SEX IN DRAGONS:
AN INVESTIGATION OF THE EVOLUTION AND ECOLOGY OF SEX DETERMINATION MECHANISMS IN AGAMID LIZARDS (SQUAMATA: AGAMIDAE)

by

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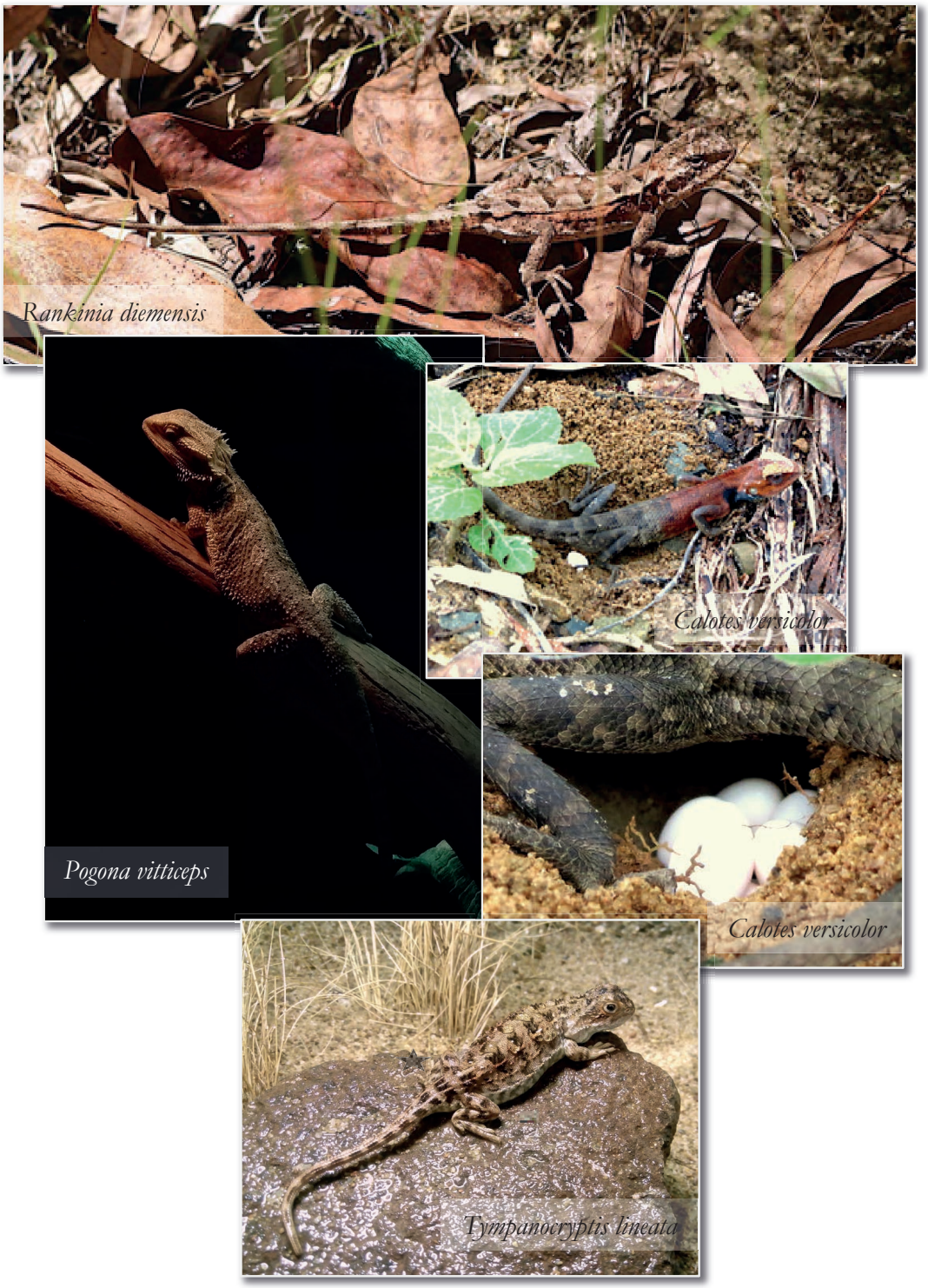
Rankinia diemensis
Calotes versicolor
Pogona vitticeps
Calotes versicolor
Tympanocryptis lineata

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ABSTRACT

Sex in vertebrates can be determined by genes on specific chromosomes (genotypic sex determination, GSD), by environmental factors such as temperature (e.g., temperature-dependent sex determination, TSD) or by the combination of both. Reptiles, especially lizards, exhibit the greatest diversity in sex-determining mechanisms (SDM) among amniotes. This diversity suggests frequent transitions between sex-determining modes. Among the lizards, the Agamidae family (commonly known as agamids) is known to show diverse modes of reproduction as well as modes of sex determination mechanism, possibly even among congenerics. This family contains more than 500 species across Africa, Asia and Australia under six subfamilies and includes species in which both temperature and genes interact to determine sex. The multiple modes of sex determination in agamid lizards have evolved many times, suggesting multiple and independent evolutions of sex-determining modes within the animal kingdom.

Most of the studies of sex determination in agamids have focused on the species under one sub-family from one continent, i.e., Australian species of the sub-family Amphibolurinae. Only little is known about the agamids from other subfamilies. As a result, the diversity and evolution of sex determination mechanisms remain unidentified among a significant group of agamid lizards, yet these have the potential to uncover novel sex determination mechanisms, including sex chromosomes. Filling in this knowledge gap would provide insight into the overall understanding of the phylogenetic relationship and evolutionary history of sex determination mechanisms. In my thesis, I examined aspects of evolution and ecology of sex determination across the family Agamidae with a combination of incubation experiments, cytogenetics and genomics.

As part of this study, I conducted an extensive literature review on the background of the current knowledge of sex determination and sex chromosome in reptiles in the General Introduction chapter (chapter 1). I published a review as a first author (chapter 2) on lizards focussing on what makes this group unique among reptiles in terms of sex determination and sex chromosome evolution. The sex chromosomes in lizards are known for remarkable diversity in terms of morphology and degree of degeneration. The presence of TSD species together with such diversity implies multiple and independent origins of sex chromosomes and, in turn, the lability in sex determination mechanisms within lizard lineages. The review

perceived that such lability in sex determination in lizards are largely due to following independent trajectories in sex chromosome evolution coupled with multiple lineage divergences within this group. This may have contributed to the diversified systems ranging from species with sex chromosomes (homomorphic, XY/ZW heteromorphic or multiple sex chromosomes) to species in which sex chromosomes are absent, that is, where there is a weak genetic determinant at most, involving autosomal genes acting differently on sex-determining pathways. I then examined the chromosomes of agamids from multiple lineages within the family Agamidae with the aim of identifying the sex determination mechanisms (i.e. sex-determining modes) across different subfamilies of Agamids lizards. My key findings are as follows:

Chapter 3 has been published as a research article. In this chapter, I investigated the possible synteny of the sex chromosomes of *P. vitticeps* across agamid subfamilies. I used cross-species two-colour FISH with two BAC clones from the pseudo-autosomal regions of this species against representative species from all six subfamilies as well as two species of chameleons, the sister group to agamids. I found that one of the BAC sequences is conserved in macrochromosomes and the other in microchromosomes across the agamid lineages. However, within the subfamily Amphibolurinae, multiple chromosomal rearrangements were evident. No hybridisation signal was observed in chameleons for either BAC. Overall, my study showed lineage-specific evolution of sequences/syntenic blocks and successive rearrangements and revealed a complex history of sequences leading to their association with critical biological processes such as the evolution of sex chromosome and sex determination.

In chapter 4, a published article, I identified a pair of microchromosomes as sex chromosomes in the Canberra grassland earless dragon *Tympanocryptis lineata,* a threatened grassland specialist species endemic to Australia. This brings to five the number of Australian agamid species (*Pogona vitticeps, P. barbata, Diporiphora nobbi, Ctenophorus fordi* and *T. lineata*) for which sex chromosomes have been identified. All five species have micro sex-chromosomes and female heterogamety (ZZ/ZW). The study included further investigation of the sex determination mode in *T. lineata* (Chapter 5) through incubation experiments and identification of sex-linked loci (markers) through DArTseq^{TM}, a genome complexity reduction and high throughput sequencing method. Incubation experiments conducted at five different constant temperatures (24°, 26°, 28°, 30° and 32 °C) confirmed this species to be a GSD species. DArTseq identified female-biased single nucleotide polymorphism (SNP) and

presence-absence (PA) loci in *T. lineata,* supporting a female heterogamety (ZZ/ZW). Based on the results from Chapter 4 and 5, it can be concluded that *T. lineata* determine their sex through genotypic sex determination (GSD) with female heterogametic (ZZ/ZW) system but cannot, as yet, rule out higher incubation temperatures than those tested.

In Chapter 6, I investigated the evidence of variation in sex determination modes in Oriental Garden lizards, *Calotes versicolor*, a species with a wide distribution range from Iran through south and southeast Asia. The taxon is considered to be a complex of cryptic species. *C. versicolor* samples were collected from Bangladesh and Thailand, at three locations in each country. A two-step study was undertaken. First, I used genomic (SNP data from methylation-sensitive DArTseq analysis) and mitochondrial (Sanger sequencing) DNA data to test for population and phylogenetic structuring within this species complex and second, I used DArTseq (SNP) and SilicoDArT (PA) data to identify potential sex-linked markers in this species. I showed that the samples collected from different localities were genetically distinct, providing evidence that the taxon currently recognised as *C. versicolor* is a complex of cryptic species. My analyses of sex-linked markers revealed variation in sex determination modes among these different forms, implying that different sex determination mechanisms have evolved in closely related species and possibly even lineages within this species.

The studies conducted under this thesis have expanded the knowledge of labile sex-determination mechanisms in reptiles, keeping agamid lizards as models. The studies on the sex determination in this group were previously concentrated mainly on the Australian clade of Amphibolurinae (subfamily), while this research went beyond this boundary and initiated an investigation including species from other subfamilies. The study identified the sex determination mode and sex chromosomes in a threatened Australian agamid species, reported variation of sex-determination modes between populations and closely related species and explored chromosomal synteny among the subfamilies of the agamid lizards using *P. vitticeps* sex-chromosome BACs. The results presented here are still preliminary, and to fully understand the process of sex determination and sex chromosome evolution in the studied species, additional studies using advanced molecular cytogenetic and genomic techniques are needed, with particular priority to gain access to samples where the gonads have been dissected.

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CHAPTER 1: GENERAL INTRODUCTION

Sex is considered one of the most intriguing biological phenomena by evolutionary biologists (Bell 1982). Most animals show gonochorism, i.e., separate sexes where members within a species are either male or female. Being specialised as a male or a female, an individual can, among other things, gain reproductive advantages (genetic recombination) (Charnov et al. 1976) and avoid the exposure of recessive deleterious alleles that arise through self-fertilisation (Charlesworth and Charlesworth 1978). Determination of sex is one of the most fundamental and yet highly variable mechanisms in the animal kingdom (Bull 1983) that can be considered as a rapidly evolving trait. Variation in this mechanism can be observed within many lineages (for example, reptiles, fishes, crustaceans and angiosperms) and even within closely related species or populations (Bull 1983; Charlesworth 1996).

1.1 Sex determination and sexual differentiation

For species with genotypic sex determination (GSD), sex is considered to be determined at conception by the chromosomal complement of the zygote (Georges et al. 2010). In a second sense, sex determination is said to occur when the master sex gene(s) on the sex chromosomes are expressed to give effect to the earlier decision made at conception. There are two different terms, i) sex determination and ii) sex differentiation, which may be confusing, often used in different ways, sometimes interchangeably (Valenzuela 2008). However, these two terms have fundamental differences (Hayes 1998).

Sex determination can be defined as the mechanism that directs sex (gonadal) differentiation (Hayes 1998), herein referred to as the molecular changes that direct and commit gonadal development to proceed down one of the two alternative pathways of differentiation, male or female (Quinn 2008). Sexual differentiation, on the other hand, refers to the regulatory processes that follow sex determination to govern the development of testes or ovaries from the undifferentiated or bipotential gonad. That is, sex differentiation refers to the series of developmental events in an embryo leading to the formation of a functional testis or ovary from an undifferentiated gonadal ridge (Hayes 1998) (Fig. 1.1). The pathway by which gonad (testis or ovary) is differentiated is conserved across vertebrates, but the trigger (genetic or environmental) that initiates the male or female pathway varies considerably between lineages.

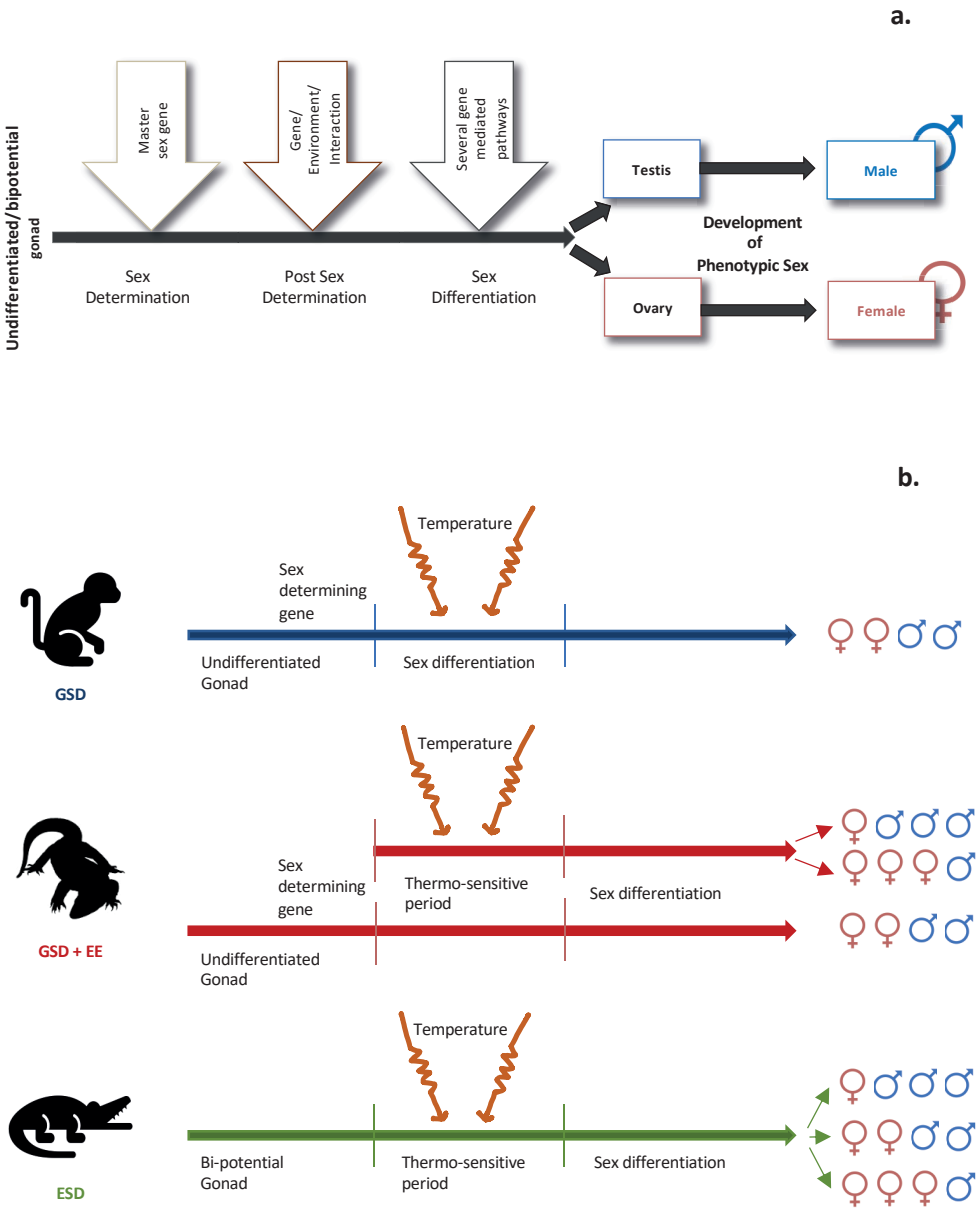

Figure 1.1 Process of sex determination and sexual differentiation simplified. Sex is pre-determined by genotype during fertilisation and the subsequent formation of a zygote (panel a). In many species (GSD), a master sex-determining gene (e.g., *SRY* by dominance in mammals, *dmrt*1 by dosage in birds) initiates the process of sex differentiation, the trajectory towards becoming male or female. In other species (GSD+EE and ESD), this effect is influenced by different environmental factors (e.g., temperature) that can cause a switch between the male and female developmental pathways leading to functional sexual phenotypes (panel b). Sex determination in GSD species is dictated by a dedicated master sex gene that triggers the downstream pathways towards gonadal differentiation either to the testis or ovary and ultimately the phenotypic male or female. In GSD species with temperature effects (GSD+EE), this pathway is interrupted if only a certain threshold level is violated. While, in TSD species, the phenotypic sex of the individual is decided during certain stages in the embryo depending on the exposure to the temperature gradient. In all cases, the sex of the individual may have been determined during conception, i.e., fertilisation as coded in the newly formed DNA, by a primordial gene. This gene remains functional in strict GSDs but may be interrupted by temperature (or other environmental factors) in TSD or temperature influenced GSD species (GSD+EE).

Sex in vertebrates can be determined by (1) genetic factors (genotypic sex determination, GSD), or (2) environmental factors (environmental sex determination, ESD), or (3) by an interaction between genotype and environment (Bull 1983; Charnier 1966; Conover and Kynard 1981; Hayes 1998; Pieau 1971; Quinn et al. 2007b; Radder et al. 2008). In many vertebrates (GSD), sex is determined by a specific gene(s) located on a particular pair of chromosomes (the sex chromosomes) that provides the initial trigger for sex differentiation, male or female, and under random assortment and equal viability, assumptions results in male: female ratio of 1:1. Sex in many other species, depends on an environmental variable (ESD) experienced during embryonic development, such as temperature, pH or salinity or even by social factors. GSD and ESD is viewed as two ends of a continuum of sex-determining mechanisms (Sarre et al. 2004) where the continuum between ESD and GSD can be explained by the existence of GSD with environmental effects (GSD+EE) (Valenzuela et al. 2003). Indeed, it is unlikely that sex in any species is determined purely by GSD without any environmental influence or ESD without any underlying predisposition. For example, in species with temperature-dependent sex determination (TSD) and no chromosomal or evident genetic differences between sexes, eggs incubated at a pivotal temperature (where male: female ratio is 1:1) appear to have an underlying genetic propensity (e.g., a multigenic system with minor cumulative variants and gene dosage) to be one sex or the other (Capel 2017).

1.2 Sex determination mechanism and modes of sex determination

While sex determination mechanism refers to the underlying molecular mechanisms, sex determination mode, herein, is referred to the process in general, i.e., GSD, TSD and GSD+EE. Among vertebrates, all birds and mammals have genetically determined sex (female heterogamety or ZZ/ZW and male heterogamety or XX/XY, respectively). In contrast, all crocodiles and the Tuatara exhibit TSD, while lizards, turtles, and bony fishes have a wide variety of sex determination mechanisms (Alam et al. 2018; Bachtrog et al. 2014; Ezaz et al. 2009c; Gamble and Zarkower 2012). Such variety has provoked investigation into the reticulate transitions of sex determination modes among different taxa. Questions arise, including what are the evolutionary forces behind such changes, why sex determination is labile in some taxa and not in others, and what are the molecular mechanisms causing the different modes of sex determination (Bachtrog et al. 2014). However, the various reports of somatic sexual dimorphisms preceding the gonadal development calls for a more considered definition of sex determination (Cutting et al. 2013).

1.2.1 Genotypic sex determination (GSD)

GSD is more of a clear-cut phenomenon where the genes are the sole factors determining the sex of the offspring (gonads differentiate into testes or ovaries without external (environmental) influences) (Hayes 1998). Organisms such as mammals, birds, many reptiles, amphibians, fishes, many insects and plants, have sex chromosomes with certain degrees of synteny (Ezaz et al. 2017; Voss et al. 2011). In humans and other therian mammals, the presence or absence of a single master sex-determining gene, *SRY*, on the Y sex chromosome determines the sex of the offspring – its presence initiates testis differentiation, while ovaries develop in its absence (Koopman 1995). It has been shown in mouse and assumed in other species that sex-determining genes, inherited at fertilisation, become active in the gonads during early embryonic or larval life. Sex determination in birds, on the contrary, is controlled by the dosage of a gene on the Z chromosome known as doublesex and mab-3 related transcription factor 1 (*DMRT1*): males have two copies of *DMRT1* and females have only one. However, no sex-determining gene has yet been discovered in any reptiles.

1.2.1.1 Sex chromosome and its evolution in amniotes

Sex chromosomes are a dynamic entity in any genome, displaying unique morphology, gene content, and evolution (Charlesworth 1991; Deakin and Ezaz 2014, 2019; Furman et al. 2020; Graves 2006; Muller 1914; Ohno 1967). The diversity of sex chromosome morphologies among amniotes is truly remarkable, with sex chromosomes ranging from cryptic to highly heteromorphic. It is generally accepted that sex chromosomes originate from an autosomal ancestor following a mutation that affords a particular allele at a locus a defining influence on the sex developmental trajectory and also prevents recombination of that locus (Charlesworth and Charlesworth 1980; Graves 2006, 2008; Ohno 1967). Once established, other genes with male advantage and female disadvantage or cost are recruited to the proto- Y or W chromosome, and the non-recombining region progressively expands. In the absence of recombination, what would be otherwise deleterious mutations, the proliferation and accumulation of repeats take place. Progressive loss of genes from the Y or W and an increase in heterochromatin results in a sex-specific chromosome. Deletions of heterochromatin occur in some Y or W chromosomes that resulted in their smaller sizes compared with the X or Z chromosomes (Modi and Crews 2005; Ohno 1967).

In organisms with heteromorphic sex chromosomes, sex is set at fertilisation by the differential inheritance of sex chromosomes (Arnold et al. 2013; Cutting et al. 2013). Germ cells/gonads

receive either a male or female determining chromosome through meiosis, and during fertilisation, these combine to determine the sex of the zygote. Vertebrate GSD species are either male heterogametic where males have an XY and females an XX pair of sex chromosomes, or female heterogamety where females have a ZW and males a ZZ pair of sex chromosomes. Sex-specific Y or W chromosomes are sometimes more or less degraded versions of the X or Z and are shorter because of deletion or longer because of insertion and amplification. The XX/XY sex chromosome pair is conserved in therian mammals, and a ZZ/ZW pair is conserved in birds. However, there are exceptions to mammalian XY systems including the monotremes (platypus and echidnas) that have a multiple XY sex chromosome system. Platypus and echidna males have 10 unpaired sex chromosomes (X_1Y_1, X_2Y_2, X_3Y_3, X_4Y_4, X_5Y_5) (Rens et al. 2004; 2007). Another exception is the spiny rat *Tokudaia osimensis* and the mole vole *Ellobius lutescens* that have lost their Y chromosome, and both males and females are XO (Kuroiwa et al. 2010; Mulugeta et al. 2016; Soullier et al. 1998). In different species of *Akodon*, the South American grass mice, females can be either XX or XY (Fredga 1988; Fredga et al. 1976). In contrast, many reptiles, amphibians and fish lineages exhibit remarkable variation in the sex chromosome pair and in the system of heterogamety, sometimes even among closely related species or even populations (Ezaz et al. 2009b; Ezaz et al. 2006a; Graves 2006).

1.2.2 Environmental sex determination (ESD)

In contrast to GSD, external stimuli (such as temperature, pH, salinity, photoperiod and social factors) experienced by the developing embryos control sex determination in ESD species (Charnier 1966; Devlin and Nagahama 2002; Guler et al. 2012; Hayes 1998; Kobayashi et al. 2013; Merchant-Larios and Diaz-Hernandez 2013; Pieau 1971; Walker 2005; Warner et al. 1996) with each individual having the full capacity to develop into males (testicular differentiation) or females (ovarian differentiation), or in some cases, to reverse with age or under the influence of social cues. Fishes, amphibians and reptiles are the vertebrates where sex determination is influenced by the environmental factors in some species. Temperature is the most common among environmental factors to play a role in sex determination in fishes (Devlin and Nagahama 2002) and reptiles (Bull 1980; Harlow 2004). pH and dissolved oxygen level are among other factors known to influence sex ratios in different fish species (Devlin and Nagahama 2002), and social factors to cause sex reversal in coral reef fishes (Robertson 1972). In amphibians, discordance between sexual genotype and phenotype has been observed

in exposure to extreme temperatures (Wallace and Wallace 2004; Witschi 1929) and certain chemicals in the environment (Tamschick et al. 2016). The actual mechanism that acts behind environmental sex determination remains unresolved. However, Castelli et al. (2020) proposed a model where sex determination is initiated, male or female, by the balance of cellular calcium (Ca^{2+}) and redox (reactive oxygen species; ROS), both of which are subject to environmental influence. These CaROS-sensitive regulatory pathways may be a crucial link between sex determination and the environment that have been adopted independently and repeatedly in different vertebrate lineages.

1.2.3 Gene-environment interaction in sex-determination

Environmental sex determination is likely to be favoured over genetic/chromosomal sex determination, where a particular environment is more beneficial to the fitness of one particular sex (Bull 1983). Genotypic sex determination, on the other hand, is likely to be favoured over ESD when the environment is unpredictable or not variable enough, and there are chances of producing skewed sex ratios or intersex individuals (Bull 1983). There is growing evidence that environmental influences can affect offspring sex ratio in GSD species, especially in lizards (Quinn et al. 2007a; Quinn et al. 2011). Temperature has been found to influence the offspring sex ratio in GSD species as the eastern three-lined skink, *Bassiana duperreyi*, (XX/XY heterogamety) (Shine et al. 2002), and the Australian central bearded dragon, *Pogona vitticeps* (ZZ/ZW heterogamety) (Quinn et al. 2007a). In both cases, shifts from the 1:1 sex ratios were observed, resulting in either male or female-biased sex ratios.

More recent researches have shown that the spotted snow skink *Carinascincus ocellatus* has different sex-determining modes (Hill et al. 2018; 2021; Pen et al. 2010). For example, GSD+EE occurs at low altitudes with low variance in temperature where early birth is an advantage, and it is expected that females with environmental (temperature) influence could produce optimal sex ratios. In contrast, GSD skinks prevail at high altitudes where the variation in temperature over the year is high, and there is no advantage for early-born females, and extreme sex ratios are prevented (Pen et al. 2010; Wapstra et al. 2009). Another study by Cornejo-Páramo et al. (2020) showed that the yellow-bellied water-skink *Eulamprus heatwolei*, a viviparous skink regarded to have temperature-dependent sex determination, has more than 100 million years old XY chromosomes, and concluded that viviparity in reptiles might be strictly associated with GSD systems.

1.2.3.1 Genotype-phenotype discordance

In GSD species, individuals undergoing development may not have sex based on expectation at conception (Georges et al. 2010). Environmental factors (e.g., temperature) can influence during the developmental stages that may alter gene expression and cause mismatches between the phenotypic and genetic sex. Such phenomena are commonly known as sex reversal and have been observed in fishes (Chan and Yeung 1983; McNair et al. 2015), in amphibians (Dournon et al. 2003; Miura 1994) and in reptiles (Holleley et al. 2015; Quinn et al. 2007b).

Such discordance between the genotypic and phenotypic sex can occur in either direction: 1) an individual can be genetically male but anatomically and functionally female or 2) genetically female but anatomically and functionally male. Sex reversal is now regarded to be common in reptiles (Holleley et al. 2016). It is likely to be a powerful evolutionary force responsible for generating and maintaining lability and diversity in reptile sex-determining modes. Sex reversal can have both positive and negative impacts on the individual. Sex-reversed female bearded dragons were found to lay almost twice as many eggs than normal females (Holleley et al. 2015) and are bolder than male and normal female individuals (Li et al. 2016). However, it can lead to an abnormality or compromised fitness of individuals (Holleley et al. 2016) and a sex-biased ratio in the natural population that may lead to the extinction of the species (Boyle et al. 2014).

1.3 Sex determination in reptiles

Reptiles are a familiar group of vertebrates, having existed for more than 300 million years. Although these animals became the most dominant group during the Jurassic and Cretaceous periods, today they are represented by only four orders (turtles, crocodilians, squamates - snakes and lizards and sphenodontians - tuatara). However, not only do these animals occupy a pivotal position in the phylogeny of vertebrates, they also share the most recent common ancestor with birds and mammals (Modi and Crews 2005).

There is debate on the ancestral sex-determining state in reptiles. Analysis of the tetrapod vertebrate phylogeny (amphibians, mammals, reptiles and birds) reveals that the ancestor was likely to be a GSD (Janzen and Phillips 2006) with ZW heterogamety (Gruetzner et al. 2006). On the other hand, the examination of Squamata reveals a putative TSD ancestor (Bull 1980; Pokorna and Kratochvíl 2009), with the distribution of different sex-determining modes in this lineage acquired through multiple transitions in sex-determining systems. All snakes are known

to show GSD, while all Crocodiles and Tuataras show TSD (Cree et al. 1995). Many lizards and some turtles also exhibit genotypic sex determination (GSD), and both male and female heterogamety (XY/ XY, and ZZ/ZW) may be found within the same taxa (Alam et al. 2018; chapter 2). Existence of species with TSD, GSD (both ZW and XY heterogamety), and obligate and facultative parthenogenesis among squamates (Bull 1980; Deeming et al. 1988; Ewert and Nelson 1991; Moritz et al. 1990; Moritz et al. 1992; Raynaud and Pieau 1985; Wibbels et al. 1991) suggest a complex evolutionary history of sex-determining mechanisms in this group.

1.3.1 Genotypic sex determination in Reptiles

1.3.1.1 Sex chromosomes in Reptiles

Reptiles exhibit extraordinary variability in sex chromosome structure, and patterns reported among vertebrates range from homomorphic to heteromorphic in structure (Alam et al. 2018; Ezaz et al. 2009b; Ezaz et al. 2009c; Olmo and Signorino 2005). Several species of turtles and lizards have male heterogamety (XY males and XX females). In contrast, some turtles and lizards and all snakes have female heterogamety (ZZ males and ZW females) except boids (Gamble et al. 2017). Some turtles and lizards have no noticeable heteromorphic sex chromosomes. Additional experiments are needed to determine if these species have GSD or TSD, as species that have TSD also do not reveal karyotypic differences between males and females (Crews et al. 1994).

Evidence from comparative gene mapping shows that ancient (ancestral/ plesiomorphic) states may be retained among the sex chromosomes of mammals, birds, and reptiles. A conserved homology can be observed in sex chromosomes between birds and lizards (Ezaz et al. 2009a; Kawai et al. 2009; Matsubara et al. 2006). However, the sex chromosomes and modes of sex determination in reptiles are evolutionary labile where different genes on different chromosome regions may take over the role of sex determination (in different lineages or species), so are likely to have been through multiple and independent events (Ezaz et al. 2009c; Ezaz et al. 2017).

1.3.1.2 Sex chromosome evolution in reptiles

Unlike most vertebrates, both macro and microchromosomes have been identified as sex chromosomes in various species of reptiles (Badenhorst et al. 2013; Ezaz et al. 2005; Ezaz et al. 2006b; Gamble et al. 2014; Kawagoshi et al. 2009; Rovatsos et al. 2017a; Rovatsos et al. 2014; Srikulnath et al. 2014; Zeng et al. 1997). In addition, multiple sex chromosomes are also

common in some groups, particularly in iguanids and lacertids, suggesting sex chromosome-autosome translocations. Morphological variation between sex chromosomes ranges substantially, spanning from homomorphic to highly heteromorphic, representing various stages of evolutionary degradation (Ezaz et al. 2009c; Ezaz et al. 2017; Graves 2008). However, this does not correlate with the evolutionary age of those taxa. Identification of sex microchromosomes is challenging but is made possible by the recent advancement of cytogenetic techniques (such as CGH). The evolution of heteromorphic sex chromosomes has involved the gradual degradation of macrochromosomes (Charlesworth 1991; Charlesworth and Charlesworth 1980; Deakin and Ezaz 2019; Graves 2006, 2008, 2016; Ohno 1967); however, whether similar mechanisms are involved in the evolution of micro sex chromosomes, has rarely been discussed. The molecular and cytogenetic mechanisms behind the evolution of micro sex chromosomes are unknown. They may have evolved as a result of chromosome fission or fusion events. Microchromosomes might also followed the same pathway as proposed for vertebrates, or entirely different molecular mechanisms were involved in the evolution of sex microchromosomes. One possibility is that macro and microchromosomes have evolved independently, involving new pair of sex microchromosomes driven by frequent turnovers, either via translocation of sex-determining factors via transposition, or novel sex chromosomes have evolved after each transition, involving novel genes and novel chromosomes.

In addition to the proposed pathway of vertebrate sex chromosome evolution, reptiles also achieved the evolution of sex chromosomes via other molecular mechanisms, particularly subtle gene regulatory mechanisms such as epimutations and evolution of sexually antagonistic genes (Furman et al. 2020; Mank et al. 2014; Chapter 2). This could be the mechanism of how reptiles are successful in maintaining sex ratios in the variable environments, which would also somewhat explain the maintenance of TSD, transitions between TSD and GSD, as well as temperature mediated sex reversal in GSD species with cryptic (*P. vitticeps*) and heteromorphic sex chromosomes (*Bassiana duperreyi*).

From the above discussions, it is clear that reptiles sex chromosomes have different evolutionary dynamics, unlike other amniotes. The physiology of reptiles (ectothermy), modes of reproduction and embryonic development, vulnerability to environmental changes by the developing embryos (often with longer incubation periods) or pregnant females made them resort to diverse pathways to maintain sex ratios (Bull 1980; Castelli et al. 2020a; 2020b; Deeming et al. 1988; Ewert and Nelson 1991; Moritz et al. 1990; 1992; Pyron and Burbrink

2014; Raynaud and Pieau 1985; Whiteley et al. 2021; Wibbels et al. 1991). However, the viability of any particular pathway is subject to selection, which is challenging for a stable sex chromosome to evolve following the usual pathways (i.e., recombination suppression) described for other vertebrates.

Reptiles likely employ multiple strategies for sex determination and polygenic system involving multiple sex chromosomes. Polygenic sex determination has been identified across vertebrate taxa, including eutherian mammals (Mank and Uller 2014; Moore and Roberts 2013). However, despite the high variability of reptile sex determination and reproduction, polygenic sex determination has never been investigated (Alexander et al. 2014; Bachtrog et al. 2014). Given the established interactions between gene and environment in a couple of reptiles species (e.g. bearded dragon and three-lined skink; Quinn et al. 2007a; Quinn et al. 2011; Shine et al. 2002; Young et al. 2013), systematic analysis of sex determination in reptile involving multiple genes, particularly in species where genomic, incubation data and cytogenetic resources are available would be a beneficial avenue of research. TSD species, though they produce skewed sex ratios at temperature extremes, may produce a 1:1 sex ratio among offspring like GSD species if incubated at a pivotal temperature. A study, therefore, should not only focus on GSD species but should include well-characterised TSD species as well so that a clear distinction can be drawn between the influences of temperature and multiple gene effects on sex determination.

Genes from sex chromosomes of multiple reptiles have been identified (section 1.3.1.3); however, they remain candidates without appropriate functional characterisation. Reptiles are, however, a difficult group for functional characterisation of candidate genes mainly due to their embryonic development, which poses a challenge to induce mutations analysis for gain or loss of function analysis. Reptile eggs are also developmentally advanced when laid, making it particularly challenging to induce germ-line mutations.

1.3.1.3 Sex-determining genes and sex-linked sequences

In GSD species, a master sex-determining gene on the sex chromosomes likely triggers gonadal differentiation into the ovaries or testes. Very few such genes have been discovered in vertebrates (Table 1.1), and apart from mammals and birds (Koopman et al. 1991; Sinclair et al. 1990; Smith et al. 2009), these genes are apparently not conserved over a larger number of related orders, families, genera, or even species (Pan et al. 2016).

Table 1.1 Some of the known master sex-determining genes in different animal taxa

Taxon	Species	Sex determining mode	Master sex gene	Reference
Insects	Fruit flies, *Drosophila sp.*	XY	*SLX*	Cline et al. 2010
	Housefly, *Musca domestica*	ZW	*F*	Hediger et al. 2010
	Silkworm, *Bombyx mori*	ZW	*FEM*	Kiuchi et al. 2014
	Honeybee, *Apis mellifera*	Haplodiploid	*CSD*	Beye et al. 2003
	Wasp, *Nasonia vitripennis*	Haplodiploid	*NVTRA*	Verhulst et al. 2010
Fish	Medaka, *Oryzias latipes*	XY	*DMRT1Y*	Matsuda et al. 2002
	Luzon ricefish, *Oryzias luzonensis*	XY	*GSDFY*	Myosho et al. 2012
	Patagonian pejerrey, *Odontesthes hatcheri*	XY	*AMH*	Hattori et al. 2012
	Rainbow trout, *Oncorhynchus mykiss*	XY	*SDY*	Yano et al. 2012
	Tiger pufferfish, *Takifugu rubripes*	XY	*AMHR2**	Kamiya et al. 2012
	Half-smooth tongue sole, *Cynoglossus semilaevis*	ZW	*DMRT1*	Chen et al. 2014
Amphibians	African clawed frog *Xenopus laevis*	ZW	*DMW*	Yoshimoto et al. 2008
Birds	Chicken, *Gallus gallus*	ZW	*DMRT1*	Smith et al. 2009; Hirst et al. 2017
Mammals (eutherian and marsupial)		XY	*SRY*	Sinclair et al. 1990; Foster et al. 1992; Koopman 1995

***** a single SNP (C/G) is associated with phenotypic sex

1.3.1.3.1 Sex-linked sequences/markers

The identification of sex-linked markers is one approach to identify the sex-determination mode of an organism. These markers, also known as the DNA sex markers, are tightly linked to the sex-determining gene such that they are highly correlated with phenotypic sex. As a consequence, these markers will be sex-specific, located in the Y or W chromosome and

identify male or female chromosomal sex by their presence or absence in the respective sex (Quinn 2008). This approach can be particularly helpful in the absence of visually heteromorphic sex chromosomes that cannot be identified through cytogenetic analyses (Gamble and Zarkower 2014) and lends itself to several genomic approaches. For example, the use of restriction enzymes digests coupled with sequencing has enabled identifying sex-linked markers in several recent studies on agamids, varanids, skinks and iguanids (Table 1.2).

Sex-linked DNA markers are useful molecular tools for high throughput detection of genotypic sex in animals with a wide range of applications, including population ecology (demography), conservation genetics as well as evolutionary and phylogenetic studies. The development of sex-linked molecular markers is relatively straightforward in species with highly heteromorphic and conserved sex chromosomes (e.g., birds and mammals). However, it is more challenging when sex chromosomes are homomorphic, and sex reversals (genotype-phenotype discordant individuals) are common occurrences even in species with morphologically differentiated sex chromosomes (e.g., in many fishes, amphibians and reptiles). Therefore, it is particularly important to select a panel of males and females of genotype-phenotype concordant individuals to develop sex-linked DNA markers and test its efficacy in a larger sample size (13-15 individuals per sex to reduce false-positive loci; Lambert et al. 2016). The first step should include the analysis of chromosomes to detect the presence of heteromorphic sex chromosomes. If sex chromosomes are homomorphic, then sex ratio analysis through a systematic breeding experiment is critical for selecting genotype-phenotype concordant animals. This is possible in some species, such as in fish and amphibians, owing to relatively high fecundity, somewhat shorter generations, external fertilisation and amenability to captive breeding. Also, in many cases, sex-linked markers were found to be specific to a particular population, suggesting sex association (inter-population differences in the sex-linked markers, which are often repetitive elements) rather than sex linkage. Such associations can rapidly be fixed and evolved in a closed population. Therefore, it is crucial to test sex-associated molecular markers across species for their tight linkage with sex-determining locus. However, it is particularly challenging in reptiles because of their unique modes of reproduction, relatively low fecundity, often longer generation, temperature-induced sex reversal in many species, and some difficulty breeding in captivity.

Various strategies, approaches and molecular tools have been applied to identify sex-linked markers, such as analysis of mini and microsatellites, restriction fragment length analysis (e.g., AFLPs), bulked segregant analysis (BSA), transcriptomes, and more recently, next-generation

sequencing (e.g., RADseq and DArTseq) and in-silico whole-genome subtraction to isolate sex-linked molecular markers in several species of reptiles (Table 1.2). While these markers have been effective in identifying genotypic sex in relatively small sample sizes, their effectiveness in larger sample sizes and at the population level is yet to be tested.

Table 1.2 Sex-linked markers developed in different groups of reptiles

Marker type	Group	Family	No. of species	Linkage	References
Minisatellites (Bkm)	Snake	Colubride	1	W-linked	Singh et al. 1980
Microsatellites	Lizard	Scincidae	1	Y-linked	Cooper et al. 1997
	Lizard	Varanidae	1	W-linked	Matsubara et al. 2014
BSA (bulk segregant analysis)	Lizard	Varanidae	1	W-linked	Halverson and Spelman 2002
AFLPs	Lizard	Agamidae	1	W-linked	Quinn et al. 2007a; 2010; Holleley et al. 2015
		Scincidae	1	Y-linked	Quinn et al. 2009
SNPs (RADseq)	Lizard	Dactyloidae	1	Y-linked	Gamble and Zarkower 2014
		Gekkonidae	11	Y or W-linked	Gamble et al. 2015
		Xantusiidae	1	W-linked	Nielsen et al. 2020
		Agamidae	1	W-linked	Wilson et al. 2019
	Snake	Boidae	1	Y-linked	Gamble et al. 2017
		Pythonidae	1	Y-linked	Gamble et al. 2017
SNPs and PAs (DArTseq)	Lizard	Scincidae	1	Y-linked	Hill et al. 2018
Whole-genome subtraction	Lizard	Scincidae	1	Y-linked	Dissanayake et al. 2020
Transcriptome and genome	Lizard	Scincidae	1	Y-linked	Cornejo-Páramo et al. 2020
Genomic qPCR	Turtle	Trionychidae	8	Z-linked	Rovatsos et al. 2017b
	Lizard	Iguanidae	41	X-linked	Altmanova et al. 2017
	Snake	Colubridae	17	Z-linked	Rovatsos et al. 2015b
		Elapidae	1	Z-linked	Rovatsos et al. 2015b
		Lamprophiidae	3	Z-linked	Rovatsos et al. 2015b
		Homalopsidae	1	Z-linked	Rovatsos et al. 2015b
		Viperidae	3	Z-linked	Rovatsos et al. 2015b
		Pareatidae	1	Z-linked	Rovatsos et al. 2015b

Marker type	Group	Family	No. of species	Linkage	References
		Xenodermatidae	1	Z-linked	Rovatsos et al. 2015b
		Acrochordidae	1	Z-linked	Rovatsos et al. 2015b
		Pythonidae	2	Z-linked	Rovatsos et al. 2015b
		Xenopeltidae	1	Z-linked	Rovatsos et al. 2015b
		Boidae	2	Z-linked	Rovatsos et al. 2015b
		Erycidae	2	Z-linked	Rovatsos et al. 2015b
		Sanziniidae	2	Z-linked	Rovatsos et al. 2015b
Sex chromosome genes	Turtle	Trionychidae	2	W-linked	Literman et al. 2017
		Emydidae	2	Y-linked	Literman et al. 2017

1.3.2 Temperature-dependent sex determination (TSD) in reptiles

TSD was first discovered in reptiles in the African lizard *Agama agama* (Charnier 1966). In TSD reptile species, a higher temperature can produce either males or females, and the temperature ranges and lengths of exposure that influence sex determination are remarkably variable among species (Trukhina et al. 2013).

Sex is determined after fertilisation, specifically during the temperature-sensitive period (TSP), triggered by nest temperature in oviparous (egg-laying) and/or basking behaviour in viviparous (live-bearing) reptiles (Georges 1989; Shine et al. 2007; Yntema 1979). In reptiles with TSD, it was proposed that there are no sex chromosomes (Crews et al. 1994; Modi and Crews 2005), but recent studies suggest a complex association of genes in determining gonadal fate, i.e. sex differentiation in TSD species (Ge et al. 2018; Rhen and Schroeder 2017; Schroeder et al. 2016). DNA methylation induced by certain temperatures have been observed to induce the development of a particular sex, at least in part, in European sea bass *Dicentrarchus labrax* (Navarro-Martín et al. 2011), red-eared slider turtles *Trachemys scripta* (Matsumoto et al. 2016) and olive Ridley marine turtles *Lepidochelys olivacea* (Venegas et al. 2016). However, there are some species where the sexual fate of a GSD species might also be influenced by environmental factors (Sarre et al. 2004). For example, half smoothed tongue sole *Cynoglossus semilaevis* has differentiated ZW sex chromosomes, but ZW embryos develop into males when incubated at high temperatures, and sex reversal is accompanied by substantial methylation modification of genes in the sex determination pathway (Shao et al. 2014). High incubation

temperature has also been found to override genetic sex determination in the Australian dragon lizard *Pogona vitticeps* (Holleley et al. 2015; Quinn et al. 2007a) and the three-lined skink *Bassiana duperreyi* (Shine et al. 2002). The exact mechanism by which the temperature influences sex determination is still a mystery (Castelli et al. 2020).

The relationship between incubation temperature and offspring sex in reptiles with TSD follows one of three patterns, as shown in the Fig. 1.2: (i) the MF pattern with high proportions of males at low incubation temperatures and exclusively females at high temperatures (A); (ii) the FM pattern with development of exclusively females at low temperatures and high proportions of males at high temperatures (B); and (iii) the FMF pattern in which exclusively females develop at both high and low temperatures and the highest proportion of male development occurs at intermediate temperatures (C) (Bull 1983; Crews et al. 1994; Ewert and Nelson 1991; Pieau 1996; Shine 1999; Valenzuela 2004). The FM and MF patterns are considered derived conditions that evolved from the ancestral FMF type through changes in male-determining temperatures and the thermal limits of viability (Deeming et al. 1988; Ewert and Nelson 1991; Janzen and Phillips 2006; Organ and Janes 2008). However, as an exception, an FMFM Pattern of TSD has been reported in an agamid lizard, *Calotes versicolor* (Doddamani et al. 2012).

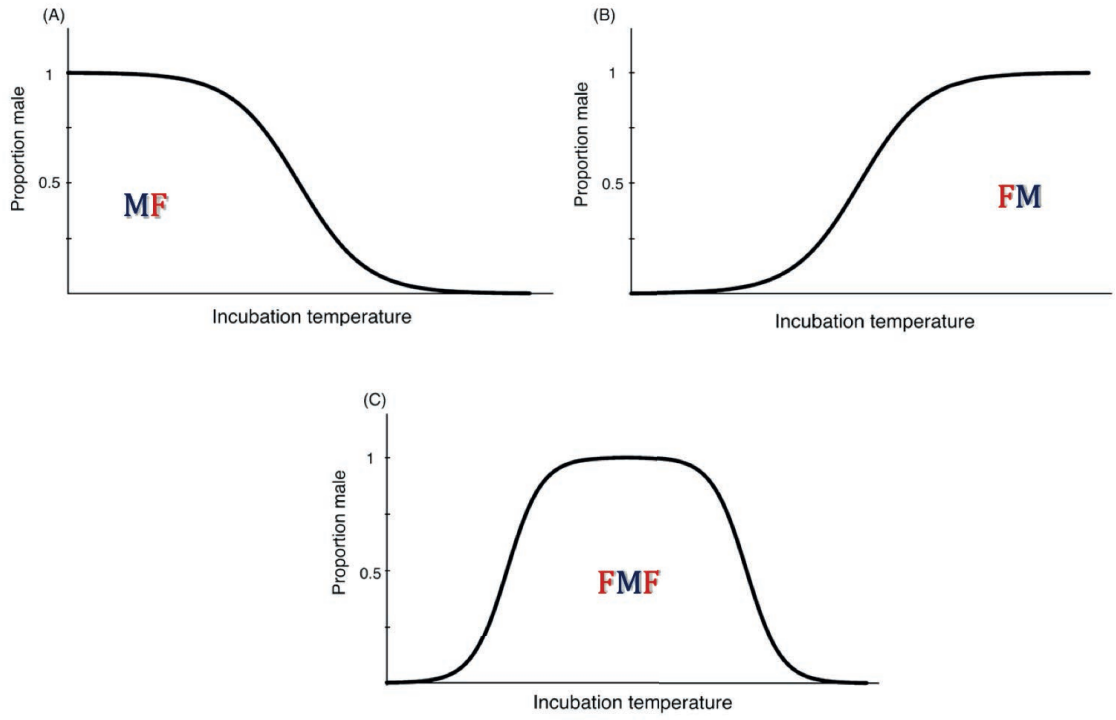

Figure 1.2 Three general patterns of offspring ratios in TSD species, adopted from Pieau (1996) and Pezaro et al. (2016).

1.3.2.1 Genetics behind sex determination in TSD reptiles

In TSD species, the phenotypic sex of the individual is decided during certain stages of embryonic development depending on the exposure to the temperature gradient. However, the sex of the individual may have been determined during conception, as in strict GSDs, but may be interrupted by temperature (or other environmental factors). The phenotypic sex in the TSD species, therefore, becomes subject to an expression of multiple genes during the sex determination and differentiation pathways, and finding a master sex-determining gene remains elusive. Indeed, such a gene may not exist rather a parliamentary system of genes (other genes of interest work unitedly and suppress any particular gene) influences determining the sex (Capel 2017; Georges et al. 2010; Scott and West 2019). Efforts have been undertaken to identify candidate sex-determining genes and discover any master sex gene in reptiles (see section 1.3.1.3). A recent study by Schroeder et al. (2016) presented multiple lines of evidence establishing cold-inducible RNA-binding protein (*CIRBP*) as functional in sex differentiation and strongly suggesting that *CIRBP* is involved in influencing the fate of the bipotential gonad in the Common Snapping Turtle, *Chelydra serpentina*, a TSD species. They adopted a wide variety of methods, including RNA extraction and cDNA synthesis, dye and probe-based PCR to measure expression, protein expression patterns using immunohistochemistry, sequencing, bioinformatics and RNA-seq analysis (to measure *CIRBP* expression in pooled RNA samples). They detected a single nucleotide polymorphism (SNP) in *CIRBP* where the 'A' allele was induced in embryos exposed to a female-producing temperature, while the expression of the 'C' allele did not differ between female- and male-producing temperatures. The first allele was associated with ovary determination and the second with testis determination. However, the role of *CIRBP* in other TSD species remains an open question. Deveson et al. (2017) observed sex-associated differential retention of the introns in *JARID2/JMJD3* genes in *P. vitticeps*, a GSD agamid lizard with temperature influence (GSD+EE). Such phenomena have also been observed in TSD alligators and turtles (Deveson et al. 2017), indicating a reptile-wide mechanism controlling TSD.

1.4 Agamid lizards

Agamid lizards are widely distributed in Asia (including the Indo-Australian archipelago), Australasia and Africa, with 526 species (Uetz et al. 2020) in six sub-families (Draconinae, Agaminae, Amphibolurinae, Hydrosaurinae, Leiolepidinae and Uromastycinae). These lizards

include thorny devils (*Moloch horridus*) and flying lizards (*Draco* spp.) and are sometimes also known as agamids, dragon lizards or dragons. The members of the family live in diverse habitat types, from tropical rain forests to deserts, in semi-aquatic to arboreal niches. This diverse group is usually identified by their well-developed legs and a mid-dorsal crest.

1.4.1 Sex determination in Agamid lizards

Agamid lizards are mostly oviparous with diverse sex-determination mechanisms (e.g. modes) including GSD (ZZ/ZW), TSD and temperature override; as well as parthenogenesis (Blackburn 1982; Ezaz et al. 2009c; Grismer et al. 2014; Harlow 2004; Harlow 2001; Holleley et al. 2015; Miller et al. 2019). About one-fifth of agamids species worldwide have been karyotyped (91/526 (Pokorná et al. 2014a; Uetz et al. 2020)), which makes them a well-studied group in this respect (Quinn 2008). Heteromorphic sex chromosomes have been identified in a few species, as morphologically differentiated Z and W macrochromosomes in the Asian species *Phrynocephalus vlangalii* (Zeng et al. 1997), and microchromosomes in the Australian species *Pogona vitticeps* (Ezaz et al. 2005). Sex determination modes in agamid lizards have also been widely studied among the lizard families (Harlow 2004), particularly Australian species mostly belonging to the subfamily Amphibolurinae. Australian species have shown evolutionary lability in sex determination mechanisms (Harlow 2004; Sarre et al. 2011), with several recent turnovers likely within GSD species (ZW⇔ZW) and transitions between GSD and TSD modes (Ezaz et al. 2009b; Georges et al. 2010). However, karyotypically, Australian agamids have been found to be conserved, having six pairs of macrochromosomes and ten pairs of microchromosomes (Witten 1983). Although reasonably well studied, the family Agamidae lacks information in subfamilies like Hydrosaurinae, Leiolepidinae and Uromastycinae (Fig. 1.3).

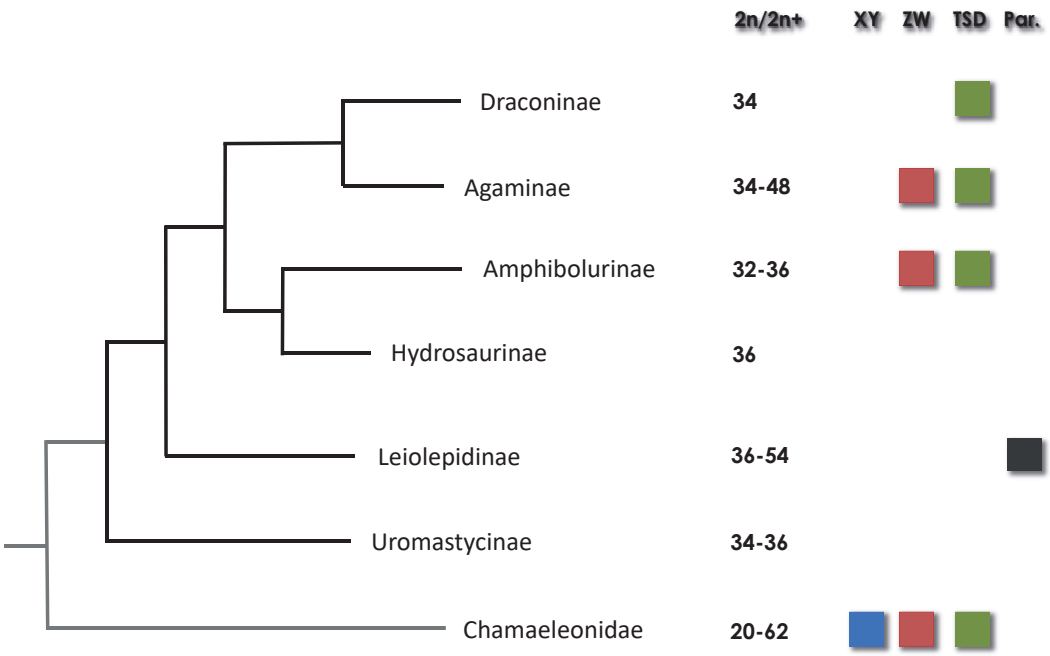

Figure 1.3 Agamid subfamilies (Reptilia: Squamata: Agamidae) showing phylogenetic relationship together with known SDM and chromosome numbers, along with their sister clade Chamaeleonidae. (Adegoke 1988; Baig et al. 2012; Ezaz et al. 2005; Grismer et al. 2014; Harlow 2004; Henle 1995; Doddamani et al. 2012; Kritpetcharat et al. 1999; Nielsen et al. 2018; Phimphan et al. 2013; Pokorna and Kratochvíl 2009; Pyron et al. 2013; Rovatsos et al. 2017a; Sharma and Nakhasi 1980; Srikulnath et al. 2009a; Uetz et al. 2020; Utong and Abukashawa 2013; Viets et al. 1994; Witten 1983; Zeng et al. 1997). 2n/2n+ - Chromosome number; XY – male heterogamety, ZW – female heterogamety, Par. – parthenogenesis.

1.4.2 Knowledge gaps and needs in agamid lizards

Figure 1.3 shows gaps in information regarding sex determination within agamid lizards. Only female heterogametic system (ZZ/ZW) has been reported, and although XX/XY system has been reported from their sister group chameleons, it is not known whether any male heterogametic (XX/XY) system exists within this group. However, most of the information is from the Australian subfamily Amphibolurinae. A remarkable diversity and evolutionary activities have taken place within this small, karyotypically conserved branch. Investigation more broadly across this group should inform whether this diversity and lability is typical of agamids and will assist in determining the mechanisms of such changes. Such an examination will also provide the opportunity to investigate the phylogenetic relationships and evolutionary history of sex determination mechanisms in this group.

1.4.2.1 Identifying the sex determination modes and sex chromosomes for understanding their evolution

Transitions between TSD and GSD are common in reptiles, particularly in lizards (Ezaz et al. 2009b; Ezaz et al. 2009c; Gamble et al. 2015), and there is an apparent association between TSD and female heterogamety (Ezaz et al. 2009c). The evolution of sex determination in agamids is both rapid and recent, suggesting frequent transitions between modes of sex determination (Ezaz et al. 2009b; 2009c). The sex chromosome - temperature interactions could be a possible mechanism for these rapid transitions between the modes of sex determination (Holleley et al. 2015).

Sex chromosomes are an inseparable part of studying genotypic sex determination. Among all the karyotyped chromosomes of agamid lizards, only six species have been found to contain sex chromosomes with female heterogamety (ZZ/ZW type). The identified sex chromosomes vary in size and location - the largest pair in *Phrynocephalus vlangali* (Zeng et al. 1997) while the smallest pair (microchromosome) in *Pogona vitticeps* (Ezaz et al. 2005; Young et al. 2013). Within Australian agamids, non-homologous chromosomes have been observed to be the sex-chromosome pair highlighting the rapid, *de novo* origin of sex chromosomes in this group of lizards (Ezaz et al. 2009b).

There are homologies between the chromosomes in different vertebrate taxa – from orders to families. It is worth studying how similar or dissimilar the sex chromosomes are in closely related species (i.e. species under the same family) that lives in different geographic locations for the complete understanding of the phylogenetic relationship and evolutionary history of sex determination mechanisms (Bull 1980; 1983; Ezaz et al. 2009b; 2017; Gamble et al. 2014; 2017; Kawagoshi et al. 2009; Kawai et al. 2009; Matsubara et al. 2006; Nielsen et al. 2018; Pokorna et al. 2009; 2014a; Rovatsos et al. 2014a; 2014b; 2015b; 2017b). Even though the Australian agamid lizard might have diverged recently and thus have a relatively short evolutionary history (Hugall et al. 2008; Hugall and Lee 2004), molecular cytogenetics and gene mapping of sex chromosomes have revealed that the sex-determining mechanisms in these lizards have evolved independently, multiple times within this short evolutionary period. If such remarkable diversity in sex determination and sex chromosomes can take place within a small branch of a larger group of animals, the diversity within the agamid lizards as a whole certainly provokes scientific interests. It is, therefore, worth investigating the evolution of sex chromosomes in species across the family Agamidae, i.e., species with common ancestors but evolved separately over time and space. Trajectories in sex chromosome evolution can be

investigated using sex chromosome-specific libraries, such as *P. vitticeps* sex chromosome BAC clones that can be used as hybridisation probes in fluorescence *in situ* hybridisation (FISH) onto metaphase chromosomes from various species having both TSD and GSD.

1.4.2.2 Sex-linked markers

Finding sex chromosomes may not be achievable if the sex chromosomes are cryptic and very close to homomorphic. These chromosomes may contain sex-specific genes or sequences that can reveal their sex determination mode and sex chromosome heterogamety (XX/XY or ZZ/ZW). This may even be true in TSD species, which lack sex-specific chromosomes but have genes or sequences expressed differentially between sexes. Besides, a GSD species may show different offspring sex ratio patterns during the incubation experiments due to gene-temperature interactions (i.e., GSD+EE species). In this case, a skewed sex ratio is observed among the offspring of a species beyond a certain incubation temperature threshold (in sections 1.1, 1.2.3 and 1.3.2). The development of sex-linked markers may provide valuable insight in understanding the genetic basis of sex determination in these species. For example, it provides information on whether the sex is determined by a sex chromosome (GSD) or by methylation mediated differential expression of genes in autosomes influenced by environmental factors. Such markers have been successfully identified in several other reptilian taxa, including agamid lizards (Gamble and Zarkower 2014; Hill et al. 2018; Lambert et al. 2016; Quinn et al. 2009). Hence, the development of such sex-linked markers is highly required to reveal the sex determination modes in species with undifferentiated sex chromosomes.

1.4.2.2.1 Methylation and sex determination

It is speculated that the differential methylation of the promoters of genes is involved in sex determination at sex-specific temperatures, yet the mechanism is not well understood (Deakin et al. 2014). Differences in gene methylation between genetic males and females have been observed in both adults (Baroiller and D'Cotta 2016; Navarro-Martín et al. 2011; Shao et al. 2014) and during the developmental stages (Ramsey et al. 2007) in different animal groups. However, epigenetic changes do not involve a change in nucleotide sequence facilitated by DNA methylation (Matsumoto et al. 2013). Restriction site-associated DNA sequencing (RADseq and DArTseq) has been proposed as a method for developing sex-linked markers in taxa with homomorphic sex chromosomes (Gamble and Zarkower 2014) and has successfully been used to identify sex-linked markers and infer the sex-determining mode for several amphibian, squamate and fish species (Brelsford et al. 2016; Gamble et al. 2015; Gamble and

Zarkower 2014; Hill et al. 2018; Lambert et al. 2016; Lambert et al. 2019; Ogata et al. 2018; Palaiokostas et al. 2013; Wilson et al. 2019). Diversity Arrays Technology, a private company dedicated to genotyping by sequencing, has developed methylation-sensitive DArTseq™ that uses two different restriction enzyme isoschizomers (one CpG methylation-sensitive and other not) and can be used to identify sex-specific markers (GSD species) and has the potential to reveal any methylation mediated sex determination (GSD, GSD+EE and TSD species).

1.4.2.3 Putative yet unknown sex determination mechanisms need to be unmasked

GSD species maintain a stable 1:1 sex ratio in their offspring, while TSDs may or may not (section 1.1). Since agamid species have either TSD or GSD with temperature influenced sex determination, it needs to be investigated whether other species with unknown sex determination mechanism has either of these two types and how concordant or discordant they are with well-studied species (e.g., *Pogona* and *Bassiana*). This will fill in the gaps in the existing phylogenetic relationships and evolutionary history of sex determination mechanism in closely related species, i.e. the entire Agamidae. There are species where sex determination mechanisms have been predicted but not yet confirmed through rigorous scientific experiments. For example, no detailed study on the sex determination mode or mechanism in endangered Canberra grassland earless dragons, *Tympanocryptis lineata* has been conducted, though incubation of eggs at the different temperature indicated it to be a putative GSD species (S. D. Sarre pers. Comm.), successive analysis is yet to be done.

1.4.2.4 Sex determination mechanisms and sex chromosomes may vary geographically

Although sex determination mechanisms and sex chromosomes may vary from reptile species to species, knowledge will be improved with work on variation within species, which is poorly studied. For example, four genetic forms of the Japanese wrinkled frog, *Glandirana* (*Rana*) *rugosa* were found distributed in four different geographic regions of Japan (Miura 2007; Nishioka et al. 1994); where male and female heterogametic sexes were located in different geographic locations. This provides an excellent model for studying the similar phenomenon in the mode of sex-determining mechanisms in a closely related species with a wide geographic distribution (a species complex) in agamid lizards.

The Oriental garden lizard *Calotes versicolor* belongs to the subfamily Draconinae of the family Agamidae. It has a wide distribution and is found from Iran to Malaysia through South Asia and southeast China. This species is considered as taxonomically neglected (Gowande et al. 2016) and regarded as a complex of multiple species (Huang et al. 2013; Zug et al. 2006).

It lacks heteromorphic sex chromosomes (Ganesh and Raman 1995; Singh 1974), but its sex determination pattern was found to be closer to the genotypic sex determination in mammals than that in the environmental sex determination in reptiles (Tripathi and Raman 2010). There is speculation that this could be a GSD species with XY genotype (Chakraborty et al. 2009), while Wilson et al. (2019) reported a possible ZZ/ZW system in this species from India using RADseq, a reduced representation genome sequencing method. However, Doddamani et al. (2012) claimed it to be a TSD species with a novel FMFM pattern based on the relationship between the incubation temperature and offspring sex ratio. Whether these observed variations are due to the existence of multiple cryptic species (a species complex) or transition of sex determination mechanisms and sex chromosomes, or the existence of multiple thermosensitive points among closely related species or populations is not known. Therefore, investigating sex determination modes and sex chromosomes within this species (or species complex) is of great scientific interest.

1.5 Thesis objectives

The presence of multiple transitions (e.g., GSD ⇔ TSD) and turnovers (e.g., XY ⇔ ZW, ZW ⇔ ZW) within reptiles highlights this group as ideal for studying evolutionary transitions between sex-determining systems and understanding how temperature can influence sex. A complete understanding of sex determination evolution requires proper identification of sex determination modes and understanding sex chromosome homology among closely related species, even among populations across geographic locations. Agamid lizards hold considerable promise as a model reptile group for addressing these issues (Quinn 2008).

Although agamid lizards are gaining increasing recognition for the opportunities they present as a model reptile group, for the study of sex determination mechanism and sex chromosome evolution, it is mostly limited to one single subfamily Amphibolurinae based in Australia. Within this Australian clade, several breakthrough discoveries have been made, including micro-sex chromosomes (Ezaz et al. 2005), temperature overrides in GSD species in captivity as well as in the wild (Holleley et al. 2015), transitions among sex determination modes and evolution of *de novo* sex chromosomes (Ezaz et al. 2009b). However, section 1.4.2 and figure 1.3 clearly shows there are knowledge gaps on these issues. Therefore, investigations on sex determination with representatives across the family, at the subfamily level, will provide the potential to explore these concepts at a finer scale, among closely related species and even

between populations. An essential first step towards this initiative would be the comparative analysis of sex chromosomes in GSD agamids and the identification of their homologues in closely related TSD agamids. This needs to be complemented with incubation experiments and identification of sex-linked markers using advanced techniques as genotyping by sequencing (GBS).

This PhD research project aims to investigate several aspects of the evolution and ecology of the sex determination mechanisms within the subfamilies of agamid lizards. Following are my four research objectives; each objective is represented as a data chapter in the thesis:

Objective 1: To identify sex chromosome homologies across agamid lizard subfamilies,

Objective 2: To identify the sex determination mode in an agamid lizard using cytogenetic approaches,

Objective 3: To identify sex determination modes in a representative agamid lizard using incubation experiments and sex-linked molecular markers, and

Objective 4: To identify sex determination modes within a species complex.

1.6 Thesis structure

The thesis contains seven chapters (Fig. 1.4). Chapter 1 is this general introduction, and Chapter 7 is a synopsis. Chapter 2 involves a review of the existing literature on lizard sex chromosomes and their evolution. Chapter 3 to 6 each represent one of the objectives as mentioned in section 1.5.

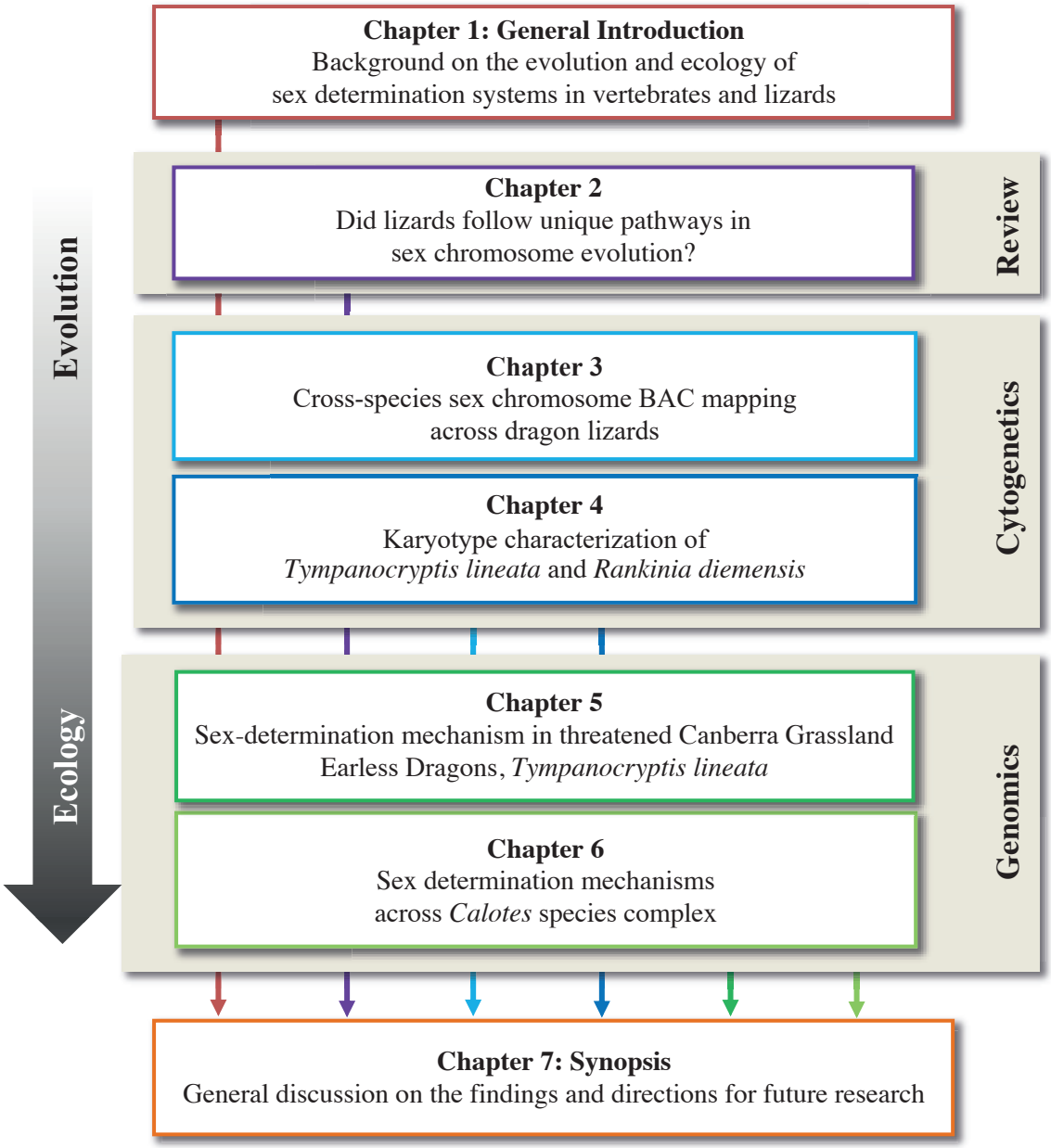

Figure 1.4 Schematic diagram of the thesis structure.

CHAPTER 2: Did lizards follow Unique Pathways in Sex Chromosome Evolution?

This paper has appeared in print.

Alam SMI, Sarre SD, Gleeson D, Georges A, Ezaz T: Did Lizards Follow Unique Pathways in Sex Chromosome Evolution? Genes 2018, 9(5): 239; https://doi.org/10.3390/genes9050239

Preface

Lizards show no particular pattern of sex chromosome degeneration of the kind observed in mammals, birds and or snakes. Besides, the remarkable diversity in modes of reproduction and sex determination, together with the co-existence of GSD with TSD within and among sister clades makes lizards an attractive model to study and understand the evolution of sex chromosomes. The question then remains what enables lizards to maintain such diversity in sex-determining modes and sex chromosomes. This article explored this issue speculating that sex chromosome evolution in lizards is labile and rapid, and mostly follows independent trajectories. Existing knowledge on the evolution of sex chromosomes in lizards was reviewed for this article and discussed how sex chromosome evolution within this group differs from other amniote taxa, facilitating unique evolutionary pathways.

Justification of inclusion

As first author, I was responsible for writing this paper which included reviewing the literature, writing and revising the draft and designing the diagrams.

Declaration for Thesis Chapter 02

DECLARATION BY CANDIDATE

In the case of Chapter 02, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contributions (%)
• Literature review • Writing and revising draft • Designing the diagrams • Correspondence with the journal	75%

The following co-authors contributed to the work:

Name	Nature of Contribution	Contributor is also a UC student (Yes/No)
Tariq Ezaz	Idea development, draft writing, reviewing and supervision	No
Stephen D. Sarre	Reviewing and supervision	No
Dianne Gleeson	Reviewing and supervision	No
Arthur Georges	Reviewing and supervision	No

31/08/2020

Candidate's Signature and Date

DECLARATION BY CO-AUTHORS

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

[Please note that the location(s) must be institutional in nature, and should be indicated here as a department, centre or institute, with specific campus identification where relevant.]

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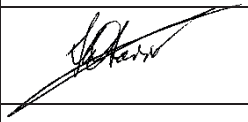
Stephen D. Sarre	
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genes

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Review

Did Lizards Follow Unique Pathways in Sex Chromosome Evolution?

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Abstract: Reptiles show remarkable diversity in modes of reproduction and sex determination, including high variation in the morphology of sex chromosomes, ranging from homomorphic to highly heteromorphic. Additionally, the co-existence of genotypic sex determination (GSD) and temperature-dependent sex determination (TSD) within and among sister clades makes this group an attractive model to study and understand the evolution of sex chromosomes. This is particularly so with Lizards (Order Squamata) which, among reptiles, show extraordinary morphological diversity. They also show no particular pattern of sex chromosome degeneration of the kind observed in mammals, birds and or even in snakes. We therefore speculate that sex determination sensu sex chromosome evolution is labile and rapid and largely follows independent trajectories within lizards. Here, we review the current knowledge on the evolution of sex chromosomes in lizards and discuss how sex chromosome evolution within that group differs from other amniote taxa, facilitating unique evolutionary pathways.

Keywords: lizards; genotypic sex determination (GSD); sex-chromosome evolution

1. Introduction

Sex chromosomes, modes of sex determination and reproduction in reptiles (non-avian: tuatara, lizards, snakes, turtles and crocodilians) are among the most diverse of the amniotic vertebrates (reptiles, birds and mammals), often showing little regard to phylogeny, which in turn implies multiple and independent origins as well as frequent transitions and reversals [1–7]. For example, reptile sex chromosomes vary greatly in their level of degeneration, ranging from cryptic to highly differentiated [6,8–11] and are the only amniotes where genotypic sex determination (GSD) and temperature-dependent sex determination (TSD) appear to have evolved independently many times with evidence of both modes at the level of order through to species and even within a single species [12–17]. Moreover, in some species, like the Australian bearded dragon lizard *Pogona vitticeps* [17–19], genes on the sex chromosomes interact with the incubation environment to determine sex. Reptiles also epitomize the variability of modes of reproduction and fertilization among amniotes, with many species exhibiting oviparity, ovoviviparity or placental viviparity, and both facultative and obligate parthenogenesis have been reported [4,20–28]. Apart from fishes, no other vertebrate group shows such diversity and variability in the mode and mechanism of sex determination or in sex chromosomes and modes of reproduction. This diversity, all within a single taxonomic order, provides a fertile field for the discovery of novel mechanisms that define the most fundamental of phenotypes, sex. It is therefore remarkable that little is known of the molecular mechanisms that have

enabled this diversity. In comparison to other amniote groups, little effort has been made to better understand the mode and mechanisms of sex determination in reptiles, and how they have evolved over 300 million years since they diverged from other amniotes [29,30].

In this review, we present a brief overview of the current understanding of sex chromosomes and sex determination in reptiles, particularly in lizards and highlight the aspects that are unique to them. Specifically, we ask: did reptile sex chromosomes follow the same evolutionary pathway as proposed for birds and mammals?

2. Sex Chromosome Evolution in Amniotes—The Classical Concept

Sex chromosomes are the most dynamic entity within a genome, being characterized by a unique morphology and specialized evolution [1–3,7,31]. The morphological diversity of amniotic sex chromosomes is truly remarkable ranging from cryptic to highly heteromorphic. Amniote GSD species are either male heterogametic where males and females have XY/XX sex chromosomes (respectively—as in most mammals), or female heterogametic (male/female: ZZ/ZW) as in all birds. Among mammals, monotremes (platypus and echidnas) present as rare exceptions, possessing a remarkable multiple XY system that is not homologous with therian XY systems, but rather show homology with chicken Z chromosomes [32,33]. In contrast to the conservative nature of mammals and bird sex chromosomes, many reptiles exhibit remarkable variation in the sex chromosome pair, and in the system of heterogamety, sometimes even among closely related species or even within populations [6,12,31].

It is generally accepted that heteromorphic sex chromosomes originate from an autosomal ancestor following mutational acquisition of a sex determining allele [2,31,34–38]. This can happen within all systems that already involve sex chromosomes or sex determination genes or within those that require environmental factors such as temperature to resolve sex. Additional sex-linked mutations and the subsequent suppression of recombination (either by chromosomal rearrangements or accumulation and expansion of repetitive sequences) in these proto sex chromosomes result in morphologically, as well genomically, specialized sex-specific chromosomes. It has also been proposed that sex chromosome formation may start with the acquisition of sexually antagonistic alleles close to the sex-determining locus which would suppress recombination and pseudogenize genes (i.e., multiply and accumulate these genes, with mutations that cause loss of functionality) that do not have sex-specific benefits [39]. Additional sexually antagonistic alleles could cause the expansion of the non-recombining region and further suppression of recombination [40–42] leading to an increase in the size of the Y or W. However, these increases are often reduced through large-scale deletions [43,44], resulting in a chromosome that is smaller than the X or Z chromosome [2,45].

Sex chromosomes are the most rapidly evolving structural entities of the genome in many groups of animals [12,34,36,46]. One aspect of sex chromosome evolution, recombination suppression, is known to trigger several evolutionary processes, such as Muller's Ratchet [47,48] and genetic hitchhiking (see below in Section 2.1), that cascade through to the loss of gene activity and pseudogenization, particularly where sex chromosomes are heteromorphic. However, it is less clear what happens in the case of homomorphic sex chromosomes [39]. All sex chromosomes in the heterogametic systems (X, Y, Z and W) have differences in their evolutionary environments including background selection pressure, effective population sizes, mutation rates, and genomic imprinting as well as meiotic drive [45–48]. These factors influence chromosomal rearrangements, and thereby play a role in changes in genome structure [39,49]. For example, genes on the mammalian Y chromosome are subject to selection via expression in the male phenotype, whereas genes on the W chromosome in birds do not appear to be subject to the same level of selection primarily because Z chromosomes exhibit differential expression in males [49–52].

2.1. Hitchhiking, Meiotic Drive and Imprinting

Evolution of sex chromosomes and their ability to spread within populations have been explained using different mechanisms, such as genetic hitchhiking, meiotic drive and imprinting. However, none of these explain how initial processes such as the acquisition of sex-specific genes or the suppression of recombination occurs or how derived lineages end up in two genetically distinct sex chromosomes [53].

Genetic hitchhiking, in the context of sex chromosome evolution, is the process whereby genes or mutations in genes not involved in sex determination are carried along with the chromosome through linkage. In this process, deleterious mutations on the Y chromosome cannot be removed by recombination and are therefore able to spread through a population because they are linked to, and hitchhike with, sex-specific beneficial genes. The net result of this is that the heterogametic chromosome becomes less and less genetically active resulting in locus-specific dosage tolerance or compensation [3,54]. The homogametic chromosomes (X or Z) escape this fate because they are still able to recombine [43]. However, selective advantage can be gained by the heterogametic chromosome when beneficial mutations are more pronounced than the linked deleterious ones [43]. The hitchhiking effect is most distinct when Muller's ratchet is in place, that is, when these mutations/changes are irreversible.

Maintaining an even sex ratio among offspring is seen as being critical for many species, and it is common for the heterogametic sex to produce equal numbers of male and female gametes (X and Y or Z and W). Nevertheless, unequal transmission of X and Y chromosomes from individuals of the heterogametic sex have been observed during meiosis [55,56]. Such phenomena are referred to as sex chromosome 'meiotic drive' and result in biased sex ratios among offspring and even within populations [56]. Such phenomena may also occur in TSD species where skewed sex ratios can be caused by exposure to nest incubation temperatures that are biased towards one sex or the other [13,56]. However, the unequal distribution of sex chromosomes can reduce mean fitness within a population by interfering with the sex chromosome—autosome relationship (intragenomic conflict between the X, the Y chromosomes, and the autosomes) and altering the intensity or mode of sexual selection [56]. Nevertheless, such phenomena are neither evolutionary stable nor easily detectable, hence these might be a more common occurrence than reported (only to occur in the insect order Diptera and mammalian Rodentia) [57,58].

It has been proposed that differences between sexes may be determined by differential methylation in nuclear DNAs of males and females. Methylation suppresses recombination and increases mutation rates that drive Muller's ratchet. As a result, selection pressures are created to remove these areas of Y or W chromosomes, ultimately playing a role in their degeneration [53]. Genomic imprinting is the epigenetic marks imprinted on genes owing to chromosomal transmission through the female and male germlines and this often results in gene expression differences between maternally and paternally inherited alleles [59]. These epigenetic marks are established during spermatogenesis and oogenesis by DNA methylation and histone modifications and carried from parents to offspring via sperm or egg cells. These are continued within the offspring somatic cells by mitosis [60]. As a result, gene expression in offspring depends on a parent-of-origin manner rather than from both homologous chromosomes—some genes are expressed that are only from the father and some others are expressed that imprinted only from the mother [61]. But a balanced contribution between these maternal and paternal expressions is required for development of such lineages [59].

2.2. Multiple Origin, Rapid Transitions and Turnovers

Sex determination in amniotes shows a sharp contrast between the extraordinary conservation of mammals and birds and the astonishing lability of reptiles [62]. Sex determination in therian mammals depends almost universally on the presence or absence of the Y chromosome and the master sex determining gene *SRY*, as demonstrated through manipulative studies of development and gene expression [63–65]. In marsupials too, the formation of the testes is determined by a Y-dominant mechanism, although other traits that are also characteristic of males and females

depend on X-chromosome dosage [66]. Deterioration of the Y chromosome in mammals is ubiquitous leading to pronounced heteromorphy of the X and Y chromosomes or, in the case of mole voles (*Ellobius* sp.) and spiny rats (*Tokudaia* sp.) [67–69], the total loss of the Y. Birds too appear highly conserved in their sex determination mechanism. ZW sex chromosome heteromorphy occurs in most birds [70] and female development depends on the presence or absence of a W chromosome, since it carries female-determining genes. In contrast to mammals, male sex determination in chickens (sensu birds) have shown to be influenced by the dosage of the Z-linked gene *DMRT1* (two copies of the gene in males, one copy in females) [70] rather than through a dominance as in most eutherian mammals (Y-linked *SRY*, [63,65,71]). However, existence of ZZW females in birds (e.g., [72]) may indicate a more complex system, including a genic balance system, could not be ruled out.

Against this background, sex determining modes among reptiles are diverse. Among those with GSD, both male and female heterogamety (XY and ZW) are known in turtles, female heterogamety (ZW, Z_1Z_2W, or ZW_1W_2) is known in snakes and both are known in lizards including XXY [73,74]. Many species have TSD where sex is determined by the temperature of incubation [75–78] and temperature and genotype can co-exist within species or even interact to reverse chromosomal sex [79–82] and influence sex ratios and drive divergence in sex determining mechanisms [82]. In contrast to mammals and birds, the sex chromosomes in most GSD reptiles are cryptic, lacking detectable heteromorphic chromosomes and in many cases involving micro-chromosomes.

The diversity of sex determining mechanisms in the ectothermic reptiles, compared to that of the homeothermic birds and mammals, and amphibians and fish (typically poikilothermic), may have arisen because of a unique predisposition to the development of TSD, acting as an intermediary in the evolution of GSD in its various forms [83]. The diversity and haphazard distribution of sex determination mechanisms among reptiles and the lack of sex chromosome homology suggests that transitions between modes has occurred many times (Figure 1) [6,84] and may occur extremely rapidly [17]. The interaction between temperature seen in bearded dragons provides a mechanism for rapid switching among chromosomal states and TSD [17,19]. Among reptiles, lizards are perhaps the most diverse and labile. Of the 181 species for which sex chromosomes have been detected, about two thirds have male heterogamety [6], while both male and female heterogamety occur in at least one family (Gekkonidae) along with TSD [85]. This fascinating diversity of sex-determining mechanisms shows no clear phylogenetic segregation [13,14,86].

3. Overview of Current Understanding of Sex Determination and Sex Chromosomes in Lizards

3.1. Temperature-Dependent Sex Determination in Lizards

Unlike other amniotes, the class reptilia is the only vertebrate group where both GSD and TSD have been described (450 species examined) and where there is evidence of frequent evolutionary transitions between these two modes (Figure 1) [12,14,62]. Although not all species have been subjected to systematic incubation experiments to identify cases of TSD, it has been likely that all crocodiles (25 species), marine turtles (seven species), and tuatara (one species), and several freshwater turtles and tortoises (49 of 260 species) and many lizards (45 of 6459 species) have TSD. So far, TSD has not been reported in any snakes (3619 species), but only three species have been subjected to laboratory-based systematic incubation experiment [87–90], and at least one publication (though not focused on TSD) reported sex ratio biases in response to variable incubation temperatures in stripe-tailed rat snake *Elaphe taeniura* [91]. In that case, both high and low temperatures produced more males while intermediate temperatures produced females resulting in a biased sex ratio. TSD has also been described in both viviparous and ovoviviparous reptiles with three out of the four viviparous (lizards) and one ovoviviparous species (snake) that have been tested through incubation experiments reported to have TSD [87,88,92–95]. Most species remain untested for their sex determination system, highlighting the fact that what we know about reptilian TSD is only the tip of the iceberg. More lab-based systematic incubation experiments as well as field

based studies will be required to obtain a clearer picture of reptilian TSD (for details see available literatures [5,12,13,18,21,22,36,38,45,62,84,86,96–106] and references therein).

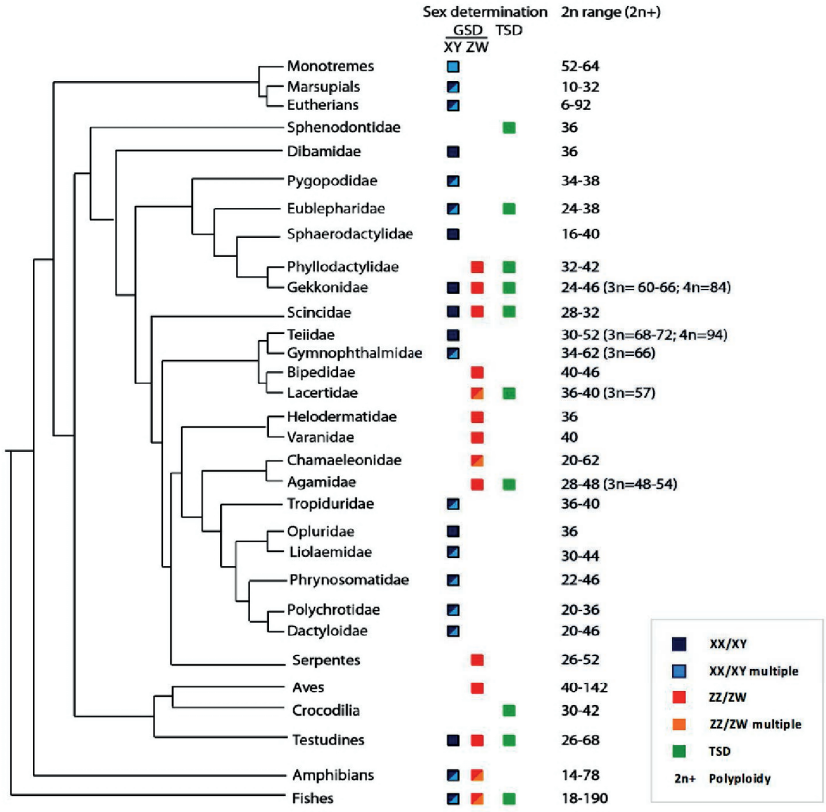

Figure 1. Truncated phylogeny (not according to scale) showing modes of sex determination and number of chromosomes in major lineages of vertebrates, with a particular emphasis on major families of lizards where modes of sex determination and sex chromosome systems are known and show high diversity. This figure includes only those lizard families where sex chromosomes have been identified cytogenetically. Adopted from [6,62,107–117] and references therein.

3.2. Karyotypes, Genotypic Sex Determination and Sex Chromosomes in Lizards

Reptilians are also karyotypically diverse, with macro- and micro-chromosomes occurring (with the exception of crocodilians which have all macrochromosomes) in the genomes of most reptile species that have been karyotyped to date. They are also karyotypically heterogeneous group [108] with varying numbers of micro- and macro-chromosomes (Table 1). The lowest number of diploid chromosomes occurs in a lizard ($2n$ = 20, Cameroon stumptail chameleon, *Rampholeon spectrum*), while the highest diploid number ($2n$ = 68) occurs in a freshwater turtle (twist-neck turtle, *Platemys platycephala*) and the highest number of macro- and micro-chromosomes are observed in crocodilians and freshwater turtles respectively (42 and 56 respectively, Table 1). Although diploidy is common in reptiles, a considerable number of parthenogenetic species have triploidy and the occurrence of triploid individuals in populations of diploid species is not uncommon [118].

Reptiles exhibit considerable genomic variation across different organizational levels of reptiles ranging from 1.03 to 5.3 Gb (Table 1) [119]. The lowest range of genome size is found in a lizard, in Mionecton skink *Chalcides mionecton* (1.03 Gb) and largest in Mediterranean spur-thighed tortoise

Testudo graeca (5.3 Gb). The largest genome size in lizards is 3.80 Gb in Slow worm *Anguis fragilis*. However, consistence with different plant and animal groups [120], genome size and the number of micro and macro chromosomes are not evolutionarily correlated in reptiles.

Table 1. Ranges of diploid chromosomes numbers and numbers of macro and microchromosomes and genome sizes in major groups of reptiles.

Taxon	Chromosome			Genome Size (Gb) [119]	
	2*n* Range	**Macro Range**	**Micro Range**	**Low**	**High**
Tuatara	36	28	8	4.9	
Lizards	20–62	10–38	0–28	1.03	3.8
Snakes	26–50	10–38	0–36	1.3	3.7
Crocodilians	30–42	30–42	0	1.3	3.9
Freshwater Turtles	26–68	10–36	0–56	1.4	5.3
Marine turtles	56	24–32	24–32	2.6	

3.3. Sex Chromosome Differentiation in Lizards

In many lizards, as in most mammals and birds, the heterogametic Y or W chromosomes are highly differentiated morphologically and in sequence composition. In contrast, there is great variability in the degree of differentiation between the sex chromosome homologues in reptiles, particularly in lizards [121]. In addition, some lizards have been found to possess complex male or female heterogametic systems involving multiple sex chromosomes from varying evolutionary stages of differentiation [84]. In particular, heterochromatinization of one sex chromosome varies greatly in GSD lizards, ranging from a small block to the entire chromosome. Additionally, chromosomal rearrangements such as fusions, inversions and translocations have also contributed to sex chromosomal differentiation in lizards [122].

Heteromorphic sex chromosomes are differentiated at the level that can usually be detected cytologically, while in the case of homomorphic sex chromosomes, this differentiation is most likely at the gene level. It has been found that the W chromosome of *P. vitticeps* is heterochromatic and more heavily C-banded than its Z chromosome. This heterochromatinization is thought to be an early change that initiated sex chromosomal differentiation in lizards [121]. On the other hand, deletion events are likely to be involved in the differentiation of W chromosomes in multiple agamid lizard species including *P. vitticeps, Pogona barbata, Amphibolurus nobbi* and *Ctenophorus fordi* [84].

It is generally considered that highly differentiated sex chromosomes are a barrier to the subsequent evolution of TSD, and that homomorphic sex chromosomes are a necessary prerequisite for such a transition in a sex-determining mechanism [4,36,123]. However, GSD reptiles with highly differentiated sex chromosomes can indeed switch to TSD as in the case of *P. vitticeps* and *Bassiana duperreyi* [121,124]. Moreover, Chromosome rearrangements may well play a major role in sex chromosome differentiation in the reptilian lineages [121]. Shifts have even been observed from one form of agamid lizard ZW sex chromosome system to a different ZW system in a short evolutionary time [84].

4. Unique Pathway of Sex Chromosome Evolution in Lizards—A Different Pathway from the Classical Model (as That Proposed for Birds and Mammals)?

4.1. Temperature Dependent Sex Determination and Sex Chromosome Evolution

Sex in TSD species is determined by temperature experienced by developing embryos and any involvement or association of sex chromosome is unknown [4,13,36]. It is predicted that the common ancestral mechanism for all amniotes was GSD with ZW heterogamy, although the TSD found in present day squamates have been known to evolve multiple times and independently from a common

TSD ancestor [13,14]. However, it seems unlikely that sex in any species is determined purely by TSD and transitions between TSD and GSD may involve loss and gain of sex chromosomes as described in Section 2. Transitions between these two mechanisms are more likely to occur in species with poorly differentiated sex as in some reptiles and it is also likely that well developed sex chromosomes will resist such transitions owing to their accumulation of beneficial sexually antagonistic genes that maintain and regulate sexual fitness, meiosis and dosage compensation [111].

However, in reptile species with well-differentiated heterogametic sex chromosomes, as in Australian bearded dragon *P. vitticeps*, sex reversal has been observed both in the laboratory and the field, and has been attributed to the influence of temperature during embryonic development [17,19]. Normal ZZ males incubated at high temperatures became sex-reversed to fertile ZZ females that are able to breed with ZZ males. The homomorphic sex chromosomes in ZZ male and female offspring have effectively become autosomal [17]. This finding shows how temperature could cause a rapid transition from GSD to TSD and in doing so, eliminate the W chromosome. A similar case was observed in the Eastern three-lined skink, *B. duperreyi*, with a well-differentiated XX/ XY GSD system where sex reversed XX were found in cold conditions [16]. In both lizard species, sex reversal took place in homogametic sexes and there was no sex reversal in the heterogametic sexes apparently. This prevents mating between individuals of heterogametic genotype and ultimately eliminates the chances of producing nonviable WW or YY offspring [81]. How this works in nature remains problematic and is in need of intense field-based studies to unravel.

4.2. Multiple Sex Chromosomes in Lizards

The existence of non-homologous sex chromosomes in closely related lineages implies that sex chromosomes in lizards can evolve independently and will be little constrained by past evolutionary events [125,126]. As a result, deviation from the typical XY/XX or a ZW/ZZ systems that includes absence of a sex chromosome from the system (X0/XX or Z0/ZZ systems) or multiple sex chromosomes ($Z_1Z_2W/Z_1Z_1Z_2Z_2$ and XY_1Y_2/XX systems) are possible. The number of chromosomes is considered as an important feature of eukaryote genomes which may have potential consequences for processes such as recombination and segregation [49]. Chromosome number may vary between closely related species and even within species that can contribute to adaptation and speciation [1–5,49]. Differences in chromosome numbers are usually caused by reciprocal translocation between two chromosomes—by the fusion between two acrocentric chromosomes or the split (fission) of a metacentric chromosome into two [127]. Fixation of chromosomal rearrangements through random genetic drift, changes in recombination rate and meiotic drive are the evolutionary forces that may be involved in such chromosome number variations [128,129] however, what allows fusion and fission to become fixed within a population is not yet known [49].

The fusion of a Y chromosome with an autosome creates an X_1X_2Y system with the unfused homologue segregating as a neo-X chromosome and causes an odd number of chromosomes in one sex [2,18]. Instances of such fusions have been found in a number of lizard families Gekkonidae (e.g., ♂33♀34 in *Phyllodactylus lanei*), Gymnopthalmidae (e.g., ♂57♀58 in *Calyptommatus* spp.), Chamaeleonidae (e.g., ♂34♀35 in *Bradypodion ventrale*), Iguanidae (e.g., ♂31♀32 in *Sceloporus* spp.) and Pygopodidae (e.g., ♂33♀34 in *Lialis burtonis*). Likewise, X-autosome fusions generate XY_1Y_2 systems, as may be in family Scincidae (e.g., ♂31♀30 in *Mabuya mabouya*), W-autosome fusions generate Z_1Z_2W systems as in Lacertidae (e.g., ♂36♀35 in few populations of *Lacerta vivipara*) and Z-autosome fusions generate ZW_1W_2 systems that have not yet been observed in lizards but probably occur in another reptile group, sea snake family Hydrophiidae (e.g., ♂34♀35 in *Hydrophis fasciatus*) [108]. Many species with sex chromosome-autosome fusions have been discovered as these multiple sex-chromosome systems can be easily identified [2,19–22]. Such phenomena can predominantly be found in reptiles among amniotes and especially among lizards.

4.3. Sex Chromosome Evolution in Lizards May Involve yet Undescribed Gene Regulatory Mechanisms

The evolution of heteromorphic sex chromosomes in vertebrates has been thought to involve a process of gradual degradation of macro-chromosomes [2,3,11,31,35,38]. However, both macro- and micro-chromosomes have been identified as sex chromosomes in reptiles [121,130–136]. In addition, multiple sex chromosomes are common in some groups, particularly in Iguanids and Lacertids, suggesting that the translocation of sex chromosomes to autosomes has occurred. These variations present a challenge for interpretation under the conventional theory of vertebrate sex chromosome evolution, while morphological variations between sex chromosomes also range quite substantially from homomorphic to highly heteromorphic, representing various stages of evolutionary degradation [8,11,121]. However, this does not often correlate with the evolutionary age of those taxa. The identification of sex micro-chromosomes has previously been challenging but is now made possible owing to recent advancements of cytogenetic techniques (such as CGH—Comparative Genomic Hybridization and FISH—Fluorescence in situ Hybridization). Whether similar mechanisms are involved in the evolution of micro sex chromosomes has yet to be discussed. Therefore, molecular and cytogenetic mechanisms behind the evolution of sex micro-chromosomes remain unknown, such as whether they have evolved as a result of chromosome fission or fusion events or micro-chromosomes also followed the same pathway as proposed for vertebrates, or whether completely different molecular mechanisms are involved in evolution of sex microchromosomes, is yet to be determined. One possibility could be that both macro and microchromosomes have evolved independently, involving new pairs of sex microchromosomes driven by frequent transitions, either via translocation of sex determining factors via transposition or novel sex chromosomes have evolved after each transitions, involving novel genes and novel chromosomes.

We argue that, in addition to the proposed pathway of vertebrate sex chromosome evolution, the evolution of sex chromosomes in reptiles has also occurred via other molecular mechanisms, particularly subtle gene regulatory mechanisms (e.g., epimutations, i.e., abnormal transcriptional repression of active genes and/or abnormal activation of usually repressed genes caused by errors in epigenetic gene repression [137], evolution of sexually antagonistic genes, e.g., [53,138]). This perhaps suggests reptiles possess a plasticity in maintaining sex ratios in highly labile environments (Figure 2), which would somewhat explain the maintenance of TSD, transitions between TSD and GSD as well as temperature mediated sex reversal in GSD species with cryptic (*P. vitticeps*) and heteromorphic sex chromosomes (*B. duperreyi*). This also explains the numbers of homomorphic sex chromosomes (in about 70 spp.) as well undetected sex chromosomes (1185 spp.) in described karyotypes (1562 spp.) [139].

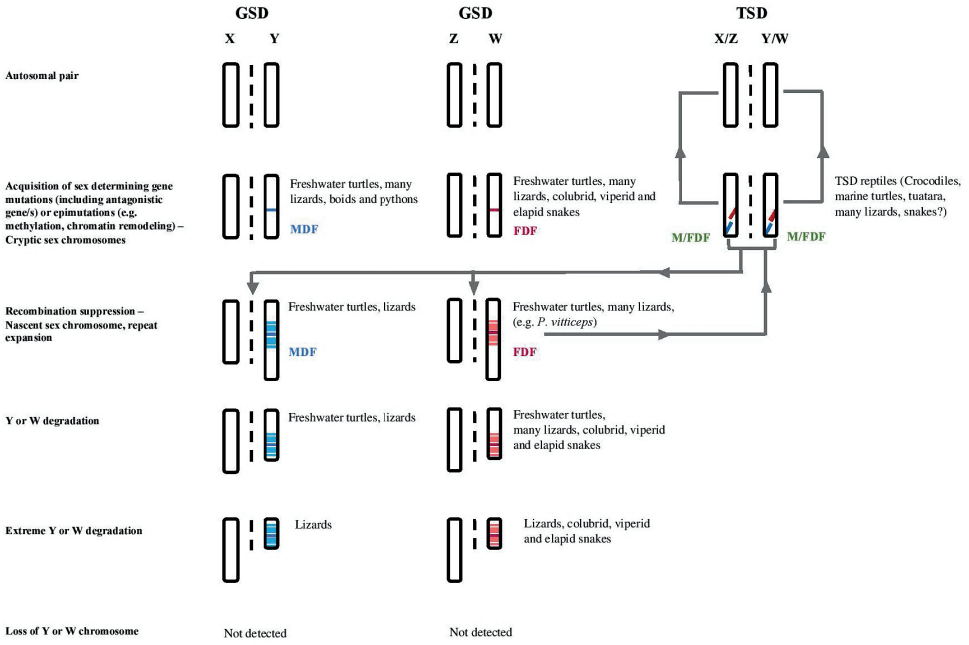

Figure 2. Proposed model for lizard sex chromosome evolution, modified from [11]. Many Reptiles are likely to have followed the currently held view of sex chromosome evolution as proposed for vertebrates but may also involve other regulatory molecular mechanisms (e.g., epigenetic). The sex determination (sensu sex chromosomes) in genotypic sex determination (GSD) and temperature-dependent sex determination (TSD) with environment influence is bipotential and could involve polygenic or epigenetic mechanisms, hence retaining the homomorphic sex chromosomes and high diversities. MDF = Male determining factor, FDF = Female determining factor.

5. Conclusions

Reptiles have diverse modes of reproduction, sex determination mechanisms as well as diversity of sex chromosomes—from GSD to TSD as well as GSD with temperature influences. Independent evolution and multiple lineage divergence in reptiles than other amniotes (mammals and birds) may have contributed in the diversified systems ranging from species with devoted sex chromosomes (homomorphic, XY/ZW heteromorphic or multiple sex chromosomes) to none, that is, non-strong genetic determinant, autosomal genes acting differently on sex-determining pathways. However, it has been observed that sex chromosomes have degenerated and novel sex chromosomes have evolved to resolve sex determination. There are also numerous instances of convergent evolution of sex chromosomes across distantly related taxa if certain genes are particularly adept at taking on a sex-determining role [140,141].

Several species of reptiles have been studied in respect to evolution of sex chromosomes but unlike other amniotes as birds and mammals, reptiles still lack a functional model that represents the overall reptilia. Species with well-characterized GSD, such as green anole or *P. vitticeps* should be given priorities in identifying master sex genes in reptiles, as TSD in reptiles might be polygenic involving multiple sex chromosomes or even autosomes [142]. Reptilian sex chromosomes have similar evolutionary history but have taken different pathways with differential temperature influences, and particularly in lizards.

Recent advanced technologies such as comparative gene mapping and whole genome sequencing have shown surprising relationships among different groups of reptiles, as well as with other amniotes that share common ancestry. Therefore, it will be valuable to compare maps and sequences and

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CHAPTER 3: Cross-species BAC mapping highlights conservation of chromosome synteny across dragon lizards (Squamata: Agamidae)

This paper has appeared in print.

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Preface

Previous comparative studies based on cytogenetically well-characterized Australian central bearded dragon (*Pogona vitticeps*) and other species have revealed chromosomal rearrangements involving sex chromosomes and turnovers in sex chromosomes within the Australian clade of Amphibolurinae. This article explored the level of synteny of the sex chromosomes of *P. vitticeps* across agamid subfamilies using cross-species two-colour FISH with two bacterial artificial chromosome (BAC) clones from the pseudo-autosomal regions of *P. vitticeps* across representative species from all six subfamilies as well as species of chameleons, the sister group to agamids. Conservation of these sequences across the agamid lineages were observed along with multiple chromosomal rearrangements. The study revealed an agamid lineage-specific evolution of sequences/syntenic blocks and successive rearrangements leading to their association with important biological processes such as the evolution of sex chromosomes and sex determination.

Justification of inclusion

As the first author, I wrote this paper which included reviewing the literature, conducting experiments, writing and revising the draft, designing the diagrams and corresponding with the co-authors and the journal.

Declaration of Co-Authored Publications

UNIVERSITY OF CANBERRA

Declaration for Thesis Chapter 03

DECLARATION BY CANDIDATE

In the case of Chapter 03, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contributions (%)
• Conceptualization • Animal and sample collection and preparation • Conducting experiment and analysis • Literature review • Writing and revising draft • Designing the figures/diagrams • Correspondence with co-authors and the journal	70%

The following co-authors contributed to the work:

Name	Nature of Contribution	Contributor is also a UC student (Yes/No)
Marie Altmanová	Reviewing and sample preparation	No
Tulyawat Prasongmaneerut	Reviewing and sample preparation	No
Arthur Georges	Reviewing, supervision and resources	No
Stephen D. Sarre	Reviewing, supervision and resources	No
Stuart V. Nielsen	Reviewing and sample collection	No
Tony Gamble	Reviewing, sample collection and resources	No
Kornsorn Srikulnath	Reviewing and resources	No
Michail Rovatsos	Reviewing, sample preparation and resources	No
Lukáš Kratochvíl	Reviewing, sample preparation and resources	No
Tariq Ezaz	Conceptualization, draft manuscript, reviewing, supervision and resources	No

31/08/2020

Candidate's Signature and Date

DECLARATION BY CO-AUTHORS

The undersigned hereby certify that:

(7) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.

(8) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(9) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(10) there are no other authors of the publication according to these criteria;

(11) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

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Tariq Ezaz	
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* This paper came out as an international collaboration and hence, only the signatures from the supervisory panel have been incorporated.

genes

MDPI

Article

Cross-Species BAC Mapping Highlights Conservation of Chromosome Synteny across Dragon Lizards (Squamata: Agamidae)

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Abstract: Dragon lizards (Squamata: Agamidae) comprise about 520 species in six subfamilies distributed across Asia, Australasia and Africa. Only five species are known to have sex chromosomes. All of them possess ZZ/ZW sex chromosomes, which are microchromosomes in four species from the subfamily Amphibolurinae, but much larger in *Phrynocephalus vlangalii* from the subfamily Agaminae. In most previous studies of these sex chromosomes, the focus has been on Australian species from the subfamily Amphibolurinae, but only the sex chromosomes of the Australian central bearded dragon (*Pogona vitticeps*) are well-characterized cytogenetically. To determine the level of synteny of the sex chromosomes of *P. vitticeps* across agamid subfamilies, we performed cross-species two-colour FISH using two bacterial artificial chromosome (BAC) clones from the pseudo-autosomal regions of *P. vitticeps*. We mapped these two BACs across representative species from all six subfamilies as well as two species of chameleons, the sister group to agamids. We found that one of these BAC sequences is conserved in macrochromosomes and the other in microchromosomes across the agamid lineages. However, within the Amphibolurinae, there is evidence of multiple chromosomal rearrangements with one of the BACs mapping to the second-largest chromosome pair and to the microchromosomes in multiple species including the sex chromosomes of *P. vitticeps*. Intriguingly, no hybridization signal was observed in chameleons for either of these BACs, suggesting a likely agamid origin of these sequences. Our study shows lineage-specific evolution of sequences/syntenic blocks and successive rearrangements and reveals a complex history of sequences leading to their association with important biological processes such as the evolution of sex chromosomes and sex determination.

Keywords: agamid lizards; sex chromosomes; BACs; synteny; evolution; FISH

1. Introduction

Reptiles are well known for their diverse modes of sex determination and sex chromosomes [1,2]. They exhibit large variability in the degree of differentiation of sex chromosomes ranging from homomorphic to heteromorphic in structure [2–5]. Squamate reptiles (lizards, snakes and amphisbaenians) are the most diverse reptile group in terms of species diversity and mode of sex determination [1,6]. The variability seen among squamate sex chromosomes suggests that sex chromosome and sex determination systems have evolved independently many times. Non-homologous sex chromosomes have been reported even among relatively closely related species [2,5,7]. The same parts of the genome (i.e., homologous regions) have been found to play the role of sex chromosomes in different vertebrate taxa [5,8–11]. A high degree of synteny has been observed between birds and squamate reptiles owing to a relative low degree of chromosomal rearrangements in this group [5,12–18]. Temperature-dependent sex determination (TSD), genotypic sex determination (GSD), and GSD with temperature influences between relatively closely related species make squamate lizards an interesting group to study and understand the evolution of sex chromosomes.

Agamid lizards (Squamata: Agamidae), commonly known as dragons in Australasia, are notorious for their variability in forms of sex determination [19–23]. Together with chameleons (Chamaeleonidae), they form the iguanian clade Acrodonta, sister to iguanas (Pleurodonta) [24,25]. Acrodonts are an interesting group in terms of the evolution and diversity of sex determination [7,26,27], while the iguanas have, with one exception (basilisks), conserved XX/XY sex chromosomes [28,29]. There are about 520 currently described agamid species [6] comprising six subfamilies that diverged around 70–120 million years ago [25,30]. Most agamid species are oviparous [6], and the groups includes species with obligate and facultative parthenogenesis [31–33]. Sex determination mode is relatively well studied in a few species from the subfamily Amphibolurinae [7,19], but not in the other five subfamilies (Figure 1), highlighting a significant gap in our understanding of how sex chromosomes evolved in this widespread and chromosomally variable family.

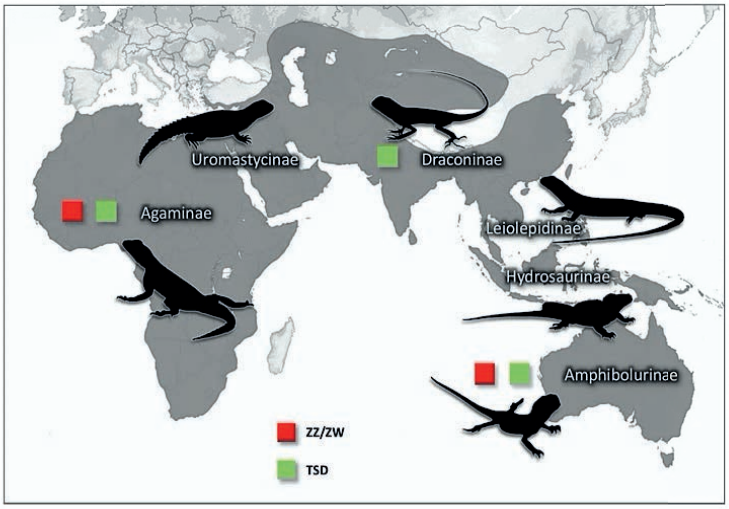

Figure 1. Estimated distribution of the agamid subfamilies together with known sex determination mechanisms [6,17,20,21,24,31,34–49]. The species of the subfamily Draconinae are distributed over South and Southeast Asia, Agaminae across Africa and Asia, Amphibolurinae across Australia, Papua New Guinea and Southeast Asia, Hydrosaurinae across Papua New Guinea, the Philippines and Indonesia, Leiolepidinae across Southeast Asia and Uromastycinae across Africa and South Asia. TSD—temperature dependent sex determination, ZZ/ZW—female heterogamety. Obligatory parthenogenesis has been reported in several species of the subfamily Leiolepidinae, although the sex determination system is not known in this lineage.

Only about one fifth (91 species) of Agamid species have been karyotyped, with diploid chromosome numbers ranging from 2n = 32 to 2n = 54 [4,6,50]. Agamids exhibit a diverse array of sex-determination mechanisms that include TSD, GSD and GSD with sex reversal [1,2,22,39,51]. Sex chromosomes have only been identified in five species, all with a female heterogametic system (ZZ male/ZW female). Sex chromosomes in an Asian species *Phrynocephalus vlangalii* from the subfamily Agaminae are macrochromosomes [39], whereas the four Australian species from the subfamily Amphibolurinae, namely, *Pogona vitticeps*, *P. barbata*, *Diporiphora nobbi* and *Ctenophorus fordi*, have micro sex chromosomes [7,20]. The karyotypes of the Australian species are highly conserved, comprising six pairs of macrochromosomes and ten pairs of microchromosomes [35]. Nevertheless, they show considerable evolutionary lability in sex determination mechanisms [19,52] with a number of likely transitions reported within GSD forms and between GSD and TSD [2,7,30,53].

Molecular cytogenetics is a powerful tool for discovering homology and evolutionary trends in reptile sex chromosomes [54,55] and has provided evidence that the sex chromosomes of lizards are extremely varied in terms of morphology and homology [2,5]. The Australian central bearded dragon, *Pogona vitticeps*, has a well-annotated genome with well-characterized ZZ/ZW sex microchromosomes, homologous to chicken chromosomes 17 and 23 [20,56–60]. Comparative studies based on this and other species have revealed chromosomal rearrangements involving sex chromosomes and transitions in sex chromosomes within the Amphibolurinae [7,61,62], including the rapid evolution of non-homologous ZW sex chromosomes. Here, we evaluate the synteny of sex chromosomes across the dragons of the family Agamidae using fluorescence in situ hybridization (FISH) [7]. We used two BAC clones (Pv03_L07 and Pv150_H19) derived from *P. vitticeps* ZW sex chromosomes as probes and hybridized them to the metaphase chromosomes of 14 acrodont taxa (12 agamids from all six subfamilies, and two chameleons), comprising species that span the spectrum of sex determination, including TSD, GSD and obligatory parthenogenesis.

2. Materials and Methods

2.1. Animal and Sample Collection

In total, 22 individuals of 14 species of acrodont lizards—12 agamids (from six subfamilies) and 2 chamaeleonid species—were chosen for the study (Table 1). Animal collection, handling, sampling and all other relevant procedures for the Australian species (*P. vitticeps*, *Tympanocryptis lineata* and *Rankinia diemensis*) were performed following the Animal Ethics Guidelines of the University of Canberra (approval number CEAE 16-21), with permits issued by the ACT Government (license number LT2017960). Fieldwork conducted for *Agama picticauda* was under Miami-Dade County Parks and Recreation Scientific Research Permit number 263-2016 and Marquette University IACUC AR-288. *Calotes versicolor* and *Leiolepis reevesii rubritaeniata* specimen collection, animal care and procedures were approved by the Animal Experiment Committee, Kasetsart University, Thailand (approval number ACKU61-SCI-021). *Phrynocephalus* cf. *guttatus*, *Bronchocela cristatella*, *Leiolepis* cf. *ngovantrii*, *Saara loricata* and *Chamaeleo calyptratus* were sampled in collaboration with breeders in Czech Republic. Samples of *Hydrosaurus weberi* and *Trioceros johnstoni* were provided by Czech zoological gardens (Zoo Plzeň and Zoopark Zájezd, respectively). All experimental procedures in Czech Republic were approved by the Committee for Animal Welfare of the Ministry of Agriculture of the Czech Republic, permissions No. 29555/2006-30 and 8604/2019-7.

2.2. Cell Culture and Chromosome Preparation

Fibroblast cells were cultured from the tail tissues of *P. vitticeps*, *T. lineata*, *R. diemensis* and *A. picticauda* for the cytogenetic analyses. Cells were cultured, and metaphase chromosomes were harvested following the procedures as described by Ezaz et al. [63]. *C. versicolor* and *L. reevesii rubritaeniata* cells were also cultured from tail tissues. Cell culture and chromosome harvesting followed the procedures as described by Chaiprasertsri et al. [64]. Mitotic chromosomes of *P.* cf.

guttatus, *B. cristatella*, *L.* cf. *ngovantrii*, *S. loricata*, *C. calyptratus*, *H. weberi* and *T. johnstoni* were obtained by cultivation of leukocytes and the preparation of the cell cultures and chromosome harvesting followed a detailed protocol described in Mazzoleni et al. [65].

Table 1. Results of the bacterial artificial chromosome (BAC) clone fluorescence in situ hybridization (FISH) experiments. SDM = sex determination mechanism, 2n = diploid chromosome number, M + m = number of macrochromosomes and microchromosomes, GSD = genotypic sex determination, TSD = temperature-dependent sex determination, UNK = unknown, OP = unisexuality with obligatory parthenogenesis, qtel—telomeric region of the large arm of a macrochromosome.

Taxon	SDM	2n	M + m	Sex	Mapping	
					Pv03_L07	Pv150_H19
Family: Agamidae						
Subfamily Amphibolurinae						
Pogona vitticeps	GSD—ZW	32	12 + 20	1 F	2*qtel* + ZW micro sex chromosome	ZW micro sex chromosome
Tympanocryptis lineata	UNK	32	12 + 20	1 M, 1 F	2*qtel* + 1 pair of micros	1 pair of micros
Rankinia diemensis	UNK	32	12 + 20	1 M, 1 F	2*qtel* + 2 pairs of micros	1 pair of micros
Subfamily Agaminae						
Agama picticauda	TSD	44	20 + 24	1 M, 1 F	1*qtel*	1 pair of micros
Phrynocephalus cf. *guttatus*	UNK	46	22 + 24	1 M, 1 F	1*qtel*	No hybridization
Subfamily Draconinae						
Calotes versicolor	TSD	34	12 + 22	1 M, 1 F	2*qtel*	1 pair of micros
Bronchocela cristatella	UNK	34	14 + 20	1 F	5*qtel*	No hybridization
Subfamily Hydrosaurinae						
Hydrosaurus sp.	UNK	36	12 + 24	1 UNK	No hybridization	1 pair of micros
Hydrosaurus weberi	UNK	36	12 + 24	1 M	2*qtel*	No hybridization
Subfamily Leiolepidinae						
Leiolepis reevesii rubritaeniata	UNK	36	12 + 24	1 F	2*qtel*	1 pair of micros
Leiolepis cf. *ngovantrii*	OP	36	12 + 24	1 F	2*qtel*	1 pair of micros
Subfamily Uromastycinae						
Saara loricata	UNK	36	12 + 24	1 M, 1 F	2*qtel*	No hybridization
Family: Chamaeleonidae						
Chamaeleo calyptratus	XY	24	12 + 12	1 M, 1 F	No hybridization	No hybridization
Trioceros johnstoni	UNK	36	14 + 22	1 M, 1 F	No hybridization	No hybridization

2.3. Fluorescence In Situ Hybridization (FISH) and Image Analysis

Two *P. vitticeps* ZW sex chromosome BAC clones (Pv03_L07 and Pv150_H19) from the *P. vitticeps* Bacterial Artificial Chromosome (BAC) library (6.2x, Amplicon Express, Pullman, WA, USA) [56] were mapped onto the metaphase chromosomes of all 14 species (Table 1). The sex chromosomes of *P. vitticeps* have been found to be highly repetitive in nature [56]. The BAC Pv03_L07 (about 98 kb) contains 41% of repetitive sequences of which 43% are non-LTR (long terminal repeat) retrotransposons and includes at least two genes, *ZNF135-like* and a fragment of *ORPRD1* [56]. BAC Pv150_H19 (size not estimated and repeat content not known) contains the *NR5A1* gene [59], which is known to play an important role in sex differentiation [59]. These two BAC clones share homologous sequences with chicken chromosome 17 and were chosen because they were previously mapped in few agamid species, and their sequence content is known [59]. The two BACs, Pv03_L07 and Pv150_H19, represent the two ends of Z and W chromosomes of *P. vitticeps*. In addition, BAC Pv03_L07 hybridizes onto the telomeric region of the second-largest chromosome (chromosome 2) of *P. vitticeps* [56,57,59]. The two BAC clones were mapped using FISH, following the protocols described in Ezaz et al. [7] and Young et al. [58].

All slides were observed, and images of metaphases were captured using a Zeiss Axio Scope A1 epifluorescence microscope fitted with a high-resolution microscopy camera AxioCam MRm Rev. 3 (Carl Zeiss Ltd. Oberkochen, Germany). Images were analyzed using Metasystems Isis FISH Imaging System V 5.5.10 software (Metasystems, Altlussheim, Germany).

3. Results

In line with the previous observations [56,59,61], the BAC clone Pv03_L07 hybridized onto the Z and W chromosomes as well as onto the telomeric region of the long arms of the chromosome pair

2 in *P. vitticeps* (Figure 2a). This BAC probe hybridized onto the telomeric region of the long arms of the chromosome pair 2 in all species under the subfamilies Amphibolurinae (*P. vitticeps*, *T. lineata* and *R. diemensis*; Figure 2a–c), Uromastycinae (*S. loricata*; Figure 2d) and Leiolepidinae (*L. reevesii rubritaeniata* and *L.* cf. *ngovantrii*; Figure 2e,f). A similar hybridization pattern was also observed in *H. weberi* (Hydrosaurinae, Figure 2h), but no hybridization signal was detected in *Hydrosaurus* sp. (Figure 2g). The probe also hybridized onto chromosome 2 in *C. versicolor* from the subfamily Draconinae, (Figure 2i) but onto the fifth-largest chromosome pair in another member of that family, *B. cristatella* (Figure 2j). Hybridization signals from BAC clone Pv03_L07 were observed in the largest chromosome pair in members of the subfamily Agaminae (*A. picticauda* and *P.* cf. *guttatus*; Figure 2k,l). Additional to chromosome 2, BAC Pv03_L07 only hybridized onto microchromosomes in the subfamily Amphibolurinae (Figure 2a–c), onto one pair in *P. vitticeps* and *T. lineata* and two pairs in *R. diemensis*. In *P. vitticeps*, the BAC Pv03_L07 hybridization signal varied between Z and W with a brighter signal in the W [56,59]. The only other species in which we observed a similar pattern was *R. diemensis*. In this species, BAC Pv03_L07 hybridized to an additional pair of microchromosomes and the hybridization signals in one pair are brighter than the other. However, no inter-sex pattern variation was observed either in this species.

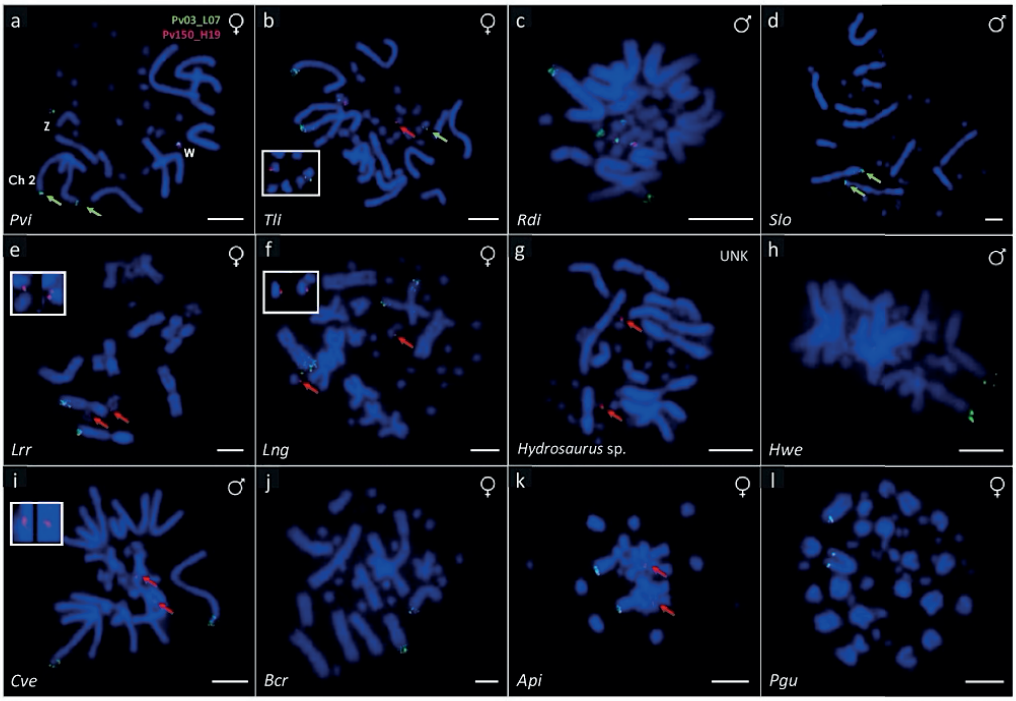

Figure 2. FISH (fluorescence in situ hybridization) using *P. vitticeps* BAC clones (Pv03_L07 in green and Pv150_H19 in red) on different agamid species. Pvi—*P. vitticeps* (**a**); Tli—*T. lineata* (**b**); Rdi—*R. diemensis* (**c**); Slo—*S. loricate* (**d**); Api—*A. picticauda* (**k**); Pgu—*P.* cf. *guttatus* (**l**); Cve—*C. versicolor* (**i**); Bcr—*B. cristatella* (**j**); Lrr—*L. reevesii rubritaeniata* (**e**); Lng—*L.* cf. *ngovantrii* (**f**); *Hydrosaurus* sp. (**g**); Hwe—*H. weberi* (**h**); UNK—unknown sex. Arrows and insets showing very low hybridization signals. Scale bars equal 5 μm.

The hybridization patterns formed by the BAC probe Pv150_H19 across agamid lizards are presented in Figure 2. This BAC probe hybridized onto the Z and W chromosomes of *P. vitticeps* (Figure 2a), as previously observed [57,59]. The hybridization signals from Pv150_H19 co-localized with the signals from Pv03_L07 in this species (Figure 2a) and hybridized onto a pair of microchromosomes

in all species of the subfamilies Amphibolurinae (*P. vitticeps*, *T. lineata* and *R. diemensis*; Figure 2a–c) and Leiolepidinae (*L. reevesii rubritaeniata* and *L.* cf. *ngovantrii*; Figure 2e,f). However, the hybridization signals were observed in only one species from each of the subfamilies Hydrosaurinae (*Hydrosaurus* sp.; Figure 2g), Draconinae (*C. versicolor*; Figure 2i) and Agaminae (*A. picticauda*; Figure 2k) while no hybridization signal was observed in *S. loricata* (subfamily Uromastycinae; Figure 2d). Both BAC clones hybridized onto microchromosomes in all species of the subfamily Amphibolurinae (*P. vitticeps*, *T. lineata* and *R. diemensis*; Figure 2a–c). Nevertheless, in *T. lineata*, the BACs did not colocalize on the same pair of microchromosomes. No inter- or intra-sex variation of the BAC Pv150_H19 hybridization signal was recorded from *P. vitticeps* [59], nor was variation detected in any of our studied species. No hybridization signal was observed from any of the BAC clones in any of the chameleon species (*C. calyptratus* and *T. johnstoni*; Table 1). A summary of the overall BAC mapping is presented in Table 1.

4. Discussion

Our data revealed the conservation of macro- and microchromosome specific sequences across Agamidae. The *P. vitticeps* sex chromosome derived BAC probe Pv03_L07, which hybridizes onto the sex microchromosomes and telomeric region of chromosome 2 in *P. vitticeps*, hybridized to a pair of macrochromosomes across agamid lineages in all but one species (*Hydrosaurus* sp.; Figure 2g). This suggests that chromosomal synteny is retained across agamid lineages. In contrast, none of the BACs hybridized to chameleon chromosomes. Together, our findings indicate that the sequence is conserved in macrochromosomes across the Agamidae but has most likely been secondarily lost in the ancestor of *Hydrosaurus* sp. (Figure 3).

The BAC Pv03_L07 exhibits a conserved hybridization pattern on the telomeric region of macrochromosome 2 in members of the subfamilies Amphibolurinae, Hydrosaurinae, Leiolepidinae and Uromastycinae. However, it is localized in the largest chromosome pair in both members of the subfamily Agaminae, which might represent a synapomorphy. The localization of the hybridization signal of Pv03_L07 on chromosome 5 in *B. cristatella* suggests a chromosomal rearrangement in its ancestor. Additionally, it hybridizes onto two pairs of microchromosomes in *R. diemensis* and one pair in *T. lineata*. Both of those species are representatives of the subfamily Amphibolurinae, and so, these data lend support to the chromosomal rearrangements such as duplication near the telomeric region of ancestral chromosome 2 and successive translocation to microchromosomes as previously reported by Matsubara et al. [61].

The second BAC clone, Pv150_H19, was derived from *P. vitticeps* Z and W sex chromosomes only, and its sequences are located onto the opposite ends of the Z and W micro sex chromosomes in relation to Pv03_L07 (Figure 2a). This probe also showed somewhat conserved distribution in a pair of microchromosomes across the agamid phylogeny. The probe hybridized to all species of Amhibolurinae and Leiolepidinae, to one of two species in Hydrosaurinae, Draconinae and Agaminae and did not hybridize to the only species from Uromastycinae (Figure 3). This suggests a haphazard distribution across the lineages. The absence of signal in *S. loricata* and presence in all other agamid subfamilies indicate that BAC Pv150_H19 sequence might have evolved after the split of the other lineages from Uromastycinae (Figure 3). The lack of hybridization signal in *B. cristatella*, *P.* cf. *guttatus* and *H. weberi* suggests an independent loss in these three species. Alternatively, there could be a mutation in the target sequence so that the probe was washed away from the less complementary target, and/or shrinkage of the target sequence, so it was no longer detectable. Since BACs are usually predominately composed of repeats which evolve quickly, it is possible that the sequences are still present in all the species but no longer detectable with the approach used. The sequence content of both BACs is enriched on repetitive elements [52,55], which—due to their fast-evolution nature—may have diverged significantly since agamid and chameleon lineages split approximately 90–125 million years ago (MYA) [25,26,62]. Therefore, the homologous sequences might exist in the genome of chameleons but the BACs could not hybridize because of significant divergence from *P. vitticeps*. (These results must be viewed with some caution, however, as they are based on a limited number of chameleon

species, which have also been shown to harbour transitions between sex determining systems [27].) Pv150_H19 hybridized to a microchromosome pair in both TSD and GSD species, but we were unable to determine whether these microchromosomes (with Pv150_H19 signals) were sex chromosomes. Nevertheless, since this BAC contains a gene associated with sex differentiation function (*NR5A1*), it is possible that the microchromosome pair with Pv150_H19 could be a sex chromosome in the GSD species. If so, those same homologous chromosomes could be autosomes in the TSD species while still contributing to the sex-differentiation cascade or pathways. Further investigation is required on this aspect.

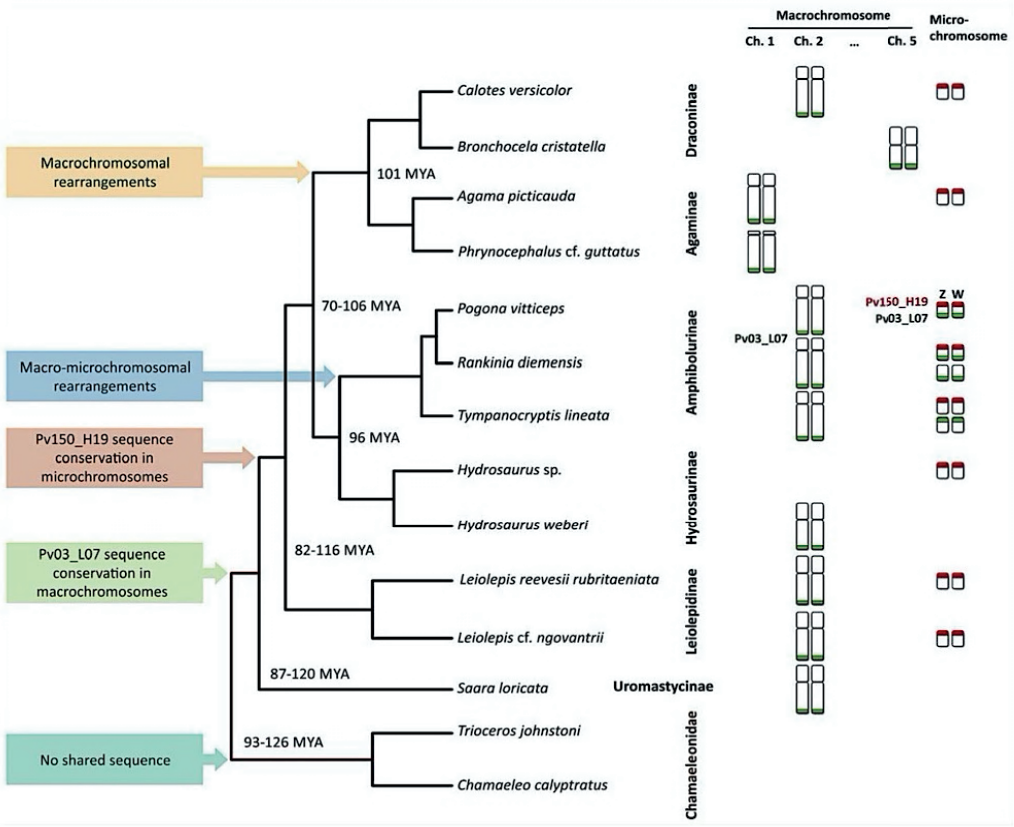

Figure 3. Cross-species chromosome mapping of *P. vitticeps* sex-chromosome-derived BAC probes Pv03_L07 (in green) and Pv150_H19 (in red) highlighting hypothetical evolutionary scenarios of chromosome rearrangements within the subfamilies of Agamidae. Truncated phylogeny (not according to scale) is adopted from Pyron, et al. [24]. Known divergence times are provided in million years ago (MYA) [25,30,66].

The ancestral vertebrate karyotype has remained relatively stable over the last ~370 million years as large segments of ancestral chromosomes are still retained among all lineages [67]. These segments have been rearranged, but their synteny has been maintained together with increases and decreases of genomic content and genome sizes. Chromosome painting has been used to determine such homologies, as well as rearrangements among and between different reptilian species [8]. For example, karyotype and genome organization have been found to be conserved in monitor lizards (Varanidae) [18,68]. Conservation of several homologous syntenic regions has been found to be retained within different groups of fishes [69,70] and birds [71,72] as well. Comparative painting has also revealed chromosome homologies between bird groups [73] and also between vertebrate groups as observed between turtle

sex chromosomes and amphibian autosomes [74]. The data presented in agamids show that this group has quite conserved karyotypes as well, and many rearrangements can be putatively dated a long time ago (Figure 3). The broad distribution of our *Pogona vitticeps* derived BAC sequences among agamids indicate that there has been conservation of chromosome segments across agamid lineages. The BAC sequence contained in the BAC Pv150_H19 is largely conserved in microchromosomes across the agamid phylogeny, while the other (BAC Pv03_L07) in macrochromosomes appears to have been only translocated to microchromosomes in the ancestor of the studied members of the subfamily Amphibolurinae. It was then likely later duplicated to a microchromosome containing the BAC sequence Pv150_H19, while the original microchromosome copy of the BAC Pv03_L07 has been lost in the ancestor of *P. vitticeps* (Figure 3). Since nearly half of the BAC Pv03_L07 consists of mobile elements, another explanation of the co-occurrence of this BAC signal in the microchromosomes could be as a result of the propagation of these mobile elements. The co-occurrence of both sequences in the ZW sex microchromosomes in *P. vitticeps* is thus likely a result of a rather complex history of rearrangements [59]. Future investigations that include more agamid lizards will better test the proposition that the reconstruction of events suggested here was important for the establishment of cytogenetically distinguishable sex chromosomes in *P. vitticeps* and its relatives.

Author Contributions: T.E. and S.M.I.A. conceptualized the study. M.A., T.P., S.M.I.A., M.R. and L.K. prepared different chromosome samples. S.M.I.A., T.P., T.G. and S.V.N. conducted field works. S.M.I.A. did the cytogenetic experimentations. S.M.I.A. and T.E. designed and co-drafted the manuscript. T.E., A.G. and S.D.S. supervised S.M.I.A. All authors contributed to reviewing and editing the manuscript. All authors have read and agreed to the published version of the manuscript.

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CHAPTER 4: Karyotype Characterisation of Two Australian Dragon Lizards (Squamata: Agamidae: Amphibolurinae) Reveals Subtle Chromosomal Rearrangements between Related Species with Similar Karyotypes

This paper has appeared in print.

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Preface

Even though Australian agamids under the subfamily Amphibolurinae are comparatively well studied regarding the sex chromosome, no comparative cytogenetic research has yet been performed. This article focusses on characterising the chromosomal landscapes in two Australian agamid lizards – the Canberra grassland earless dragon *Tympanocryptis lineata* and the Australian mountain dragon, *Rankinia diemensis* using molecular cytogenetic techniques (differential staining and C-banding procedures along with fluorescence *in situ* Hybridisation (FISH) including cross-species BAC mapping). The results suggest that although both species have karyotypes similar to that of *P. vitticeps*, they also exhibit subtle rearrangements in the chromosomal landscapes that suggest dynamic chromosomal processes.

Justification of inclusion

As the first author, I wrote this paper which included reviewing the literature, conducting the experiments and the analyses, writing and revising the draft and preparing the figures. I also collected the animal samples for the experiments.

UNIVERSITY OF CANBERRA

Declaration of Co-Authored Publications

Declaration for Thesis Chapter 04

DECLARATION BY CANDIDATE

In the case of Chapter 04, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contributions (%)
• Conceptualization • Animal and sample collection • Conducting experiment and analysis • Literature review • Writing and revising draft • Designing the figures/diagrams • Correspondence with the journal	80%

The following co-authors contributed to the work:

Name	Nature of Contribution	Contributor is also a UC student (Yes/No)
Tariq Ezaz	Conceptualization, draft writing, reviewing, resources and supervision	No
Stephen D. Sarre	Reviewing, resources and supervision	No
Arthur Georges	Reviewing, resources and supervision	No

31/08/2020

Candidate's Signature and Date

DECLARATION BY CO-AUTHORS

The undersigned hereby certify that:

(13) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.

(14) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(15) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(16) there are no other authors of the publication according to these criteria;

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Karyotype Characterisation of Two Australian Dragon Lizards (Squamata: Agamidae: Amphibolurinae) Reveals Subtle Chromosomal Rearrangements Between Related Species with Similar Karyotypes

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Keywords
Amphibolurinae · Karyotype · Chromosomal rearrangement · Heterochromatin · FISH

Abstract
Agamid lizards (Squamata: Agamidae) are karyotypically heterogeneous. Among the 101 species currently described from Australia, all are from the subfamily Amphibolurinae. This group is, with some exceptions, karyotypically conserved, and all species involving heterogametic sex show female heterogamety. Here, we describe the chromosomes of 2 additional Australian agamid lizards, *Tympanocryptis lineata* and *Rankinia diemensis*. These species are phylogenetically and cytogenetically sisters to the well-characterised *Pogona vitticeps*, but their sex chromosomes and other chromosomal characteristics are unknown. In this study, we applied advanced molecular cytogenetic techniques, such as fluorescence in situ hybridisation (FISH) and cross-species gene mapping, to characterise chromosomes and to identify sex chromosomes in these species. Our data suggest that both species have a conserved karyotype with *P. vitticeps* but with subtle rearrangements in the chromosomal landscapes. We could identify that *T. lineata* possesses a female heterogametic system (ZZ/ZW) with a pair of sex microchromosomes, while *R. diemensis* may have heterogametic sex chromosomes, but this requires further investigations. Our study shows the pattern of chromosomal rearrangements between closely related species, explaining the speciation within Australian agamid lizards of similar karyotypes.

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Introduction

Reptiles are a karyotypically heterogeneous group. Their diploid chromosome number ($2n$) varies between 20 and 68, including variable numbers of macro- (10–42) and microchromosomes (0–56) [Olmo and Signorino, 2005; Alam et al., 2018; Deakin and Ezaz, 2019]. Some exhibit triploidy ($3n$), and triploid individuals can occur in populations of typically diploid species (especially in lizards) [Spangenberg et al., 2017; Alam et al., 2018]. Sex chromosomes have been described in about 24% of karyotyped reptile species [Olmo, 1986; Janzen and Paukstis, 1991; Olmo and Signorino, 2005; Pokorná et al., 2014a; Uetz et al., 2020] and include both male (XX/XY) and female (ZZ/ZW) heterogamety. Several families have species with sex-determining systems involving multiple chromosomes in both male and female heterogamety

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Fig. 1. Global distribution (in grey) of agamid lizards, and sample collection locations of *T. lineata* and *R. diemensis*. Agamid distribution map from Midtgaard [2019], Australia including ACT map from DEPPRO [2020].

[Olmo, 1986; Janzen and Paukstis, 1991; Olmo and Signorino, 2005; Grosso et al., 2017; Alam et al., 2018; Altmanová et al., 2018]. These karyotypic features make reptiles an exceptional group for studying chromosome evolution [Deakin and Ezaz, 2019]. Among reptiles, lizards are particularly of interest in terms of chromosomal evolution because of their diversity in the number of species and chromosomes.

Within lizards, agamid lizards (Squamata: Agamidae) are karyotypically heterogeneous ($2n$ = 20–54), with about 20% (91/526) [Pokorná et al., 2014a; Uetz et al., 2020] of the species worldwide having been karyotyped. However, heteromorphic sex chromosomes have been identified in only 5 of those karyotyped species, with 4 of the 5 sex chromosomes being microchromosomes and 1 being a macrochromosome [Zeng et al., 1997; Ezaz et al., 2005, 2009b]. Agamid lizards have also been widely studied for GSD (genotypic sex determination) and TSD (temperature-dependent sex determination) mechanisms among the lizard families [Harlow, 2004], particularly the Australian species mostly belonging to the subfamily Amphibolurinae.

In Australia, 106 species of agamid lizards are currently described from 16 genera, all within the subfamily Amphibolurinae [Uetz et al., 2020]. Most Australian dragons are karyotypically conserved with $2n$ = 32, comprising 6 pairs of macrochromosomes and 10 pairs of microchromosomes (20 species out of 23 karyotyped) [Witten, 1983; Ezaz et al., 2008]. Heteromorphic sex chromosomes have been identified in only 4 Australian species [Ezaz et al., 2005, 2009b], all of which exhibit female heterogamety with ZZ/ZW sex chromosomes and are all microchromosomes. This karyotypic conservatism is widely considered to be the result of the recent and rapid radiation of agamids into the Australian continent around 22 million years ago [Witten, 1983; Hugall et al., 2008]. Exceptions to the conserved karyotype number are *Lophosaurus spinipes* and *Physignathus lesueurii*, each with $2n$ = 36 including an additional pair of microchromosomes and assumed to be the representatives of the agamid species group that have arrived in Australia in relatively recent times and possess a primitive iguanian karyotype, and *Lophognathus gilberti centralis* ($2n$ = 40) [Witten, 1983]. Despite the overall karyotypic conservatism, Australian agamids have diverse sex-determining mechanisms that

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Table 1. Number of individuals and cells examined in this study. The numbers shown here indicate the least number of cells examined under respective experiments

Experiment	*T. lineata*				*R. diemensis*	
	Male		Female			
	Individuals	Cells	Individuals	Cells	Individuals	Cells
Karyotyping	2	11	2	13	4	34
C-banding	3	95	3	49	4	181
CMA_3	1	46	1	18	2	64
Methylation	1	60	1	35	2	79
FISH						
$(TTAGGG)_7$	1	62	1	40	2	172
$(AAGG)_8$	1	62	1	28	2	64
BAC 3L7-150H19	1	12	1	21	2	36
BAC 3L7-116G15	1	46	1	18	2	96

include GSD, TSD, and GSD with thermally induced sex reversal of the ZZ genotype to a female phenotype [Harlow, 2001; Quinn et al., 2007; Holleley et al., 2015]. A recent study [Matsubara et al., 2019] suggested that rearrangements in sex chromosomes may have driven speciation in this group of lizards, and comparative cytogenetic analysis between closely related species might be one way of explaining such recent variations.

Even though several studies regarding the sex chromosomes of this family have been published, no comparative cytogenetic research has yet been performed. Here, we used molecular cytogenetic techniques (differential staining and C-banding procedures along with FISH including cross-species BAC mapping) to characterise the chromosomal landscapes and to identify sex chromosomes of 2 Australian agamid lizards, the Canberra grassland earless dragon *Tympanocryptis lineata* Peters, 1863 and the Australian mountain dragon, *Rankinia diemensis* Gray, 1841. We compared our findings with the cytogenetically well-characterised close relative, the central bearded dragon *Pogona vitticeps* Ahl, 1926 [Witten, 1983; Ezaz et al., 2005; Young et al., 2013; Deakin et al., 2016]. Our data suggest that although both species have karyotypes similar to that of *P. vitticeps*, they also exhibit subtle rearrangements in the chromosomal landscapes that suggest dynamic chromosomal processes.

Materials and Methods

Animals, Sample Collection, and Sexing

The Canberra grassland earless dragon *T. lineata* and the Australian mountain dragon *R. diemensis* are endemic to Australia (Fig. 1) and are closely related to the well-characterised central bearded dragon *P. vitticeps* but adapted to different ecological niches and are found within the Australian Capital Territory (ACT), but not sympatric. *T. lineata* [Melville et al., 2019] faced a dramatic decline in population over the last decade and is at high risk of extinction [Dimond et al., 2012; Carlson et al., 2016; Melville et al., 2019]. On the other hand, *R. diemensis* is of Least Concern (LC) under IUCN criteria [Melville et al., 2018]. Both species have the same karyotype compared to the closely related *P. vitticeps* ($2n = 32$) [Witten, 1983; Ezaz et al., 2008; Hugall et al., 2008; Pyron et al., 2013].

The University of Canberra houses a captive population of *T. lineata* for research and conservation purposes, and samples for this study were collected from these animals. Samples of *R. diemensis* were collected from the forested area of Namadgi National Park in north-western Australian Capital Territory (ACT). A total of 6 individuals of *T. lineata* (3 males and 3 females) were used for the study purpose and 4 individuals of *R. diemensis* (sex not known) (Table 1). No animal was killed, and every individual was released at its point of capture after sample collection. Each animal was observed at least 10 min after release, where possible. All *T. lineata* individuals were sexed phenotypically by the presence of an extruded hemipenis in the adult males and absence in females following Harlow [1996, 2004].

Cell Culture, Chromosome Preparation, and Staining

Fibroblast cells were cultured from the tail tissues of the sampled individuals following the procedures described by Ezaz et al. [2008]. Metaphase chromosomes were harvested as described by Ezaz et al. [2005, 2008]. Visualising blocks of constitutive heterochromatin was achieved by C-banding according to Sumner [1972] with slight modifications as described in Ezaz et al. [2009b] and Pokorná et al. [2014b]. Chromomycin A_3 (CMA_3, DNA dye specific for GC-rich regions) fluorescent staining was performed to reveal the GC genome composition as described by Sola et al. [1992] but using DAPI (4′, 6-diamidino-2-phenylindole, AT-specific) in anti-fade medium Vectashield (Vector Laboratories, Burlingame, CA, USA) as a mounting reagent [Majtánová et al., 2017].

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Immunostaining with a 5-methylcytosine (meC) antibody was used to visualise the global DNA methylation state of metaphase chromosomes following Ingles and Deakin [2018].

Repeat and Cross-Species Gene Mapping

Telomeric repeats were FISH-mapped using a conserved vertebrate telomeric repeat Cy3-labelled $(TTAGGG)_7$ oligonucleotide probe to allow documentation of interstitial telomeric sites (ITSs) as evidence of chromosomal rearrangements. We also carried out mapping with a simple sequence repeat $(AAGG)_8$ to the chromosomes of both sexes of *R. diemensis* and *T. lineata* as this motif has been found to accumulate in the heterochromatinized W chromosome of *P. vitticeps* [Holleley et al., 2015; Matsubara et al., 2016]. The telomere probe and microsatellite motif experiments were performed following the protocol by Matsubara et al. [2013] and were purchased from GeneWorks (Hindmarsh, Australia).

We mapped 2 BAC clones to *T. lineata* and *R. diemensis* metaphase chromosomes from both sexes. All these (Pv03_L07 and Pv116_G15) were from *P. vitticeps* Z and W micro sex chromosomes [Ezaz et al., 2013; Domaschenz et al., 2015; Deakin et al., 2016; Alam et al., 2020], while Pv03_L07 also hybridises at the telomeric region of chromosome 2 of *P. vitticeps*. Therefore, hybridisation of these BAC clones to study species metaphases would identify sex chromosome homologies. We also confirmed the locations of the 2 *P. vitticeps* clones, Pv03_L07 as previously mapped and sequenced by Ezaz et al. [2013], Young et al. [2013], Domaschenz et al. [2015] and Alam et al. [2020], and also keeping *P. vitticeps* female metaphase chromosomes as control during the experimentation. BAC Pv116_G15 contains the *SF1* gene (Janine Deakin, pers. comm.) that falls under the *NR5A1* gene family, which are considered to have important roles in the early stages of male sex differentiation. Positive BAC clones were cultured following the protocols described in Ezaz et al. [2009b] and Young et al. [2013]. The chromosomal locations of isolated sex chromosomal BAC clones were verified by physical mapping using FISH following protocols described in Ezaz et al. [2009b].

Microscopy and Image Analyses

All slides were observed and images of metaphases were captured using a Zeiss Axio Scope A1 epifluorescence microscope fitted with a high resolution microscopy camera AxioCam MRm Rev. 3 (Carl Zeiss Ltd.). Images were analysed using Metasystems Isis FISH Imaging System V 5.5.10 software for both fluorochrome (FISH, CMA_3) and grey-scale images (C-banding). The CMA_3 signal was inserted into the green and the DAPI signal into the red channel to enhance the contrast between these 2 types of signals. Sizes of the chromosomes were measured using the analysing software Metasystems Isis FISH Imaging System V 5.5.10 to arrange them accordingly (Fig. 2). The number of individuals and cells examined in this study are provided in Table 1.

Results

Karyotypes and C-Banding in T. lineata and R. diemensis

The DAPI-stained mitotic karyotypes of 2 males and 2 females of *T. lineata* and 4 individuals of *R. diemensis* were examined. At least 10 cells were karyotyped from mitotic chromosome spreads at metaphase (Table 1). The diploid chromosome complements of both *T. lineata* and *R. diemensis* is $2n = 32$, and the karyotype is represented by 12 macrochromosomes and 20 microchromosomes with a distinct break in size between the macro- and microchromosomes in both species (Fig. 2). In both species, all 12 macrochromosomes are metacentric except for the second-largest pair, which is submetacentric. In both studied species, chromosomes 1, 2, 5, and 6 could be distinguished morphologically by size and centromere positions, whereas chromosomes 3 and 4 were relatively similar in morphology. The centromeric positions of the microchromosomes could not be determined accurately because of their small sizes. A comparison of the DAPI-stained mitotic karyotypes between males and females did not reveal any morphologically differentiated sex chromosomes in either sex in *T. lineata* (Fig. 2a,b), while the phenotypic sex of *R. diemensis* specimens was not known (Fig. 2c).

C-banding revealed the presence of small centromeric bands in only 1 pair of microchromosomes and a few of prominence in macrochromosomes in both *T. lineata* (3 males and 3 females) and *R. diemensis* (4 individuals) (Fig. 3). At least 49 cells were observed from mitotic chromosome spreads at metaphase. A heavily C-banded microchromosome was observed in all females ($n = 3$) in *T. lineata* (Fig. 3b) but not in any males (Fig. 3a). This suggests the presence of a putative W sex microchromosome and a ZZ/ZW sex chromosome system in this species. In contrast, a large constitutive heterochromatic C-band was observed in 1 of the microchromosomes in 3 out of 4 individuals of *R. diemensis* (Fig. 3c, d).

Reverse Fluorescence (DAPI/CMA_3) and Methylation Staining

Reverse fluorescence staining with DAPI and CMA_3 was conducted in 2 individuals from both *T. lineata* (1 male and 1 female) and *R. diemensis* (sex unknown) (Fig. 4). For each individual, at least 18 cells were observed from the mitotic chromosome spreads at metaphase (Table 1). CMA_3 binds to GC-rich DNA (in green) and DAPI preferentially to AT-rich DNA (in red), and the method revealed mostly homogeneously stained chromosomes with a balanced proportion of AT-GC in macrochromosomes and GC-rich sequences in microchromosomes in both *T. lineata* (Fig. 4a, b) and *R. diemensis* (Fig. 4c). Moderately GC-rich centromeric and telomeric regions were observed in all cells studied in individuals in both species with an interstitial GC-rich re-

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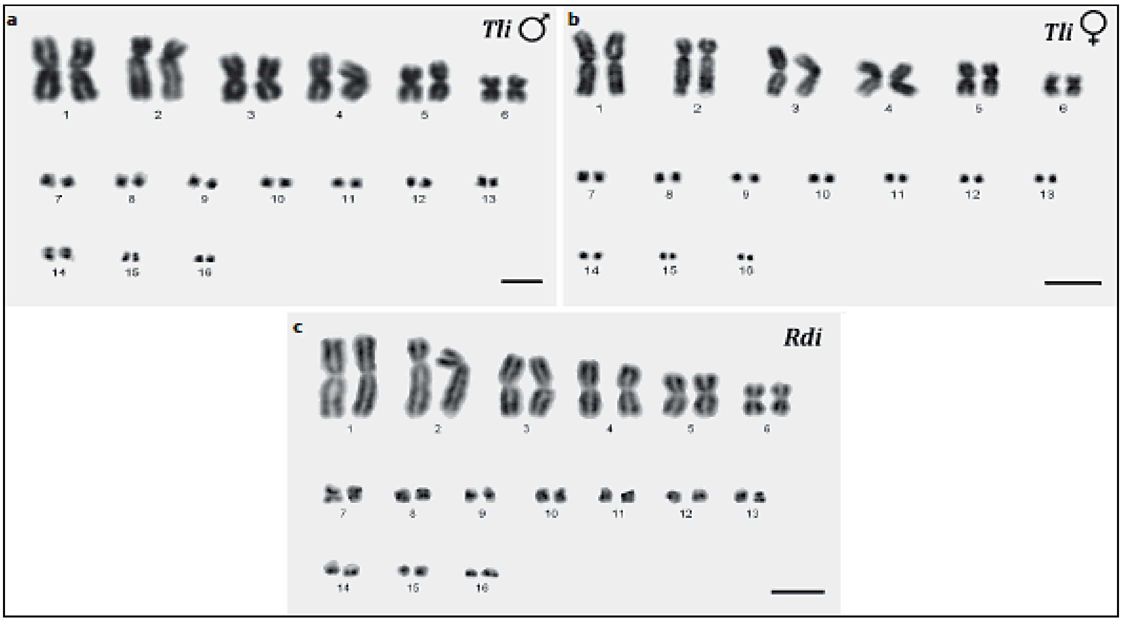

Fig. 2. DAPI-stained karyotypes of *T. lineata* (**a**, **b**) and *R. diemensis* (**c**). The chromosome numbers in both species are $2n = 32$. Scale bars, 5 μm.

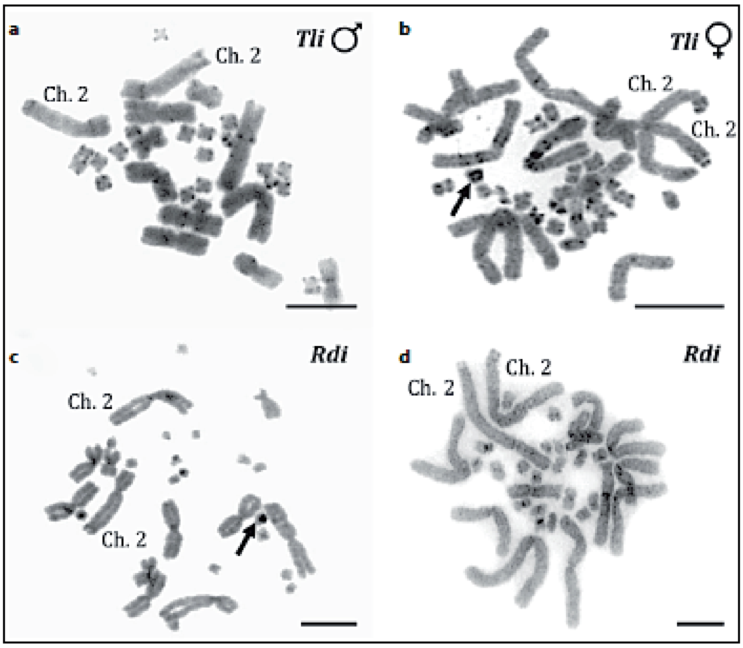

Fig. 3. C-banding in *T. lineata* (**a**, **b**) and *R. diemensis* (**c**, **d**). Arrows show the heterochromatinized microchromosomes. Scale bars, 5 μm.

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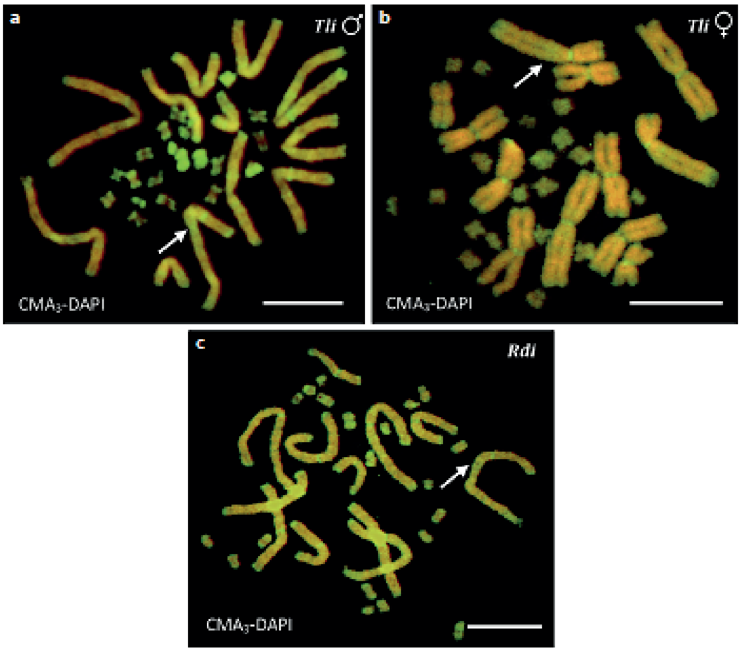

Fig. 4. CMA_3-DAPI fluorescence in *T. lineata* (**a**, **b**) and *R. diemensis* (**c**). Red denotes CMA_3- and green denotes DAPI-staining. Arrows show interstitial GC-rich regions in chromosome 2. Scale bars, 5 μm.

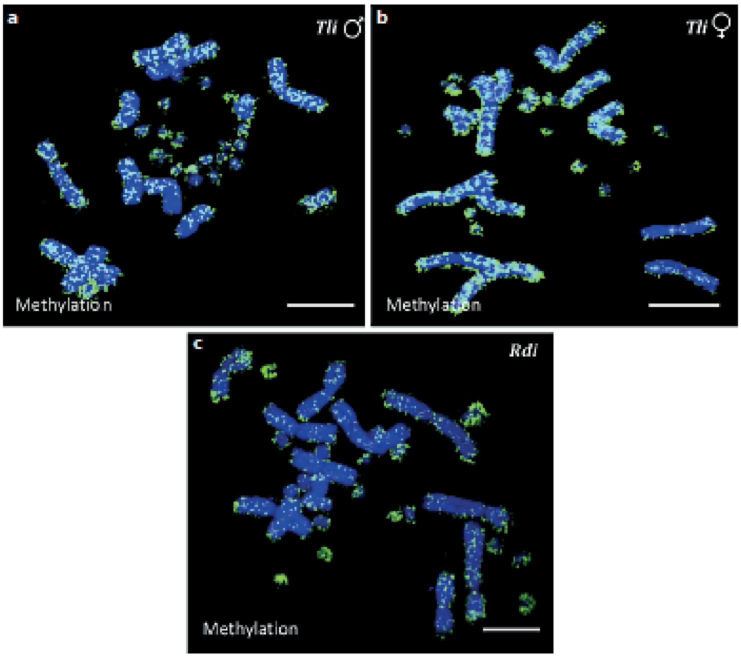

Fig. 5. DNA methylation immunofluorescence staining in *T. lineata* (**a**, **b**) and *R. diemensis* (**c**). Green denotes methylation signals on top of the blue DAPI background. Scale bars, 5 μm.

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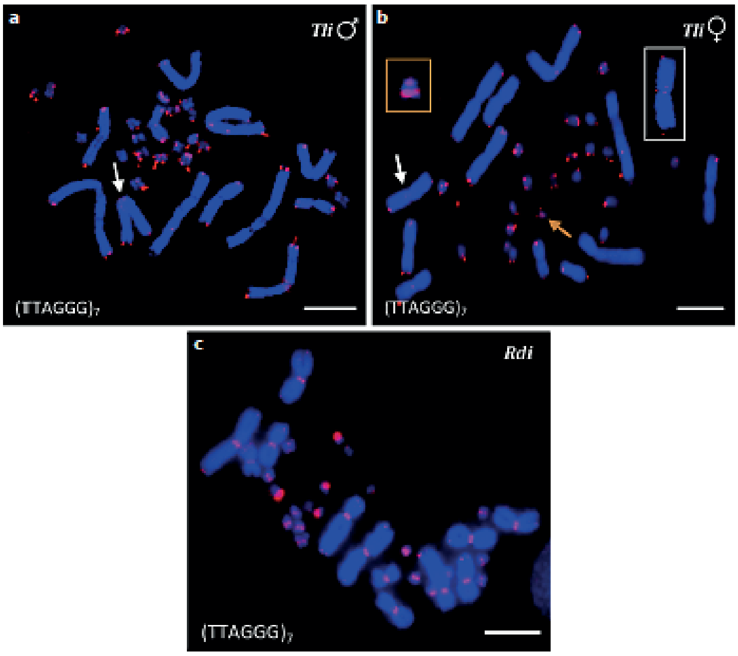

Fig. 6. FISH with Cy3-labelled oligonucleotide telomeric probe (TTAGGG)$_7$ in *T. lineata* (**a**, **b**) and *R. diemensis* (**c**). White arrows indicate ITSs in macrochromosomes and the orange arrow indicates the unpaired microchromosome (enhanced in **insets**) with ITS in *T. lineata.* Scale bars, 5 µm.

gion in the long arm of chromosome 2. DAPI/CMA_3 did not reveal any difference between the sexes in *T. lineata* and between individuals of *R. diemensis* where phenotypic sexes were not known (Fig. 4).

Immunostaining with a 5-methylcytosine (meC) antibody was used to visualise the global DNA methylation state of metaphase chromosomes in 1 male and 1 female from *T. lineata* and 2 individuals of *R. diemensis* (Fig. 5). For each individual, at least 35 cells were observed from mitotic chromosome spreads at metaphase. Telomeric regions of most *T. lineata* (Fig. 5a, b) and *R. diemensis* (Fig. 5c) chromosomes showed stronger methylation staining than the rest of the chromosome, a pattern that has been observed in *P. vitticeps* by Domaschenz et al. [2015]. All observed metaphase spreads from both species showed more intense staining of micro- than macrochromosomes. This is consistent with the observation from DAPI/CMA_3 staining that both *T. lineata* and *R. diemensis* microchromosomes are GC rich. Males and females did not reveal any staining differences between sexes in *T. lineata* and between individuals of *R. diemensis* where phenotypic sexes were not known (Fig. 5).

Telomere and Simple Sequence Repeat (SSR) Mapping

The Cy3-labelled oligonucleotide telomeric probe (TTAGGG)$_7$ was mapped by FISH onto the metaphase chromosomes in 1 male and 1 female of *T. lineata* and 2 individuals of *R. diemensis*. For each individual, at least 40 cells were observed from mitotic chromosome spreads at metaphase (Table 1). In *T. lineata*, the probe hybridised to the terminal ends of all chromosomes in all studied individuals (Fig. 6a, b). ITSs were observed in a pair of microchromosomes and chromosome 4, as seen in *P. vitticeps* by Young et al. [2013]. The hybridisation signals were weaker on macrochromosomes than on almost all microchromosomes. This is maybe due to the smaller arm lengths of the microchromosomes where both telomeres lie within proximity and signals were visualised combinedly. In *R. diemensis*, on the other hand, the telomeric repeats (TTAGGG)$_7$ identified very strong signals in the centromeric regions of all chromosomes (as ITS). Centromeric signals in micro- and macrochromosomes did not vary in intensity; however, 6 microchromosomes were very bright compared with the other 14 microchromosomes (Fig. 6c). An unpaired microchromosome with ITS was observed in cells of female *T. lineata* (Fig. 6a, b). Such a phenomenon could not be identified in *R. diemen-*

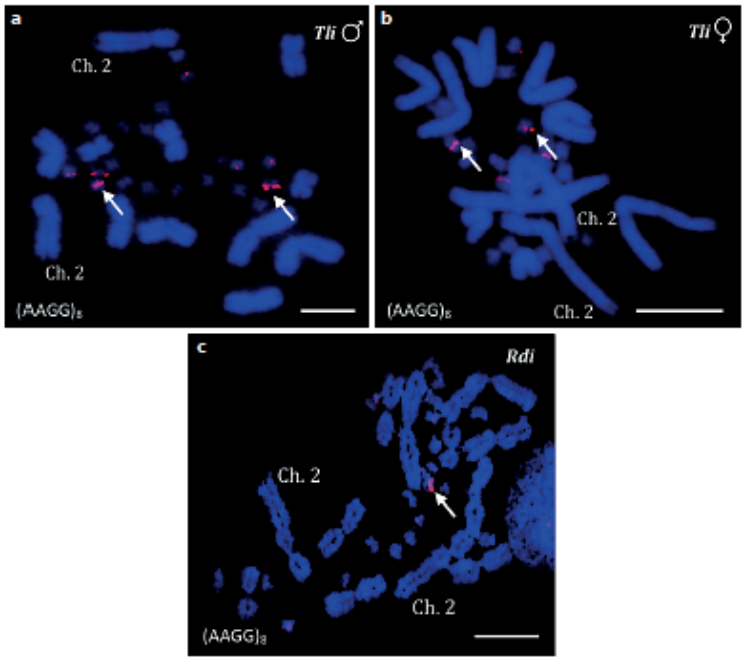

Fig. 7. FISH with Cy3-labeled simple sequence repeat $(AAGG)_8$ in *T. lineata* (**a**, **b**) and *R. diemensis* (**c**). Arrows show intense hybridisation signals. Scale bars, 5 µm.

sis where phenotypic sex of the individuals was not known (Fig. 6c).

The Cy3-labelled simple sequence repeat $(AAGG)_8$ was mapped by FISH onto the metaphase chromosomes of 1 male and 1 female of *T. lineata* and 2 individuals of *R. diemensis* (Fig. 7). For each individual, at least 28 cells were observed from mitotic chromosome spreads at metaphase. The $(AAGG)_8$ sequence showed strong signals in 2 pairs of microchromosomes in both sexes of *T. lineata*, stronger in 1 pair and weaker in another (Fig. 7a, b). $(AAGG)_8$ showed an intense hybridisation signal in 1 microchromosome in all examined cells of *R. diemensis* (Fig. 7c) where phenotypic sex was not known.

Cross-Species Gene Mapping with P. vitticeps Sex Chromosome BAC Clones

The intensity and pattern of hybridisation signals of BAC clones (Pv03_L07 and Pv116_G15) in *P. vitticeps* were different between 2 microchromosomes (Z and W) (Fig. 8a, b). Also, BAC clone Pv03_L07 showed terminal hybridisation signals on the long arms of chromosome 2 pair in both sexes as reported by Ezaz et al. [2013] and Young et al. [2013] (Fig. 8a, b). In *T. lineata*, BAC clone Pv03_L07 primarily hybridised to chromosomes 2 with a very low intensity of hybridisation signals to a pair of microchromosomes in both sexes (Fig. 8c, d). In *R. diemensis*, BAC Pv03_L07 hybridised to an additional pair of microchromosomes (Fig. 8e, f). The other BAC clone from *P. vitticeps* ZW (Pv116_G15) hybridised to a pair of microchromosomes in both sexes of *T. lineata* and in all individuals of *R. diemensis* (phenotypic sexes not known), as well as another *P. vitticeps* ZW BAC clone, Pv150_H19 [Alam et al., 2020]. However, in *T. lineata*, these BACs do not co-localise with Pv03_L07 signals, and in *R. diemensis*, they co-localise only with 1 of the 2 pairs, indicating possible chromosomal rearrangements.

Discussion

T. lineata and *R. diemensis* are phylogenetically closely related to *P. vitticeps* [Hugall et al., 2008; Pyron et al., 2013], and cytogenetically they share the similar karyotype ($2n$ = 32; 12 macro- and 20 microchromosomes) [Witten, 1983]. Our study revealed the subtle differences within the similar chromosomal landscapes (identical karyotypes), including differential organisation of GC-rich regions and telomeric and repeat sequences as well

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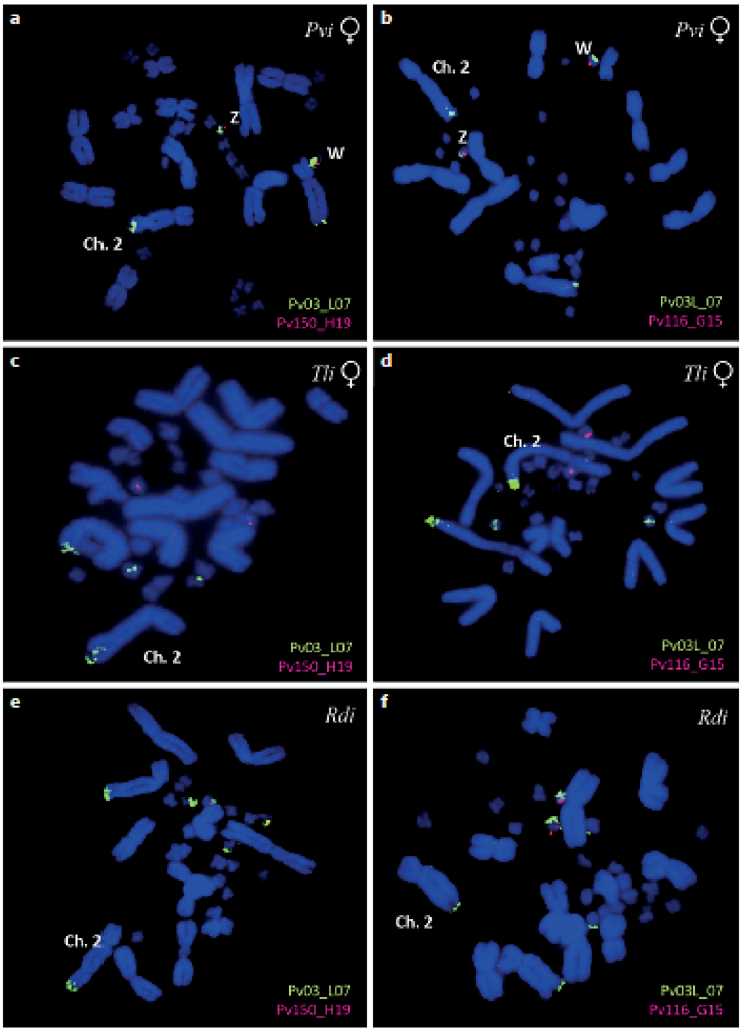

Fig. 8. FISH using *P. vitticeps* BAC clones on control *P. vitticeps* (**a**, **b**), *T. lineata* (**c**, **d**), and *R. diemensis* (**e**, **f**). The pseudo-autosomal BAC Pv03_L07 (in green) hybridises to the telomeric region of chromosome 2 of *T. lineata* (**c**, **d**) and *R. diemensis* (**e**, **f**) as in *P. vitticeps* (**a**, **b**). In addition, it also hybridises to Z and W of *P. vitticeps*, a pair of microchromosomes of *T. lineata* (**c**, **d**), and 2 pairs of microchromosomes of *R. diemensis* (**e**, **f**). BAC Pv150_H19 and Pv116_G15 (in red), both from *P. vitticeps* Z and W microchromosomes (**a**, **b**) also hybridises to a pair of microchromosomes in both sexes of *T. lineata* (**c**, **d**; only female is shown) and *R. diemensis* (**e**, **f**; phenotypic sex not known). Pvi, *P. vitticeps*; Rdi, *R. diemensis*; Tli, *T. lineata*.

as chromosomal rearrangements, possibly through duplication and translocation. We could also identify a female-specific heterochromatinized microchromosome in *T. lineata*, indicating female heterogamety (ZZ/ZW system) in this species.

C-Banding Revealed Micro Sex Chromosome in Female T. lineata

C-bandings has been found to be effective in revealing sex chromosomes in several different species [Traut et al., 1999, 2001; Barzotti et al., 2000], including different lizards [Ezaz et al., 2005; Olmo and Signorino, 2005; Matsubara et al., 2016] and identified a heterochromatinized microchromosome specific to females in *T. lineata*. This female-specific chromosome, therefore, is designated as a W chromosome, implying a ZZ/ZW sex chromosome system in this species as in *P. vitticeps* [Ezaz et al., 2005; Young et al., 2013]. Harlow [2004] reported that *R. diemensis* is a GSD species, but we did not detect sex chromosomes using standard karyotyping. Our experiments

involving C-banding identified a highly heterochromatinized microchromosome, the largest microchromosome pair in the karyotype in all individuals (Fig. 3c, d), suggesting that heterochromatin might have accumulated in the sex chromosomes. Whether this heavily C-banded microchromosomes in *R. diemensis* is the micro sex chromosome as discovered in other reptile species [Ezaz et al., 2005, 2006, 2009b] needs further investigation since the sex of the individuals was unknown.

Our finding of sex chromosomes in *T. lineata* brings the number of Australian agamid species to 5 (*P. vitticeps, P. barbata, Diporiphora nobbi, Ctenophorusfordi,* and *T. lineata*) for which sex chromosomes have been identified. All 5 sex chromosomes have been micro sex chromosomes and exhibit female heterogamety (ZZ/ZW system) [Ezaz et al., 2005, 2009b]. The other single agamid species known to have heteromorphic sex chromosomes is the Qinghai Toad-head Agama, *Phrynocephalus vlangalii*, which has the largest macrochromosome pair as sex chromosome with female heterogamety [Zeng et al., 1997]. In many recent studies, microchromosomes have been emphasised in the karyotype because of their recent identification as sex chromosomes and their role in sex chromosomal evolution [Ezaz et al., 2005, 2006, 2009a, b, c]. Chromosomal rearrangements involving microchromosomes have played a significant role in sex chromosome differentiation and evolution in the reptilian lineages, particularly in Australian agamid lizards [Ezaz et al., 2009b; Matsubara et al., 2019].

GC- and Methylation Pattern in T. lineata and R. diemensis

The fluorochrome chromomycin A_3 specifically binds to the nucleotide guanine, thus highlighting chromosome sites which contain highly repeated GC-rich sequences [Schweizer, 1976; Wrigley and Graves, 1988]. The GC-rich pattern in *T. lineata* and *R. diemensis* were found to be in concordance with the results from methylation (Fig. 5) and telomere sequence (Fig. 6) analyses. Methylation seemed to occur throughout the chromosomes, but stronger methylation signals were observed at the telomeric regions of most *T. lineata* and *R. diemensis* chromosomes, a pattern that has been observed in closely related *P. vitticeps* [Domaschenz et al., 2015] and also in many mammalian species [Barbin et al., 1994; Rens et al., 2010; Ingles and Deakin, 2015] and even plants [Frediani et al., 1996]. However, the centromeric region was not observed to be hypermethylated even though the telomeric sequence highly hybridised to the centromere in *R. diemensis* (Fig. 6c). The telomeric repeat sequence $(TTAGGG)_n$ in vertebrates does not contain the GC dinucleotide required for methylation to occur, but the adjacent subtelomeric regions in mammals are known to be GC-rich and hypermethylated [Brock et al., 1999; Gonzalo et al., 2006]. It clearly explains why centromeric regions in *R. diemensis* were not observed to be methylated. All observed metaphase spreads from both species showed more intense staining of micro- than macrochromosomes (Fig. 5), which is consistent with previous observations of the GC-rich nature of microchromosomes in lizards and birds [Grützner et al., 2001; Domaschenz et al., 2015], suggesting that both *T. lineata* and *R. diemensis* microchromosomes are gene rich. It has been found that hypermethylation of gene bodies is associated with gene activity [Harlow, 1996; Villasante et al, 2007Harlow, 1996; Villasante et al, 2007; Zilberman et al, 2007], and therefore, it can be suggested that these hypermethylated microchromosomes may be connected to important gene activity, including sex determination, in these species. A combination of gene expression and a sequencing-based approach could be adopted to validate this explanation [Domaschenz et al., 2015].

Chromosomal Rearrangements Revealed through FISH Mapping

Chromosome mapping of telomeric sequences has been widely used to identify chromosomal rearrangements between the karyotypes of different vertebrate lineages [Tsipouri et al., 2008; Schmid et al., 2010; Scacchetti et al., 2011; Nagamachi et al., 2013; Suárez et al., 2013; da Costa et al., 2016; de Araújo et al., 2016; Barros et al., 2017; Cavalcante et al., 2018], and ITS signals could represent remnant DNA telomeres from chromosome fusion processes involved in the karyotype evolution or latent telomeres present in the ancestral karyotype [Meyne et al., 1990]. We observed ITSs in the centromeric region of all chromosomes of *R. diemensis* (Fig. 3; 6c), while only in chromosome 4 in *T. lineata* (Fig. 6b). ITSs have also been observed in 2 pairs of microchromosomes (Fig. 6b) in *T. lineata* as in *P. vitticeps* which might be representing the remnants of chromosomal fusions that reduced the diploid number from their Asian ancestors [Young et al., 2013]; but this needs further experimentations. In addition, ITSs were observed in 1 of the unpaired microchromosomes in females of this species (orange arrow and inset in Figure 6b), indicating the putative W chromosome as in *P. vitticeps* [Young et al., 2013]. It has been suggested that regions rich in repetitive DNA act as hotspots for double-strand breaks and chromosomal reorganisation [Huang et al., 2008; Farré et al., 2011; Barros

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et al., 2017]. Srikulnath et al. [2019] reported ITSs in Australian dragon lizards supporting the frequent chromosome fusions between acrocentric and microchromosomes in the infraorder Iguania from ancestral squamate reptiles. They found ITSs in macrochromosomes of the *Amphibolurus* lineage but not in the *Ctenophorus* lineage, but in microchromosomes in both lineages. *P. vitticeps*, *T. lineata*, and *R. diemensis* fall into the *Amphibolurus* lineage [Hugall et al., 2008] and therefore support their findings as well. It is also likely that these ITSs originated via inversions or simply via an accumulation of telomere-like satellite sequences.

One interesting finding from *R. diemensis* telomere FISH was its intense hybridisation signals to all centromeric regions and no ITS. This pattern has also been observed in *Amphibolurus muricatus* and *A. norrisi* by Srikulnath et al. [2019]. The explanation may lay within satellite DNAs that are collectively found most highly concentrated in the centromeric and pericentromeric regions of chromosomes and have a high degree of variation among species in both sequence diversity and overall content. These centromeric repeats are integral to centromere function and stability, as well as the evolution of novel karyotypes [Hartley and O'Neill, 2019].Villasante et al. [2007] proposed a telomeric origin of the centromeres, and Rovatsos et al. [2015] reported that ITSs in centromeric and pericentromeric regions is rather common in squamates with conserved karyotypes, suggesting frequent and independent cryptic chromosomal rearrangements. Therefore, we support the proposition of accumulation of telomeric repeats as satellite DNAs that occurred independently during the chromosomal evolution of this species [Bolzan, 2017]. Besides, we found 6 microchromosomes with intense telomeric hybridisation signals at the centromere with a pair being the brightest (Fig. 6c). We suggest that 2 pairs of these microchromosomes may also harbour ITSs as observed in *P. vitticeps* [Young et al., 2013] and the remaining pair acquired telomeric-like repeats in centromeres, similar to that observed on macrochromosomes or be the sex chromosome pair in this species.

Amplification of simple repetitive sequences played a significant role in the evolution of Y and W chromosomes (differentiation and heterochromatinization of sex chromosomes) in vertebrates, including reptiles and birds [Pokorná et al., 2011; Ezaz and Deakin, 2014; Matsubara et al., 2015, 2016] and may differ among groups and even species. In *P. vitticeps*, SSR $(AAGG)_8$ hybridised onto the W microchromosome [Holleley et al., 2015; Matsubara et al., 2016] whereas, in our study, we found that $(AAGG)_8$ strongly hybridised to a pair of microchromosomes in both sexes of *T. lineata* and a single microchromosome of *R. diemensis*. Whether this single hybridised microchromosome is the heterochomatinized micro sex chromosome in *R. diemensis* has not been tested. An explanation could be that the accumulation of $(AAGG)_8$ is not sex-specific in *T. lineata*, while in the case of *R. diemensis* the more probable explanation is that the specimens were not sexed correctly.

In this study, BAC Pv116_G15 showed similar hybridisation patterns as Pv150_H19 (Fig. 8) by Alam et al. [2020]. The sex microchromosomes of *P. vitticeps* were formed by a translocation of the region containing Pv03_L07 sequences from the ancestral chromosome 2 [Matsubara et al., 2019]. A similar situation might have also occurred in the case of *R. diemensis* and *T. lineata*. Alam et al. [2020] mapped BAC Pv03_L07 and Pv150_H19 on 12 different species of agamid lizards from all 6 subfamilies, including *T. lineata* and *R. diemensis*. They found evidence of multiple chromosomal rearrangements within the Australian Amphibolurinae. The probe Pv03_L07 is known to possess high density and frequency of repetitive sequences [Ezaz et al., 2013], showing the repetitive accumulation sites in sex chromosomes that can be observed through C-banding (Fig. 3). In *R. diemensis*, the probe Pv03_L07 hybridises to an additional pair of microchromosomes. Whether this is simply a chromosomal rearrangement or accumulation of repeats of non-sex determination function or evolution of neo sex chromosomes or multiple sex chromosomes is not clear. The weak signal produced by the Pv03_L07 probe in the microchromosome of *T. lineata* suggests that while the accumulation rate of this sequence was retained in the *Pogona* and *Rankinia* lineage, it was reduced in *Tympanocryptis*. Alam et al. [2020] reported that the BAC Pv03_L07 is conserved across the agamid macrochromosomes and appeared to have been only translocated to microchromosomes in the ancestor of *P. vitticeps*, *T. lineata*, and *R. diemensis* of the subfamily Amphibolurinae because of its high content of mobile elements through a complex history of rearrangements[Ezaz et al., 2013].

Conclusions

In this study, we investigated the karyotype evolution of 2 Australian agamid species with the well-characterised species *P. vitticeps* by karyotyping, C-banding, and comparative mapping of sex chromosome BACs, telomeric, and simple sequence repeats. Both *T. lineata* and *R. diemensis* have identical karyotypes to that of *P. vitticeps* and many other Australian agamids [Witten, 1983;

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Ezaz et al., 2009b], and we provided the pieces of evidence of rearrangements within chromosomal landscapes among closely related species of identical karyotypes. This showed that speciation within the Australian agamid clade involved subtle chromosomal rearrangements, both micro- and macrochromosomes [Irwin, 2018]. We could identify the sex chromosome in *T. lineata* but not in *R. diemensis*. Detailed investigation of heterochromatinized microchromosomes of this species may lead to new information on sex chromosome evolution among Australian agamid species. Of particular priority is to gain access to samples where the gonads have been dissected. Sexing through external morphology (as was the case with *R. diemensis*) can result in misidentification of sex, which immediately compromises the hunt for sex chromosomes. However, the results presented here are still preliminary, and to fully understand the process of karyotype evolution in these species, additional studies using advanced molecular cytogenetic and genomic techniques are needed. This may prove of immense benefit to our understanding of the evolution of chromosomes in vertebrates.

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Statement of Ethics

Animal collection, handling, sampling, and all other relevant procedures were performed following the guidelines of the Australian Capital Territory Animal Welfare Act 1992 (Section 40), the permit issued by the ACT Government (License number LT2017960) and under the approval of the University of Canberra Animal Ethics Committee (CEAE 16–21).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

T.E. and S.M.I.A. conceptualised the study. S.M.I.A. collected the animal and tissue samples and did the cytogenetic experimentations. S.M.I.A. and T.E. designed and co-drafted the manuscript that was edited and proofread by S.D.S. and A.G.

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CHAPTER 5: Egg incubation and sex-linked marker suggest a genotypic sex determination (GSD) in the Canberra Grassland Earless Dragons, *Tympanocryptis lineata* Peters, 1863

5.1 Abstract

Australian agamid lizards (Squamata: Agamidae: Amphibolurinae) show diversity in sex determination mechanisms (SDM), including genotypic (GSD) and temperature-dependent (TSD) sex determination as well as GSD with temperature overrides (GSD+EE) and are known to vary in these mechanisms even among closely related species. Turnovers in sex chromosomes within this group of lizards have also been reported. Establishing how sex is determined in different species is essential for understanding the origins and phylogenetic history of these transitions in sex-determining mechanisms. Here, I used two approaches to investigate the sex determining modes in the Canberra grassland earless dragon (*Tympanocryptis lineata*), a threatened grassland specialist agamid endemic to Australia. First, I applied incubation experiments at five different constant temperatures (24°, 26°, 28°, 30° and 32 °C) to test whether the sex of the offspring is influenced by temperature in this species. I found that when all mortalities are conservatively scored as males (the rarest of the two sexes), sex ratios did not differ significantly from 1:1 male to female sex ratio at all temperatures except for 24 °C, a response consistent with GSD. Second, I used DArTseq™, a genome complexity reduction and high throughput sequencing method, to search for evidence of heterogamety in this species by identifying sex-linked single nucleotide polymorphism (SNP) and restriction fragment presence/absence (PA) markers in 171 phenotypically sexed *T. lineata* individuals (82 males and 89 females). Using this approach, I identified SNP and PA loci associated with females indicating a female heterogamety (ZZ/ZW system) in this species. Based on these two analyses, I conclude that *T. lineata* determine their sex through genotypic sex determination (GSD) with a female heterogametic (ZZ/ZW) system. Since sex determination modes influence sex ratios, a crucial demographic population parameter for the existence of any species, my findings, therefore, have direct relevance to the future conservation and management of this threatened species.

5.2 Introduction

Sex is considered one of the most intriguing biological phenomena (Charlesworth 2006; King 1912), and sex determination is the process that controls the fate of an individual's sex, whether male or female, and the mechanisms of that determination are diverse (Capel 2017; Ezaz et al. 2006a; O'Meally et al. 2012). Incubation experiments can provide important information about the nature of sex determination in a species (Ballen et al. 2016; Doddamani et al. 2012; Hansson and Olsson 2018; Quinn et al. 2007a; Steele et al. 2018). Species with genotypic sex determination (GSD), where genes within the chromosome determine the sex of any offspring, are expected to maintain a stable 1:1 sex ratio in their clutches. Sex ratios in TSD (temperature-dependent sex determination) species may be subject to variation in the thermal environment of their nests (Harlow 2004; Schwanz 2016), and sex ratios can be skewed towards male- or all female-biased clutches at extreme temperatures during early developmental stages, with different combinations of males and females at a very narrow pivotal temperature (the constant temperature that produces both males and females in equal proportions) range (Ewert et al. 2005; Lang and Andrews 1994). Conversely, GSD species with environmental influence (GSD+EE) will present a 1:1 sex ratio at most temperatures but show skewed sex ratios at one or both extremes (Holleley et al. 2015; Shine et al. 2002). This influence of temperature on species with sex chromosomes can result in sex reversals, generating discordance between genotypic and phenotypic sexes (Alho et al. 2010; Tamschick et al. 2016). If sex ratios are 1:1 at all incubation temperatures, then a chromosomally based system of sex determination is likely. Deviations from that ratio suggest an environmental influence that overrides the influence of sex chromosomes at certain temperatures or operates in the absence of sex chromosomes (Sarre et al. 2004).

Sex chromosomes, carrying sex-determining genes, are an integral part of genotypic sex determination. Finding sex chromosomes may not be easy if the sex chromosomes are cryptic and very close to homomorphic. In that case, the presence of sex-specific genes or sequences in these chromosomes can reveal the sex determination mode (XX/XY or ZZ/ZW) identified in several reptilian taxa, including agamid lizards (Gamble and Zarkower 2014; Hill et al. 2018; Quinn et al. 2009). A variety of methods have been applied to genotypically assign sex from uncertain sex chromosome complements. These include genotyping-by-sequencing (GBS) approaches such as RADseq and DArTseq™, in which sex-linked markers that distinguish male- from female-heterogametic systems have been identified (Berset-Brändli et al. 2006;

Brelsford et al. 2016; Gamble and Zarkower 2014; Hill et al. 2018; Lambert et al. 2016; Lambert et al. 2019; Matsuba et al. 2008; Ogata et al. 2018; Sopniewski et al. 2019). These approaches can be applied without information on offspring sex and therefore represent an approach that is complementary to incubation experiments and provides the potential for establishing the mode of heterogamety.

The Canberra grassland earless dragon *Tympanocryptis lineata* is an endemic species to Australia (Melville et al. 2019), confined to the natural temperate grasslands around Canberra and is closely related to the central bearded dragon (*Pogona vitticeps*), a species with a well characterised GSD + EE sex-determination system. The Canberra grassland earless dragon has experienced a dramatic decline in population size over the last two decades and is at a high risk of extinction (Carlson et al. 2016; Dimond et al. 2012; Melville et al. 2019). I conducted this study on this species since sex determination is known to directly affect population sex ratios, a demographic parameter essential for population persistence (Boyle et al. 2014). Here, my primary goal was to determine the mode of sex determination in this species and hence build a better understanding of sex determination within the bearded dragon clade. Results from the cytogenetic analysis reported in chapter three suggest that this species has a female heterogametic (ZZ/ZW) sex-determination system as in other Australian agamid species (Ezaz et al. 2009b). Here, I use a combination of incubation experiments (Quinn et al. 2007a) and genotyping by sequencing DArTseq™ (Kilian et al. 2012) of known males and females to identify sex-linked markers to test the proposition that this species has a GSD mode of sex determination driven by a ZZ/ZW heterogametic sex chromosome system.

5.3 Methods

5.3.1 Incubation experiment

The University of Canberra houses a captive population of *T. lineata* for research and conservation purposes, and most of the eggs and tissue samples used for this study came from these animals. The cages containing gravid lizards were checked daily for eggs, collected as soon as possible after laying and placed into incubation. A number of the eggs (n=66) used were taken from wild nests as part of the establishment of a captive colony. A total of 138 *T. lineata* eggs were incubated in glass pots at five different temperatures - 24°C (n = 9), 26°C (n = 23), 28°C (n = 48), 30°C (n = 31) and 32°C (n = 27). Each pot was filled to two-thirds with

moist vermiculite and maintained at an unmeasured humidity by keeping water-filled trays inside the incubators and top-up as required. The temperatures used were within the range for incubation experiments used for the sister species *P. vitticep*s (Quinn et al. 2007a). Since *T. lineata* has experienced a recent population decline (Dimond et al. 2012), incubation temperatures excluded the high (34-36 °C) and low extremes (22 °C) used for *P. vitticeps* (Quinn et al. 2007a) to minimise the risk of low hatching success. Before incubation, the eggs were wiped clear of sand or moisture, weighed, and placed individually in glass pots. Eggs from a single clutch were allocated sequentially across the temperature treatments such that if there were six eggs in a clutch and five temperature treatments, then the eggs were allocated randomly at one per treatment with the sixth egg allocated at random to one of the five temperature treatments. Therefore, the treatments were replicated, i.e., several eggs (at least nine eggs per temperature) in each incubation temperature during the breeding season over the years of experimentation (2013-2018). Incubators were checked daily for hatchlings or overtly unhealthy or dead eggs. Following the placement of the eggs, the cups were sealed using plastic cling wrap and a rubber band to preserve moisture, and the cups were placed in incubators set at constant temperatures. The realised temperature in each incubator was measured using data loggers (ibuttons) throughout all incubations within a glass pot with similar conditions but without any egg. Phenotypic sex was determined by everting hemipenes in male hatchlings following the method of Harlow (1996, 2004).

5.3.1.1 Statistical analyses

Sex ratio data were compared across the constant incubation temperature treatments in order to determine whether this species displays a sex ratio pattern typical of GSD (no departure from 1:1 sex ratio), TSD (sex ratios depart from 1:1 at temperature extremes), or GSD+EE species (sex ratios depart from 1:1 at one extreme temperature but not the other). To account for the possibility of differential mortality between the sexes, all eggs that had died during incubation or died before they could be sexed were scored as the lesser of the two sexes (specimens that could not be sexed with confidence were considered as males within the respective incubation temperature, assuming male-biased mortality within that respective temperature) for the treatment (Georges 1988; Georges et al. 1994) and then analysed using a chi-square test. My expectations, based on previous cytogenetic works (chapter 3 and Ezaz et al. 2009b), is that *T. lineata* has a ZZ/ZW system. It is likely that temperature effects within such a system might take the form of a skew towards females at high temperatures, as seen in *P. vitticeps* (Quinn et al. 2011), with ZZ male genotypes being reversed to female phenotypes.

Thus, I expect a skew towards females at high temperatures as male genotypes are reversed to female phenotypes and a 1:1 sex ratio at lower temperatures as phenotypic sex correlates with genotypic sex. As a consequence, I considered all eggs that died before they could be sexed to be male. Statistical analyses were performed in Microsoft Excel. The incubation data were further tested by fitting to GSD versus TSD (logistic) models in R using the `tsd()` function of the package *embryogrowth* version 8.1 (Girondot 2021). The models were created both with the unsexed individuals excluded and included. These models were then compared based on the AIC (Akaike Information Criterion), and the model with the lowest AIC value was selected as the best-fitted model.

5.3.2 Sex-linked marker analysis using genotyping-by-sequencing (GBS)

A total of 171 individuals (82 males and 89 females) with confirmed sexes (phenotypic sex was determined by everting hemipenes following the method of Harlow, 1996 and 2004) were used in the search for the sex-linked marker (Annex II). DNA extraction and sequencing of the tissue samples (tail snips from live animals or liver tissues from dead animals) was conducted through Diversity Arrays Technology Pty Ltd. using DArTseq™, a methodology combining DArT genome complexity reduction methods and next-generation sequencing technologies (Kilian et al. 2012) that employs genomic complexity reduction using restriction enzyme pairs. This method has been successfully deployed in microorganisms (Talamantes-Becerra et al. 2019; 2020), many plants (Baloch et al. 2017; Sardos et al. 2016) and animals (Couch et al. 2016; Hill et al. 2018; Lambert et al. 2016; Lambert et al. 2019; Lind et al. 2017; Melville et al. 2017; Ogata et al. 2018; Shams et al. 2019a; Sopniewski et al. 2019). The DArTseq approach assigns a lower density of high-quality markers with reasonable coverage of the genome and a low level of excluded data (Baloch et al. 2017). These markers can be grouped based on sex and, thereby, have the potential to identify loci/markers linked to sex chromosomes. This approach, thus, provides a useful molecular tool for detecting sex-linked sequences, which in turn may be indicative of the sex-determining modes in non-model species with cryptic sex chromosomes or species with unknown sex determination (Hill et al. 2018; Lambert et al. 2016; Sopniewski et al. 2019).

A detailed description of the DArTseq™ methodology can be found in Kilian et al. (2012). The DNA samples were digested using two different adaptors corresponding to two different Restriction Enzyme (RE) overhangs - *Pst*I and *Sph*I before ligation reaction were performed.

The *Pst*I compatible adaptor consisted of an Illumina flow cell attachment sequence, sequencing primer sequence and a unique barcode sequence, and the *Sph*I compatible adaptor consisted of an Illumina flow-cell attachment region. The resultant ligated fragments then run for under 30 rounds of PCR amplification (PCR conditions: 94 °C for 20 seconds, 58 °C for 30 seconds and 74 °C for 45 seconds, followed by an extension of seven minutes at 72 °C). The equimolar amplicons obtained from each individual were pooled together, run in Illumina's proprietary cBot bridge PCR and sequenced on an Illumina Hiseq2000 for 77 cycles.

These sequences were then processed using proprietary DArTseq™ analytical pipelines. Poor quality sequences were filtered from the FASTQ files generated from Hiseq2000, including parameters for any results with reproducibility >90% and read depth >3.5 for SNPs and >5 for PA markers, as well as the application of more stringent selection criteria to the barcode region (compared to the remainder of the sequence). All identical sequences were then collapsed into 'FASTQCOL' files that were run through a secondary pipeline (DArTsoft14) for differentiating single nucleotide polymorphism (SNP) and SilicoDArT (presence/absence of restriction fragments in representation; PA data). With a reference-free algorithm (optimised clustering algorithm), DArTsoft14 identified and clustered each unique sequence by sequence similarity from the FASTQCOL file (three base pair variation was used as the distance threshold). These clustered SNPs and SilicoDArT markers (sequences) were labelled with several metadata parameters based on the quantity and distribution of each sequence within all samples analysed. The genotyping process also included high levels of technical replication that allowed the parameter of 'reproducibility' to be calculated for each marker. Additional features of the output by DArTsoft14 are the average count for each sequence (sequencing depth), the balance of average counts for each SNP allele, and the call rate (proportion of samples for which the marker is scored) for each marker.

5.3.2.1 Marker selection

I used DArTseq™ to identify sequences that were different between the sexes. I identified sex-linked SNP (single nucleotide polymorphism) and silicodart (presence-absence, PA) loci following criteria set by Lambert et al. (2016) with modifications. I ensured data quality by filtering the 58,245 SNP and 116,490 PA sites identified by DArTseq against the following criteria on i) Call rate (at least 80% i.e., 0.8 – 1.0) (for SNP and PA Markers), ii) Average read depth (≧10) (for PA Markers) and iii) Reproducibility/RepAvg (at least 80% i.e., 0.8 – 1.0) (for SNP and PA Markers).

To identify potential sex-related markers, I modified the method used by Lambert et al. (2016) to filter loci against the criteria required for ZZ/ZW or XX/XY systems (Table 5.1). I primarily selected loci that had restriction fragments sequenced in at least 80% of one sex and not sequenced in at least 90% of the other sex. I then selected against the loci showing at most 15% discordance using the equation:

$$((t-p) + q)/n) *100$$

Where,

t = total number of individuals of the target sex against a marker

p = number of individuals with a positive score against the marker for the target sex/total number of heterozygote individuals in the target sex

q = number of individuals with a positive score against the marker in the opposite sex/total number of heterozygote individuals in the opposite sex

n = total number of treatments/individuals for both sexes against a marker.

Table 5.1 Selection criteria for sex-linked SNP markers, modified from Lambert et al. 2016

Parameter	Male heterogamety (XX/XY)		Female heterogamety (ZZ/ZW)	
	Female	Male	Female	Male
Homozygosity for the reference allele	≥0.80	≤0.10	≤0.10	≥0.80
Homozygosity at the SNP allele	≤0.10	≥0.90	≥0.90	≤0.10
Heterozygosity	≤0.20	≥0.75	≥0.75	≤0.20

Previous studies have identified that at least 13–15 individuals per sex are required to minimise the occurrences of false-positive sex-linked loci (Brelsford et al. 2016; Lambert et al. 2016; Ogata et al. 2018; Sopniewski et al. 2019). I adopted a conservative approach in considering a maximum of 15% discordance in the occurrence of sex-specific loci based on the assumptions of random recombination between sex chromosomes, and that this is less than the rate of sex reversal (22%) seen in wild *P. vitticeps* (Holleley et al. 2015).

5.4 Results

5.4.1 Incubation experiment

Egg incubations for most temperatures were conducted across five years (2013-2018) as provided in Annex I. However, incubations were conducted at 24 °C for the clutches of only

one year (2014) and at 26 °C for three years (2013-2015) as a precautionary measure due to the declining population status of this species since the incubation period was much longer (Table 5.2) at this temperature than the other temperatures.

Table 5.2 Incubation period and the percentage of hatchlings surviving across time from the different incubation experiments

Set incubation temperature (°C)	Average ibutton incubation temperature (°C)	Average incubation period (## days)	Hatchling survival (%)			
			21 days	90 days	180 days	1 year
24	24.4	73	66.67	66.67	55.56	55.56
26	26.5	51	92.31	65.38	61.54	57.69
28	28.4	43	100.00	100.00	96.67	96.67
30	30.0	40	100.00	100.00	100.00	84.62
32	32.1	32	100.00	100.00	100.00	72.73

Out of the 138 eggs incubated, 118 individuals (46 males and 72 females, Annex I) hatched and survived to the point where morphological sex could be determined. The percentages of females were between 58-65% across the temperatures 26 °C and 32 °C (Table 5.3) but 22% at 24 °C. Sex ratios did not differ significantly from 1:1 at any of the temperatures between 26-32 °C, a response consistent with GSD (Table 5.3).

Table 5.3 Sex ratio during incubation experiments. Mortalities were assigned to reduce observed sex bias resulting in non-significant differences (p=<0.05) at different incubation temperatures (Chi-Square Goodness of Fit test). The low $\chi 2$ value is an indication that the sex of an individual is independent of its incubation temperature.

Incubation temperature (°C)	**Not hatched/ sexed** (n)	**Males** (n)	**Females** (n)	**% Females**	**Males + mortalities** (n)	**$\chi 2$**	**Significance** (p)
24	0	7	2	22.2	7	2.777	0.095
26	3	7	13	65.0	10	0.416	0.518
28	11	12	25	67.6	23	0.083	0.773
30	5	11	15	57.7	16	0.032	0.857
32	1	9	17	65.4	10	1.814	0.177
Total	**20**	**46**	**72**	**61.0**	**66**	**0.260**	**0.609**

These data (Table 5.3) were then modelled to fit any thermal reaction norms, i.e., TSD and GSD (Fig. 5.1). Upon comparing both models based on the lowest AIC value, the GSD model considering all unsexed individuals as males was the best model (Table 5.4).

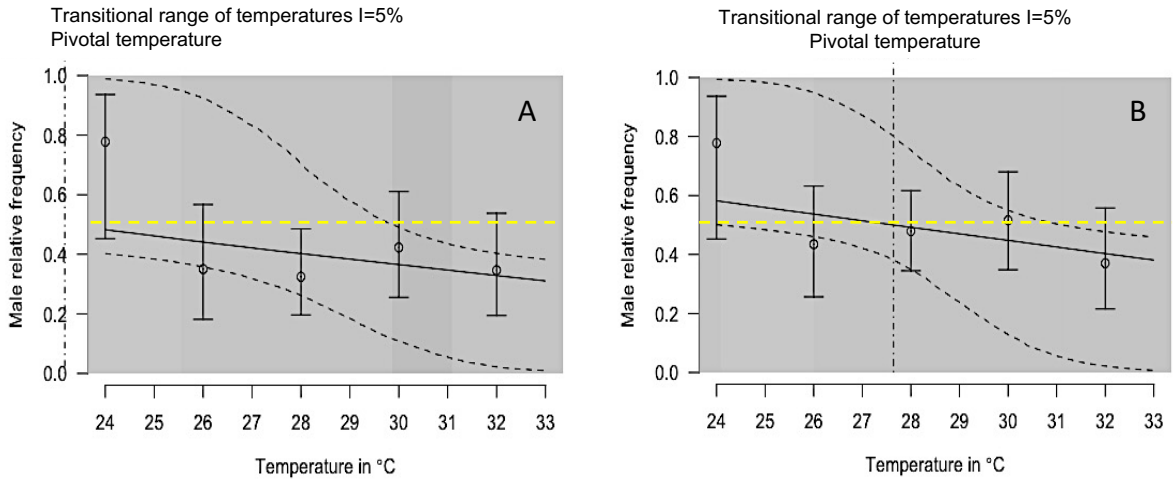

Figure 5.1 Sex ratio patterns among hatchlings modelled. A. only confirmed sexed males included, B. all unsexed individuals included as males. The points correspond to observations and the bars to their 95% confidence intervals. The plain line shows the maximum likelihood model and the 95% confidence interval shown as dashed lines. The vertical dash-dotted line indicates the pivotal temperature. The horizontal yellow dashed lines indicate the point where the relative abundance of males is 50%.

Table 5.4 Summary of TSD (logistic) and GSD model tested for *T. lineata* incubation data. Line with bold text indicates the best model with the lowest AIC value.

Condition	Model	AIC
Confirmed males only	TSD (logistic)	26.40586
	GSD	29.58968
Males + unsexed individuals	TSD (logistic)	25.12534
	GSD	**23.17581**

5.4.2 Sex-linked markers

Both SNP data (58,245 loci) and SilicoDArT (Presence-Absence, PA) data (44,488 loci) from 82 males and 89 females were examined for sex-specific SNPs. After filtering, I retained 26,208 SNPs (45% of the total SNP data; 1,808,352 bp) and 7,628 PAs (17% of the total silicoDArT data; 526,332 bp) loci, that were analysed in all 82 males and 89 females (171 individuals). I did not find any loci that were 100% concordant with any sex. However, one SNP (Annex III) and 26 PA (Annex IV) loci were identified that were 85-88% concordant with female phenotypes (Fig. 5.2), and none of these loci was concordant with males.

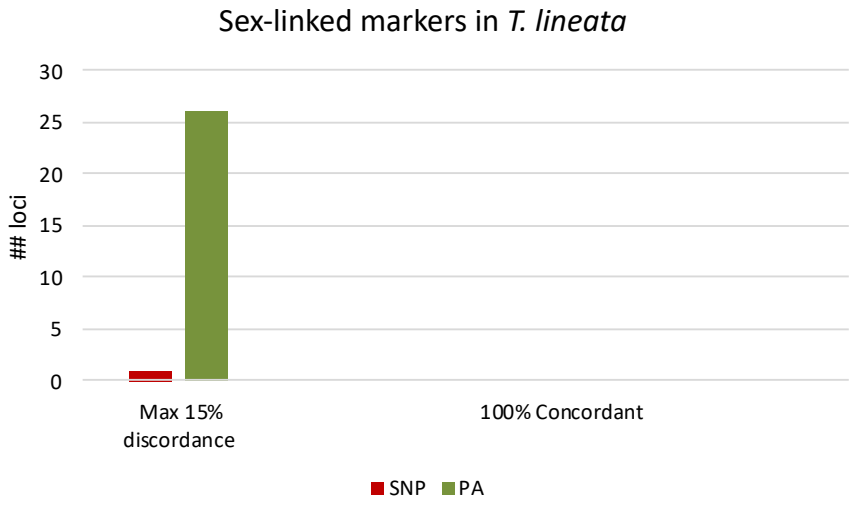

Figure 5.2 Sex-specific markers identified in *T. lineata*. The number of sex-markers indicated female heterogamety (ZZ/ZW system) in this species.

5.5 Discussion

5.5.1 Sex Determination mode in *T.lineata*

I applied two complementary approaches to examining the sex determination mode in *T. lineata* and taken together, provide strong evidence that this species has a ZZ male/ZW female system of genetic sex determination. In the first, I conducted egg incubations across a number of temperatures and could detect no departures from the 1:1 sex ratio (considering all mortalities were male) that would be expected under a GSD (chromosomally determined) system. Second, I applied the DArTseq™ method to search for sex-linked markers in *T. lineata* and found a female bias in both SNPs and presence/absence markers in this species.

5.5.1.1 Egg incubation experiment

I explored the effects of incubation temperatures on sex ratio to provide an assessment of how natural nest temperatures affect variation in offspring sex ratios in *T. lineata*. The hatchling sex ratios in this species do not vary with incubation temperature in a way that is similar to that shown in its closely related GSD+EE species *P. vitticeps* (Quinn et al. 2007a). In *P. vitticeps*, a 1:1 sex ratio was observed only up to 32 °C, although skewed towards female-biased in high temperature, between 34-37 °C. *T. lineata* is different from *P. vitticeps* in the way that it produced unbiased phenotypic sex ratios only when all the mortalities were considered males.

Offspring sex ratios of 1:1 (male: female) at different incubation temperatures imply that incubation temperature does not influence offspring sex and provided evidence of a genetic form of sex determination (Sarre et al. 2004). However, because of the endangered status of this lizard species, I was unable to test fully the upper limits of incubation temperatures that have been shown to influences sex in *P.vitticeps* (Quinn et al. 2007a), so the potential for an environmental effect in this species remains unresolved. As a consequence, more research is required to investigate sex ratios produced at temperatures over 32 °C to identify if sex reversal occurs, as seen in *P. vitticeps*. The lower percentage of female production (22%) at lower incubation temperature (24 °C) suggests that there could be a male-biased sex ratio at this extreme ($p=0.095$); as observed in bird species due to temperature-dependent sex-biased embryonic mortality (DuRant et al. 2016) but greater sample sizes are required to test that proposition.

To identify GSD using egg incubation studies, I could not exclude the potential for sex-biased mortality of embryos under different incubation treatments. Sex biases in embryo sensitivity to incubation temperature could provide results similar to TSD (Steele et al. 2018), yet this does not prove that sex is determined by temperature. In this study, egg/hatchling mortality was 20 out of 138 (14.5%). Assuming that all dead eggs and unsexed hatchlings[1] were males, the sex less represented in each treatment (Quinn et al. 2007a), the sex ratio was found not to be influenced by incubation temperature (Table 5.3), and therefore, the possibility of sex-biased mortality cannot be ruled out in this species. Besides, the results show higher ratios of females ranging from 58-68% in most experiments (26-32 °C) that contradicts the known TSD norms (MF, FM or FMF) and therefore, considering all the unsexed individuals as less represented sex (males) seemed more rational. When modelled, the incubation experiment data did not show any sigmoid curve as expected from a TSD pattern (Fig. 5.1); instead, my model comparison indicates male-biased mortality is the likely key factor behind the high female ratio within a GSD pattern (Table 5.4). The potential for sex-biased mortality across treatments, on the other hand, must be ruled out during the egg incubation experiments to identify TSD (El Mouden et al. 2001; Steele et al. 2018).

5.5.1.2 Sex-linked markers

In my study, I applied DArTseq™ method to search for the sex-linked marker in *T. lineata.* I identified a number of markers, both SNPs and presence/absence, significantly associated with a female heterogametic sex-determining system (ZZ/ZW).

I could not identify 100% female-specific markers; however, several SNP and PA loci were identified as moderately sex-linked (not 100% concordant), with females expressing homozygosity to the reference/SNP alleles. Such a scenario suggests that these markers are distributed in proximity to the sex-specific region of the W chromosome and have undergone recombination with the Z chromosome to low degrees, hence no longer exhibit perfect female concordance. Such potential recombination has been reported in amphibians (Lambert et al. 2016; Sopniewski et al. 2019) and humans (Cotter et al. 2016). The absence of 100% concordance of presence/absence markers could be due to the presence of null alleles, as described by Sopniewski et al. 2019. A null allele is a non-zero count that appears as a dash in

[1] Data used for the analysis have been recorded between 2013 and 2018 from the conservation breeding colony at the University of Canberra, and no data regarding the sex of the dead hatchlings that could not be sexed using the hemipenes were ever recorded.

the silicoDArT data of the DArTseq file, can be indicative of heterozygotes. Alternatively, it could also be through mistakes in phenotypic sexing or higher rates of sex reversal than assumed.

Out of the 171 individuals used for sex-linked marker analysis, only 28 could be identified as belonging to a family group (Annex II and Annex V). Family relationships of the remaining individuals were not known since the tissue samples were collected from the individuals in the wild and were released back after collecting the samples. Furthermore, some samples were collected from the individuals that were trapped from the wild, raised within the colony but did not breed. However, I calculated the concordance of the identified SNP and PA markers in the six family groups and found 0-33% discordance while considering individual groups (Annex V). When all family groups were considered, 3.6-14.3% discordance were observed. Out of these family groups, the group with the highest number of members (nine individuals of four females and five males) were selected and spurious sex association were assessed utilizing the formula $P_i = 0.5^n$ (Lambert et al. 2016); where P_i is the probability that any locus was sex-linked by chance and n is the sample size (male and female). P_i was multiplied by the number of SNP/PA markers (remained after filtering), which gave an estimation of the number of random sex-linked loci produced through analysis. For these nine individuals, *Pi* was 0.002, and therefore it is less likely that any marker identified in this family group is sex-linked by chance. However, based on the total sample size, i.e., 171 individuals, *Pi* was $3.34X10^{-52}$ and $8.75X10^{-48}$ loci out of the 26,208 SNP loci, and $2.55X10^{-48}$ loci out of the 7,628 PA loci were expected to spuriously show sex-linkage. Therefore, given this sample size, with this probability, it is highly unlikely that sex-linked markers would be identified by chance in the samples. Using the above equation, 15 individuals for this number of SNP markers and 13 individuals for this number of PA markers would be sufficient to be confident that less than one marker would be spuriously sex-linked.

The markers identified here do not show geographic variation since *T. lineata* is confined to the Canberra region of the Australian Capital Territory. I did not detect any clear evidence of sex reversal in my samples, as sex reversal would be diagnosed by the discordance between the phenotypic sex and the majority of sex-linked markers. This may be due to a relatively low frequency of sex reversal in this species since I had a relatively large sample size (Lambert et al. 2016). However, since no perfectly sex-linked marker was observed, it strengthens the likelihood that sex-reversal occurs in this species.

I conclude that DArTseq data collected on *T. lineata* support differentiated sex chromosomes in this species. All my markers conform to a ZZ/ZW system in *T. lineata,* as previously inferred through cytogenetic analysis as described in chapter 4. Incubation experiments described in this chapter provides further evidence that a genetic sex determination occurs in *T. lineata*. My results, therefore, underpin the usefulness of DArTseq™ in identifying sex-linked markers for the identification of sex-determining modes in non-model organisms, without a priori sequence information.

5.6 Conclusion

In this study, I investigated the sex determination mechanism in a threatened Australian agamid lizard species – the Canberra grassland earless dragon, *Tympanocryptis lineata.* Through egg incubation experiments, I was unable to disprove the null hypothesis that constant incubation temperatures do not influence the hatchling sex ratio in *T. lineata*. In addition, I was able to identify a number of sex-linked markers, despite given the small portion of the genome represented through DArTseq analysis. However, the markers identified should be validated using PCR-based tests. Additional evidence may be achieved by using these markers as probes in fluorescence *in-situ* hybridisation (FISH) onto *T. lineata* metaphase chromosomes. My findings, therefore, provide further support for the findings of cytogenetic analysis presented in chapter 3 of a species with sex chromosomes and, therefore, most likely to be a predominantly GSD species. However, whether this species is a pure GSD species or has temperature influence (as in *P. vitticeps*), requires further laboratory studies with a broader range of temperature variations (e.g., 22–36 °C). Reduced representation genotyping methods such as DArTseq™ that combine genome complexity reduction with high throughput sequencing are regarded as valuable options for studying the genetic basis of sex determination.

Sex ratio is considered an essential factor for the existence of a species or population since a biased sex ratio may result in population decline by reducing the chance of finding a potential mate or eliminating one sex from the population (Grayson et al. 2014; Janzen 1994; Kallimanis 2010). With the discovery of GSD+EE species, it is no longer a valid notion that vertebrate sex-determining mechanisms are simply dichotomous - GSD in one end and ESD on the other (Sarre et al. 2004). Identification of sex-determination mechanisms and any factor influencing sex ratio such as temperature (Boyle et al. 2014) would, therefore, guide the conservation and

management practices of threatened species like *T. lineata*, especially in the face of global climate change.

CHAPTER 6: Sex determination mechanisms between species and populations within *Calotes* cryptic species complex (Squamata: Agamidae: Draconinae)

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Preface

Variation in sex determination modes and mechanisms can be observed between closely related species or populations in different groups of animals. Such variation between closely related species has been reported from agamid lizards but not from a species complex or population perspective. This manuscript explored this issue in the Oriental garden lizard *Calotes versicolor*, an agamid species with a wide distribution range and is considered as a complex of different cryptic species. The results suggest that different sex-determining modes, such as GSD and TSD, have been evolved within *C. versicolor* species complex and even between populations.

Justification of inclusion

As the first author, I was responsible for writing this paper which included reviewing the literature, sample collection and preparation, analysis, writing and revising the draft and designing diagrams.

Declaration for Thesis Chapter 06

DECLARATION BY CANDIDATE

In the case of Chapter 06, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contributions (%)
• Conceptualization • Animal and sample collection and preparation • Literature review • Analysis • Writing and revising draft • Designing the figures/diagrams • Correspondence with the co-authors	70%

The following co-authors contributed to the work:

Name	Nature of Contribution	Contributor is also a UC student (Yes/No)
Tulyawat Prasongmaneerut	Analysis, sample collection and preparation, and draft preparation	No
Dianne Gleeson	Reviewing and supervision	No
Arthur Georges	Reviewing and supervision	No
Stephen D. Sarre	Reviewing and supervision	No
Kornsorn Srikulnath	Conceptualization, reviewing and resources	No
Tariq Ezaz	Conceptualization, reviewing, supervision and resources	No

31/08/2020

Candidate's Signature and Date

DECLARATION BY CO-AUTHORS

The undersigned hereby certify that:

(19) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.

(20) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(21) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(22) there are no other authors of the publication according to these criteria;

(23) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(24) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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6.1 Abstract

Sex determination mechanisms and sex chromosomes vary between species and within populations. Evidence for such variation in sex determination mechanisms in the Oriental garden lizard *Calotes versicolor* was investigated in this study. This species has a wide distribution and is considered a cryptic species complex. Samples were collected from three locations in Bangladesh and three in Thailand, and a population level genetic analysis was conducted to determine whether these samples of *C. versicolor* comprise different species or populations. Both genomic (SNP data) and mitochondrial DNA data were used for population analysis. The samples collected were genetically distinct and provided evidence that within its range *C. versicolor* exists as a complex of multiple cryptic species. Sex-linked markers were then analysed to identify sex determination modes within these samples. DArT sequencing data (SNP and presence-absence loci) were used to analyse sex determination modes. Analysis of sex-linked markers revealed variation in sex determination mechanisms (*sensu* sex determination modes). Overall results suggested that different sex determination modes have evolved between closely related species and within populations in Agamid lizards.

Keywords: Sex determination mode, *ND*2 gene, Sex-linked loci, SNP, Sex chromosome heterogamety

6.2 Introduction

Determination of sex is one of the most fundamental and yet highly variable mechanisms in the animal kingdom (Bull 1983) and is a rapidly evolving trait. Variation in this mechanism can be observed within many lineages of reptiles, fishes, crustaceans and angiosperms and even in closely related species or populations (Bull 1983; Charlesworth 1996). For example, in the Japanese wrinkled frog, *Glandirana* (*Rana*) *rugosa,* four genetic forms are distributed in different geographic regions of Japan (Miura 2007; Nishioka et al. 1994) with male and female heterogametic sexes. Although sex determination modes and sex chromosomes may vary between reptile species (Alam et al. 2018; Ezaz et al. 2009b; Ezaz et al. 2009c; Harlow 2001), variation within species is poorly studied. Determining heterogametic sex by identifying sex chromosomes is not easy if the sex chromosomes are cryptic and very close to homomorphic. These chromosomes may contain sex-specific genes or sequences that can reveal their sex determination mode (XY or ZW) (Gamble et al. 2017; Gamble et al. 2015; Gamble and

Zarkower 2014; Hill et al. 2018; Lambert et al. 2016; Nielsen et al. 2020; Ogata et al. 2018). This may even be true in the case of species with temperature-dependent sex determination (TSD), which lack sex-specific chromosomes. Different species may show diverse sex ratio patterns in offspring during incubation experiments, further complicated by gene-temperature interactions. In these cases, development of sex-linked markers may provide valuable insight. Such markers have been successfully identified in several reptilian taxa (Cornejo-Páramo et al. 2020; Gamble et al. 2017; Gamble and Zarkower 2014; Hill et al. 2018; Lambert et al. 2016; Nielsen et al. 2020; Quinn et al. 2009) and the development of sex-linked markers is therefore required in order to reveal sex determination modes in species with undifferentiated sex chromosomes.

It is speculated that differential methylation of the promoters of genes is involved in sex determination at sex-specific temperatures, but a full understanding of the mechanism is unknown (Deakin et al. 2014). Differences in gene methylation occurring between genetic males and females have been observed in European sea bass, *Dicentrarchus labrax* (Navarro-Martín et al. 2011), Nile tilapia, *Oreochromis niloticus* (Baroiller and D'Cotta 2016) and the half-smooth tongue sole, *Cynoglossus semilaevis* (Shao et al. 2014). Differential methylation patterns associated with phenotypic sex have also been reported in different developmental stages of red-eared slider turtle, *Trachemys scripta* (Ramsey et al. 2007). However, epigenetic changes do not involve a change in nucleotide sequence facilitated by DNA methylation (Matsumoto et al. 2016). Restriction site-associated DNA sequencing, such as RADseq and DArTseq, has been proposed as a method for developing sex-linked markers i.e. sex-linked loci in taxa with homomorphic sex chromosomes (Gamble and Zarkower 2014; Lambert et al. 2016). This has successfully been used to develop sex-linked markers and to infer the sex-determining mode for several amphibian, squamate and fish species (Brelsford et al. 2016; Gamble et al. 2017; Lambert et al. 2016; Nielsen et al. 2020; Palaiokostas et al. 2013; Utsunomia et al. 2017; Wilson et al. 2014). Diversity Arrays Technology, the developer of DArTseq™, has developed a methylation-sensitive DArTseq (DArTseqMet) that uses two different restriction enzyme isoschizomers (one CpG methylation-sensitive and the other not) to identify sex-specific markers. Therefore, it has the potential to reveal any methylation mediated sex determination considering the same age and tissue type being investigated.

The Oriental garden lizard, *Calotes versicolor* (Daudin, 1802), belongs to the subfamily Draconinae of the family Agamidae. It has a wide distribution and is found from Iran to Malaysia through South Asia and Southeast China (Uetz et al. 2020). It has been introduced to

different countries, including the USA (Florida), Celebes, Maldives, Seychelles and Kenya and is found across highly heterogeneous habitats in different elevation ranges. This species is considered taxonomically neglected (Gowande et al. 2016) and is comprised of a complex of multiple species (Huang et al. 2013; Zug et al. 2006). It lacks heteromorphic sex chromosomes (Ganesh et al. 1997; Singchat et al. 2020; Singh 1974), but gene expression analysis has shown that the sex determination mechanism in this species appears closer to genotypic sex determination in mammals than birds and environmental sex determination in reptiles (Chakraborty et al. 2009; Tripathi and Raman 2010). However, Doddamani et al. (2012) claimed that *C. versicolor* is a TSD species with a novel FMFM pattern of offspring sex ratio. It is not known whether this observed variation is due to the existence of multiple cryptic species, or a transition of sex determination mechanisms and sex chromosome turnovers, or the existence of multiple thermosensitive points among closely related species or populations. Therefore, investigation of sex determination modes and sex chromosomes within this species (or species complex) potentially provides a good model for examining variation in the mode of sex-determination mechanisms in a species with a wide geographic distribution.

The primary aim of this study was to identify sex-determining mechanisms (i.e. modes of sex determination) across the *Calotes* species complex. The initial task was to determine whether *C. versicolor* individuals collected from Bangladesh and Thailand are diverse genetic populations or a species complex. We then identified sex determination modes according to the species/populations. Both of these forms of data were used to reveal whether *C. versicolor* consists of a cryptic species complex.

6.3 Methods

6.3.1 Specimen collection

C. versicolor samples were collected from three locations in Bangladesh – Dhaka (23.664722 N, 90.420833 E), Feni (23.00376 N, 91.27464 E) and Habiganj (24.12475 N, 91.44459 E) and three locations from Thailand – Bangkok (13.691695 N, 100.671882 E), Samut Prakan (13.669499 N, 100.795996 E) and Khon Kaen (16.46662 N, 102.84771 E). A total of 49 samples of *C. versicolor* were captured and sexed (Table 6.1). Phenotypic sexes of Bangladesh samples were determined by everting hemipenes in males following Harlow (1996; 2004), while the sexing of Thai samples was done by dissecting gonads in the lab. Tail snips from the

Bangladesh samples were collected in the field using sharp scissors (sterilised between samples using hydrogen peroxides) and immediately transferred to 5 ml 1x Hanks's balanced salt solution (Sigma-Aldrich), kept at room temperature (~25°C) and transported to the University of Canberra within five days. Lizards from Thailand were collected by the local people and the DNAs were extracted from either tail or liver tissues at the Kasetsart University, Thailand and transported to the University of Canberra.

Figure 6.1 Collection locations of *Calotes versicolor*. Numbers within the parentheses represent the number of samples representing each location.

Table 6.1 *Calotes versicolor* sample details

Sl. No.	Sample id	Location	Sex	Used for		Comment
				DArT sequencing	Sanger sequencing	
1.	TL_CAV5	Bangkok, Thailand	Female	X		
2.	TL_CAV6	Bangkok, Thailand	Male	X		
3.	TL_CAV7	Khon Kaen, Thailand	Male	X	X	
4.	TL_CAV8	Khon Kaen, Thailand	Male	X	X	
5.	TL_CAV9	Khon Kaen, Thailand	Female	X		
6.	TL_CAV10	Khon Kaen, Thailand	Female	X	X	
7.	TL_CAV11	Khon Kaen, Thailand	Female	X		
8.	TL_CAV12	Khon Kaen, Thailand	Female	X		
9.	TL_CAV13	Samut Prakan, Thailand	Male	X		
10.	TL_CAV15	Samut Prakan, Thailand	Male		X	
11.	TL_CAV17	Samut Prakan, Thailand	Male		X	
12.	TL_CAV19	Samut Prakan, Thailand	Male	X		
13.	TL_CAV20	Samut Prakan, Thailand	Male	X		
14.	TL_CAV22	Samut Prakan, Thailand	Male		X	
15.	BD_DHK01	Dhaka, Bangladesh	Male	X	X	
16.	BD_DHK02	Dhaka, Bangladesh	Male	X		
17.	BD_DHK03	Dhaka, Bangladesh	Male	X		
18.	BD_DHK04	Dhaka, Bangladesh	Female	X		
19.	BD_DHK05	Dhaka, Bangladesh	Female	X		
20.	BD_DHK06	Dhaka, Bangladesh	Male	X		
21.	BD_DHK07	Dhaka, Bangladesh	Female	X		
22.	BD_DHK08	Dhaka, Bangladesh	Male	X	X	
23.	BD_DHK09	Dhaka, Bangladesh	Male	X		
24.	BD_DHK11	Dhaka, Bangladesh	Male	X		
25.	BD_DHK12	Dhaka, Bangladesh	Male	X	X	
26.	BD_DHK13	Dhaka, Bangladesh	Female	X		
27.	BD_DHK15	Dhaka, Bangladesh	Female	X		
28.	BD_DHK16	Dhaka, Bangladesh	Male	X		
29.	BD_DHK18	Dhaka, Bangladesh	Female	X		
30.	BD_DHK19	Dhaka, Bangladesh	Female	X		
31.	BD_DHK20	Dhaka, Bangladesh	Female	X		
32.	BD_FEN02	Feni, Bangladesh	Female	X		
33.	BD_FEN03	Feni, Bangladesh	Female	X		
34.	BD_FEN06	Feni, Bangladesh	Female	X		
35.	BD_FEN07	Feni, Bangladesh	Female	X	X	
36.	BD_FEN10	Feni, Bangladesh	Male	X		

Sl. No.	Sample id	Location	Sex	Used for		Comment
				DArT sequencing	Sanger sequencing	
37.	BD_FEN15	Feni, Bangladesh	Female	X	X	
38.	BD_FEN17	Feni, Bangladesh	Male	X		
39.	BD_FEN18	Feni, Bangladesh	Male	X		
40.	BD_FEN19	Feni, Bangladesh	Male	X	X	
41.	BD_HBJ02	Habiganj, Bangladesh	Female		X	
42.	BD_HBJ08	Habiganj, Bangladesh	Male	X		
43.	BD_HBJ09	Habiganj, Bangladesh	Female	X	X	
44.	BD_HBJ10	Habiganj, Bangladesh	Female	X		
45.	BD_HBJ11	Habiganj, Bangladesh	Female	X		
46.	BD_HBJ14	Habiganj, Bangladesh	Female	X	X	
47.	BD_HBJ16	Habiganj, Bangladesh	Male	X		
48.	BD_HBJ17	Habiganj, Bangladesh	Male	X		
49.	BD_HBJ20	Habiganj, Bangladesh	Male	X		

6.3.2 DNA extraction and sequencing

DNA samples of *C. versicolor* tissues from Bangladesh were extracted by Diversity Arrays Technology (DArTseq™) following their standard protocol. DNA from the Thailand samples were extracted either from the tail tip or liver following the methods of Srikulnath et al. (2010). Extracted DNA was used for two different DNA sequencing approaches i) Sanger sequencing for mitochondrial DNA and ii) DArTseqMet for nuclear DNA.

6.3.2.1 Sanger sequencing

We randomly selected three individuals from each of the sampling locations Dhaka (BD_DHK), Feni (BD_FEN) and Habiganj (BD_HBJ) from Bangladesh and Bangkok, Samut Prakan and Khon Kaen from Thailand (TL_CAV). Total genomic DNA was extracted from muscle tissues only from the Bangladesh samples using DNeasy Blood & Tissue Kit (Qiagen). The DNA concentration was measured on a NanoDrop™ Spectrophotometer (Thermo Fisher).

A region of the mitochondrial genome spanning *tRNATrp*, the *ND2* gene and the *COI* gene was targeted, and all samples were amplified and sequenced with primers designed by Huang et al. (2013), L3705 (5'-ATT AGG GTC TGC TAC ACA AGC AGT TGG-3') and H5162 (5'-GGT TGA RAG TAR TCA TCG AGT TAA GAA CGAC-3'), which were synthesised by Integrated DNA Technologies, Inc. (IDT). Standard polymerase chain reactions (PCR) were performed

in 25 µl reactions, including approximately 1 µl of template DNA (25 ng/µl), 1 µl of each primer (10 pmol/ µl), 12.5 µl of MyTaq™ HS Red Mix, 2x (Bioline) and 9.5 µl of nucleus-free water (Ambion). PCR was conducted following the conditions set by Huang et al. (2013) as an initial denaturing step at 95 °C for 4 min, 35 cycles of denaturing at 94 °C for 35 s, annealing at 65 °C for 45 s with an extension at 72 °C for 90 s and a final extension step at 72 °C for 8 min. The PCR products were electrophoresed in 0.8% agarose gels and visualised with SYBR® Safe DNA gel stain (Invitrogen). The PCR products (amplicons) were purified by PureLink™ PCR Purification Kit (Invitrogen) and sequenced at the Biomolecular Resource Facility (BRF) of the Australian National University using the corresponding PCR primers.

6.3.2.2 DArTseqMet – Methylation analysis

Tissues from the Bangladesh samples were preserved in 95% ethanol and transported to Diversity Arrays Technology Pty Ltd (Bruce, ACT, Australia) for genetic sequencing. DNA was extracted, sequenced and informative SNP and silicoDArT presence-absence markers were identified by Diversity Arrays Technologies (Kilian et al. 2012). Genotyping by sequencing was performed by DArTseq™ using a combination of DArT complexity reduction methods and next-generation sequencing following protocols described in section 5.3.2.1 (Kilian et al. 2012; Lambert et al. 2016). Out of 49 samples, a total of 45 (23 males and 22 females) were used for data analysis (Table 6.1). The four samples that were not sequenced were due to the lack of optimal quantity and quality of the DNAs.

Diversity Arrays Technology has developed a dedicated methylation analysis (DArTseqMet) using the DArTseq™ platform. In this study, two methods of complexity reduction were created for each sample. Both methods used the same 'rare cutting' restriction enzyme, while two isoschizomer restriction enzymes that differed in sensitivity to cytosine methylation were used as 'frequent cutting' enzymes for the DArTseqMet. The two different restriction enzymes that recognised the same sequence (5′-C|CGG- 3′) were 1) *Hpa*II as CpG methylation-sensitive and 2) *Msp*I as CpG methylation not sensitive. Comparison of the sequence composition of the two resulting representations (libraries) revealed differences in methylation pattern across the genome and had the potential to reveal any methylation mediated sex determination (Fig. 6.2). In this study, two rare cutting restriction enzymes were used as *Sbf*I (recognition sequence 5′-CCTGCA|GG- 3′) and *Pst*I (recognition sequence 5′ -CTGCA|G- 3′). The primary goal was to develop a series of sex-linked markers in *Calotes* spp. and infer their sex determination modes based on sex bias within these markers.

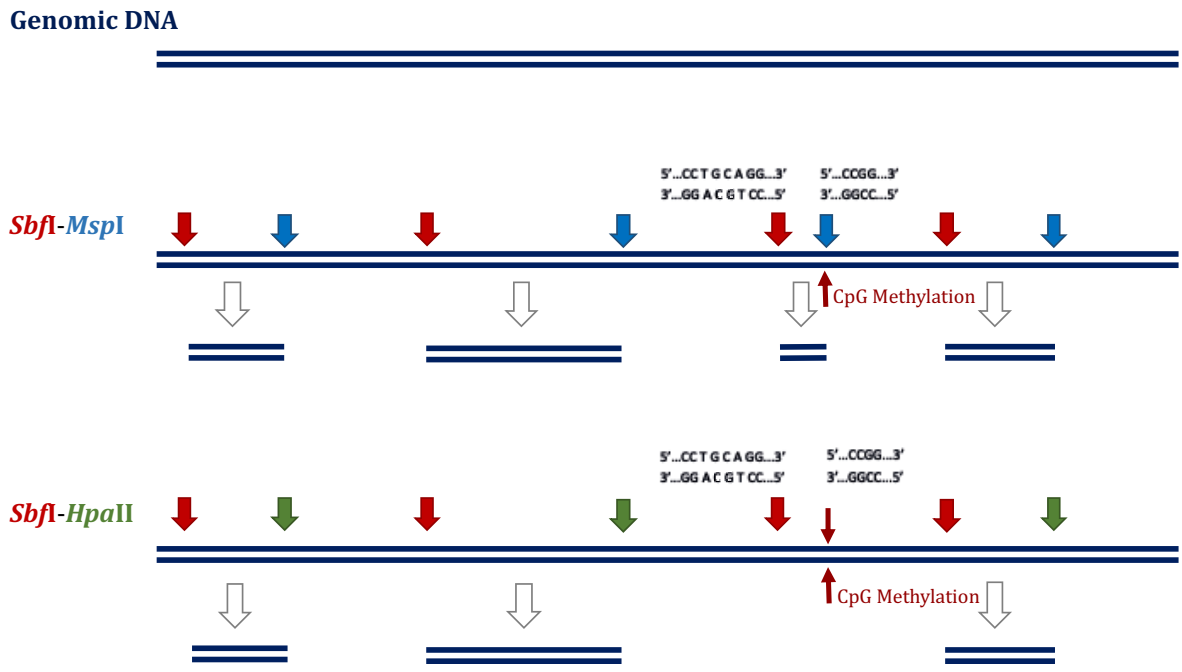


Figure 6.2 Schematic view of the use of DArTseqMet process. The two isoschizomer restriction enzymes, *Hpa*II and *Msp*I, differ in sensitivity to cytosine methylation and result in two different representations based on methylation patterns across the genome.

Sex-linked markers were identified using the Genotyping by Sequencing (GBS) method, i.e. DArTseq™ and analysed using MS 'Excel' (Sopniewski et al. 2019) and 'R' packages rdist and ggplot2.

6.3.3 Population and phylogenetic analysis

For population genetic analysis, both nuclear (SNP data) and mitochondrial DNA data were used. Genetic dissimilarity of individuals and populations were visualised using PCoA (principal coordinate analysis) constructed by the R package dartR (Gruber et al. 2018). Population differentiation due to genetic structure or fixation indices were calculated using the R package hierfstat and StAMPP (pairwise F*st* values). We also calculated the fixed differences (i.e. number of private alleles; Unmack et al. 2019) using R packages dartR, reshape and adegenet. Data obtained from Sanger sequencing were edited and aligned, and consensus sequences created using BioEdit software (version 7.2.5). We also used BLAST (Basic Local Alignment Search Tool) for the consensus sequences (NCBI BLASTN 2.10.0+). We downloaded hit sequences with the highest scores from GenBank to construct maximum likelihood (1,000 bootstraps) and Bayesian (1,10,0000 iterations) phylogeny using MEGAX

(10.1.8) and MrBayes plugins of Geneious Prime (version 2020.2.3) software, respectively. The consensus sequences were further edited and aligned, and phylogenetic trees were constructed considering only a region of the *ND2* gene.

6.3.4 Sex-linked marker analysis

We identified sex-linked SNP (single nucleotide polymorphism) and silicodart (presence-absence, PA) loci independently for each population, following criteria set by Lambert et al. (2016) with modifications. For the sex-linked markers, we filtered the total 65,652 SNP and 741,396 PA sites identified by DArTseq based on i) call rate (at least 80% i.e. 0.8 – 1.0 and recalculated for each population) (for SNP and PA markers), ii) average read depth (≧10) (for PA markers) and iii) reproducibility/RepAvg (at least 90% i.e. 0.9 – 1.0) (for SNP and PA markers).

Screening for candidate sex-linked SNP markers was performed by filtering loci meeting criteria for ZZ/ZW or XX/XY systems (Table 6.2). For the silicoDArT presence-absence (DArTseqMet) data, loci with restriction fragments sequenced in at least 75% of one sex and not more than 25% of the other sex were primarily selected.

Table 6.2 Selection criteria for sex-linked SNP markers, modified from Lambert et al. 2016

Parameter	Male heterogamety (XX/XY)		Female heterogamety (ZZ/ZW)	
	Female	Male	Female	Male
Homozygosity for the reference allele	≥0.80	≤0.10	≤0.10	≥0.80
Homozygosity at the SNP allele	≤0.10	≥0.90	≥0.90	≤0.10
Heterozygosity	≤0.20	≥0.75	≥0.75	≤0.20

Previous studies identified that at least 13 – 15 individuals per sex are required to minimise the occurrences of false-positive sex-linked loci (Brelsford et al. 2017; Lambert et al. 2016; Ogata et al. 2018). Since our sample population sizes were small (n = 4 – 9 per sex per population for SNP and 1-8 for PA marker analysis), we considered only loci that were perfectly sex-linked, especially for silicoDArT presence-absence (PA) data. Due to the small sample size, spurious sex association is most likely, and hence the formula $P_i = 0.5^n$ (Lambert et al. 2016) was utilised; where P_i is the probability that any locus is sex-linked by chance, n is the sample size (male and female together). P_i was multiplied by the number of SNP/PA markers (remained

after filtering), which gave an estimation of the number of random sex-linked loci produced through the analysis.

6.4 Results

6.4.1 Population analysis with DArTseq results

The PCoA plot revealed three distinctive clusters within the sampled populations of *Calotes versicolor,* which comprise of 1) Dhaka and Feni samples together, 2) Habiganj samples, and 3) Thailand samples together (Fig. 6.3). Most of the variation was represented in axis 1 (71.4%), while axis 2 represented lower variation (6.1%).

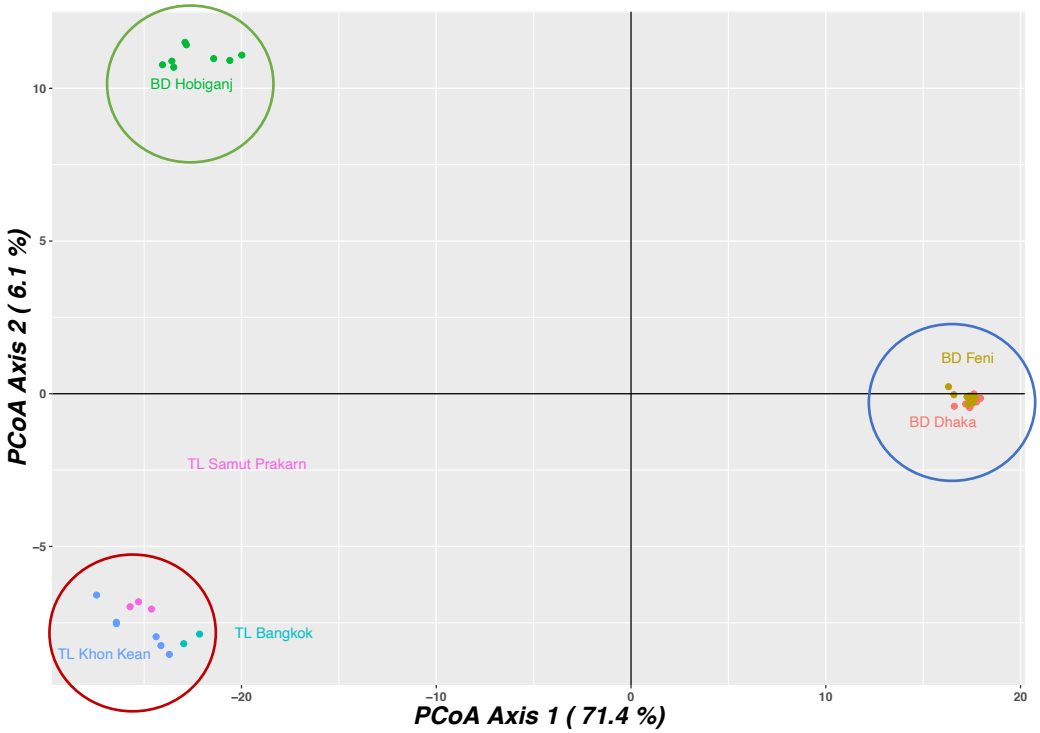


Figure 6.3 PCoA plot using DArTseq SNP data showing samples clustered in three distinct lineages as Dhaka-Feni (blue), Habiganj (green) and Thailand (red)

Pairwise genetic differentiation (F_{st}) between populations/localities ranged between 0.2 and 0.86 (Table 6.3). Genetic structure based on different localities in Thailand showed low significant differences, with F_{st} ranging between 0.09 and 0.22. By contrast, F*st* values were extremely high within the Bangladesh localities, as suggested by the PCoA. Genetic structure based on different localities in Bangladesh showed low significant differences between Dhaka and Feni, with F_{st} value of 0.21, to highly significant differences between Dhaka and Habiganj and between Feni and Habiganj, with F_{st} values of 0.81 and 0.84 respectively.

Table 6.3 Pairwise F_{st} values between the clusters

Fst values		Thailand			Bangladesh		
		Khon Kaen	Samut Prakarn	Bangkok	Dhaka	Feni	Habiganj
Thailand	Khon Kaen	NA	NA	NA	NA	NA	NA
	Samut Prakan	0.221	NA	NA	NA	NA	NA
	Bangkok	0.201	0.0945	NA	NA	NA	NA
Bangladesh	Dhaka	0.838	0.814	0.798	NA	NA	NA
	Feni	0.864	0.844	0.829	0.206	NA	NA
	Habiganj	0.548	0.509	0.491	0.813	0.837	NA

Analysis of fixed differences confirmed the diagnosability (a significant number of fixed differences) of each of the six clades as Dhaka, Feni and Habiganj from Bangladesh and Bangkok, Khon Kaen and Samut Prakan from Thailand. Fixed difference refers to the number of loci where the populations share no alleles at that locus (Georges et al. 2018). Lower fixed differences between populations indicate recent divergence or higher gene flow, and higher fixed differences indicate periods of long isolation and lack of gene flow (Unmack et al. 2019). Divergences in pairwise comparisons ranged from 338 to 13,900 fixed differences between the clades (Table 6.4). Among the Bangladesh samples, the divergence of populations ranged from 652 to 13,900 fixed differences. However, among the Thailand samples, the divergence of populations was comparatively lower, ranging from 338 to 439 fixed differences. Between the Bangladesh and Thailand samples, the divergence of populations ranged from 1,544 to 12,753 fixed differences.

Table 6.4 Fixed difference analyses between the sample locations.

Fixed differences		Thailand			Bangladesh		
		Khon Kaen	Samut Prakarn	Bangkok	Dhaka	Feni	Habiganj
Thailand	Khon Kaen	0	439	420	12753	12606	1974
	Samut Prakan	439	0	338	11044	10923	1748
	Bangkok	420	338	0	8889	8709	1544
Bangladesh	Dhaka	12753	11044	8889	0	652	13895
	Feni	12606	10923	8709	652	0	13900
	Habiganj	1974	1748	1544	13895	13900	0

6.4.2 Phylogenetic analysis with Sanger sequencing results

For each of the *C. versicolor* samples, we expected the overall region of the mitochondrial DNA sequenced to be 2,663 bp in total, spanning $tRNA_{Trp}$, *ND2* and *COI* as found by Huang et al. (2013). However, our consensus sequences were found to be much shorter and instead ranged between 882 and 1,377 bp long. The BLASTN of these consensus sequences resulted in hits of Dhaka (BD_DHK) and Feni (BD_FEN) samples with *C. calotes*, *C. htunwini* and *C. versicolor*, while sequences from Habiganj (BD_HBJ) and Thailand (TL_CAV) had hits only with *C. versicolor* sequences as shown in Table 6.5). However, we could not get any target amplicon from the Bangkok samples using this method. Thus, only the Samut Prakan and Khon Kaen samples from Thailand were included in the analysis.

Table 6.5 BLASTN results against *Calotes* spp. mitochondrial DNA consensus sequences.[2]

	Sample ID	Species 1	Accession No.	Species 2	Accession No.	Species 3	Accession No.
Bangladesh	BD_DHK 01M	*C. calotes*	AF128482.1	*C. versicolor*	GQ373016.1	-	-
	BD_DHK 08M*	-	-	-	-	-	-
	BD_**DHK 12M**	*C. calotes*	**AF128482.1**	*C. htunwini*	DQ289463.1	*C. versicolor*	KC875798.1
	BD_FEN 07F	*C. calotes*	AF128482.1	-	-	-	-
	BD_**FEN 15F**	*C. calotes*	AF128482.1	*C. htunwini*	**DQ289463.1**	*C. versicolor*	KC875798.1
	BD_**FEN 19F**	*C. calotes*	AF128482.1	*C. htunwini*	DQ289463.1	*C. versicolor*	DQ289476.1
	BD_**HBJ 02F**	*C. versicolor*	**DQ289476.1**	*C. versicolor*	KC875647.1	-	
	BD_**HBJ 09F**	*C. versicolor*	DQ289476.1	*C. versicolor*	DQ289471.1	*C. versicolor*	KC875647.1
	BD_**HBJ 14F**	*C. versicolor*	DQ289476.1	*C. versicolor*	DQ289471.1	*C. versicolor*	KC875647.1
Thailand	**TL_CAV 07M**	*C. versicolor*	DQ289474.1	*C. versicolor*	KC875776.1	-	-
	TL_CAV 08M	*C. versicolor*	DQ289474.1	*C. versicolor*	**KC875647.1**	-	-
	TL_CAV 10F	*C. versicolor*	DQ289474.1	*C. versicolor*	KC875647.1	-	-
	TL_CAV 15M	*C. versicolor*	DQ289474.1	*C. versicolor*	DQ289472.1	*C. versicolor*	KC875773.1
	TL_CAV 17M	*C. versicolor*	DQ289469.1	*C. versicolor*	KC875798.1	-	-
	TL_CAV 22M	*C. versicolor*	DQ289474.1	*C. versicolor*	DQ289472.1	*C. versicolor*	KC875773.1

We aligned the sequences with the BLAST hit sequences, edited further and considered only the sequences spanning the *ND2* gene (Annex VI) for constructing the phylogenetic trees. The maximum-likelihood and Bayesian dendrograms derived from the consensus sequences are shown in Fig. 6.4. Three distinct clades were observed consisting of the TL_CAV samples and two from the Bangladesh samples as Dhaka-Feni (BD_DHK – BD-FEN) and BD-HBJ, supporting the findings observed from the DArTseq SNP (genomic DNA) data. The trees were constructed using *P. vitticeps ND2* gene sequence as an outgroup. The *C. versicolor* KC875609.1 sequence was taken from a study by Huang et al. (2013), which was also found as a hit during BLAST analysis. The BD_HBJ and TL_CAV samples shared a common root within the clades of different *C. versicolor* populations. By contrast, the BD_DHK and

[2] Few of the sequences had multiple hits. The table shows the top three based on their highest total scores and lowest E-values. Accession numbers in bold were retrieved from GenBank and used to construct phylogenetic trees. The *C. calotes* sequence was from Macey et al. (2000), *C. htunwini* sequence was from Zug et al. (2006), and *C. versicolor* sequences were from Zug et al. (2006) and Huang et al. (2013). Only the 16 sequences in bold were used for phylogenetic tree construction. * No BLAST hit obtained for this sequence.

BD_FEN samples from Bangladesh fell within the clade with a common root from *C. calotes* or *C. htunwini* or both (Fig. 6.4, Annex VII). This indicated that *C. versicolor* samples comprised three genetically distinct clades as two distinct species under the genus *Calotes*.

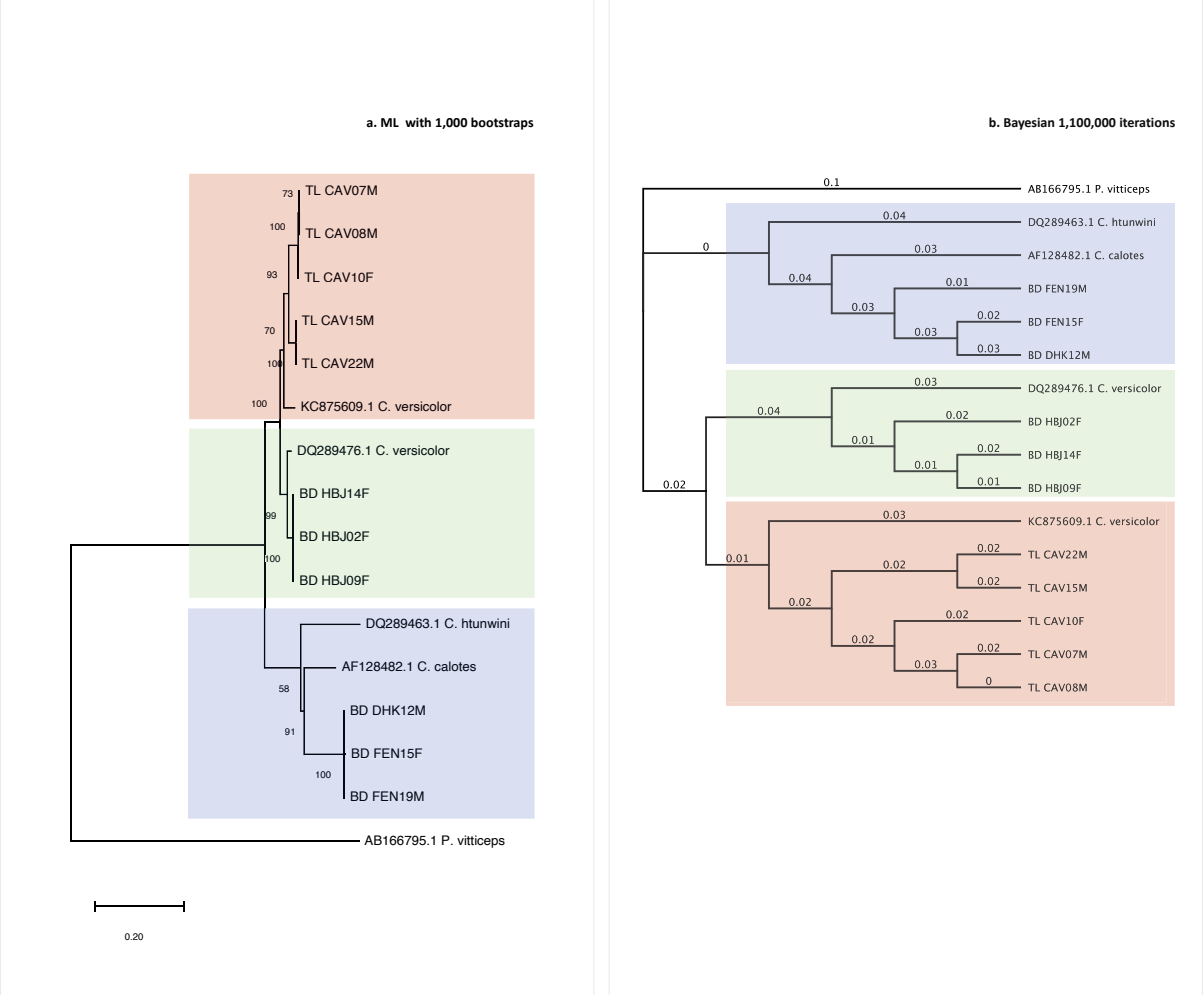


Figure 6.4 Relationship of all *Calotes* individuals inferred from mtDNA (*ND2* gene) consensus sequences using maximum-likelihood (a, with bootstrap values) and Bayesian (b, with substitutions per site) trees. These trees indicate three distinct clades – 1) Thailand (TL_CAV in red), 2) Dhaka-Feni (BD_DHK – BD-FEN in blue) and 3) Habiganj (BD-HBJ in green), supporting the findings from the DArTseq SNP (genomic DNA) data. The trees were constructed using *P. vitticeps ND2* gene sequence as an outgroup.

6.4.3 Sex-linked marker analysis using SNP data

From a total of 65,652 loci, 7,136 were retained for sex-linked screening after quality filtering. Screening for loci sex-linked criteria using samples from all populations yielded no loci; however, separate specific screening for each population resulted in 88 loci altogether with sex bias (Fig. 6.5a). Of these, the BD_DHK population had 3 loci (100%) correlating to females, the pattern expected for a sex chromosome system with ZW females and ZZ males. Likewise,

for the BD_FEN population, out of 41 loci, 4 were assigned to a ZZ/ZW and 37 (90%) to a XX/XY system. The BD_HBJ population had 38 loci with 10 assigned to a XX/XY system and another 28 loci (74%) to the ZZ/ZW system, while for the TL_CAV population 4 out of 6 loci (66%) indicated ZZ/ZW and the other 2 loci suggested a XX/XY system.

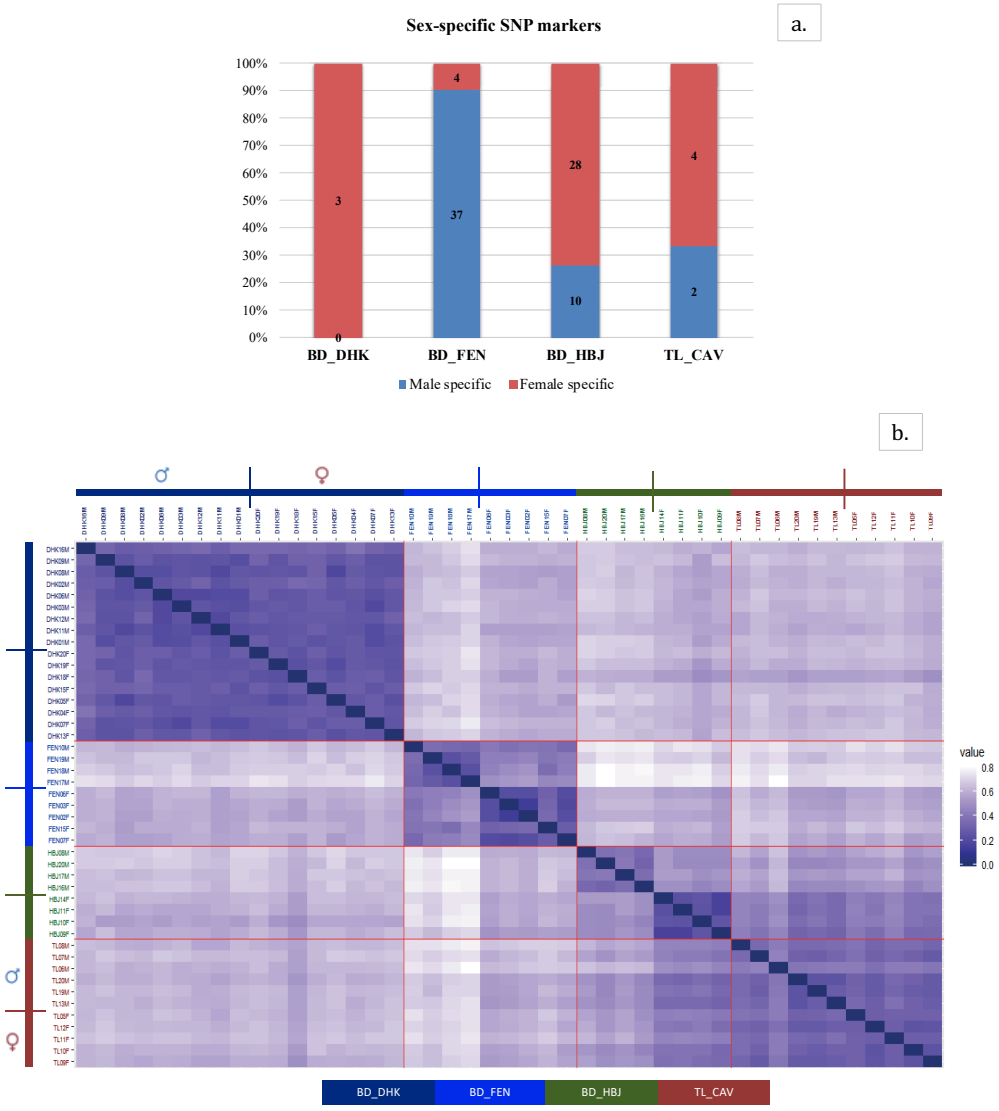


Figure 6.5 Sex-specific SNP markers identified in different populations/lineages (a) and Hamming distance matrix (b) illustrating proportional differences across the analysed adults using all 88 sex-biased SNP loci. In panel a, the numbers on the corresponding-coloured bars represent the number of sex-specific markers. In panel b, values closer to zero (dark purple) signify high similarity, whereas values closer to one (light purple) are more dissimilar across the SNP loci.

Hamming distance analysis of sex-linked markers from each population (Fig. 6.5b, Annex VIII) revealed that sex-linked markers were able to distinguish male and female phenotypes but only within their own populations, especially in the BD_FEN and BD_HBJ populations, with no sex-linked loci shared between populations.

Our filtered dataset of 7,136 SNP markers, for a sample range of 8-17 phenotypically sexed individuals, revealed that 0.05 - 28 markers are likely to be spuriously sex-linked following the equation of Lambert et al. (2016). Using this equation for this number of SNP markers, 13 individuals would be sufficient to be confident that less than one marker would be spuriously sex-linked.

6.4.4 Sex-linked marker analysis using silicoDArT presence-absence data

From the initial sequenced 741,396 PA markers (45 individuals), we retained 277,926 PA loci after filtering. The data belonged to four different treatments with four diverse restriction enzyme combinations as 1) *Sbf*I-*Hpa*II, 2) *Sbf*I-*Msp*I, 3) *Pst*I-*Hpa*II, and 4) *Pst*I-*Msp*I (described in section 6.3.2.2). Not all treatments worked for all populations. Each population revealed different numbers of markers based on the type of treatment (Fig. 6.6, Annex IX). Considering markers identified using *Sbf*I-*Hpa*II will be a subset of the markers identified using *Sbf*I-*Msp*I (and *Pst*I-*Hpa*II of *Pst*I-*Msp*I), methylation mediated marker profiles could only be achieved from BD_DHK and BD_FEN populations. In BD_DHK, no marker from the *Sbf*I-*Hpa*II treatment was identified, denoting all 2972 markers identified using *Sbf*I-*Msp*I are methylated at their recognition sites. While in BD_FEN, 44 loci out of 295 identified by *Sbf*I-*Msp*I were identified by *Sbf*I-*Hpa*II denoting the rest 251 markers to be methylated at their recognition sites.

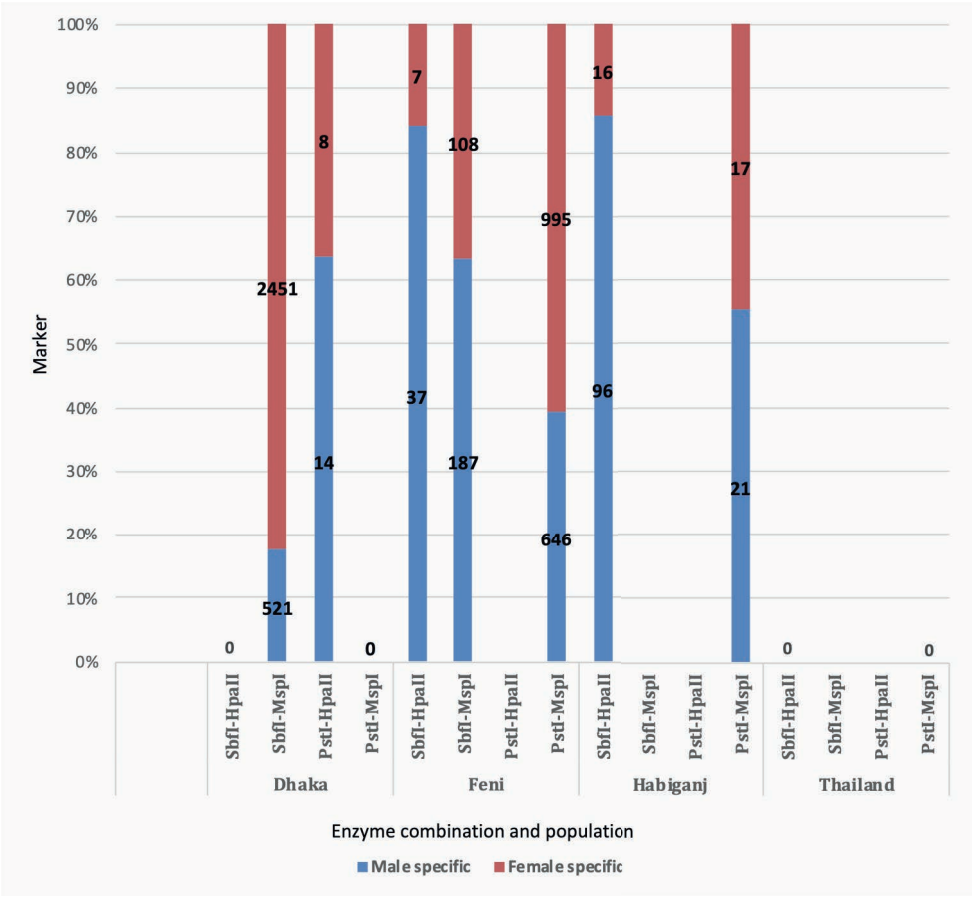


Figure 6.6 Sex-specific silicoDArT presence-absence (PA) markers identified in different populations. The number of markers is shown on the corresponding bar. Zero refers to scenarios where no sex-specific loci were identified, whereas empty spaces refer to scenarios where the particular enzyme combination did not produce any initial data.

In our filtered dataset of 277,926 PA markers, for a sample range of 4-16 phenotypically sexed individuals, it was revealed that 11 – 46,337 markers are likely to be spuriously sex-linked following the equation of Lambert et al. (2016). For this number of PA markers, 20 individuals would be sufficient to be confident that less than one marker would be spuriously sex-linked.

6.5 Discussion

Calotes versicolor is an agamid species with wide distribution and regarded as a species complex (Huang et al. 2013; Zug et al. 2006). It is apparent from different previous studies that this species may vary in terms of its sex determination mechanisms across its range (Chakraborty et al. 2009; Doddamani et al. 2012; Ganesh et al. 1997; Singh 1974; Singchat et al. 2020; Tripathi and Raman 2010). In this study, *C. versicolor* has been found to be a combination of three divergent lineages, at least within the sampled locations, and potentially

of different species, which might have evolved different modes of sex determination from TSD to GSD (ZW and XY).

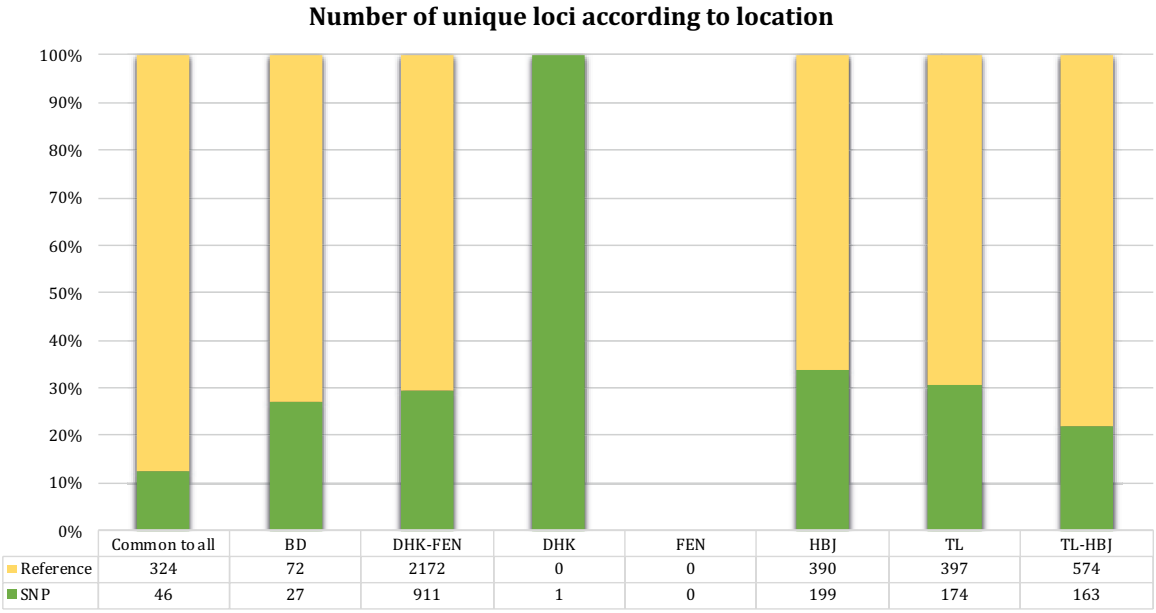
6.5.1 Population and phylogenetic analysis

Our results from PCoA, F*st*, and fixed difference analysis using DArTseq data and phylogenetic analyses of mtDNA revealed three distinct clades. These comprised of clade 1 (Dhaka-Feni; BD_DHK – BD_FEN), clade 2 (Habiganj; BD_HBJ) and clade 3 (Thailand; TL_CAV) across the sample populations. Based on the F*st* and fixed difference values, the Habiganj clade associated more closely with the Thailand clade than the other Bangladesh clades (Dhaka-Feni). The F*st* values between those two clades were at least four times as seen between the two major clusters from Bangladesh and tended to be less affected by geographic distance than within-cluster comparisons, as would be expected among highly divergent evolutionary lineages (McCartney-Melstad et al. 2018). Our phylogenetic analysis also revealed that the Thailand and Habiganj sequences clustered together with other specimens of *C. versicolor*, while the Dhaka-Feni sequences clustered together with *C. htunwini* and *C. calotes* sequences. A neighbour-joining tree also conforms with these results (Annex VII).

It has been previously suggested that adaptation to local environments might play an important role in diversification in *C. versicolor* (Huang et al. 2013). We found 370 loci common to all location samples (both SNP and reference alleles, Fig. 6.7) and also identified SNP loci that were unique to sampling locations. The straight-line distance between Bangladesh and Thailand is approximately 1,500 km, while distances between Dhaka, Feni and Habiganj range from 116 to 128 km and between Bangkok, Samut Prakan, and Khon Kaen distances are 20 to 394 km. The levels of sequence divergence and frequencies of private haplotypes are high for intraspecific data within the Bangladesh lineages, i.e. across a small geographical area. Our results, therefore, do not suggest that patterns in *C. versicolor* in Bangladesh are consistent with the isolation-by-distance model (Wright 1943), whereby gene flow decreases with increasing geographical distance because of limited dispersal.

Given that there has been limited gene flow occurring between the Dhaka-Feni and Habiganj lineages, one plausible scenario could result from their different geographic positions. Geologically, Dhaka and Feni are floodplain areas affected by seasonal flooding, predominantly caused by human-modified landscapes, whereas the Habiganj sampling location consists of hilly tracts with tropical evergreen forests (Nishat et al. 2002). Both the Habiganj and Thailand sampling locations fall under the Indo-Burma biodiversity hotspot area (Myers

et al. 2000) within the Indo-Chinese sub-region of the Oriental zoogeographic region. Dhaka and Feni, on the other hand, fall within the Indian sub-region of the Oriental region (Wallace 1876).



	Common to all	BD	DHK-FEN	DHK	FEN	HBJ	TL	TL-HBJ
Reference	324	72	2172	0	0	390	397	574
SNP	46	27	911	1	0	199	174	163

Figure 6.7 Number of population-specific loci. The high number of unique loci in Dhaka-Feni samples and lower individual number indicate that these two populations are a single population that is unique from the Habiganj and Thailand samples. BD-Bangladesh, TL-Thailand, DHK- Dhaka, FEN-Feni, HBJ-Habiganj.

Zug et al. (2006) found evidence of a species complex with high genetic differentiation without a differentiated morphotype in *C. versicolor*. They postulated that *C. versicolor* represents multiple species and at least two clades, one from India-Myanmar and another from Myanmar-Southeast Asia. Our data also provides evidence of high genetic divergence between the samples reflected in clustering into three distinct groups. These three groups can be considered as three distinct evolutionarily significant units (ESUs) and potentially supports the presence of *C. versicolor* species complex. Based on our population and phylogenetic analysis, it can be predicted that the Thailand and Habiganj samples could be *C. versicolor*, while the Dhaka-Feni samples could be *C. htunwini* or *C. calotes*, or even a new species within a cryptic species complex. Zug et al. (2006) suggested that *C. htunwini* represented an early branching within the *C. versicolor* group with affinities to Indian species and populations. Conversely, *C. calotes* is commonly known as green forest calotes across South India and Sri Lanka (Uetz et al. 2020), with a completely different phenotypic appearance and green coloration (Pal et al. 2018), therefore, do not comply with our samples' phenotypes. However, it can be suggestive that

samples collected from Habiganj and Thailand were *C. versicolor* but represented different populations of this species.

6.5.2 Sex determination modes across *Calotes* species complex

Our results identified a total of 88 SNP loci that were linked to sex phenotypes but extended across different lineages. None of these loci was shared either between or among lineages. We also identified a range of 0 to 2,972 sex-linked loci that varied according to the lineages.

The SNP DArT results implied that either *C. versicolor* had a TSD system since no sex-linked markers were found or that a multiple sex determination system exists in this species. From the population-specific analysis, the Dhaka and Thailand populations could either have TSD or ZZ/ZW systems due to low marker counts, while the Habiganj population showed a line of evidence for the ZZ/ZW system and Feni towards XX/XY system. In this scenario, *C. versicolor* exhibits multiple sex determination systems of both XY and ZW, including environmental sex determination (ESD), i.e., temperature-dependent sex determination (TSD). The sex determination system of *C. versicolor* is elusive due to the lack of clear heteromorphic sex chromosomes and contradictory research results regarding sex determination. However, Wilson et al. (2019) found four weakly sex-associated RAD-tag markers in *C. versicolor*. They concluded that their data did not support the presence of strongly differentiated sex chromosomes in their population of *C. versicolor*.

These contradicting results may originate from possible sex reversal caused by temperature overriding the original sex-determination system as recorded in other agamid lizards such as *P. vitticeps* (Quinn et al. 2007b), or the existence of multiple sex determination systems within the same species as in the Japanese wrinkled frog *G. rugosa* (Miura 2007; Nishioka et al. 1994). In previous studies, most *C. versicolor* samples were from India, while in this study, the samples were from Bangladesh and Thailand. Our results suggest that multiple systems exist based on population and locality. *C. versicolor* has a very large distribution area from Iran to Southeast Asia, with the potential for each population to evolve a different sex-determination system. Our sample results from Bangladesh and Thailand identified the existence of a *Calotes* cryptic species complex as previously suggested by Zug et al. (2006). Therefore, we propose that variation in sex determination mechanisms in *C. versicolor* found in previous studies are due to cryptic species undergoing the process of speciation. This can also be true where Doddamani et al. (2012) raised the possibility that *C. versicolor* has a novel FMFM pattern of TSD based on multi-year experiments over several seasons. However, they have not included

the locality from where the *C. versicolor* individuals or eggs were collected. Based on my results, it can be presumed that the pattern inferred from incubation experiments by Doddamani et al. (2012) might came from different populations or even species that were assumed to be single species with the same sex-determination system.

Methylation DArTseq data identified dominant sex-specific markers as loci that were either present or absent according to sex. However, not all combinations of restriction enzymes worked for all populations. We obtained results from both methylation-sensitive and non-sensitive restriction enzyme combinations only from Dhaka and Feni populations (from the *Sbf*I- *Hpa*II/*Msp*I combination). However, the *Sbf*I-*Hpa*II combination yielded markers from all populations, and this combination was used to generate SNP markers. Theoretically, treatment with methylation insensitive restriction enzyme (*Msp*I) should have more or equal numbers of loci than the methylation-sensitive restriction enzyme (*Hpa*II) combination. Such phenomena were observed in the Dhaka and Feni samples (only in the *Sbf*I-*Hpa*II/*Msp*I combination). Therefore, we assumed that loci identified from the *Sbf*I-*Hpa*II combination might be a subset of loci from the non-methylated *Sbf*I-*Hpa*II/*Msp*I combination. We found that all markers identified from Dhaka for this enzyme combination were CpG methylated on their restriction sites. In the Feni samples, we found that restriction sites of female loci (93.5%) were more methylated than the male loci (80.2%). By contrast, more sex-specific loci were observed in methylation-sensitive restriction enzyme combinations than insensitive combinations in Dhaka (in the *Pst*I-*Hpa*II/*Msp*I combination) and Habiganj samples (between *Sbf*I-*Hpa*II and *Pst*I-*Msp*I combinations), making it harder to predict methylation patterns in these treatments (Fig. 6.6 in section 6.4.4).

Complete *Sbf*I-*Hpa*II/*Msp*I combination data sets from the Dhaka and Feni populations (Fig. 6.6) and the number of sex-linked loci associates would suggest a ZZ/ZW system in the Dhaka (BD_DHK) population and XX/XY in Feni (BD_FEN), in accordance with the SNP data (Fig. 6.5a). However, there could be an environmental influence in both cases since the female-specific markers account for 82% and male-specific markers accounted for only 63% of the total sex-specific markers in BD_DHK and BD_FEN respectively (Fig. 6.6). Putative male-linked alleles were presented in ZZ/ZW populations (Habiganj and Thailand), while female-linked alleles in XX/XY (Feni) suggested frequent sex reversal of female-to-male phenotypes and vice versa. Such sex reversals could be caused either by environmental factors, i.e., temperature influences during the incubation period as in *P. vitticeps* (Quinn et al. 2007a) and *Bassiana duperreyi* (Shine et al. 2002). Cytogenetic analysis of *C. versicolor* revealed no

variation between male and female karyotypes (Ganesh et al. 1997; Patawang et al. 2015; Singh 1974). This did not contradict with the possibility of a genetic sex determination since other agamids such as *Phrynocephalus vlangalii* (Zeng et al. 1997) and *P. vitticeps* (Ezaz et al. 2005; Ezaz et al. 2009b) have ZW/ZZ sex chromosome systems. Therefore, it can be inferred that *C. versicolor* might have a genetic sex determination with homomorphic sex chromosomes that are in the early stage of differentiation.

6.6 Conclusions

Our findings showed variations in sex-linked markers, indicating diverse sex determination modes and mechanisms between genetically distinct lineages comprising both populations and species. The Thailand samples showed genetic differences as evidenced by both genomic and mitochondrial data, even with small sample sizes. Further studies using more individuals with confirmed morphological identification (due to other known sympatric similar *Calotes* spp., e.g., *C. emma*) and sex would enable a better understanding of the underlying mechanisms of sex determination within these closely related lineages. Additional sampling locations spanning the entire range of *C. versicolor* would provide further in-depth information regarding the intensity and diversity of this cryptic species complex. Future research is likely to open up a new window to explore the evolution of sex determination mechanisms both within this species complex, and in reptiles in general.

6.7 Acknowledgements

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CHAPTER 7: SYNOPSIS

In this research, I used non-model species to address questions on the evolutionary and ecological forces that drive sex determination in agamid lizards. In this final chapter, I review the results in the context of the broad aims and specific experimental objectives outlined at the end of the General Introduction (chapter 1) and provide a summary of the directions for future research, suggested or made possible by this study. This work has been published in one review article (chapter 2) and two research articles (chapter 3 and 4), while chapter 5 and 6 are being prepared for publication.

7.1 Review of study aim and objectives

Reptiles, particularly lizards, are well known among amniotes for their diversity in sex determination, sex chromosome numbers and modes, and even transitions between sex determination modes and mechanisms. An initiative was taken in the review (chapter 2; Alam et al. 2018) to understand this diversity within lizards by investigating their sex chromosome features from the existing literature. It was realised that lizards show no particular pattern of sex chromosome degeneration of the kind observed in mammals, birds or even in snakes. Based on the evidence, it can be speculated that sex determination, i.e. sex chromosome evolution, is labile and rapid and mostly follows independent evolutionary trajectories within lizards. This is particularly true among agamid lizards, where sex determination and sex chromosomes have evolved multiple times within a short period. In the successive data chapters (chapter 3,4,5, and 6), a combination of cytogenetics, genomics as well as incubation experiments were used to understand a few evolutionary and ecological aspects of sex determination in this group of lizards as outlined by the objectives in chapter 1.

My goal under objective 1 was to identify sex chromosome homologies across agamid lizard subfamilies. To this end, I used two *P. vitticeps* sex chromosome BAC clones and hybridised them onto 12 different species of Agamid lizards across six subfamilies, also keeping two chamaeleon species as outgroup. The study showed lineage-specific evolution of these BAC sequences and successive rearrangements within the agamid lizards. Under objective 2, which was to identify the sex determination mechanism in an agamid lizard with cytogenetic approaches, I tried to identify sex chromosomes as an indicator of the sex determination mechanism in Canberra grassland earless dragon *Tympanocryptis lineata* and Australian mountain dragon *Rankinia diemensis* using cytogenetic approaches together with

characterising their chromosomes. I identified a heterochromatinized female-specific W microchromosome in *T. lineata* but failed to identify any sex chromosomes in *R. diemensis*.

My goal for objective 3 was to identify the sex determination mechanism in a representative agamid lizard involving incubation experiments and sex-linked molecular markers. I confirmed that *T. lineata* has a female heterogametic (ZZ/ZW) sex chromosome system and that they determine their sex through genotypic sex determination (GSD). And finally, under objective 4, I investigated sex determination within the *C. versicolor* species complex, a complex with a wide distribution range and unresolved systematics and sex determination mechanisms. I used a sex-linked marker approach and provided evidence that within its range, *C. versicolor* exists as a complex of multiple cryptic species and exhibits variation in sex determination mechanisms (sex chromosome modes).

Therefore, the thesis has many novel elements using a wide range of skills and techniques. For example, I used cytogenetic, genomic, and breeding experimental data to uncover the likely sex determination mode of *T. lineata*. In addition, my thesis drives towards some big questions in sex determination system transition, with the exploration of a single species complex that may possess both GSD and ESD components. However, my thesis has several shortcomings as unconfirmed sex of the individuals, especially in *R. diemensis* and small sample sizes of *C. versicolor*, which led to falling short of proving the sex determination mode precisely in any of the populations. My findings, together with these lackings, have opened up avenues for future research. These may include, among others, the validation of the identified sex-linked markers in *T. lineata* using PCR and FISH techniques and investigate the occurrence of sex-reversal in this species, exploration of molecular and cytogenetic mechanisms behind the evolution of micro-sex chromosomes, and the utilisation of qPCR-based approaches for the identification of sex determination modes (Rovatsos et al. 2014a; 2014b; 2015b) across agamid taxa. While exploring these opportunities, identifying any individual's sex and access to samples from different species from different locations, especially of a threatened species as *T. lineata,* may remain an issue.

7.2 Implications of the study and future research needs

7.2.1 Cytogenetic analyses and sex chromosomes in agamid lizards

The findings in chapter 3 indicate conservation of chromosome segments across agamid lineages. Translocation of BAC sequences to microchromosomes observed only in the ancestor of the studied members of the subfamily Amphibolurinae, including the co-occurrence of both BAC sequences in the Z and W sex microchromosomes in *P. vitticeps.* Future research should investigate any association with these two BAC sequences or any other BAC sequences to sex chromosomes (as in *P. vitticpes*) in other agamid species. These investigations should include more agamid lizards across the subfamilies, and detailed examination should combine advanced cytogenetics and genomics (e.g., DArTseq), which will provide better information for in-depth analysis and tests whether the suggested reconstruction of events was important for the establishment of cytogenetically distinguishable sex chromosomes in *P. vitticeps* and its relatives.

While investigating *T. lineata* and *R. diemensis,* I provided evidence of rearrangements within chromosomal landscapes among closely related species with similar karyotypes. This, in turn, provides evidence that speciation within the Australian agamid clade involved subtle chromosomal rearrangements, both micro- and macrochromosomes (Irwin 2018). I failed to identify whether the heterochromatinized microchromosome in *R. diemensis* is the sex chromosome (W or Y) since the sex of individuals were unknown[3]. Identification of this heterochromatinized microchromosome is important since the detailed investigation in this species may lead to new information on sex chromosome evolution among Australian agamid species. Based on this experience, it is of particular priority to gain access to samples where the gonads have been dissected. Sexing through external morphology (as was the case with *R. diemensis*) can result in misidentification of sex, which compromises the hunt for sex chromosomes. Hence, the results presented here are still preliminary, and to fully understand the process of karyotype evolution in these species, additional studies using a combination of advanced molecular cytogenetic and genomic techniques are needed. Future cytogenetic investigations should include comparative genomic hybridisation (CGH) and successive C-

[3] *R. diemensis* were captured from the wild and released back immediately after collecting the samples (tail snips) for cell culture and subsequent cytogenetics. Sex of the individual was identified in the field using the method of Harlow (2004). Because of the confusion in few individuals thereafter, I decided to keep the sexes of *R. diemensis* unknown.

banding in CMA_3, methylation, *P. vitticeps* BAC FISH, telomere FISH, and SSR FISH experiments so that the nature of the sex chromosome could be identified in detail. This may prove beneficial to our understanding of the evolution of sex chromosomes within vertebrates as well.

7.2.2 Sex determination and sex-linked markers in agamid lizards

I found a 1:1 sex ratio among the offspring of *T. lineata*, but only after I took account of the unsexed individuals (dead eggs and hatchlings) into the analysis[4], indicating possible sex-biased mortality in this species as well. In an incubation experiment, a GSD species ends up with approximately a 1:1 male: female ratio among the offspring irrespective of environmental condition/s faced during their development. An ESD (TSD herein) species, on the other hand, shows a skewed sex bias across a particular environmental gradient. However, recent reviews have emphasised that GSD and TSD are not a simple dichotomy of the sex-determination system, and instead are two ends of a continuum (Sarre et al. 2004), established by the discovery of GSD species acting as TSD species at temperature extremes (e.g. *P. vitticeps* and *B. duperreyi;* Quinn et al. 2007; Shine et al. 2002). Future studies should examine if female dominance in *T. lineata* (among sexable individuals; Table 5.3) is a natural phenomenon or an artefact of the artificial incubation experiments.

Although I could identify the genotypic sex determination in *T. lineata* through incubation experiments, whether this species is a pure GSD species or has temperature influence requires further studies with a broader range of temperature extremes. It would be beneficial to conduct incubation experiments from 22 °C up to 36 °C with an interval of 2 °C. The incubation experiments at 22 °C and with more eggs at 24 °C are needed for a better understanding of the lower female ratio at the lower extreme (Table 5.3). Incubation experiments on the higher extreme, i.e., over 32 °C, will provide critical information regarding any sex reversal in this species (Quinn et al. 2007a; 2007b). Experiments with extreme temperatures should begin with small numbers of eggs coupled with regular measurement of heart rates using devices as the Buddy® monitor (Hulbert et al. 2017) so that the lethal and or sublethal temperatures can be determined. It is also essential to know the existence of the thermosensitive period (TSP) in

[4] The results show higher ratios of females ranging from 58-68% in most incubation experiments (26-32 °C), and therefore, the possibility of sex-biased mortality cannot be ruled out and considering all the unsexed individuals as less represented sex (males) seemed rational.

this species, if any, - the time during development when temperature influences the sexual phenotype (Girondot et al. 2018). With the low population size and the threatened status of the species, experiments at extreme temperatures may not be a valid option and can be applied only after the population is recovered, and the species status has been upgraded.

Offspring sex in many species is influenced by multiple interactive processes that can affect sex determination in any particular species (Cassey et al. 2006; Sarre et al. 2004). Future studies should, therefore, also look upon other factors known to influence offspring sex ratio in different vertebrates such as egg mass (Ballen et al. 2016), the interaction between mean nest temperatures and the degree of variation in nest temperatures (Bull 1985; Valenzuela 2001; Warner and Shine 2011), maternal and paternal body size (Lu et al. 2013), prenatal or nutritional stress of mothers (Catalano et al. 2006; Song 2012; Torche and Kleinhaus 2012; Wiebe and Bortolotti 1992), birth date (Barclay 2012), mating season duration (Elmberg 1990), transgenerational effect (Warner et al. 2013), operational sex ratios and mating behaviour (Werren and Charnov 1978; West and Godfray 1997). Since sex ratio is considered as one of the vital factors for the existence of a species or population, identification of sex-determination mechanisms and any factor influencing sex ratio such as temperature influences would provide immense benefit for the conservation and management of threatened species like *T. lineata*, and especially in the face of global climate change (Boyle et al. 2014; 2016).

The DArTseq data collected on *T. lineata* investigated here actively support differentiated sex chromosomes in this species. All my markers conform to a ZZ/ZW system in *T. lineata* as previously inferred through cytogenetic analysis in chapter 3. Our results, therefore, underpin the usefulness of DArTseq™ in identifying sex-linked markers for the identification of sex-determining modes in non-model organisms, without a priori sequence information and also used as a method for the study in chapter 6. The sex-linked markers identified should be validated using a PCR-based test using more confirmed sexed individuals.

My study with *Calotes versicolor* provides evidence that sex determination mechanisms can differ between closely related species or even between populations within a reptile species. My findings showed variations in sex-linked markers indicating variation in their sex-determination mode and mechanisms, between genetically distinct lineages, between populations if not species. However, future studies should have more individuals of confirmed identification and sex to understand the underlying mechanisms of sex determination within these closely related lineages. Particular emphasis should be given to the populations such as

BD_FEN, where the sex-linked markers indicate a probable male heterogametic (XX/XY) system, a phenomenon yet to be discovered in any agamid lizard. Cytogenetic analysis should also follow the genomic studies so that chromosome level difference between populations and cryptic species could be identified, as in the case of *T. lineata* and *R. diemensis* (different species with identical karyotype) in Chapter 3.

7.3 Concluding remarks

This thesis has concentrated on the Agamid lizards and investigated evolutionary and ecological aspects of sex determination in a few representative species from this group. This study showed subtle chromosomal rearrangements between two Australian species, identified micro sex chromosome in *Tympanocryptis lineata* but not in *Rankinia diemensis*. Incubation experiments also confirmed GSD in *T. lineata*, further supported by the identification of female-specific markers. The studies on the sex determination in agamid lizards were previously concentrated mainly on the Australian clade of Amphibolurinae (subfamily). I went beyond this boundary and investigated species from other subfamilies considering a single species to a species complex. The study revealed chromosomal synteny along with rearrangements of *Pogona vitticeps* sex chromosome BAC sequences across agamid lineages. I used DArTseq^{TM} to identify sex-linked markers in any agamid lizard for the first time. I could identify sex-linked marker in *T. lineata* (female-specific) and as well as in *Calotes versicolor* species complex, providing evidence to the variation in sex determination modes between species and population. The known variation in sex determination and sex chromosomes within Australian clades and even in a species outside this clade, i.e., *C. versicolor* (subfamily Draconinae), and paucity of information from other clades justify the need for such research. Both GSD (homomorphic and ZZ/ZW sex chromosome), GSD+EE and TSD species have been identified from agamid lizards, and no XX/XY is yet to be discovered in any agamid species. Recent discoveries of XX/XY systems in taxa with conserved ZZ/ZW systems, such as boid snakes (Gamble et al. 2017) and even their closest relative chameleons (Nielsen et al. 2018), has opened up further opportunities to explore more agamid species and investigate evolutionary novel transitions within this group of lizards. This will have immense implications in the context of the complex evolution of sex chromosomes and sex determination mechanisms in reptiles.

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ANNEXURES

Annex I: List of *T. lineata* hatchlings used for incubation experiment

Sl. No.	ID	Incubation Temperature (°C)	Sex
1	GED 22	28	Male
2	GED 23	28	Male
3	GED 27	28	Female
4	GED 28	28	Male
5	GED 29	28	Female
6	GED 30	28	Female
7	GED 31	28	Female
8	GED 34	26	Female
9	GED 35	26	Male
10	GED 42	26	Female
11	GED 43	26	Female
12	GED 44	26	Male
13	GED 49	26	Female
14	GED 50	28	Female
15	GED 51	28	Female
16	GED 52	28	Female
17	GED 53	28	Male
18	GED 56	26	Female
19	GED 57	26	Female
20	GED 58	26	Male
21	GED 59	26	Female
22	GED 60	28	Female
23	GED 61	28	Male
24	GED 62	28	Male
25	GED 63	28	Male
26	GED 64	30	Female
27	GED 65	30	Female
28	GED 66	30	Male
29	GED 67	26	Female
30	GED 68	26	Male
31	GED 69	28	Female
32	GED 70	26	Female
33	GED 71	28	Female
34	GED 72	26	Male
35	GED 73	26	Male
36	GED 74	30	Female
37	GED 75	28	Male
38	GED 76	30	Male
39	GED 81	26	Female

Sl. No.	ID	Incubation Temperature (°C)	Sex
40	GED 82	28	Female
41	GED 83	24	Female
42	GED 84	24	Male
43	GED 85	24	Male
44	GED 86	24	Male
45	GED 87	24	Male
46	GED 88	24	Male
47	GED 89	24	Male
48	GED 90	24	Male
49	GED 91	30	Female
50	GED 93	24	Female
51	GED 95	32	Female
52	GED 96	32	Male
53	GED 97	32	Male
54	GED 98	28	Male
55	GED 99	28	Female
56	GED 100	32	Female
57	GED 102	32	Female
58	GED 104	28	Male
59	GED 106	28	Female
60	GED 110	28	Female
61	GED 116	32	Male
62	GED 117	32	Male
63	GED 118	30	Female
64	GED 119	30	Female
65	GED 120	32	Male
66	GED 121	30	Female
67	GED 122	30	Female
68	GED 123	26	Female
69	GED 124	26	Male
70	GED 125	32	Male
71	GED 126	32	Male
72	GED 127	28	Female
73	GED 128	32	Male
74	GED 129	30	Male
75	GED 130	32	Female
76	GED 131	30	Female
77	GED 135	28	Female
78	GED 136	30	Female
79	GED 138	28	Female
80	GED 139	32	Female
81	GED 140	32	Female
82	GED 141	32	Female
83	GED 142	30	Male

Sl. No.	ID	Incubation Temperature (°C)	Sex
84	GED 143	30	Male
85	GED 144	28	Male
86	GED 145	32	Female
87	GED 146	30	Female
88	GED 147	30	Male
89	GED 148	28	Female
90	GED 150	32	Female
91	GED 151	32	Female
92	GED 152	30	Female
93	GED 153	30	Female
94	GED 154	32	Male
95	GED 155	30	Male
96	GED 156	28	Female
97	GED 157	28	Female
98	GED 158	32	Female
99	GED 159	32	Female
100	GED 160	32	Female
101	GED 161	32	Female
102	GED 162	30	Female
103	GED 163	32	Female
104	GED 164	30	Female
105	GED 165	28	Female
106	GED 166	28	Male
107	GED 170	32	Female
108	GED 171	30	Male
109	GED 172	32	Female
110	GED 174	30	Male
111	GED 175	28	Female
112	GED 176	28	Female
113	GED 32 (A)	28	Female
114	GED 33 (B)	28	Female
115	GED 46 (A)	26	Female
116	GED 47 (B)	26	Female
117	GED 54 (A)	30	Male
118	GED 55 (B)	30	Male

Annex II: *Tympanocryptis lineata* individuals used for sex-linked marker analysis; M – male, F – female, individuals in bold are from known family groups and individuals that are in same colour represent the same family group.

Sl. No.	ID	Sex	Origin			
			Captive breed	Wild caught	Wild Individual	Wild Egg
1	AA14537	M		X	X	
2	AA14538	M		X	X	
3	AA14541	F		X	X	
4	AA14542	F		X	X	
5	AA14587	M		X	X	
6	AA14896	F		X	X	
7	AA14900	M		X	X	
8	AA14908	F		X	X	
9	AA14933	F		X	X	
10	AA14936	M		X	X	
11	AA14939	F		X	X	
12	AA14947	M		X	X	
13	AA14949	F		X	X	
14	AA14951	M		X	X	
15	AA14952	M		X	X	
16	AA14954	M		X	X	
17	AA14959	F		X	X	
18	AA14967	F		X	X	
19	AA14978	F		X	X	
20	AA14981	F		X	X	
21	AA14987	F		X	X	
22	AA14994	F		X	X	
23	AA15245	M		X	X	
24	AA15254	M		X	X	
25	AA15256	M		X	X	
26	AA15258	M		X	X	
27	AA61566	M		X	X	
28	AA61574	M		X	X	
29	AA61576	M		X	X	
30	AA61584	M		X	X	
31	AA61588	M		X	X	
32	AA61593	M		X	X	
33	AA61599	F		X	X	
34	AA61600	M		X	X	

Sl. No.	ID	Sex	Origin			
			Captive breed	Wild caught	Wild Individual	Wild Egg
35	**AA61601**	**M**		**X**	**X**	
36	AA61602	F		X	X	
37	AA61603	F		X	X	
38	AA61607	M		X	X	
39	AA61610	F		X	X	
40	AA61615	M		X	X	
41	AA61617	F		X	X	
42	AA61618	F		X	X	
43	AA61619	F		X	X	
44	AA61620	M		X	X	
45	**AA61621**	**M**		**X**		
46	AA61623	F		X	X	
47	AA61646	F		X	X	
48	AA61657	F		X	X	
49	AA61659	F		X	X	
50	AA61661	F		X		
51	AA61665	M		X	X	
52	AA61666	F		X	X	
53	AA61667	M		X	X	
54	AA67001	F		X		X
55	AA67002	F		X		X
56	**AA67003**	**M**		**X**		**X**
57	AA67005	F		X		X
58	AA67006	M		X		X
59	AA67007	M		X		X
60	AA67008	M		X		X
61	AA67009	F		X		X
62	AA67010	F		X		X
63	AA67011	F		X		X
64	AA67012	F		X		X
65	AA67013	F		X		X
66	AA67014	F		X		X
67	AA67015	F		X		X
68	AA67016	F		X		X
69	AA67017	F		X		X
70	AA67018	M		X		X
71	AA67019	M		X		X
72	AA67020	M		X		X

Sl. No.	ID	Sex	Origin			
			Captive breed	Wild caught	Wild Individual	Wild Egg
73	AA67021	M		X		X
74	AA67022	M		X		X
75	AA67023	F		X		X
76	AA67024	F		X		X
77	AA67025	M		X		X
78	AA67026	F		X		X
79	AA67027	F		X		X
80	AA67028	M		X		X
81	AA67029	F		X		X
82	AA67030	F		X		X
83	AA67031	F		X		X
84	AA67032	F		X		X
85	AA67034	M		X		X
86	AA67035	M		X		X
87	AA67036	M		X		X
88	AA67037	F		X		X
89	AA67038	M		X		X
90	AA67042	F		X		X
91	AA67043	F		X		X
92	AA67044	F		X		X
93	AA67045	M		X		X
94	AA67046	M	X			
95	**AA67047**	**M**	**X**			
96	AA67051	F	X			
97	AA67053	F		X		X
98	AA67055	F		X		X
99	AA67056	F		X		X
100	AA67057	M		X		X
101	AA67058	M		X		X
102	AA67061	F		X		X
103	AA67081	M		X	X	
104	AA67082	F		X	X	
105	AA67084	F		X	X	
106	AA67085	M		X	X	
107	AA67087	F		X	X	
108	AA67092	F		X	X	
109	AA67093	M		X	X	
110	AA67094	F		X	X	

Sl. No.	ID	Sex	Origin			
			Captive breed	Wild caught	Wild Individual	Wild Egg
111	AA67098	F		X	X	
112	AA67101	M		X	X	
113	AA67103	F		X	X	
114	AA67105	F		X	X	
115	AA67122	F		X		X
116	AA67123	F		X		X
117	**AA67124**	**F**		**X**		**X**
118	AA67126	F		X		X
119	AA67127	M		X		X
120	AA67128	M		X		X
121	**AA67129**	**M**		**X**		**X**
122	AA67130	F		X		X
123	AA67131	M		X		X
124	AA67132	M		X		X
125	AA67133	M		X		X
126	AA67134	F		X		X
127	**AA67136**	**F**		**X**		**X**
128	**AA67137**	**F**		**X**		**X**
129	AA67138	M		X		X
130	**AA67139**	**F**		**X**		**X**
131	AA67140	F		X		X
132	AA67141	F		X		X
133	AA67142	F		X		X
134	AA67144	F		X		X
135	AA67151	F	X			
136	AA67152	F	X			
137	AA67153	M	X			
138	**AA67154**	**M**	**X**			
139	AA67155	F	X			
140	AA67156	F	X			
141	AA67157	M	X			
142	AA67158	F	X			
143	AA67159	M	X			
144	AA67161	F	X			
145	AA67162	M	X			
146	AA67163	F		X	X	
147	AA67164	M		X	X	
148	AA67166	M		X	X	

Sl. No.	ID	Sex	Origin			
			Captive breed	Wild caught	Wild Individual	Wild Egg
149	AA67168	M		X	X	
150	AA67169	M		X	X	
151	AA67170	M		X	X	
152	AA67173	M		X	X	
153	AA67185	M		X	X	
154	AA67189	M		X	X	
155	AA67190	F		X	X	
156	AA67193	F		X	X	
157	AA67200	M		X	X	
158	AA67243	F		X	X	
159	AA67250	F		X	X	
160	AA67252	M		X	X	
161	AA67259	M		X	X	
162	AA67261	M		X	X	
163	AA67263	F		X	X	
164	AA67271	M		X	X	
165	AA67272	M		X	X	
166	AA67278	F		X	X	
167	AA67321	M	X			
168	AA67322	M	X			
169	AA67323	M	X			
170	AA67325	M		X		
171	AA67366	M		X		

Annex III: Identified sex-linked SNP marker in *T. lineata*

Allele ID	Allele	Allele Sequence	Homozygocity				Heterozygocity		Discordance
			Male Reference	Male SNP	Female Reference	Female SNP	Male	Female	
17730977	REF	TGCAGCTGTAAGATCAAATCCATGTGACAGAGTGAGCGCCCGTCACCTGTCCAAGCTCCTGCCCACCTA	0.85	0.04	0.92	0.07	0.11	0.84	14.62%
	SNP	TGCAGCTGTAAGATCAAATCCATGTGACAGAGTGAGCGCCCATCACCTGTCCAAGCTCCTGCCCACCTA							

Annex IV: Identified sex-linked PA markers in *T. lineata*

Sl. No.	CloneID	Allele Sequence	Presence Male	Presence Female	% of discordance
1	17688257	TGCAGTTTTTATAGCTGAAACTAGTTGGGGTCATGGGCTTGCATGAGATCGGAAGAGCGGTTCAGCAGG	0.12	0.89	11.70
2	17724905	TGCAGTCTACATTGCCCTTTTTGTTGTTTAGCCATTAAGTCATGTCCAACTCTTTGTGACCCCATGGAC	0.13	0.89	12.28
3	17724623	TGCAGTAGCTTTGGCAATGTTGGAAAAATGTTGTCCTTTGATACTGGATGTCTGTGAAGAGCATGAGAT	0.11	0.85	12.87
4	17688206	TGCAGTTTAGCCCACAGCAAAAGCAAGCAAAAGCAAAGCAAAGCATGAGATCGGAAGAGCGGTTCAGCA	0.13	0.88	12.87
5	17725992	TGCAGATGCTGGGAGGCTCCTTACCACTACCGACCTCGCCAGGGTTGGACAGCCTGCATGAGATCGGAA	0.13	0.88	12.87
6	17722884	TGCAGCACCCAAAGGCTTCTGCGGTTTAGCCCATAGTGCCACCATGTCCCTCTTTGCTATAGTATAGCA	0.12	0.85	13.45
7	17684976	TGCAGCTTCCTCCTGAGGTTTGGCTGCTGGGGGGTAGCTATCTAGTAACTTATGGTTTGCATGAGATCG	0.12	0.85	13.45
8	13705536	TGCAGTAGAAGCTGCCCCAATCCGAAAAGAATGCATGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGA	0.13	0.86	13.45
9	13624297	TGCAGTTACACCTTTGGACTGAATAGGCAGGCATGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGA	0.13	0.86	13.45
10	13603751	TGCAGTTGAAAAACACGTTTTCCCCATCTCTAAGTAGGCAACTGTATGAACAGCATGAGATCGGAAGAG	0.13	0.86	13.45
11	17719560	TGCAGTTTCGTTTAGTCATTTAGTCGTGTCCGACTCTTTGTGACCCCATGGACCAGAGCATGAGATCGG	0.13	0.86	13.45
12	17726329	TGCAGGCACTAATTTAAAACCAAAGCCCCGCTCCCTCAGAGCATGAGATCGGAAGAGCGGTTCAGCAGG	0.13	0.85	14.03
13	17686772	TGCAGATTGTCTAATGGTTTGTTGCATGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCT	0.13	0.85	14.03
14	17720565	TGCAGCTGCATGGAATACAATCTGATAGGTTTCAATATATGCATGAGATCGGAAGAGCGGTTCAGCAGG	0.13	0.85	14.03
15	17701698	TGCAGTTCTAAGATGATGGAGATGATGGTTAAATCAATGCCACTCTGAATTTTTGTGTGTGTTCTGCAT	0.16	0.88	14.03
16	17684694	TGCAGCCACTTCAAAAGGTTTGATGTGTGCCAGAAGAGAGGGCTTCGTGTGCCAGTTGTGGCATGAGAT	0.16	0.88	14.03
17	17729891	TGCAGGAAGACTGTAGACCATAAAATGCTCAATAGCAGGGCATGAGATCGGAAGAGCGGTTCAGCAGGA	0.11	0.82	14.62

Sl. No.	CloneID	Allele Sequence	Presence Male	Presence Female	% of discordance
18	13637939	TGCAGCTGGATTCTCCCACTCACCAGCTGGCATGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGAC	0.12	0.83	14.62
19	17686319	TGCAGAAGTTGCACTTTAGATGTGTGAATACAAGCATGAGATCGGAAGAGCGGTTCAGCAGGAATGCCG	0.12	0.83	14.62
20	17687771	TGCAGTACAGCGGTTTAACCACTGCGCCACAAGGCATGGCCACGAAGCATGAGATCGGAAGAGCGGTTC	0.13	0.84	14.62
21	17721218	TGCAGTAAAAGAAAGCCAGGCAGGGTCTTCTCCAAAACGTTGCAATTCTGCATGAGATCGGAAGAGCGG	0.13	0.84	14.62
22	17732477	TGCAGAGGGAGAGTCTGGGAGTCCAGTTCATGACTGTACATTCCGGAAGAGTCAACCTGGATGATTCGG	0.13	0.84	14.62
23	17686912	TGCAGCATATCTGTGCAAGCACCACACTGCCATTTGCACATGTGCATGAGCATGAGATCGGAAGAGCGG	0.13	0.84	14.62
24	17687695	TGCAGGTGTGGCCCATCCCTTGTCTGCATGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGAT	0.16	0.86	14.62
25	13709358	TGCAGAAGCTTCTTCGTGGAGGCTCCTAGGGGAGTGGGCTGCATGAGATCGGAAGAGCGGTTCAGCAGG	0.16	0.86	14.62
26	17687867	TGCAGTCATGGTGATACCTTTTTATCTACTTGCATTTGCATGAGATCGGAAGAGCGGTTCAGCAGGAAT	0.17	0.88	14.62

Annex V: Status of the sex-linked markers in identified family groups, F = female, M = male

Marker	Marker sequence	Discordance (%)							
		All 171 individuals 89F, 82M	All six families 13F, 15M	Family groups					
				Red 4F, 5M	Dark Blue 4F, 3M	Purple 2F, 1M	Light Blue 1F, 2M	Green 1F, 1M	Orange 1F, 3M
SNP	TGCAGCTGTAAGATCAAATCCATGTGACAGAGTGAGCGCCCATCACCTGTCCAAGCTCCTGCCCACCTA	14.62	7.14	0	14.29	0	33.33	0	0
PA	TGCAGTTTTTATAGCTGAAACTAGTTGGGGTCATGGGCTTGCATGAGATCGGAAGAGCGGTTCAGCAGG	11.70	7.14	0	0.58	0	33.33	0	0
	TGCAGTCTACATTGCCCTTTTTGTTGTTTAGCCATTAAGTCATGTCCAACTCTTTGTGACCCCATGGAC	12.28	7.14	0	0.58	0	33.33	0	0
	TGCAGTAGCTTTGGCAATGTTGGAAAAATGTTGTCCTTTGATACTGGATGTCTGTGAAGAGCATGAGAT	12.87	7.14	0	0.58	0	33.33	0	0
	TGCAGTTTAGCCCACAGCAAAAGCAAGCAAAAGCAAAGCAAAGCATGAGATCGGAAGAGCGGTTCAGCA	12.87	7.14	0	0.58	0	33.33	0	0
	TGCAGATGCTGGGAGGCTCCTTACCACTACCGACCTCGCCAGGGTTGGACAGCCTGCATGAGATCGGAA	12.87	7.14	0	0.58	0	33.33	0	0
	TGCAGCACCCAAAGGCTTCTGCGGTTTAGCCCATAGTGCCACCATGTCCCTCTTTGCTATAGTATAGCA	13.45	10.71	11.11	0.58	0	33.33	0	0
	TGCAGCTTCCTCCTGAGGTTTGGCTGCTGGGGGGTAGCTATCTAGTAACTTATGGTTTGCATGAGATCG	13.45	3.57	0	0	0	33.33	0	0
	TGCAGTAGAAGCTGCCCCAATCCGAAAAGAATGCATGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGA	13.45	7.14	0	0.58	0	33.33	0	0
	TGCAGTTACACCTTTGGACTGAATAGGCAGGCATGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGA	13.45	7.14	0	0.58	0	33.33	0	0
	TGCAGTTGAAAAACACGTTTTCCCCATCTCTAAGTAGGCAACTGTATGAACAGCATGAGATCGGAAGAG	13.45	10.71	0	0.58	0	33.33	0	25
	TGCAGTTTCGTTTAGTCATTTAGTCGTGTCCGACTCTTTGTGACCCCATGGACCAGAGCATGAGATCGG	13.45	7.14	0	0.58	0	33.33	0	0
	TGCAGGCACTAATTTAAAACCAAAGCCCCGCTCCCTCAGAGCATGAGATCGGAAGAGCGGTTCAGCAGG	14.03	7.14	0	0.58	0	33.33	0	0
	TGCAGATTGTCTAATGGTTTGTTGCATGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCT	14.03	10.71	0	1.17	0	33.33	0	0
	TGCAGCTGCATGGAATACAATCTGATAGGTTTCAATATATGCATGAGATCGGAAGAGCGGTTCAGCAGG	14.03	7.14	0	0.58	0	33.33	0	0
	TGCAGTTCTAAGATGATGGAGATGATGGTTAAATCAATGCCACTCTGAATTTTTGTGTGTGTTCTGCAT	14.03	10.71	0	0.58	0.58	33.33	0	0
	TGCAGCCACTTCAAAAGGTTTGATGTGTGCCAGAAGAGAGGGCTTCGTGTGCCAGTTGTGGCATGAGAT	14.03	3.57	0	0	0	33.33	0	0
	TGCAGGAAGACTGTAGACCATAAAATGCTCAATAGCAGGGCATGAGATCGGAAGAGCGGTTCAGCAGGA	14.62	7.14	0	0.58	0	33.33	0	0
	TGCAGCTGGATTCTCCCACTCACCAGCTGGCATGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGAC	14.62	10.71	11.11	0.58	0	33.33	0	0
	TGCAGAAGTTGCACTTTAGATGTGTGAATACAAGCATGAGATCGGAAGAGCGGTTCAGCAGGAATGCCG	14.62	7.14	0	0.58	0	33.33	0	0
	TGCAGTACAGCGGTTTAACCACTGCGCCACAAGGCATGGCCACGAAGCATGAGATCGGAAGAGCGGTTC	14.62	7.14	0	0.58	0	33.33	0	0
	TGCAGTAAAAGAAAGCCAGGCAGGGTCTTCTCCAAAACGTTGCAATTCTGCATGAGATCGGAAGAGCGG	14.62	7.14	0	0.58	0	33.33	0	0
	TGCAGAGGGAGAGTCTGGGAGTCCAGTTCATGACTGTACATTCCGGAAGAGTCAACCTGGATGATTCGG	14.62	7.14	0	0.58	0	33.33	0	0
	TGCAGCATATCTGTGCAAGCACCACACTGCCATTTGCACATGTGCATGAGCATGAGATCGGAAGAGCGG	14.62	7.14	0	0.58	0	33.33	0	0
	TGCAGGTGTGGCCCATCCCTTGTCTGCATGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGAT	14.62	7.14	0	0.58	0	33.33	0	0
	TGCAGAAGCTTCTTCGTGGAGGCTCCTAGGGGAGTGGGCTGCATGAGATCGGAAGAGCGGTTCAGCAGG	14.62	10.71	0	0.58	0.58	33.33	0	0
	TGCAGTCATGGTGATACCTTTTTATCTACTTGCATTTGCATGAGATCGGAAGAGCGGTTCAGCAGGAAT	14.62	14.29	22.22	0.58	0	33.33	0	0

Annex VI: Consensus sequences (*ND2* gene) drawn from Sanger sequencing

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>AB166795.1_*Pogona vitticeps*

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CAGTAATCGCAAAACAGAAACACCCACGATCAACAGAAGCCGCAACAAAATACTTTTTAACACAAGCAACTGCCTCCGCACTTATTTTACTCTCAAGCACCCTAA
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CATCAATCGCACACCTTGGTTGAACAATAACAGTAATCTCAATTACACCAAACATTGCCCTAACAAATATTATCATCTACATAACTATCTCCACCCCATTTTTCC
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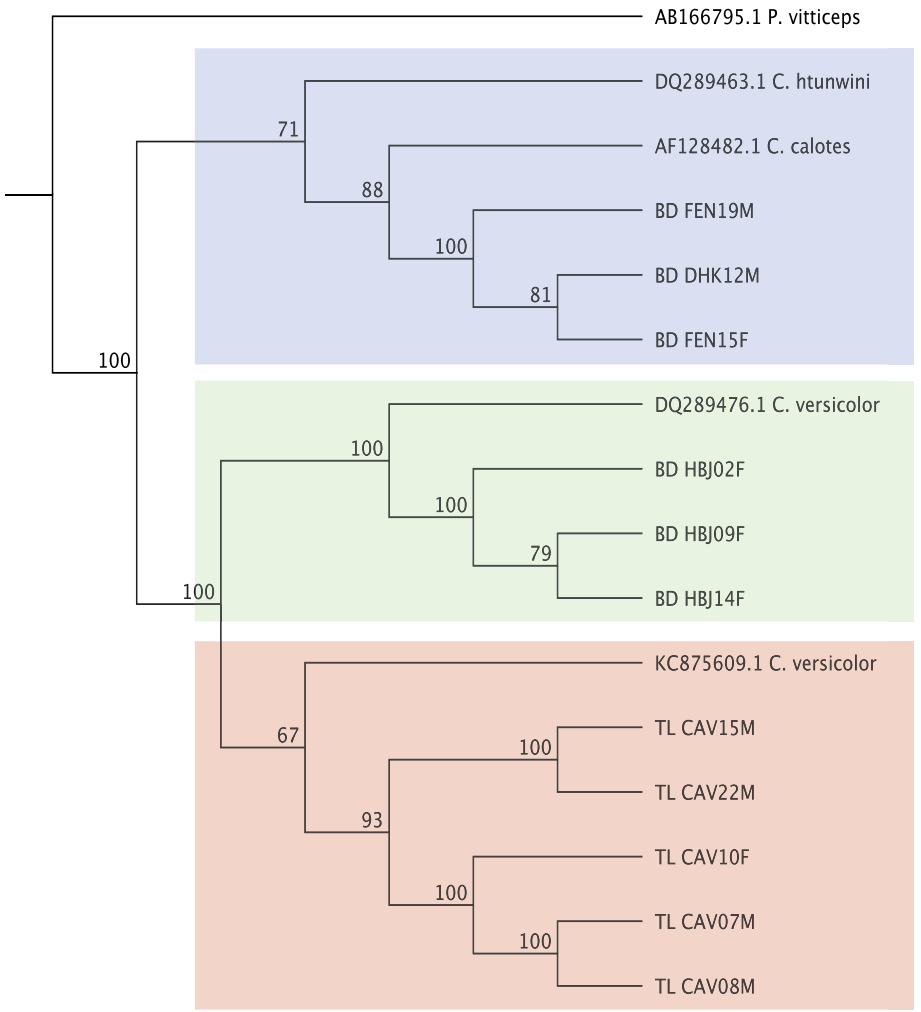
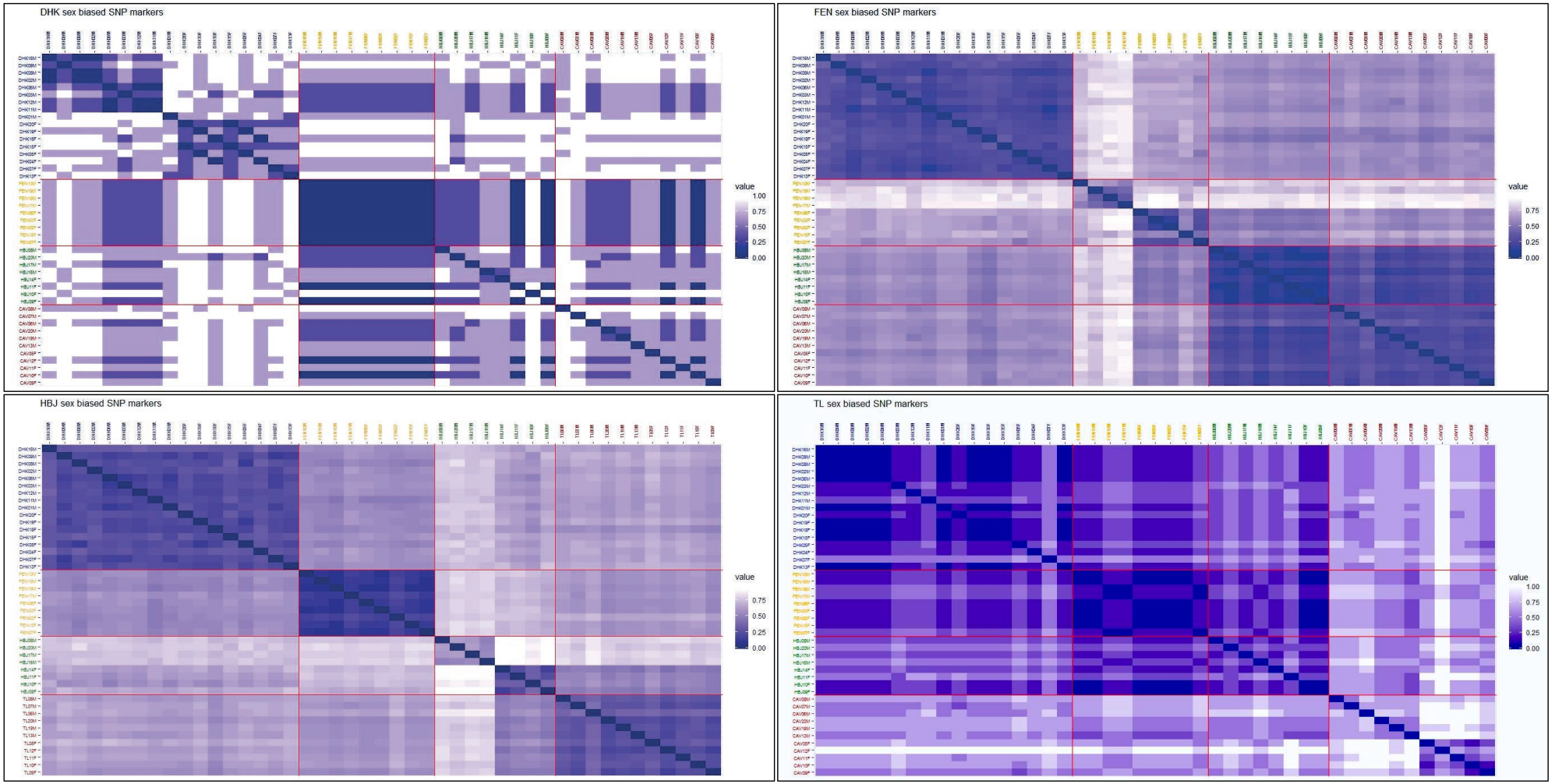
TACTAAGCCTAACTTTCTACTTACGCACGACATATCTATTAACCTCAACAGCCTCCCCAACCACAACCCAATCAACCACACTCTGACGACTAAAACCCCACCAAA
ACCAAACCACAACAATCCTAACCCCCGTTGCCTTATTTAACATTACAGTTCTGCCTGCGCTACTTACA

Annex VII: Neighbour-joining (NJ) tree with 10,000 bootstraps using the sanger sequencing data. The tree was constructed using the Geneious Tree Builder plugin of Geneious Prime (version 2020.2.3) software and the values showing the percentage of consensus support.



Annex VIII: Hamming distance matrices according to populations. DHK- Dhaka, FEN-Feni, HBJ-Habiganj and TL-Thailand



Annex IX: Number of presence-absence markers (100% concordant) in *C. versicolor* per restriction enzyme combinations together with number of individuals worked out for respective restriction enzyme treatment

Population	Enzyme combination	Male specific	Female specific	Number of individuals worked out		Enzyme combination	Male specific	Female specific	Number of individuals worked out	
				Male	Female				Male	Female
BD_DHK - BD_FEN	*Sbf*I-*Hpa*II	0	0	12	13	*Pst*I-*Hpa*II	14	8	5	3
	*Sbf*I-*Msp*I	20	32	4	4	*Pst*I-*Msp*I	0	0	9	9
BD_DHK	*Sbf*I-*Hpa*II	0	0	8	8	*Pst*I-*Hpa*II	14	8	5	3
	*Sbf*I-*Msp*I	521	2,451	3	1	*Pst*I-*Msp*I	0	0	6	7
BD_FEN	*Sbf*I-*Hpa*II	37	7	4	5	*Pst*I-*Hpa*II	-	-	-	-
	*Sbf*I-*Msp*I	187	108	1	3	*Pst*I-*Msp*I	646	995	3	2
BD_HBJ	*Sbf*I-*Hpa*II	96	16	2	5	*Pst*I-*Hpa*II	-	-	-	-
	*Sbf*I-*Msp*I	-	-	-	-	*Pst*I-*Msp*I	21	17	3	5
TL_CAV	*Sbf*I-*Hpa*II	0	0	6	5	*Pst*I-*Hpa*II	-	-	-	-
	*Sbf*I-*Msp*I	-	-	-	-	*Pst*I-*Msp*I	0	0	6	5