1U of BioTaq™ Red DNA polymerase (Bioline) and 2µl of the accompanying 10X PCR buffer were added to 20-50ng of genomic DNA template in a reaction volume of 20µl, and cycled (94°C for 2min, then 35 cycles of 94°C for 20s, 55°C for 20s, and 72°C for 2min, followed by 72°C for 5min).

Results

Detection of sex-linked AFLP markers

Twenty-five of the 96 selective primer combinations tested in phase 1 of AFLP screening were tested in phase 2, and eight combinations were tested further in phase 3 (Table 3.1). The three-phase screening process identified that the primer combination *Eco*RI-AAG/*Mse*I-CTG amplified a 72 bp fragment (designated Pv72W) in 15 of 16 females, but in none of 16 males (Figure 3.1A). Amplification with *Mse*I+4 and +5 primers (paired with *Eco*RI-AAG) established that the primers *Mse*I-CTGC and *Mse*I-CTGCT amplified the female-linked marker but produced AFLP profiles of lower complexity (fewer bands), eliminating the co-amplification of faint bands of unrelated sequence of the same size (72 bp) in some males (Figure 3.1B). A 71 bp product amplified in 15 of 16 males, but the number of females with this fragment was unclear because the size difference of only a single base pair and greater intensity of Pv72W obscured the 71 bp marker in some females (Figure 3.1). Fluorescence of the 71 bp marker was approximately twice as high in males, most noticeably in capillary electropherograms (data not shown), so the marker was suspected as Z chromosome sequence (double-copy in males, single-copy in females). The 71 bp marker (designated Pv71Z) was therefore isolated for characterisation in addition to Pv72W.

Correlation of AFLP genotype and chromosomal sex

Putative genotypic sex was determined for 15 juvenile *P. vitticeps* (which had not been part of the AFLP screening) on the basis of presence (female) or absence (male) of the AFLP marker Pv72W. Metaphase chromosome preparations of these animals were then C-banded to determine their chromosomal sex, based on the presence (ZW) or absence (ZZ) of the highly heterochromatic W microchromosome. The assignment of chromosomal sex by C-banding was performed without prior knowledge of the AFLP

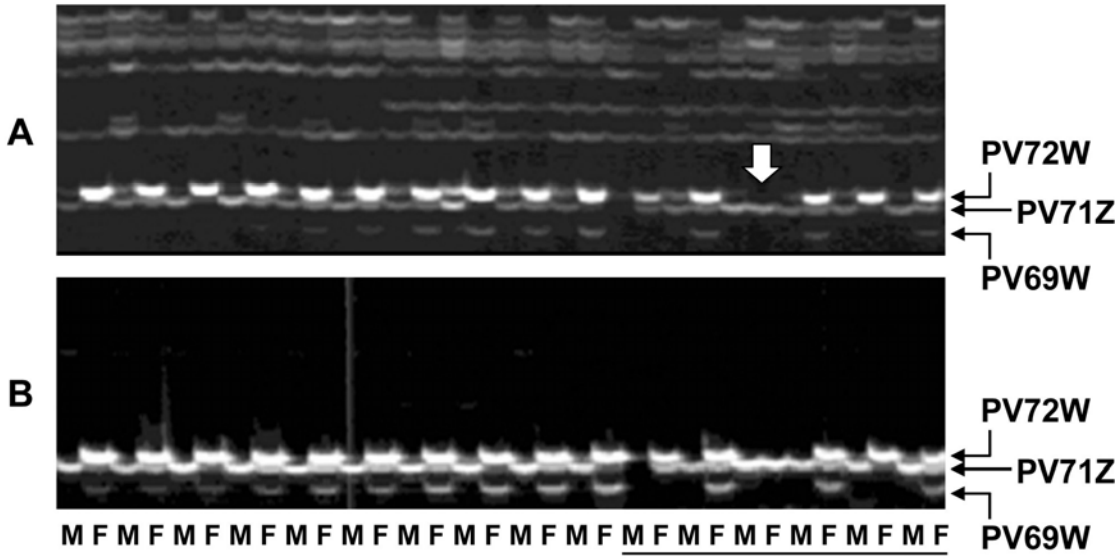

Figure 3.1 Partial AFLP profiles from polyacrylamide gels, indicating a 72 bp female-linked (W chromosome) marker (Pv72W) for *P. vitticeps*. A 71 bp Z chromosome marker (Pv71Z) shows higher intensity in males than in females. Another female-linked AFLP marker (Pv69W) is present in a majority of females. Panel A: AFLP profile generated by the +3/+3 selective primer combination *Eco*RI-AAG/*Mse*I-CTG. Panel B: AFLP profile of reduced complexity generated by the refined +3/+5 selective primer combination *Eco*RI-AAG/*Mse*I-CTGCT. Pv72W is present in 15 of 16 females, and none of 16 males. Vertical arrow indicates the single female lacking Pv72W. Wild-caught adults are underlined. All other individuals were hatchlings from the same clutch incubated at constant 28°C. All AFLP genotyping was performed by scoring electropherograms from a capillary sequencer, but polyacrylamide gel images are presented here for the purpose of illustrating the sex markers. M = male, F = female.

genotypes. AFLP genotypes and C-banding results were in 100% agreement (11 ZW; 4 ZZ), verifying that Pv72W is W chromosome-specific sequence (Figure 3.2).

Sequences of sex-linked AFLP fragments

Complete forward and reverse sequences without any ambiguity were obtained for five of eight cloned inserts from the two males and three females, all of which included the expected AFLP primer sequences at the ends of the inserts. Two of the female inserts were an identical sequence of 72 bp (i.e. Pv72W), and the two male inserts were an identical sequence of 71 bp (i.e. Pv71Z). Pv72W and Pv71Z differed in sequence at only three single nucleotide polymorphism sites plus an indel of one base pair, implying the two fragments represent homologous W and Z chromosome sequence. The cloned insert from the third female was 67 bp in length, but shared no sequence homology with the Pv71Z or Pv72W fragments (except for the primer sequences), so we assumed it to be a heterologous fragment that had been isolated accidentally. After accounting for the AFLP adaptor sequences, Pv72W and Pv71Z represented 50 bp and 49 bp of genomic sequence, respectively (Figure 3.3) (Pv72W: Accession No. ED982907).

Genome walking: Phase 1

The deduced sequences for the seven genome walking fragments were assembled into five contigs. Two were eliminated from the analysis (data not shown), leaving three contigs potentially representing sex chromosome sequence (contigs A, B and C, comprising secondary products 1-5: Table 3.3, Figure 3.3). We attempted extensive PCR experiments, using male and female genomic DNA as templates and primers internal to the contigs, to amplify products spanning the contigs (i.e. A-B, A-C, or B-C), but failed to amplify products of a size that would indicate the sequences represented by the three contigs are adjacent or near to each other in their chromosomal location (data not shown).

Assignment of genome walking sequences to Z and W chromosomes by PCR

After phase 1 of genome walking, contig C comprised two fragments of almost identical sequence, generated from the female genome walking libraries (fragments 4 and 5;

Table 3.3 Secondary PCR products for *P. vitticeps* generated in phase 1 (fragments 1-5) and phase 2 (fragments 6-12) of the genome walking experiments. Sequences obtained for the 12 fragments were assembled into three distinct contigs (A, B, C; Figure 3.3). Sizes for phase 2 fragments were approximated by agarose gel electrophoresis (data not shown), unless the exact size was determined by sequencing the whole fragment. Contigs A and C were subsequently verified as sex chromosome sequences by PCR experiments (Figures 3.4 and 3.5). Fragments 1, 2, 6 and 7 were generated from male genome walking libraries, so were presumed to be Z chromosome sequences. Fragments 4 and 5 were subsequently identified as W and Z chromosome sequences, respectively, by PCR experiments (Figures 3.4 and 3.5, Table 3.4). Fragments 8-12 were generated from a gene-specific primer specific to fragment 4, and thus were also presumed to be W chromosome sequences. No evidence was obtained for the chromosomal location of fragment 3.

Genome Walking Phase	Source genome	Genome Walking library	Secondary PCR product	Position relative to AFLP sequence	Product size (bp)	Clean sequence obtained (bp)	Contig *	Predicted chromosome
1	Male (ZZ)	*Eco*RV	1	*Eco*RI side	2186	2186	A	Z
		*Pvu*II	2	*Eco*RI side	1011	1011	A	Z
	Female (ZW)	*Stu*I	3	*Mse*I side	1675	1675	B	?
		*Dra*I	4	*Eco*RI side	1101	1101	C	W
		*Pvu*II	5	*Eco*RI side	1204	1204	C	Z
2	Male (ZZ)	*Stu*I	6	*Eco*RI side	~1600	206	C	Z
		*Stu*I	7	*Eco*RI side	~1800	1375	C	Z
	Female (ZW)	*Stu*I	8	*Eco*RI side	~1800	1767	C	W
		*Pvu*II	9	*Eco*RI side	1088	1088	C	W
		*Stu*I	10	*Mse*I side	~1600	399	C	W
		*Dra*I	11	*Mse*I side	~1000	296	C	W
		*Eco*RV	12	*Mse*I side	~1700	862	C	W

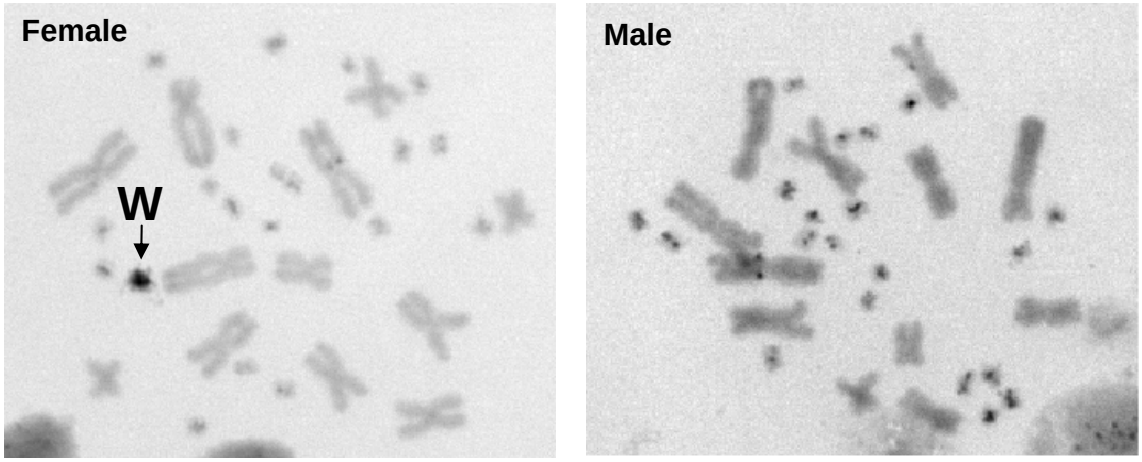

Figure 3.2 C-banding of female (left panel) and male (right panel) metaphase chromosome spreads of *P. vitticeps*. Arrow indicates highly heterochromatic W microchromosome in females only. C-banding results corroborated assignment of genotypic sex by AFLP genotyping (Pv72W amplification or non-amplification) for 15 animals (11 ZW, 4 ZZ).

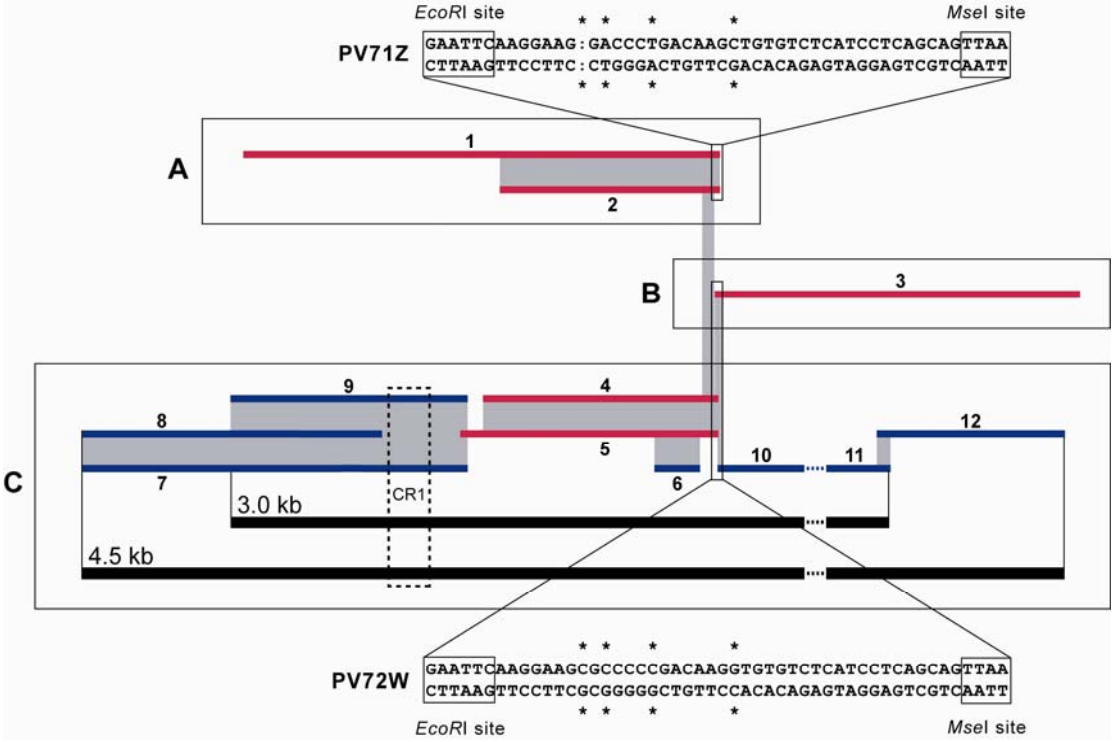

Figure 3.3 Genome walking products generated from male and female *P. vitticeps* assembled into three distinct contigs: boxes A, B and C (shown to scale). Red lines are cloned and sequenced fragments (1-5) generated in the first phase of genome walking and blue lines (6-12) are sequences obtained in a second phase of genome walking to extend contig C. Grey shading shows areas of perfect or very high sequence homology between fragments, thus the three contigs share no homology outside the AFLP sequences, except for a very short section of homology between contigs A and C immediately beyond the *EcoR*I site. Black lines indicate entire length of contig C sequence (4.5 kb), and the internal 3 kb fragment which was amplified and physically mapped by FISH. Narrow vertical boxes indicate sites of Pv71Z and Pv72W AFLP sequences. Indel and SNP differences between the two AFLP sequences are indicated by asterisks. Fragments 1 and 2 were generated from male genomic DNA using gene-specific primers designed to target Pv71Z, and fragments 3-5 were generated from female genomic DNA using gene-specific primers designed to target Pv72W. Dotted line between fragments 10 and 11 indicates short section that could not be sequenced due to the presence of mononucleotide C-G repeats. Dashed line box indicates CR1-like repetitive element.

Table 3.3, Figure 3.3). The most significant sequence discrepancy between the two fragments occurred in a section where the sequences differed at 18 nucleotide sites. A PCR primer specific to fragment 4 (J; Table 3.2) was designed to anchor within this section. PCR experiments involving this primer revealed fragment 4 as W chromosome sequence, therefore fragment 5 was presumed to be homologous Z chromosome sequence (Figures 3.4 and 3.5). Primer J was female-specific for the 28°C clutch used in AFLP primer screening, but the primer site was found to be present in two of six wild-caught males, suggesting meiotic recombination can occur in this region of the sex chromosomes (Table 3.4). PCR experiments employing a Pv72W-specific primer (F1; Table 3.2) indicated that contig A, like contig C, is homologous Z/W chromosome sequence (Figures 3.4 and 3.5). PCR experiments were also performed in an attempt to confirm contig B as sex chromosome sequence, using primers designed to be Pv72W-specific (but to extend in the opposite direction to primer F1). Multiple products were amplified, but with no indication of sex-specificity (data not shown), so it was not determined if contig B represents sex chromosome or autosome sequence.

Genome walking: Phase 2

Contig C was extended further in the second iteration of genome walking because the primer J site in fragment 4 presented the opportunity to amplify additional W (but not Z) chromosome sequence from the female genome walking libraries. Primer J, and a primer annealing at the same site but on the opposite DNA strand (I; Table 3.2, Figure 3.4), were therefore used as (W chromosome-specific) primary gene-specific primers. To amplify additional Z (but not W) chromosome sequence, primers annealing to sequences common to fragments 4 and 5 were used as gene-specific primers to amplify products from the male genome walking libraries. Sequences were obtained for seven secondary PCR products (fragments 7-12; Table 3.3) and assembled onto contig C. Additional internal primers were designed and used to sequence through the entire length of contiguous sequence; in total, sequence was obtained for a 4.5 kb region, with the exception of a 50-100 bp internal section that proved difficult to sequence due to the presence of long mononucleotide C-G repeats (Figure 3.3). A 3 kb fragment of the 4.5 kb contiguous region was amplified from the genomic DNA of a male and a female. Sequencing of internal sections of this amplified 3 kb fragment, from both the male and female, further supported the conclusion that the 4.5 kb of contig C represented highly homologous Z and W chromosomal sequence throughout its entire length.

Table 3.4 PCR 1 (see Figure 3.5) tested on the 16 males and 16 females used to screen for sex-linked AFLP markers in *P. vitticeps*. The primer J site is specific to the W chromosome within the clutch incubated at 28°C. The amplification of this "W chromosome" fragment in two of six wild-caught males implies that the primer J site is sometimes found on the Z chromosome of some animals, suggesting that recombination can occur in this region of the Z and W chromosomes.

	PCR primers (genome walk fragments including primer site)	Expected product size	Amplification in females		Amplification in males	
			28°C clutch	Wild-caught	28°C clutch	Wild-caught
Band 1	G (4 and 5) L (4 and 5)	639 bp	10 of 10	6 of 6	8 of 10	4 of 6
Band 2	J (4 only) L (4 and 5)	158 bp	10 of 10	6 of 6	None of 10	2 of 6

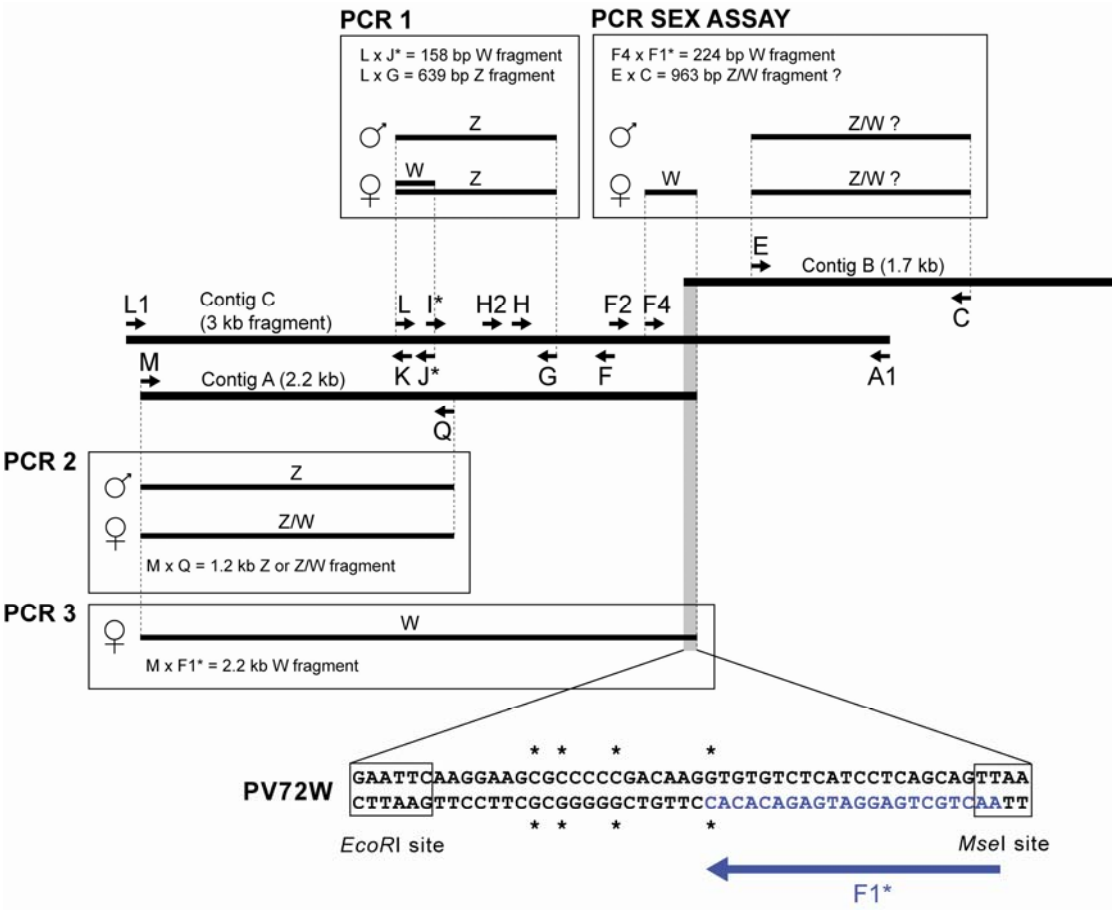

Figure 3.4 PCR primers for *P. vitticeps* mapped onto contigs A, B and 3 kb fragment of contig C (heavy black lines, shown to scale). Letters denote primers (see Table 3.2) and arrows indicate direction of primer extension (5'-3'). Grey strip indicates Pv72W AFLP sequence common to the three contigs, with the F1 primer site in blue. The final 3' nucleotide of the F1 primer aligns at one of the few nucleotide sites distinguishing the Pv72W and Pv71Z AFLP sequences. Primers marked with an asterisk (F1*, I*, J*) are W chromosome-specific (at least for the 28°C clutch used for AFLP primer screening). Boxes show four different PCRs, indicating the expected product sizes for males and females (light black lines). PCR 1 identified fragments 4 and 5 (Figure 3.3) as W and Z chromosome sequences, respectively, thus indicating that contig C is homologous Z/W sequence (Figure 3.5; Table 3.4). PCR 2 and PCR 3 indicated that contig A is also homologous Z/W sequence (Figure 3.5). Contig B could not be verified as sex chromosome sequence by PCR. In the PCR sexing assay, a 963 bp fragment of contig B served as the positive control product (amplifying in both sexes), whereas a 224bp fragment of contig C was the female-specific product (Figure 3.7).

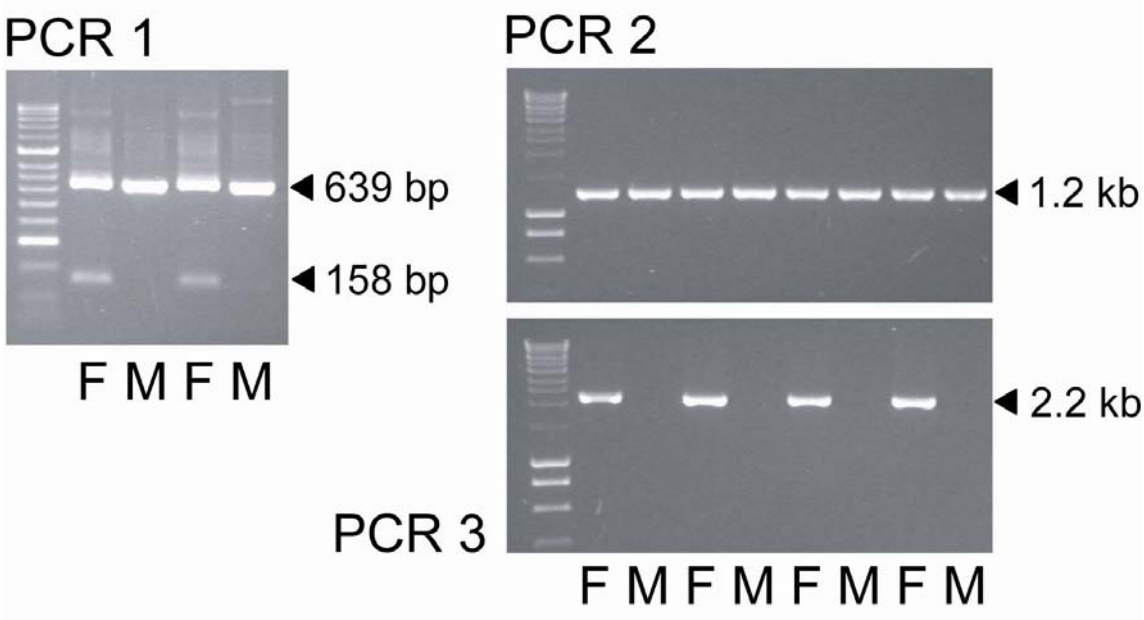

Figure 3.5 Agarose gels showing PCRs used to verify two of the genome walking contigs as *P. vitticeps* sex chromosome sequences. Templates were genomic DNA. PCR 1 was a duplex reaction incorporating one reverse primer, L (common to fragments 4 and 5), and two alternative reverse primers, J (specific to fragment 4) and G (common to fragments 4 and 5). The L x J product (158 bp, see Figure 3.4) amplified with female-specificity for the 28°C clutch used in AFLP primer screeening (Table 3.4), indicating that fragment 4 is W chromosome sequence. The L x G product (639 bp) amplified in both males and females within the clutch, indicating that fragment 5 is Z chromosome sequence. Outside the primer J site, fragments 4 and 5 have nearly identical sequence, implying that contig C is homologous Z/W sequence. PCR 2 amplified a nested fragment (1.2 kb) of the contig A sequence from four males and four females, but with an alternative, female-specific reverse primer (F1) in PCR 3, the entire contig A fragment (2.2 kb) amplified with female-specificity for the same eight individuals (Figure 3.4). This implies that contig A is homologous Z/W sequence, particularly because contig A was originally generated from male genome walking libraries (Figure 3.3), using gene-specific primers designed to target the Pv71Z sequence. Similar PCR experiments to verify contig B as sex chromosome sequence were unsuccessful. M = male, F = female.

Sequence analysis of genome walking contigs

BLAST analysis of contig A (2.2 kb), contig B (1.7 kb) and contig C (4.5 kb) revealed no sections of significant similarity to database sequences (blastn: http://www.ncbi.nlm.nih.gov/blast), with the exception of a 185 bp section of contig C which showed 68% similarity to sections of several artificial chromosome (BAC) library clones containing chicken (*Gallus gallus*) Z chromosome sequences. Repeat masking (http://www.girinst.org/cgi-bin/censor) revealed this section to be highly similar to the CR1 repeat element (Figure 3.3), first characterised as an interspersed repetitive element from the chicken genome (Stumph et al. 1981; 1983; 1984), and subsequently discovered in the genomes of almost all major vertebrate groups, including reptiles (Vandergon & Reitman 1994). We obtained the sequence for this repetitive element for three female and two male *P. vitticeps*. In both males and one female, the CR1-like element was 188 bp in length, but an identical 14 bp deletion in the other two females reduced the length of the repeat element at this locus to 174 bp.

Physical mapping of amplified 3 kb sequence by FISH to metaphase chromosomes and subsequent C-banding

FIS hybridisation of the 3 kb fragment of contig C showed a hybridisation signal on one pair of microchromosomes only. Two males and two females were analysed. The FIS hybridisation signal strength was often unequal between the homologous microchromosomes among cells (Figure 3.6). C-banding of the same female slides indicated that the heterochromatic W microchromosome was one of the pair of microchromosomes to which the 3 kb probe hybridised. Thus, the second microchromosome with the hybridisation signal in female slides, and both of the microchromosomes with the hybridisation signal in the males slides, were revealed as the Z chromosome. The 3 kb probe presumably comprised a mixture of Z and W chromosome fragments because it was amplified from female genomic DNA. Thus, hybridisation of the probe to the sex microchromosomes (on both male and female metaphase slides) further indicated that contig C represents highly homologous Z and W chromosome sequences.

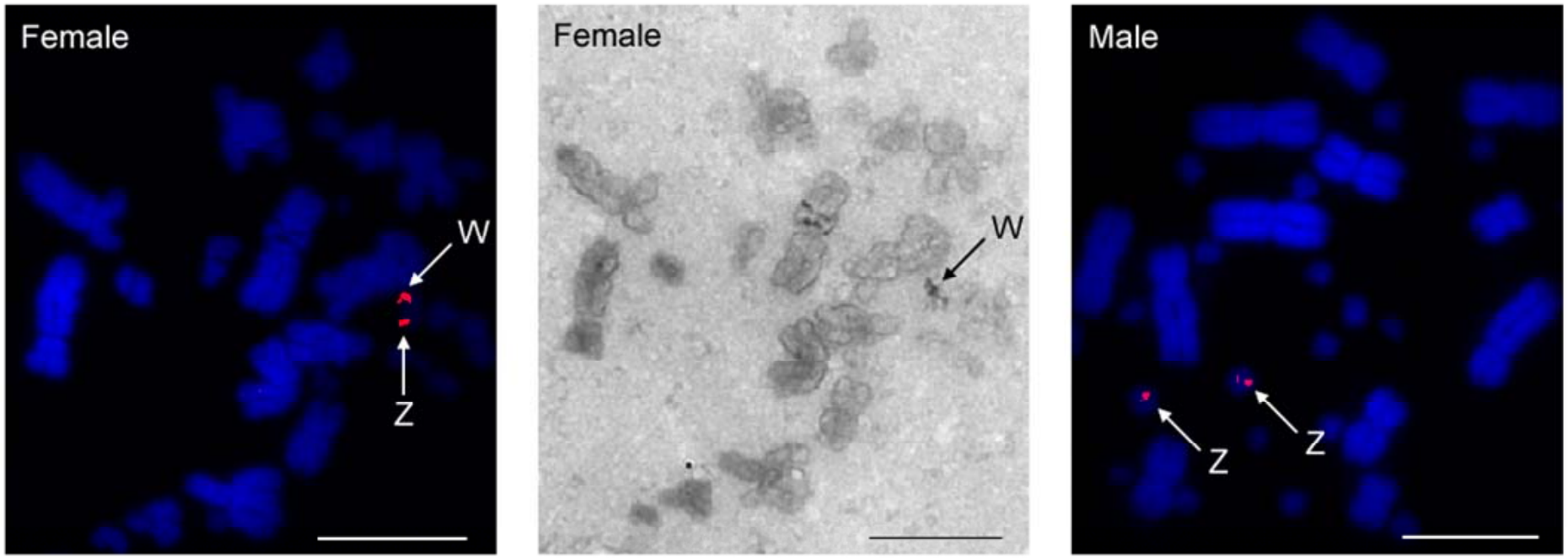

Figure 3.6 Physical mapping of *P. vitticeps* sex chromosome-specific extended AFLP fragment (amplified 3 kb fragment of contig C) to female and male metaphase spreads (left and right panels) and subsequent C-banding of same female metaphase chromosome spread (centre panel). Z and W microchromosomes are indicated with arrows. C-banding of same female metaphase spread shows heterochromatinization only on W chromosome. Scale bar represents 10μm.

Conversion of genome walking sequences into a single-locus PCR sex test and correlation with AFLP sex genotypes

The final 3' nucleotide of the Pv72W-specific primer F1 aligned with one of the single nucleotide polymorphism sites distinguishing the Pv71Z and Pv72W AFLP sequences (Figure 3.4). In the PCR sex assay, F1 was paired with primer F4 (Table 3.2) to amplify a 224 bp W chromosome fragment (Figures 3.4 and 3.7). The positive-control product for the duplex PCR was a 963 bp fragment of contig B, which amplified in both sexes (primers C and E; Table 3.2, Figures 3.4 and 3.7). Amplification was expected to be favoured for the female-specific product because of its smaller size. There were five potential outcomes for the PCR sex test based on the relative strength of amplification of the two products from genomic DNA of *P. vitticeps*, interpreted as follows: (1) 224 bp fragment stronger than (or approximately equal to) the 963 bp fragment (= ZW); (2) 224 bp fragment present, 963 bp fragment absent (= ZW); (3) 963 bp fragment present, 224 bp fragment absent (= ZZ); (4) 963 bp fragment considerably stronger than the 224 bp fragment (= ZZ); and (5) neither fragment present (= PCR failure). The most likely reason for outcome (4) was considered to be non-specific amplification of the Pv71Z sequence with the Pv72W-specific primer (or potentially, contamination of the PCR template with female DNA), and therefore this outcome was scored as a ZZ genotype. Scoring of the PCR results on agarose gels resulted in complete agreement between AFLP and PCR genotypes: 55 ZW animals (Pv72W present) presented PCR outcome (1) or (2), 45 ZZ animals (Pv72W absent) presented outcome (3), and 8 ZZ animals presented outcome (4) in which the W band was extremely faint (and therefore ignored). Four of the 112 PCRs failed (outcome 5), and so could not be compared with their AFLP genotypes.

Amplification of homologous sequences in related species

The PCR sexing assay identified chromosomal sex in *Pogona barbata* (3 males and 3 females tested), and appeared to do so for *P. henrylawsoni* (one male and one female tested) (Figure 3.8A). The positive control product also amplified in the single individual of *P. minor* (of unknown sex) available for analysis. Thus, it appears quite possible that the PCR sexing assay developed for *P. vitticeps* may be applicable to all seven species of *Pogona* (bearded dragons). The control band amplified (with the size expected for *P. vitticeps*) in *Amphibolurus muricatus* and *A. nobbi*, but not in the other

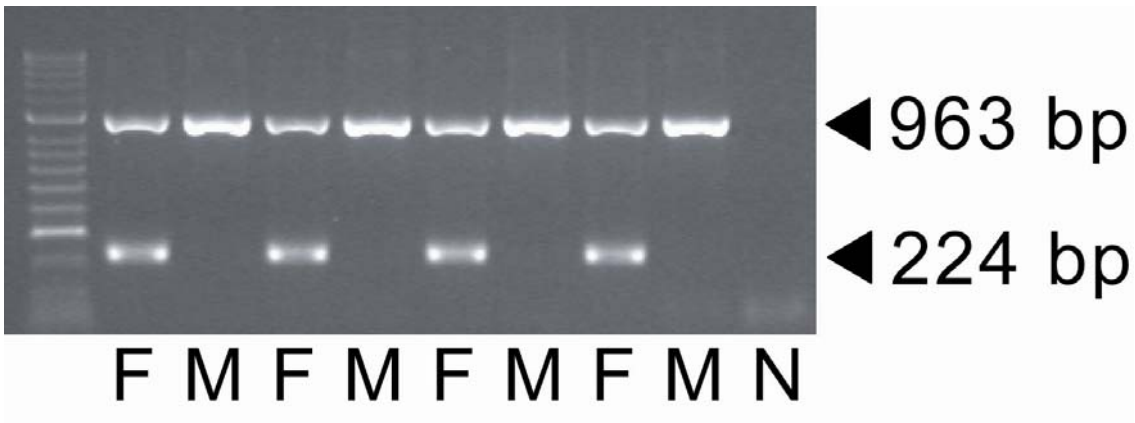

Figure 3.7 Agarose gel showing PCR sexing assay for *P. vitticeps*. Templates were genomic DNA. The duplex reaction amplified a 963 bp fragment of contig B from both sexes (as a positive control for amplification), and a 224 bp fragment of contig C from females only (see Figure 3.4). The PCR assay was tested on 112 *P. vitticeps* for which chromosome sex was ascertained by AFLP genotyping (see text), and the assay was also tested on eight other agamid species (Figure 3.8). M = male, F = female, N = negative control.

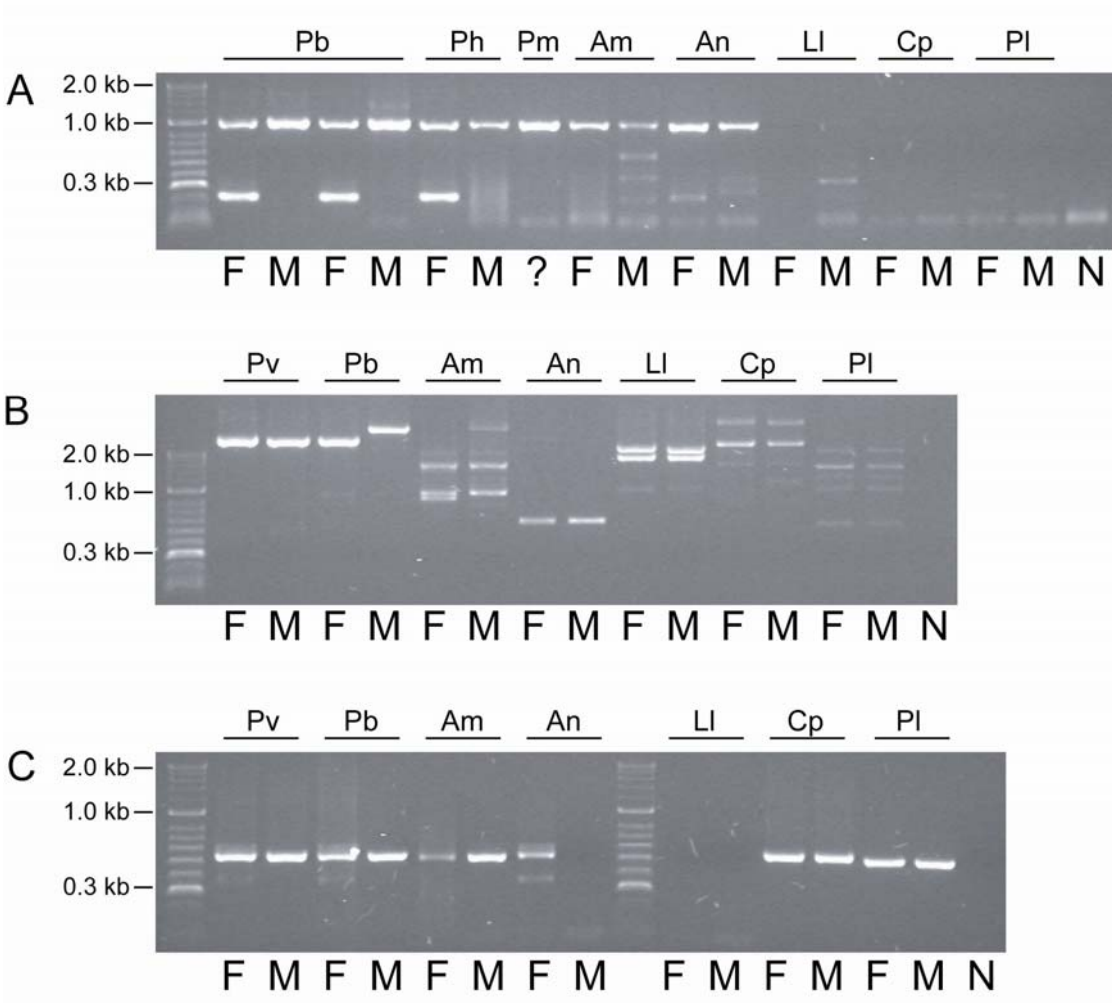

Figure 3.8 Agarose gels showing PCR experiments testing Australian agamid species for the presence of sequences homologous to the sex chromosome sequences isolated from *P. vitticeps*. Letters above gels denote species: Pv *Pogona vitticeps*, Pb *P. barbata*, Ph *P. henrylawsoni*, Pm *P. minor*, Am *Amphibolurus muricatus*, An *A. nobbi*, Ll *Lophognathus longirostris*, Cp *Ctenophorus pictus*, Pl *Physignathus lesueurii*. Panel A: *P. vitticeps* PCR sexing assay tested on eight other species. Panel B: Amplification using the primers for the 3 kb fragment of contig C (A1 x L1; Table 3.2), mapped to the sex chromosomes of *P. vitticeps*, tested on five other agamid species. Panel C: Amplification using primers for a nested 522 bp fragment of contig C (F x H2; Table 3.2). M = male, F = female, N = negative control. Molecular weight markers are shown on left.

agamid species from outside the *Pogona* genus. Interestingly, a faint band of the size expected for the W chromosome product for *P. vitticeps* amplified for the female *A. nobbi*, suggesting there may be significant homology between the sex chromosomes of *Pogona* and *A. nobbi*.

PCR amplification with the primers (A1 and L1; Table 3.2) used to amplify the 3 kb fragment of *P. vitticeps* sex chromosome sequence (Figure 3.3) gave products of quite varying sizes for the five species outside the *Pogona* genus (Figure 3.8B), but did amplify a 3 kb product for the *P. barbata* female. The amplified product was considerably larger for the male *P. barbata*, but other experiments have amplified a 3 kb product in other males of this species (data not shown), implying intraspecific variation in this sex chromosome sequence, at least for *P. barbata*. In contrast, PCR amplification with primers which amplify a 522 bp fragment in *P. vitticeps* gave quite similar sized products for all the agamid species tested (with the exception of *Lophognathus longirostris*, and the male *A. nobbi*), giving a strong indication that other Australian agamids may have sequences homologous to the sex chromosome sequences of *P. vitticeps* (Figure 3.8C).

Discussion

Agamid lizards hold considerable promise as a model vertebrate group for deciphering the processes and genomic changes underpinning the evolution of sex-determining mechanisms and sex chromosomes. The comparative analysis of agamid sex chromosomes, and the identification of their homologues in agamids with TSD, necessitates the development of comparative genomic tools, including hybridisation probes for conserved sex chromosome sequences. We used a combination of molecular genetic and cytogenetic approaches to isolate and verify sex chromosome sequences from the Central bearded dragon, *Pogona vitticeps.* From a starting point of putative W and Z chromosome AFLP markers, differing in size by a single base pair, we expanded the known sex chromosome sequence from only 50 bp to 6.7 kb (2.2 kb of contig A, plus 4.5 kb of contig C). Although we were unable to confirm an additional 1.7 kb contig (B) as sex chromosome sequence, we expect that further investigation will establish this contig as sex chromosome sequence, because it was generated from genome walking primers anchored in the W-chromosome specific Pv72W AFLP sequence.

The evolutionary lability of sex chromosomes and sex-determining mechanisms in reptiles (Sarre et al. 2004; Modi & Crews 2005) suggests that reptile sex chromosome markers are unlikely to have broad taxonomic applicability. For turtle and lizard taxa in particular, it seems likely that DNA sex markers will need to be developed *de novo* for species of interest. The PCR sex assay we have developed for *P. vitticeps*, potentially applicable to all *Pogona* species, is one of very few DNA sex tests reported for reptiles, and it will facilitate further incubation experiments aimed at elucidating the interaction between sex chromosomes and temperature in the sex determination of *P. vitticeps.* The only other W chromosome sequence reported for a lizard is a RAPD marker isolated from the Komodo dragon (*Varanus komodoensis*, Varanidae), and this was similarly converted into a PCR sex test (Halverson & Spelman 2002). The Komodo dragon sex test does not identify sex for agamid lizards (i.e. true 'dragons') (A.E. Quinn, unpublished data), but it does diagnose sex in at least one other varanid species (*V. rosenbergii*; W. Smith, pers. comm.). A PCR sex test has also been developed from a Y chromosome AFLP marker for an Australian skink lizard (*Bassiana duperreyi*) but this marker is yet to be tested beyond this species (A.E. Quinn et al., unpublished data).

Few other sex chromosome sequences have been identified for reptiles, but the notable exceptions are in snakes. Repetitive satellite sequences are interspersed throughout the chromosomes of snakes in high copy number (Singh et al. 1976; 1980), but are concentrated in particularly high density on the W chromosome, especially in those taxa where the sex chromosome pair is highly differentiated (Solari 1994). Eleven functional genes were recently mapped to the Z and W chromosomes of three snake species (Matsubara et al. 2006). These genes are not believed to be involved in sex determination, and map to autosomes in either the chicken or human genomes. There have been no reports about the gene content of the sex chromosomes in turtles and lizards, although X and Y chromosome sequences are expected to emerge from the whole genome sequencing project for the green anole lizard (*Anolis carolinensis*, Iguanidae) (http://www.broad.mit.edu/models/anole). We found no evidence for coding sequences in the isolated *P. vitticeps* sex chromosome sequences, which appeared to be mostly unique sequences with the notable exception of the CR1-like repetitive element. CR1 belongs to the non-LTR (Long Terminal Repeat) class of retrotransposons, and CR1-like elements appear to be ubiquitous in vertebrates (Vandergon & Reitman 1994; Kajikawa et al. 1997; Poulter et al. 1999; Jurka 2000), and are also present in invertebrates (Drew and Brindley 1997). It is possible that the amplified 3 kb probe,

which contained the sequence for the 188 bp CR1-like element, hybridised preferentially to CR1-like elements on the Z and W chromosomes. If so, that would imply that this repetitive element is concentrated in high copy number on the sex chromosomes of *P. vitticeps*, relative to the autosomes, or it is not present at all on the autosomes. Further FISH experiments, with a sex chromosome probe lacking this repetitive element, will be required to address this question.

This is the first time the Z chromosome has been identified for *P. vitticeps* chromosome and confirms our previous supposition (Ezaz et al. 2005) that the Z was a microchromosome because there were no unpaired chromosomes visible at meiosis, and no evidence for heteromorphism in the macrochromosomes. Our data suggest that the W and Z chromosomes of *P. vitticeps* are highly differentiated with the microdissected W chromosome probe hybridising poorly to the Z chromosome and a high degree of heterochromatinisation of the W chromosome evident. In spite of this, we have isolated Z/W sequences that are highly homologous. The hybridisation signal on a single pair of microchromosomes only is also consistent with the presumption that *P. vitticeps* has a simple ZZ/ZW sex chromosomal system, rather than a multiple sex chromosome system as exhibited by some lacertid lizards with female heterogamety (Olmo et al. 1987).

There was some indication that the 4.5 kb fragment (contig C) represents sequence from a pseudoautosomal region of the sex chromosomes, where meiotic recombination between the Z and W chromosomes still occurs. Although the primer J site was W chromosome-specific within the 28°C clutch, in two of six wild-caught males this primer site was evidently on the Z chromosome. This could be explained by Z-W recombination at a point between the AFLP locus Pv72W and the primer J site in the matrilineal ancestry of these two males. The primer J site was also present in the single female captured in the wild that was found to lack the Pv72W AFLP marker (Figure 3.1), which could be explained by Z-W recombination that broke the linkage between the Pv72W locus and the non-recombining region of the W chromosome, but retained the linkage of this region with the primer J site. The CR1-like repetitive element sequence for this particular female also included the additional 14 bp of sequence which was present in the Z chromosomes sequence of two males tested, but absent from the Z/W sequences of two other females. Taken together, these observations imply that the AFLP marker Pv72W is W-linked, rather than W-specific, in which case there will not be 100% correlation between the presence of this marker and the ZW karyotype. We

have AFLP data for 65 animals not expected to be sex-reversed by temperature (captured in the wild, or incubated at 28°C): Pv72W amplified in 32 of 34 females and none of 31 males, so Pv72W genotype did not correlate with sex for 3.1% of the 65 animals. If the Pv72W locus is pseudoautosomal, this low rate of recombination implies it is tightly-linked (in close proximity) to the non-recombining region of the W chromosome.

An alternative explanation to recombination is that mutation in the AFLP sequence caused null amplification of the Pv72W AFLP marker for the two "ZZ" females (in the 34 tested). The fact that we have not detected Pv72W amplification in any males favours mutation as an explanation, but this may reflect the small sample size (n=31); a wider survey could reveal a male with this marker, presenting a stronger case for recombination. A more intriguing possibility is that one or both of the two "ZZ" females in question truly lacked a W chromosome, and they were therefore sex reversed. Both females were euthanised prior to the AFLP genotyping, precluding cytogenetic verification of their chromosomal sex. One female was from a clutch incubated at 28°C in which all other ZZ embryos developed as male, so temperature-induced sex reversal seems unlikely. The other female was an adult captured in the wild. It is currently unknown if sex reversal occurs naturally in *P. vitticeps* populations, but the PCR sex assay we have developed will enable such questions to be addressed. Wild-caught individuals could be genotyped rapidly using the high-throughput assay; when discordance between sex phenotype and PCR genotype is detected, cytogenetic methods could be applied to verify chromosomal sex and identify genuine cases of sex reversed animals from natural nests.

The fact that contigs A, B and C are apparently distinct chromosomal fragments and not in juxtaposition, but each contains a Pv72W sequence, suggests that Pv72W is an amplified or repetitive locus on the W chromosome. The fluorescence intensity of the Pv72W marker was conspicuously higher than for all other products in the AFLP profile (Figure 3.1A), consistent with this hypothesis. If Pv72W is indeed multilocus, that would complicate inferences about linkage between this locus and the non-recombining region of the W chromosome. Further work will clearly be necessary to fully characterise the Pv72W locus. Southern hybridisation to male and female genomic DNA, and additional FISH experiments (with subsequent C-banding), using amplified fragments of the three contigs as probes, may shed light on their chromosomal location,

and confirm if the Pv72W marker is part of an amplified sequence on the W chromosome.

Several other Australian agamids with GSD also appear to have a ZZ/ZW sex chromosomal system, with a highly heterochromatic W microchromosome (T. Ezaz et al., unpublished data). The small size of microchromosomes, and the fact that the Z chromosome has not been identified in other Australian agamids, makes it difficult to make interspecific comparisons of the sex microchromosomes on the basis of chromosomal morphology or banding patterns. Our PCR experiments aimed at amplifying homologous sequences in other agamid species indicate that the amplified 3 kb fragment of *P. vitticeps* sex chromosome sequence (from contig C) will be effective as a hybridisation probe for comparative chromosome painting. The contig A and contig B sequences are also available as potential cross-species hybridisation probes, but establishing this will require further investigation. For some GSD agamid species, it may be possible to reveal whether a homologous chromosome pair, or a different pair, are the sex chromosomes, by FIS hybridisation with the 3 kb probe and then C-banding the same slides to identify the heterochromatic W chromosome. Given the highly conserved karyotype of the Australian agamids, we anticipate this approach may reveal that the sex microchromosomes are homologous in many of these species. Comparative chromosome painting of TSD agamids with the probe may also assist in identifying chromosomes that are homologous to the sex chromosomes of *P. vitticeps*. This would be an important first step towards reconstructing the genomic changes that have occurred in evolutionary transitions between GSD and TSD in the Agamidae.

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Chapter 4

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Chapter 5

The evolutionary dynamics of sex as a threshold trait

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Quinn AE, Georges A, Sarre SD, Ezaz T, Graves JAM.

Abstract

Sex is a trait of fundamental importance to the life history of individuals of all dioecious species, including vertebrates. Commonality in sex genes and similarity in gross structure of gonads and germ cells across vertebrates hint at common underlying mechanisms of sexual differentiation, yet there is great apparent diversity in the mechanisms of sex determination. Most attempts to explain this diversity invoke the capture of sex determination through the evolution of new master sex genes or mutations conferring novel environmental sensitivity. Here we show that when sex is viewed as a threshold trait, evolution in that threshold and related attributes can drive transitions between temperature-dependent sex determination (TSD) and genotypic sex determination (GSD), between various modes of reptilian TSD, and remarkably, between male (XX/XY) and female (ZZ/ZW) heterogamety. Our model thus provides a simple unified theoretical framework for evolutionary transitions between all observed modes of genotypic and temperature-dependent reptilian sex determination.

Introduction

Sexual phenotype in vertebrates was once thought to derive from a more fundamental dichotomy in the genomes of males and females. For example, mammals have heteromorphic sex chromosomes (XX female; XY male), and determine male sex by a master gene, *SRY*, on the Y chromosome (Sinclair et al. 1990). Birds also have heteromorphic sex chromosomes (ZW female/ZZ male), where the genotypic difference between the sexes is either the double dosage of a Z-borne gene which initiates male differentiation, or the presence of a dominant W-borne gene which initiates female differentiation (Smith et al. 2007). Sex determination is brought about by a primary regulatory signal that differs between the sexes by virtue of their fundamental genotypic difference. That primary signal initiates an integrated, multi-genic cascade leading to one sex or the other, a process involving great structural and regulatory complexity.

We now know that the fundamental genotypic dichotomy in the sexes of mammals and birds is not universal among vertebrates. Indeed, other major vertebrate groups show remarkable variation in sex determining mechanisms, including many variants of chromosomal sex determination (Solari 1994), polygenic sex determination (Vandeputte et al. 2007), environmental sex determination (Bull & Vogt 1979; Ferguson & Joanen 1982; Conover 2004), and cases where environment and genotype interact to determine sex (Conover & Kynard 1981; Shine et al. 2002; Quinn et al. 2007; Radder et al. 2008). In temperature-dependent sex determination, exhibited by many reptiles (Bull 1980) and some fish (Conover 2004), there may be no genomic differences between the sexes, as egg or larval incubation temperature is the critical factor that determines sex.

Any system in which a continuously varying factor (such as temperature) determines the state of a dichotomous outcome (such as sexual phenotype) necessarily involves at least one threshold that separates the two outcomes. The conclusion that TSD in reptiles is a threshold trait is well supported by incubation experiments (Ewert et al. 1994). Thresholds are also mandatory elements of systems where multiple genes interact to yield a regulatory signal that directs development to one discrete outcome over another (Wright 1934). Indeed, the involvement of genetic modifiers of *SRY* in mammalian sex determination (Lovell-Badge et al. 2002; Vilain 2002) blurs the fundamental genetic

dichotomy between the sexes, and again necessitates some form of threshold in gene expression, signaling or reception to determine sexual phenotype. The same is true of the only non-mammalian vertebrate in which a master sex gene has been identified – Japanese medaka (Osteichthyes: *Oryzias latipes*) (Matsuda et al. 2002; Nanda et al. 2002), in which early testes development appears to require *DMY* expression above a threshold level (Otake et al. 2006). Sex in vertebrates generally, even in mammals, may therefore best be regarded as a threshold trait (Mittwoch 2006). Assuming its magnitude is heritable, such a threshold is a source of continuous underlying variation subject to genetic drift and natural selection, or to the pleiotrophic effects of selection and drift on other loci.

Here we use a ZZ/ZW dosage system, combined with temperature-induced sex reversal (Devlin & Nagahama 2002; Chardard et al. 2004; Conover 2004; Eggert 2004; Quinn et al. 2007; Radder et al. 2008), to model evolutionary transitions between TSD and GSD, and remarkably, between XX/XY and ZZ/ZW heterogamety, without invoking major structural changes or the evolution of novel sex genes. Simple adjustment of parameters of the model can also reproduce all the various modes of TSD in reptiles. Our fundamental findings apply equally well to a range of other systems involving more complex gene interactions, provided sex can still be regarded as a threshold trait.

The Model

We base our model on our recent finding that female sexual differentiation in the ZZ/ZW agamid lizard *Pogona vitticeps* (Ezaz et al. 2005) can proceed in the absence of the W chromosome and our proposition that sex in this species may be a dosage system (Quinn et al. 2007). Under our model (see Materials and Methods), a Z-borne male-determining gene, when present in double dose (ZZ), initiates a regulatory signal cascade on the sex differentiation network that leads to testes development; when present in single dose (ZW), the regulatory cascade directs ovarian development. For the purposes of the model, we further postulate that the cascade is temperature sensitive, drawing from the observation that sex can be reversed by temperature in species whose sex can also be determined by the assortment of sex chromosomes (Hattori et al. 2007; Quinn et al. 2007; Radder et al. 2008). The proposition is that the efficacy of the Z-

borne master gene for directing male sexual differentiation reduces when temperatures move to extremes (Figure 5.1). The model also incorporates a threshold that the regulatory signal, combining the effects of the Z-borne male-determining gene and temperature, must exceed to initiate male development.

The factor or factors directly influenced by temperature remain unknown, and could potentially be at any of several points in the overall regulatory cascade (Sarre et al. 2004). The exact mechanism of the threshold is also obscure. In its simplest form, it may involve a threshold in the expression of a particular gene, subsequently influencing the production or activity of other regulatory elements, the efficacy of their transmission within or between cells, or the efficacy of their reception. Regardless of its exact form, there must be a critical threshold in the regulatory processes of sexual differentiation when the gonadal primordium is balanced between ovarian and testicular fates (DiNapoli & Capel 2008).

A key parameter is the magnitude of the threshold relative to the strength of the regulatory signal for male sexual differentiation for ZZ and ZW genotypes. Either the absolute magnitude of the threshold is fixed, and the strength of the regulatory signal is subject to vary via the modifying action of autosomal genes, or the signal is fixed, with the absolute magnitude of the threshold able to vary, or both are subject to variation. Either way, we assume the magnitude of the threshold relative to the strength of regulatory signal to be heritable, subject to natural variation and thus evolutionarily labile. For example, should the threshold rise above the double-dose regulatory signal for part of the temperature domain (at the extremes), under natural selection or genetic drift, then some individuals with ZZ genotypes would enter the population as female phenotypes (Figure 5.1A-C). This systematic overproduction of female phenotypes would be resisted by frequency-dependent selection for a balanced population sex ratio (Fisher 1930). This higher threshold would influence offspring sexual phenotype for only a proportion of individuals possessing the trait (those developing at temperatures that induce reversal). Thus the effectiveness of selection against individuals with a higher threshold, counteracting the sex ratio bias, would be lessened. A more rapid population response, returning the population sex ratio toward parity, would be via a reduction in the frequency of the ZW genotype in the population. Males become the rarer sex through ZZ sex reversal, affording a reproductive advantage to ZZ females

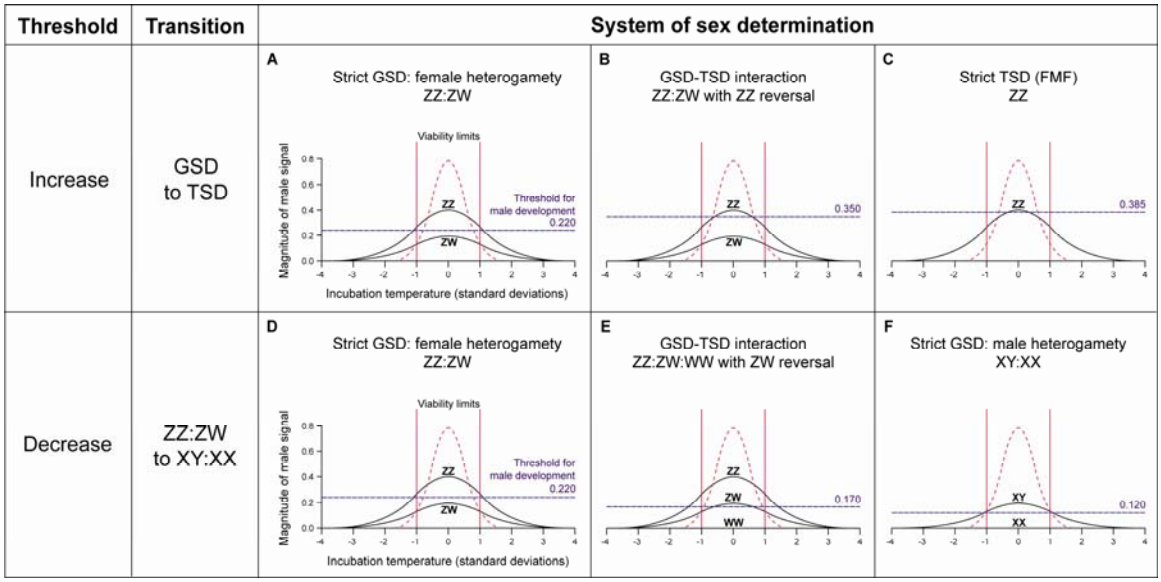


Figure 5.1 Transitions between sex-determining mechanisms through evolution in the threshold for male development. The magnitude of the sex-determining regulatory signal (black curves) is dependent on the dosage of a male-determining gene on the Z chromosome, so the magnitude in ZZ embryos is always twice that of ZW embryos. The signal magnitude also varies with temperature, with the maximum signal value (at the optimal temperature, 0) arbitrarily set at 0.4 for ZZ embryos and 0.2 for ZW embryos. Embryos develop as male only if the signal exceeds a threshold for male development (dashed blue line). Nest sites are normally distributed (dashed red curve) around the optimal temperature, and 95% of nests are incubated within the thermal limits for embryonic viability (vertical red lines). Upper panels: From an initial state of strict ZZ/ZW GSD (A; Table 5.1A), increasing the threshold causes a shift to a system of GSD-TSD interaction (B), where ZZ embryos develop as female at extreme temperatures, forcing a decrease in the frequency of the ZW genotype through frequency-dependent selection for a 1:1 population sex ratio (e.g. Table 5.1C; D). Increasing the threshold further eventually eliminates the ZW genotype (and thus the W chromosome) from the population, completing a transition to strict TSD with the *female-male-female* (FMF) pattern (C, e.g. Table 5.1E). Lower panels: From the initial state of ZZ/ZW GSD (D; Table 5.1A), decreasing the threshold causes a shift to a system of GSD-TSD interaction (E). Production of ZW males allows WW genotypes to arise in the population, which are assumed to be viable, and to have a signal magnitude of zero (because they lack the Z chromosome gene) and therefore always female. In this intermediate system, three genotypes coexist in the population (ZZ:ZW:WW, equivalent to YY:YX:XX). Decreasing the threshold further eliminates the ZZ (YY) genotype from the population, completing a transition to strict XY:XX GSD (F; e.g. Table 5.1H).

over ZW females (ZZ females have twice as many sons). Thus, evolution in the mean population value for the threshold for male development will influence both the frequency of production of sex-reversed individuals and the frequency of the ZW genotype within the population, given time to reach equilibrium.

These ideas are used in our simulation model, implemented in Microsoft Excel and Visual Basic (refer to supplementary material). Parameters that could be altered in the simulations to investigate responses were the initial genotype frequencies, the threshold value, the temperature limits for embryo survivorship, and the distribution of nests across temperatures in relation to the reaction norm of the overall male-determining regulatory signal. The responses were trends and equilibrium states for the ratio of genotypes ZZ, ZW and WW, and for the relative frequencies of concordant and sex reversed individuals.

Evolutionary dynamics of sex determination

Increasing the relative magnitude of the threshold from the starting point of the standard ZZ/ZW state reproduces different patterns of TSD (Ewert et al. 1994). The proportion of ZZ sex-reversed embryos progressively increases, with a compensating progressive reduction in the frequency of the ZW genotype at equilibrium. Initially, a modest change in the threshold causes sex reversal at extreme temperatures and dominance of genotypic sex determination (approximately 1:1 sex ratios) at intermediate temperatures (Table 5.1C). The resulting pattern of sex ratio in relation to temperature resembles that observed in the lizard *Agama impalearis* (El Mouden et al. 2001). Increasing the threshold further drives down the frequency of the ZW genotype and so diminishes the influence of genotype and the production of females at intermediate temperatures (Table 5.1D). This pattern resembles that observed in the agamid lizard *Physignathus lesueurii* (Harlow 2004; Doody et al. 2006). Ultimately, the ZW genotype, and thus the W chromosome, is eliminated and sex becomes determined entirely by temperature, yielding a pattern qualitatively consistent with the *female-male-female* (FMF) pattern of TSD that is exhibited by *Alligator mississippiensis* (Table 5.1E) (Deeming 2004). If we restrict or shift the thermal window of embryo survivorship relative to the regulatory signal, in addition to increasing the threshold, we can generate all known patterns of

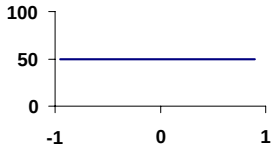
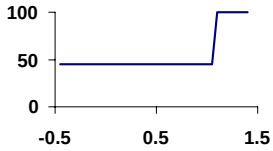
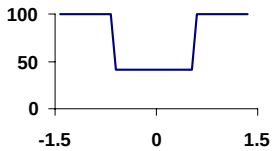
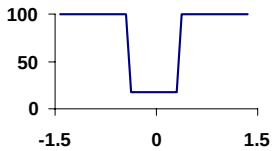
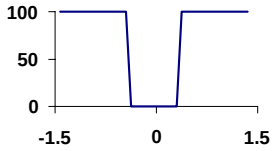
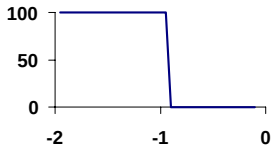
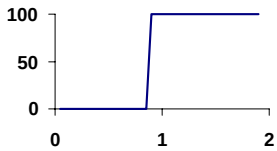
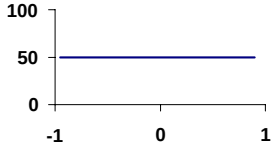
temperature influence on sex ratio in reptiles – high temperature sex reversal in *Pogona vitticeps* (Table 5.1B) (Quinn et al. 2007), the *male-female* (MF) pattern of TSD in the pig-nosed turtle *Carettochelys insculpta* (Young et al. 2004) and many other species (Table 5.1F), and the *female-male* (FM) pattern of TSD of the tuatara *Sphenodon punctatus* (Cree et al. 1995) (Table 5.1G).

Reducing the relative magnitude of the threshold, from the starting point of the standard ZZ/ZW state, results in an evolutionary transition from female heterogamety to male heterogamety (Figure 5.1D-F). If the threshold falls below the maximum level of the male-determining regulatory signal for ZW individuals, those individuals for whom the signal exceeds the threshold will develop as male. This opens the possibility of ZW females mating with sex-reversed ZW males, and the production of WW female offspring. If the WW offspring are viable, as they may well be in species showing little differentiation between sex chromosomes, the population will establish an equilibrium in the frequencies of ZZ, ZW and WW genotypes that will interact with temperature to yield a 1:1 equilibrium population sex ratio. Once the threshold falls to a level where all surviving ZW individuals have a male-determining regulatory signal that is above the threshold, only WW individuals will be female. Production of ZZ individuals will fall to zero, and the population then has ZW/WW male heterogamety, differing only semantically from XX/XY heterogamety. Remarkably, progressive reduction in the relative magnitude of the threshold has shifted the population from a ZZ/ZW dosage system to an XX/XY system with a master sex determining gene on the Y chromosome. The intermediate state remains a two-factor system (e.g. ZZ/ZW/WW; Figure 5.1E) rather than a multiple factor system. By simply manipulating the threshold for male-determination, we have charted a transitional pathway between what were previously regarded as distinct chromosomal modes of sex determination.

Transitions along the continuum of states linking TSD to ZZ/ZW GSD to XX/XY GSD are impeded by processes that may well result in increased frequency of particular states in nature. For example, once a population has evolved strict TSD, a downward shift in the male (or female) threshold cannot reverse the loss of the W chromosome. A switch back to a system with a major genotypic component would require the evolution of a new sex-determining gene, presumably through mutation or duplication of a gene in the sexual differentiation network. The population might re-acquire the previous W

Table 5.1 (facing page) Model responses for various values of the male-determining threshold. By adjusting the threshold value from the starting point of the standard model (A), the thermal window of embryo survivorship, and the nest site distribution, we are able to generate patterns of sex determination qualitatively similar to all known patterns in reptiles. References: 1) Matsubara et al. 2006; 2) Kawai et al. 2007; 3) Quinn et al. 2007; 4) Harlow & Taylor 2000; Warner & Shine 2005; 5) El Mouden et al. 2001; 6) Harlow 2004; 7) Deeming 2004; 8) Ewert et al. 2005; 9) Young et al. 2004; 10) Ewert & Nelson 1991; 11) Mitchell et al. 2007; 12) Georges 1988; Ezaz et al. 2006b. References 1 and 2 are reports of heteromorphic sex chromosomes in these species, and the reaction norm is assumed in the absence of incubation data.

Table 5.1

	System of sex determination	Threshold (viability limits)	Genotypic frequencies (male:female sex ratio)	Nest site distribution: std deviation (nest survival)	Reaction Norm % Females vs Temperature	Examples
A	Classic ZZ:ZW GSD	0.22 (-1.0, 1.0)	50% ZZ 50% ZW (1:1)	0.5102 (95%)		*Elaphe quadrivirgata* [1] *Pelodiscus sinensis* [2]
B	ZZ:ZW GSD with ZZ sex reversal	0.21 (-0.5, 1.5)	55% ZZ 45% ZW (1:1)	0.5102 (95%)		*Pogona vitticeps* [3]
C		0.32 (-1.5, 1.5)	58% ZZ 42% ZW (1:1)	0.4559 (99.9%)		*Amphibolurus muricatus* [4] *Agama impalearis* [5]
D		0.37 (-1.5, 1.5)	82% ZZ 18% ZW (1:1)	0.4559 (99.9%)		*Physignathus lesueurii* [6] *Crocodylus porosus* [7] *Chelydra serpentina* [8]
E	FMF-type TSD	0.37 (-1.5, 1.5)	100% ZZ 0% ZW (41:59)	0.7653 (95%)		*Alligator mississippiensis* [7]
F	MF-type TSD	0.265 (0, -2.0)	100% ZZ 0% ZW (42:58)	0.5102 (95%)		*Carettochelys insculpta* [9] *Trachemys scripta* [10]
G	FM-type TSD	0.265 (-2.0, 0)	100% ZZ 0% ZW (42:58)	0.5102 (95%)		*Sphenodon punctatus* [11]
H	Classic XX:XY GSD	0.11 (-1.0, 1.0)	50% ZW 50% WW (1:1)	0.5102 (95%)		*Chelodina longicollis* [12]

chromosome (conceivably even the same sex-determining locus), or an entirely novel sex chromosome pair may arise. A strict TSD system can thus serve as a viable transitionary stage in the evolution of an entirely new mechanism of genotypic sex determination (Bull 1983; Sarre et al. 2004).

In a similar vein, once a population has evolved strict GSD, various processes lead to the degeneration of the Y chromosome (or the W chromosome in the case of female heterogamety) (Charlesworth & Charlesworth 2000). The loss of active genes whose functional complements occur only on the X (or Z) leads to reduced viability or lethality in YY (or WW) individuals and creates a barrier to transitions between XX/XY and ZZ/ZW systems along the transitional continuum. Indeed, widespread prevalence of homomorphic sex chromosomes in the lower vertebrates (Ezaz et al. 2006a) may indicate relatively frequent rearrangements in the sex determining genes and the chromosome pairs involved because homomorphy implies that there has been insufficient time for degeneration of the W or Y chromosomes (Tanaka et al. 2007). In our model, heterogametic transitions exchange the Z and Y chromosomes and the X and W chromosomes, which would disrupt the processes leading to loss of functionality of genes on the Y or W chromosome. Regular transitions beween male and female heterogamety, resulting from evolutionary flux in the threshold, or alternatively, the maintenance of three genotypes within a population (ZZ/ZW/WW), may explain in part the widespread occurrence of homomorphic sex chromosomes in these groups.

The qualitative behaviour of our model is not sensitive to the genic mechanism of sex determination. We have modelled a system in which a Z-borne male-determining gene doubles the male-determining regulatory signal in ZZ embryos. Alternatively, the master sex gene could be a female-determining W-borne gene that down-regulates the regulatory cascade, ensuring the signal is lower (not necessarily half) in ZW embryos (Table 5.2). The model can be further generalized to systems in which male differentiation is the default developmental pathway and a threshold must be exceeded for female sexual development, in which case an analogous continuum of sex-determining modes emerges. An XX/XY system becomes the standard state, and increasing the threshold for female differentiation produces a *male-female-male* (MFM) pattern of TSD, as opposed to the FMF pattern of TSD that emerges in the male threshold system (Figure 5.2; Table 5.2). The FMF pattern is widespread in crocodiles,

Table 5.2 Shifts in the genic mechanism of sex determination during evolutionary transitions between male and female heterogamety. Under the model, the W and X chromosomes are homologous, as are the Y and Z chromosomes, and equal fitness of genotypes within a sex is assumed. In heterogametic transitions caused by evolution in the sex-determining threshold, the genic mechanism of sex determination always changes from the double dosage (2:1) of a master gene in the homogametic sex (XX or ZZ) to the presence/absence (dosage of 1:0) of that same gene on the heterogametic chromosome (Y or W), or vice versa. This occurs irrespective of the direction of the transition, whether the model incorporates a male or female threshold for development, or whether the gene acts to determine sex by upregulating or downregulating the sex-determining signal. The three-genotypes present in the intermediate system of sex determination (ZZ:ZW:WW, equivalent to YY:YX:XX) have a 2:1:0 (or 0:1:2) dosage ratio of the sex-determining gene. In principle, this route of heterogametic transition could occur via a shift in the mean population value for the threshold, even in the absence of thermosensitivity.

Master sex gene	**Dosage of master sex gene**			**Action of master sex gene**	
	Female heterogamety ZZ : ZW	Intermediate system ZZ : ZW : WW YY : YX : XX	Male heterogamety XY : XX	Threshold for male development	Threshold for female development
Female-determining gene on W / X	0 : 1	0 : 1 : 2	1 : 2	Downregulates male signal	Upregulates female signal
Male-determining gene on Z / Y	2 : 1	2 : 1 : 0	1 : 0	Upregulates male signal	Downregulates female signal

Heterogametic transition

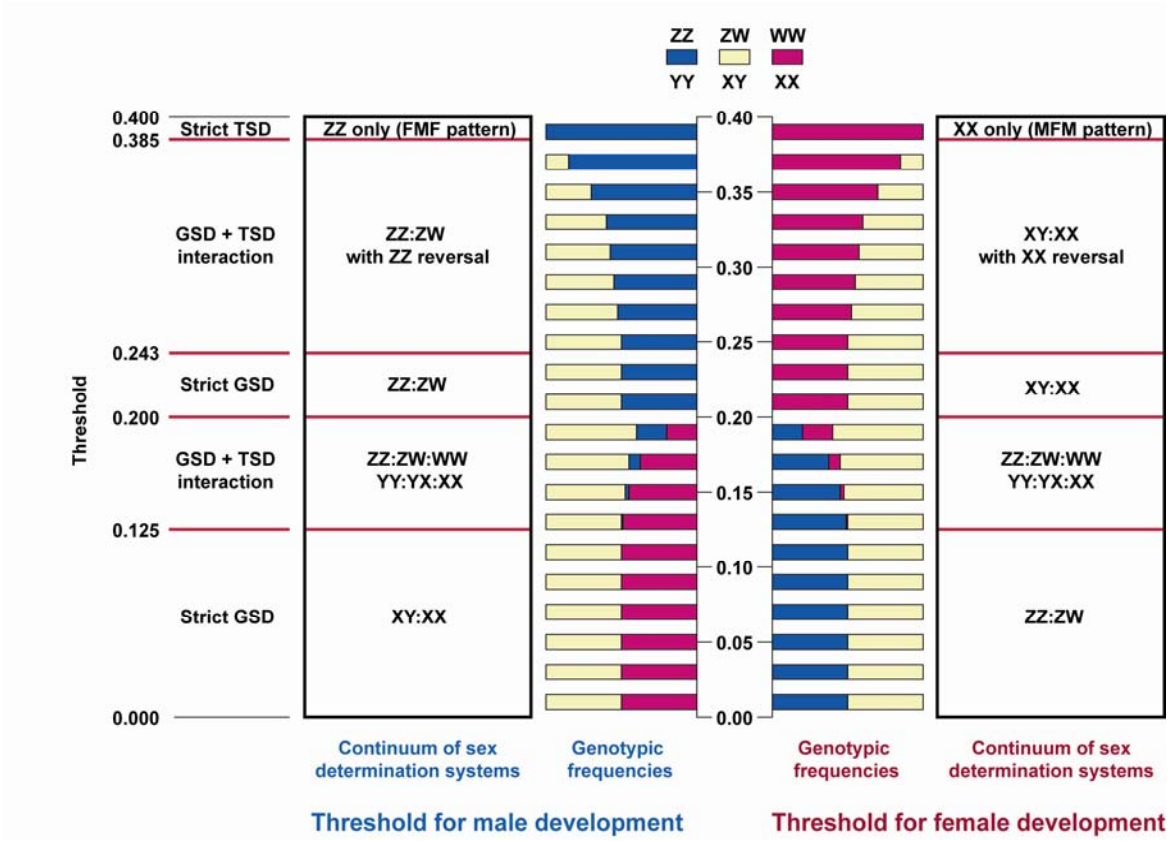


Figure 5.2 Analogous continua of sex determination systems arise from evolution in a threshold value for male or female development. In the male-threshold model (blue text), XY:XX GSD and strict TSD (FMF pattern) are at opposite ends of the continuum, with ZZ:ZW GSD as a midpoint system. In the female-threshold model (red text), ZZ:ZW GSD and strict TSD (MFM pattern) are at opposite ends of the continuum, with XY:XX GSD as a midpoint system. Threshold values (left hand side, arbitrarily scaled from zero to 0.4) indicate critical transition points (red lines) between sex-determining systems. Coloured bars show relative genotypic proportions at specific values of the threshold. Genotypic frequencies and threshold values for transition points were determined for the following model conditions (consistent with figure 5.1): viability limits = -1.0 and 1.0, nest survival = 95%, initial genotypic frequencies (ZZ:ZW:WW) = 1:1:1. Adjustment of these parameters changes the threshold values of the transition points.

turtles, and lizards (Deeming 2004; Ewert et al. 2004; Harlow 2004). The MFM pattern of TSD has not been reported for any reptile but has been reported in flatfishes (Yamamoto 1999; Luckenbach et al. 2003), which have an XX/XY system as expected under our model. At the other extreme, female heterogamety is the endpoint of the continuum, as opposed to male heterogamety that emerges in the male threshold system. For simplicity, we have and will consider ZZ/ZW heterogamety with a male-determining threshold as the initial standard condition, with corresponding argument assumed to apply to XX/XY heterogamety with a female-determining threshold as the initial standard condition.

Model Predictions

Several predictions amenable to testing by experiment and in natural systems arise from our model. One prediction is that sex determination via male and female heterogamety amongst closely related forms will involve homologous genes and chromosomes. Encouraging indications arise from studies of the only vertebrate known to have both ZZ/ZW and XX/XY populations, the Japanese frog *Rana rugosa* (Miura et al. 1998; Ogata et al. 2003). The X chromosome and the W chromosome are indeed homologous, as are the Z and Y chromosomes, a finding consistent with our model. As a test of our model, we would predict that in one population, sex is determined by the presence of a sex-determining gene on the heterogametic chromosome (Y or W), with sex determination in the other population depending on double-dosage *of that same gene* in the homogametic sex (Table 5.2). A test of this hypothesis awaits molecular characterisation of the sex chromosomes in these two populations of ***R. rugosa***.

A second prediction of our model is that there should be two pivotal temperatures, not one as is minimally required of any threshold system governing a dichotomous outcome. This is indeed observed in many reptile species (Ewert et al. 1994; Deeming 2004; Harlow 2004). Those with only one pivotal temperature can be explained by constraints on embryo survivorship with temperature. A third prediction is that an evolutionary shift in our threshold should result in a coordinated shift in both upper and lower pivotal temperatures. They would converge or diverge in concert. In the only study to date to examine this, latitudinal variation in the pivotal temperatures of the

turtle *Chelydra serpentina* shows that very pattern (Ewert et al. 2005), until now with no clear explanation. A final prediction emerging from our model is that there will be an underlying system of genotypic sex determination in many reptile species currently regarded as exhibiting TSD, such that the distinction between TSD and temperature-induced reversal of genotypic sex will be difficult to sustain. We can expect genotype and sexual phenotype to be correlated, with a magnitude varying with temperature, and this correlation should be detectable experimentally.

Discussion

We have presented a simple unified theoretical framework for evolutionary transitions between temperature-dependent and genotypic sex determination, between male and female heterogametic GSD and between all the observed modes of reptilian TSD. Transitions between heterogametic systems have long been thought to occur via various intermediate states including, ESD, haplodiploidy, polyfactorial sex determination and in particular, multifactorial sex determination, and invariably involve the acquisition of a novel master sex gene and a novel sex chromosome pair (Bull & Charnov 1977; Bull 1983, 1985; Hillis & Green 1990). What is remarkable about our model is not that these transitions are possible (Bull & Charnov 1977; Charnov & Bull 1977; Bull 1980; Bull 1981), but that they can occur without substantive genotypic innovation. They occur largely through changes in the relative magnitude of the threshold that distinguishes a male developmental trajectory from a female trajectory, without substantial structural change to the underlying genetic machinery. Under our model, the W and X chromosomes, and the Y and Z chromosomes, are homologous, and the dominant master gene involved in one heterogametic system is the same as the dosage-dependent master gene in the closely-related, but opposite system of heterogamety (Table 5.2). Thus, in transitions between ZZ/ZW and XX/XY GSD, the physical network of genes involved in sex determination remain largely unchanged. It is the magnitude of interactions among those genes governing the strength of the sex-determining regulatory signal, relative to a threshold, that alters during the transitions. This is a major departure from previous models of the transition between modes of heterogametic sex determination.

Our model considers not only strict TSD, but a range of states involving the interaction of temperature and genotype to determine sex, as has been demonstrated in two reptiles (Shine et al. 2002; Quinn et al. 2007; Radder et al. 2008) and several fish (Goto et al. 1999; 2000; Yamamoto 1999; Luckenbach et al. 2003). We have reproduced the qualitative properties of every known mode of reptilian TSD by incorporating the effects of temperature on an underlying genotypic sex determination. Examples include the agamid lizards *Agama impalearis* (El Mouden et al. 2001) and *Amphibolurus muricatus* (Harlow & Taylor 2000; Warner & Shine 2005) which produce 100% females at extremes but otherwise unexplained 1:1 sex ratios at intermediate temperatures. With the frequency of the ZW genotype free to vary, such interaction of temperature and genotype may also be the case in the very many reptiles that produce a strong but not absolute bias in male offspring at intermediate temperatures.

Commonality in sex genes and similarity in gross structure of gonads and germ cells across vertebrates hint at common underlying mechanisms of sexual differentiation, yet there is great apparent diversity in the primary mechanisms of sex determination. Certainly, much of this diversity could arise through the capture of sex determination by novel genes and chromosome pairs (Matsuda et al. 2002; Nanda et al. 2002; Takehana et al. 2007). We have shown that such genetic rearrangements, though sufficient, are not necessary to effect transitions between any of the major modes of sex determination in vertebrates. As with sex differentiation, there is potentially great commonality in the genes involved in sex determination, whether it be TSD, male heterogamety or female heterogamety. We believe that this, together with the continual discovery of new forms of fish and reptile where environment and sex genes interact, heralds a paradigm shift from viewing evolution of sex determination as transitions between disjunct modes to viewing it as a continuum of states.

Materials and Methods

Simulation Model

The overall signal, though initiated by dosage of a Z-borne male-determining gene, is governed by the activity of many genes and gene products in the sex differentiation

network each with their own particular thermal reaction norms. We therefore model the overall male-determining signal ε for ZZ individuals and ZW individuals respectively as normal functions of temperature *T* where, without loss of generality, temperature *T* is measured in units of standard deviation.

$$\varepsilon_{ZZ}(T) = N(\mu,\sigma) = N(0,1) \quad \text{..... (1)}$$

$$\varepsilon_{ZW}(T) = \frac{\varepsilon_{ZZ}(T)}{2} \quad \text{..... (2)}$$

Females select their nest sites to be distributed normally with respect to temperature

$$\eta(T) = N(0,(T_H - T_L)/5.16) \quad \text{..... (3)}$$

We assume that female nest site choice and thermal tolerance of embryos to temperature have co-evolved to ensure that the temperatures embryos usually experience (arbitrarily set in Equation (3) at 99% of nests) fall within the range conducive to embryo survivorship defined by upper and lower limits T_H and T_L, respectively.

The first phase of the simulation begins with 100 individuals at the point of conception. Of these, *m* have the ZZ genotype and $f = 1 - m$ have the ZW genotype. Their sexual phenotype depends upon whether the signal exceeds a threshold τ. For the ZZ individuals, we have

If $\tau \geq \max(\varepsilon_{ZZ}(T))$ then

$$M = 0$$

$$F = m\int_{T_L}^{T_H} \eta(T)dt$$

If $\tau < \max(\varepsilon_{ZZ}(T))$ then

$$M = m\int_{T_0}^{T_1} \eta(T)dt$$

$$F = m\left(\int_{T_L}^{T_H} \eta(T)dt - \int_{T_0}^{T_1} \eta(T)dt\right)$$

where

$$T_0 = \max\left(-\varepsilon_{ZZ}^{-1}(\tau), T_L\right)$$

$$T_1 = \min\left(\varepsilon_{ZZ}^{-1}(\tau), T_H\right)$$

$$0 \leq \tau \leq \max(\varepsilon_{ZZ}(T))$$

The equations for the ZW individuals are similarly defined, with the genotypic frequency *f* replacing *m* and ε_{ZW} replacing ε_{ZZ}. Using these formulae we can calculate the proportion of surviving individuals that are phenotypic ZZ males or phenotypic ZW females (i.e. concordant) and those that are phenotypic ZW males or phenotypic ZZ females (i.e. sex reversed).

The second phase of the analysis was to simulate random mating between the genotypes in strict accordance with their phenotypic sex, that is, assuming that concordant and sex reversed individuals are equally fit. The offspring from these matings were reduced to 100 while maintaining relative genotypic proportions, to simulate a constant population size. Phase 1 of the simulation was then repeated on these new 100 genotypes. The whole process was repeated for 30 generations to allow the system to come to an equilibrium state. In this way, both the action of temperature-induced sex reversal and frequency-dependent selection operating on the population sex ratio were incorporated into the analysis. In some analyses, WW individuals were generated. These simulations required seeding with a small initial non-zero frequency for the WW genotype, and we assumed that WW individuals were viable phenotypic females of equal fitness to the other genotypes.

Chapter 6

Synopsis

In this chapter, I review the results of the study in the context of the broad aims and specific experimental objectives outlined at the end of the Introduction. I then discuss the implications of the study for the evolution of reptile sex determination and outline specific experiments to further this research. The synopsis concludes with a summary of the directions for future research, suggested or made possible by this study.

Review of study aim and objectives

My primary aim in this study was to advance understanding of the genetic basis of evolutionary transitions between sex-determining mechanisms in reptiles, in particular, transitions between GSD and TSD. I addressed this aim through both experimental and theoretical approaches.

In the former approach, I tested the hypothesis that incubation temperature and genotype interact to determine sex in some reptiles. The first experimental objective, which was critical to enabling such a test, was to develop DNA sex markers for reptile species in which GSD and TSD might co-occur. The study organisms were a pair of distantly-related species of Australian lizards, one exhibiting male heterogamety and the other exhibiting female heterogamety. I isolated a Y chromosome-linked marker for *Bassiana duperreyi* (Scincidae), by applying an AFLP screening strategy designed to facilitate detection of sex-linked markers, and subsequently developed the Y chromosome sequence into a single-locus PCR sexing assay for this species (Chapter 2). This approach was applied again to isolate homologous Z and W chromosome markers for *Pogona vitticeps* (Agamidae), and a single-locus PCR sexing assay was developed from the W chromosome sequence (Chapter 3). I applied the PCR sex assay to genotype hatchlings of *B. duperreyi* from controlled incubation treatments, and demonstrated that low incubation temperature can cause female-to-male sex reversal in some XX embryos of this lizard (Appendix 3). I applied the W chromosome AFLP marker to genotype hatchlings of *P. vitticeps* from controlled

incubation treatments, and demonstrated that high incubation temperatures can cause male-to-female sex reversal in ZZ embryos of this lizard (Chapter 4).

In the theoretical approach, I extended these empirical findings to advance a novel theory for the evolution of reptile sex determination (Chapter 5). A model was derived from the observation that in both lizards, an extreme of incubation temperature causes sex reversal of one genotypic sex only, implying that there is a threshold for the development of one of the sexes (females in *Bassiana*, males in *Pogona*), and that there is temperature-sensitivity in that sexual differentiation pathway. By viewing sex as a threshold trait, it was possible to integrate GSD (with temperature-sensitivity at extremes) and strict TSD into a single framework. In this model, increasing the threshold for sexual development causes an evolutionary transition between GSD and TSD. Decreasing this threshold causes a transition between female and male heterogamety. Simulation modelling supported my hypothesis, showing that simple quantitative shifts in the threshold can chart a continuous evolutionary pathway between XY, ZW, and TSD mechanisms, conventionally considered to be disjunct modes of sex determination.

Another aim of my study was to develop further the potential of the Australian agamid lizards as a model system for investigating the molecular and evolutionary basis of reptile sex determination. In this context, the demonstration of thermosensitivity in the sex determination of *P. vitticeps* (Chapter 4), and the development of a DNA sex assay for the *Pogona* genus (Chapter 3), are important advances. The DNA sex assay will facilitate further experiments to elucidate the interaction between temperature and genotype in the sex determination of this genus (I have preliminary data which indicates that high temperature sex reversal of ZZ embryos also occurs in *P. barbata*).

To further the utility and characterisation of the *P. vitticeps* sex marker, I extended the AFLP sex marker sequences into larger fragments of the sex chromosomes by genome walking (Chapter 3). Physical mapping of an extended 3 kb sequence identified the W microchromosome, and for the first time in this species, the Z microchromosome. PCR amplification indicated the presence of homologous sequences in other Australian agamid species, implying that the extended sex chromosome sequence from *P. vitticeps* should prove effective as a comparative genomic tool for investigating the relationship of sex

Chapter 4

Temperature sex reversal implies sex gene dosage in a reptile

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Sex is determined by genes on sex chromosomes in many vertebrates (genotypic sex determination; GSD), but may also be determined by temperature during embryonic development (temperature dependent sex determination; (TSD) (Bull 1983). In reptiles, sex determination can involve GSD with XX/XY sex chromosomes (male heterogamety; as in mammals), GSD with ZZ/ZW sex chromosomes (female heterogamety; as in birds), or TSD (Bull 1983; Valenzuela & Lance 2004). The distribution of TSD and GSD across reptiles suggests several independent evolutionary transitions in sex determining mechanism (Valenzuela & Lance 2004; Sarre et al. 2004), but transitional forms have yet to be demonstrated. We show that high incubation temperatures reverse genotypic males (ZZ) to phenotypic females in the Australian central bearded dragon lizard (*Pogona vitticeps*), which, like birds, has GSD with female heterogamety (Ezaz et al. 2005). Temperature thus overrides gene(s) involved in male differentiation.

We incubated eggs of *P. vitticeps* at constant temperatures between 20-37°C. No embryos survived to hatching at 20°C. Between 22-32°C, sex ratios did not differ significantly from 1:1, a response consistent with GSD (Figure 4.1A). However, between 34-37°C, there was an increasing female bias, suggesting that temperature was overriding genotypic sex in some males. Differential mortality cannot explain the deviation of the sex ratio from 1:1 at temperatures where survivorship allowed a test (34.5, 35, and 36°C), as the deviation remains significant even when all mortalities are conservatively scored as male (Table 4.1).

We isolated a female-specific DNA marker for *P. vitticeps* by screening Amplified Fragment Length Polymorphisms (AFLP) (Vos et al. 1995), to enable a test for sex reversal (Figure 4.1B). To verify that the AFLP marker (designated Pv72W, accession no.

chromosomes in the Australian agamids. Physical mapping of this sequence in TSD agamids should identify chromosomes which are homologous to the sex chromosomes of *P. vitticeps*. That will be a first step towards reconstructing the chromosomal and genomic changes that occur in evolutionary transitions between GSD and TSD.

Implications of the study

DNA sex markers in reptiles

Very few sex-linked DNA sequences have been reported for reptiles prior to this study. The *Bassiana* Y chromosome marker is the first reported Y chromosome sequence for a reptile. Outside snakes, the *Pogona* sex chromosome sequences are the first Z chromosome sequence isolated, and the second W chromosome sequence isolated for reptiles. This study is also the first report of AFLP being applied to isolate sex markers in reptiles, reinforcing that it is an effective technique for detecting sex markers in a taxonomic group where variation in the sex chromosome pair amongst lineages is to be expected. In *B. duperreyi*, the X and Y sex chromosomes are distinguishable on the basis of gross morphology (reduced Y size) (Donnellan 1985), indicating an extremely advanced stage of differentiation. The Z and W microchromosomes of *P. vitticeps* are not morphologically distinguishable, but chromosome banding identifies the W chromosome (Ezaz et al. 2005). The apparent difference in sex chromosome differentiation between the two lizards was reflected in the number of AFLP primer combinations required to detect a sex-linked marker: 44 for *B. duperreyi* and 96 for *P. vitticeps.* In many reptiles with cryptic sex chromosomes, the level of differentiation may be very slight, necessitating screening of hundreds of AFLP primer combinations to detect sex-linked sequences. Other molecular genetic approaches such as subtractive hybridisation techniques may be a more practical alternative if the sequence difference between the male and female genomes is expected to be extremely subtle.

The DNA sex test developed for *P. vitticeps* appeared to be applicable to congeneric species, whereas the DNA sex test developed for *B. duperreyi* is yet to be tested on other species. Y and W sex chromosome sequences may diverge rapidly in distinct evolutionary lineages, which means that sex chromosome sequences isolated for a species of interest

may not have wide taxonomic applicability as sex markers. Evolutionary lability of sex-determining mechanisms is likely to exacerbate this issue for reptiles.

Temperature-induced sex reversal in reptiles

An important assumption made in this study was that the system of sex determination in reptiles with temperature-induced reversal of chromosomal sex is in some respects representative of transitional forms intermediate to GSD and TSD. This is not to say that species such as *Bassiana* and *Pogona* are necessarily in the process of an evolutionary transition, but that temperature-induced sex reversal in these species reveals something of the nature of genotype-temperature interaction that could pervade transitional systems. The thermosensitivity exhibited by these species may be a relic of an ancestral state of strict TSD (e.g. Valenzuela 2008). Alternatively, instability of genotypic sex determination at extreme temperatures may be a universal characteristic of vertebrate sex determination (*because* of the threshold nature of sexual differentiation) and it may provide the raw material for selection for TSD. Three conditions must be met for the evolution of TSD via this path: (1) heritable variation in the degree of thermosensitivity, such that a proportion of individuals are sex reversed under natural incubation conditions; (2) equal (or higher) fitness of sex reversed genotypes; and (3) selection for increased thermosensitivity (directly or via selection for pleiotropic effects). I will consider each of these conditions in turn.

First, direct selection for increased thermosensitivity cannot occur if sex reversal is never realised under natural conditions. Sex reversal may not occur for most organisms simply because the thermal limits for embryonic viability are too narrow (possibly the case for mammals and birds), or the requisite temperatures are not naturally encountered during embryogenesis. In the case of *P. vitticeps*, sex reversal was demonstrated for eggs incubated at constant temperatures above 33°C. There are no data available on the thermal conditions in natural nests of this species, but it is plausible that some nests in the Australian arid zone habitat of this lizard may be exposed to high temperatures. Notably, in the Australian agamid *Lophognathus temporalis* (which has FMF-type TSD), constant 33.0°C incubation produces a sex ratio with no significant bias, but a cyclical incubation temperature of 33.7 ± 5°C is sufficient to produce females only (Harlow 2004). This suggests that cyclical temperature treatments with a less extreme nominal mean

temperature than constant 35°C treatment may be sufficient to induce 100% female production in *P. vitticeps*. Further work is therefore required to determine natural nest conditions of *P. vitticeps*, and to test for sex reversal under more realistic cyclical temperature conditions in the laboratory. It is premature to presume that TSD can occur naturally in *P. vitticeps*, but there does appear to be variation in thermosensitivity, since not all ZZ embryos were sex reversed in the high temperature clutches. Whether or not this variation is genetic, and thus heritable, warrants further investigation. The particular significance of demonstrating temperature-induced sex reversal for this lizard is that it has revealed an important similarity with the pattern shown by TSD relatives – females only at the highest viable temperatures – implying commonality in the underlying molecular pathways of sex determination. The evidence for natural co-occurrence of GSD and TSD is much stronger for *B. duperreyi*, since sex reversal was demonstrated for cool incubation conditions which emulate field nests of this species at the highest elevations of its range. Again, there appears to be (possibly heritable) variation in thermosensitivity, since only a proportion of XX embryos were sex reversed by cool incubation treatment. Future experiments could be directed at elucidating the basis of this variation in *B. duperreyi*, and verifying the incidence of XX reversal in natural nests, by applying the PCR sex assay.

Second, the sex chromosomes in *Pogona* and particularly in *Bassiana* are well differentiated (Ezaz et al. 2005; Donnellan 1985). The accepted theory for sex chromosome degeneration posits that sex reversed individuals of these species will have reduced fitness, hence there should be selection against thermosensitivity, impeding any transition to TSD. It may be no coincidence that in both these lizards, it is the homogametic sex that is reversed by temperature. Production of potentially inviable YY or WW embryos are unlikely to occur. XX males are also expected to have reduced fitness when the sex chromosomes are heteromorphic, arising from the accumulation of male-advantage genes on the Y chromosome. Consequently, XX males are infertile or have lower fitness than XY males, which implies selection against thermosensitivity. The corresponding argument applies to ZZ females. (The gross morphology of gonads and reproductive ducts appear normal in sex reversed hatchlings of both *Bassiana* and *Pogona*, and histologically normal in the case of *Bassiana* (Radder et al. 2008), but the fertility and relative reproductive fitness of these animals are unknown. This question needs to be addressed).

In this context, it is puzzling that XX sex reversal is expected to occur naturally in *Bassiana*. Why do XX males arise at all, unless they are fertile and have sufficient reproductive fitness to avoid negative selection? The Y chromosome in this lineage may have degenerated in such a way that XX males have only a slight disadvantage relative to XY males. Degeneration of the Y chromosome can occur when sexually-antagonistic genes linked to the male-determining locus afford only a very slight advantage to males, but are detrimental to females (Rice 1987). Interestingly, empirical data indicates differential fitness of the sexes in *Bassiana* with respect to incubation temperature: warmer temperatures are optimal for female development and lower temperatures are optimal for male development (Shine et al. 1995). Thus, at lower incubation temperatures, XX embryos may have higher relative fitness if they develop as male instead of female, eliciting selection for XX sex reversal (thermosensitivity). Under such a scenario, there may be a complex interaction of opposing evolutionary pressures on the XX and XY genotypes. The result could be that the progress of Y degeneration is impeded, or constrained in such a way that the disadvantage of XX males is minimised or negated. Alternatively, the benefit afforded to XX embryos through temperature-induced sex reversal may be sufficient to counteract any disadvantage XX males suffer because they lack male-benefit genes on the Y chromosome. An evolutionary balance may be struck in GSD species with natural sex reversal, such that GSD and TSD are maintained at an equilibrium where strict TSD does not evolve, and sex chromosome differentiation is limited in how far, or how rapidly, it can proceed.

If *Bassiana* is in the process of a transition to TSD, it appears destined for a sex ratio pattern unlike that seen in most TSD lizards. Male-biased production at the lowest viable temperatures is exhibited by many TSD turtles (MF-type), but in crocodilians and TSD lizards, the lowest temperatures produce a female bias. *Bassiana* is possibly an exception because it is a montane species and therefore experiences relatively cold developmental temperatures for a lizard. Alternatively, this apparent irregularity may simply reflect the paucity of robust data on lizard TSD patterns – production of males at low temperatures may be common, but unreported, for lizards.

Evolutionary transitions between GSD and TSD

The third condition for the evolution of TSD is that the level of thermosensitivity in the population must increase. Under the transition model I have proposed, two evolutionary processes could drive a non-adaptive transition to TSD by increasing the mean population value of the sex-determining threshold: drift in the frequency of genes governing the threshold magnitude, or selection for pleiotropic effects of those genes. It is usually assumed, however, that the level of thermosensitivity in a population will increase through direct selection for this trait.

The empirical evidence for the adaptive significance of TSD in the Australian agamid *Amphibolurus muricatus* (Warner & Shine 2008) is consistent with the evolutionary transition model, if it is assumed that the ancestral condition for this species is ZW GSD, as in the related *Pogona* genus. Male fitness in *A. muricatus* is optimal at intermediate incubation temperatures, so at extreme temperatures, ZZ embryos would fare better reproductively if they developed as female, rather than male, resulting in selection for ZZ reversal. As modelled, ZZ reversal would force a decrease in the ZW genotype in the population, leading to the FMF pattern of TSD exhibited by *A. muricatus*. Although female fitness was optimal at the highest incubation temperatures in this species, providing further impetus for FMF TSD to evolve, this process could still occur if female fitness was invariant with respect to temperature, provided that male fitness was optimal at intermediate temperatures. The upper pivotal temperature, and high temperature females, may rarely be realised in natural nests of *A. muricatus* (D.A. Warner, pers. comm.). Thus, the crucial fitness differential may be for males, between low and intermediate temperatures, across which, female fitness does not vary (Warner & Shine 2008).

The apparent absence of the *male-female-male* (MFM) pattern of TSD in reptiles, and the prevalence of the FMF pattern, is striking. This could indicate that evolution of TSD is usually adaptive, and occurs because intermediate temperatures are optimal for male fitness, but rarely for female fitness (at least in lizards). The model implies that evolution of the MFM pattern from XY GSD is as straightforward as the evolution of the FMF pattern from ZW GSD, but XY species can only evolve to FMF TSD via an intermediate state of female heterogamety. Although weak sex chromosome differentiation was assumed for the

purposes of the model, the ZW GSD to TSD transition may still be possible in species where the sex chromosomes are moderately differentiated, for the reasons discussed in regard to persistence of homogametic sex reversal in *Bassiana*. The pathway from XY GSD to FMF TSD (via ZW GSD) would be impeded, however, because the initial heterogametic transition involves production of the YY (ZZ) genotype, assumed to be inviable when the Y chromosome has degenerated. It follows that XY species, but not ZW species, may be constrained from evolving to FMF TSD when the sex chromosomes are more than just weakly differentiated. This hypothesis predicts that female heterogamety and FMF TSD systems will be associated with each other in evolutionary lineages more often than male heterogamety and FMF TSD. Conversely, MFM-type TSD should be more often associated with male heterogamety. Available data on the occurrence of these three mechanisms provide some support for these predictions. In lizards, female heterogamety and TSD occur in the Agamidae, and there are unsubstantiated reports of FMF TSD in the Lacertidae (ZW) and the Varanidae (ZW) (Harlow 2004). All three mechanisms have been reported in the Gekkonidae, offering little information. In the Scincidae, XY GSD is widespread, and the reported incidences of TSD do not match the FMF pattern. In the viviparous skink *Eulamprus tympanum*, for instance, the highest temperatures produce males only but lower temperatures produce an even sex ratio (Robert & Thompson 2001) (even lower temperatures have not been tested, but could conceivably also show a male bias, producing the MFM pattern). In *Bassiana*, the bias is towards males at the lowest temperatures, implying that a transition from its XY system to strict TSD would result in either an MF or MFM pattern. In the lizard families Teiidae, Gymnophthalmidae and Iguanidae, where only XY GSD has been reported, there is no clear evidence for TSD. On a broader phylogenetic scale, birds and crocodilians are sister groups in the archosaurian lineage, and they display ZW GSD and FMF TSD, respectively. Beyond reptiles, the MFM pattern of TSD is apparent in flatfishes, which have male heterogamety.

Although the available data are therefore consistent with these model predictions, the data are very limited; the system of TSD or heterogamety has been determined reliably for only a small fraction of reptiles. The hypothesis that XY GSD is constrained from evolving to FMF TSD in lizards could be tested indirectly through investigation of taxonomic groups exhibiting alternative mechanisms of heterogamety, but containing species inhabiting equivalent ecological niches. The Agamidae and Iguanidae present such an opportunity

(Pianka & Vitt 2003; Vitt & Pianka 2005; Melville et al. 2006). No evidence exists for TSD in the iguanids (XY), but few species have been tested thoroughly. Iguanids that are ecological equivalents of agamids with TSD (and presumably subject to similar selective pressures) could be targeted specifically in a search for the existence of TSD within this group. Failure to detect FMF TSD in such species, or indeed any lizard group where XY GSD is widespread, would lend support to the hypothesis.

Evolutionary transitions between male and female heterogamety

Modelling the evolution of sex as a threshold system revealed a novel pathway for heterogametic transitions, via an intermediate state of GSD-TSD interaction. This novel transitional route has four fundamental differences to previous models:

1) The transition occurs through changes in the relative magnitude of the threshold that distinguishes a male developmental trajectory from a female trajectory, without substantial structural change to the underlying genetic machinery.
2) The intermediate state is a two-factor (rather than a multifactorial) system, where three genotypes coexist in the population (e.g. XX:XY:YY).
3) Rather than a novel sex chromosome pair arising, the sex chromosome pair is retained. The W and X, and the Z and Y chromosomes, are homologous.
4) Previous models describe shifts between systems governed by a single copy of the sex-determining gene in the heterogametic sex only, such that a system involving a Y-borne male-determining gene is replaced by a system involving a W-borne female-determining gene, or vice versa. In the model I have proposed, the transition switches between a system governed by the presence of a master gene in the heterogametic sex (i.e. a dominant Y or W gene) and a system of dosage of that same gene in the homogametic sex (i.e. X or Z gene dosage). This is true for either a male or female threshold system.

In effect, the model implies that ZW and XY systems are two sides of the same coin. Some support is provided by the observation that the Z and Y chromosomes, and the W and X chromosomes, are indeed homologous in the only vertebrate species (the frog *Rana rugosa*) reported to have distinct ZW and XY populations (Miura 2007). No direct evidence for the

hypothesised transitional pathway exists, but a testable prediction is that sex will be determined by a master sex-determining gene on the heterogametic chromosome in one of the *R. rugosa* populations, and dosage of that *same gene* in the homogametic sex of the other population. Intriguingly, this route of heterogametic transition may even occur in the absence of thermosensitivity of the sexual differentiation pathway. All that is required, in principle, is heritable variation in the threshold value, and a sufficient shift in the mean value for a population.

For reptiles, the principal implication is that simple quantitative shifts in the threshold for sex determination can chart a continuous evolutionary pathway between male heterogamety, female heterogamety, and TSD. Thus, these supposedly disjunct mechanisms of sex determination may be fundamentally more similar than previously supposed. A further prediction is that the widespread occurrence of homomorphic sex chromosomes in reptiles (and fish and amphibians) may be partly explained by evolutionary flux in the threshold for sex determination, causing regular heterogametic transitions, and impeding the process of sex chromosome degeneration.

Intermediate systems of sex determination in reptiles: GSD-TSD interaction

Perhaps the most striking implication of the transition model is that species with FMF-type TSD that produce a mixed sex ratio at intermediate temperatures, such as the agamid *A. muricatus*, have an underlying system of ZW genotypic sex determination. Potentially, this may include many lizards, crocodilians and turtles. The proposition of GSD-TSD co-occurrence seems to conflict with the conventional descriptions of GSD and TSD – sex determination cannot be simultaneously genotypically-directed and temperature-dependent (e.g. Valenzuela at al. 2003). In the model, for species such as TSD agamids there is neither a consistent genotypic difference between the sexes (i.e. GSD), nor does every embryo have the potential to develop as male or female, depending on temperature (i.e. TSD). Populations are in fact a mixture of GSD and TSD ‘genotypes’: ZW embryos are always female, irrespective of incubation temperature (GSD), whereas the sex of ZZ embryos is temperature-dependent. If viewed in this way, the apparent conflict with the conventional definitions dissolves; at the level of the individual, sex determination is not simultaneously genotypic and temperature-dependent. At the level of the population or species, however,

there is coexistence of GSD and TSD, so an appropriate description for the system of sex determination for the species might be *GSD-TSD interaction*. The model therefore challenges the traditional view of reptilian sex determination as dichotomous (Bull 1980). It implies that rigidly classifying reptile species as either GSD or TSD (*sensu* Valenzuela et al. 2003) could conceal a continuum of reptile sex determination systems with varying degrees of genotypic and temperature influence on sex determination (Sarre et al. 2004), where that variation is manifested in the relative proportions of embryos exhibiting *either* GSD or TSD.

To explain the mechanism of FMF TSD in crocodiles, Deeming and Ferguson (1988; 1989) similarly hypothesised that a male-determining factor is produced in sufficient quantity to exceed a threshold dose for male development only within an optimum temperature range. There is no GSD component to the sex determination system in their model. In the model I have proposed, the GSD component is lost once the male threshold increases to the point where the W chromosome is eliminated from the population. Thus, for FMF-type species which produce 100% males at intermediate temperatures, such as *Alligator mississippiensis*, the two hypotheses are qualitatively equivalent. The two hypotheses differ, however, in their explanation for why no constant temperature induces 100% males in other FMF-type species. Under the Deeming-Ferguson model, the small proportion of females arising from male-biased temperatures are presumably non-inducible as males since the threshold of these embryos is too high, even at optimal temperature. In contrast, the GSD-TSD interaction model implies that the proportion of females produced at the 'maximum-male' temperature reflects the frequency of the ZW genotype in the population. In real populations, frequency of the ZW genotype would depend on a complex interaction between the mean threshold value (i.e. pivotal temperatures) and the distribution of nest-site temperatures, which in turn, would reflect local environmental conditions and the accuracy and heritability of nest-site selection by females. Frequency of the ZW genotype could vary between generations and between populations. A potential example of such variation is found in another TSD agamid, *Physignathus lesueurii*, where the sex ratio at the maximum-male temperature varies considerably (56-100%) between four populations spanning a wide latitudinal gradient, even though the pivotal temperatures appear to be stable (Doody et al. 2006).

It may prove difficult to distinguish between the explanations provided by the GSD-TSD interaction hypothesis and the Deeming-Ferguson hypothesis for the occurrence of females at the maximum-male temperature. The GSD-TSD interaction hypothesis could be tested by incubation of entire clutches of FMF species at the maximum-male temperature, with the expectation that some clutches will be 100% male (ZZ mothers), but others will have a mixed sex ratio not statistically different from 1:1 (ZW mothers). This approach would be facilitated by investigating species with large clutch sizes, and by testing repeated clutches from individual females, because the detection of Mendelian sex ratios could be hindered by yolk steroid levels and intraclutch genetic variation in the sex-determining threshold (possibly as a result of multiple paternity).

DNA sex markers in reptiles with GSD-TSD interaction

A complementary approach to test for GSD-TSD interaction could be to identify sex chromosomes or sex-linked sequences in such species. In the model, the W chromosome is present in females only, but not all females are ZW. W chromosome markers may therefore be detectable as DNA markers present in a proportion of females, but never present in males. This approach would be facilitated by screening for markers using only individuals incubated at the maximum-male temperature, to minimise the possible inclusion of ZZ females. The GSD-TSD interaction model could be tested by examining the magnitude of the correlation between candidate sex-linked marker(s) and female sex, across the range of incubation temperatures. High correlation at intermediate temperatures, but low correlation outside this range, would provide empirical support for the model.

The likelihood of detecting DNA sex markers depends on the degree of sex chromosome differentiation, and this may be minimal in species where temperature-induced reversal of genotypic sex occurs naturally. In species with subtle differences between the sex chromosomes, detection of DNA sex markers by molecular genetic approaches might be achievable through considerable investment in time and effort, or it may be the proverbial needle-in-a-haystack search. If, however, sex chromosome differentiation can proceed or be maintained in populations subject to temperature-induced reversal of the homogametic sex, as may occur in *Bassiana*, it could be feasible to detect sex markers in species exhibiting the system of GSD-TSD interaction proposed by the model.

Directions for further research

Suggestions for further experiments that have arisen in the context of this discussion are summarised here, along with some additional suggestions for research.

1) The *Pogona* DNA sexing assay could facilitate incubation experiments to elucidate the interaction between genes and temperature in the sex determination of this genus. The degree of variation and heritability of the 'threshold' temperature required to induce ZZ sex reversal should be examined. Temperature-shift experiments could establish the minimum amount of thermal exposure and the critical period of development for inducing ZZ sex reversal (for constant and cyclical temperatures), to enable comparison with the thermosensitive period of TSD agamids. The possibility of natural sex reversal in *Pogona* species could be examined by establishing thermal conditions of field nests, and testing for sex reversal in wild populations.
2) The *Bassiana* DNA sexing assay could be applied to (a) test the degree of variation (and heritability) in the 'threshold' temperature for XX reversal; (b) verify that XX males arise in natural nests through temperature reversal; (c) test for variation in temperature sex reversal throughout the geographical range of this species; and (d) establish the potential of the sex test for other lygosomine skink species. The fertility and fitness of XX males in *Bassiana* should also be examined.
3) The hypothesis that XY systems are constrained from evolving to FMF TSD, but ZW systems are not, could be tested by searching for evidence of this TSD pattern in lizard families where heterogametic systems are established. Species in XY families with ecological counterparts that exhibit TSD (in other families) could be targeted specifically in the search for TSD (e.g. XY iguanids/TSD agamids).
4) The hypothesis for heterogametic transitions is inherently difficult to test, but support would include further demonstrations of sister populations or species with opposing systems of heterogamety but a homologous sex chromosome pair. Demonstration of the same sex-determining gene in the two groups, operating by dominance in one group, and dosage in the other, would be strong evidence. Experimental evolution approaches using established model systems such as *Drosophila* may be a means of testing the hypothesis.

5) The hypothesis for GSD-TSD interaction in FMF-type reptiles could be tested by identifying sex-linked markers for the W chromosome, by applying the outlined screening strategy. Agamid lizards such as *Amphibolurus muricatus* and *Agama impalearis*, in which intermediate temperatures produce a sex ratio close to 1:1, are obvious candidates for such an approach.
6) The extended sex chromosome sequences from *P. vitticeps* could be physically mapped in related GSD and TSD agamids, with subsequent C-banding to test if the W chromosome in GSD species is homologous to that of *P. vitticeps.* This may reveal conservation, or variation, of sex chromosome pairs in this family, and may identify homologous chromosomes in TSD species.

Final remarks

The Z and W chromosome sequences isolated from *P. vitticeps* in this study are now being used as the launching point for probing a recently constructed Bacterial Artificial Chromosome (BAC) library of the female genome of this lizard (T. Ezaz et al., unpublished data; Amplicon Express, Washington, USA). Overgo primers designed to anchor within these sequences (but avoiding repeat sequences, such as the CR1 element) will provide an entrée into the BAC library, enabling the identification of clones containing large (>120 kb) Z and W chromosome fragments. This research program will be directed at characterising the structure and functional gene composition of the Z and W microchromosomes of *P. vitticeps*, with the ultimate aim of discovering the sex-determining gene in this lizard, a goal which has not been achieved for any reptile species. The finding that high incubation temperature induces female development of ZZ embryos in *P. vitticeps* suggests the underlying molecular mechanism of sex determination in this GSD species may have considerable commonality with that of related TSD agamids, in which females are also produced exclusively at high temperatures. In this context, further characterisation of the sex chromosomes, and the ultimate identification of a primary sex-determining gene for *P. vitticeps*, will be important advances towards a more complete picture of the evolution and molecular mechanisms of GSD and TSD, not only for the agamid lizards, but potentially for all reptiles.

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Appendix 1

The ends of a continuum: genetic and temperature-dependent sex determination in reptiles

Manuscript published as:

Sarre SD, Georges A, Quinn AE (2004) *Bioessays* 26: 639-645

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Appendix 2

The dragon lizard *Pogona vitticeps* has ZZ/ZW micro-sex chromosomes

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Appendix 3

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