Sex reversal in the alpine skink *Bassiana duperreyi* – response to natural environment

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Institute for Applied Ecology

University of Canberra, Australia

“The whole of life is coming to terms with yourself and the natural world. Why are you here? How do you fit in? What’s it all about?”

— *Sir David Attenborough*

I dedicate this dissertation to Aboriginal Australians and especially to the Ngunnawal, Wiradjuri & Wolgalu, Ngyimpa, and Monaro Ngarigo peoples, whose lives are spiritually and culturally bound to the marvellous natural environment of the Australian high-country and who use their ancient traditional knowledge and cultural practices to conserve this unique nature.

I would like to acknowledge the Ngunnawal people who are the traditional custodians of the land upon which I conducted my PhD research and pay respect to the Elders of the Ngunnawal Nation, past, present and emerging.

Statement of originality

Except where clearly acknowledged in footnotes, quotations and the bibliography, I certify that I am the sole author of the thesis submitted today entitled -

"Sex reversal in the alpine skink *Bassiana duperreyi* – response to natural environment"

I further certify that to the best of my knowledge the thesis contains no material previously published or written by another person except where due reference is made in the text of the thesis.

The material in the thesis has not been the basis of an award of any other degree or diploma except where due reference is made in the text of the thesis.

The thesis complies with university requirements for a thesis as set out in the Examination of Higher Degree by Research Theses Policy:
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16/07/2021

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Declaration of Co-Authored Publications

Declaration for Thesis Chapter 2

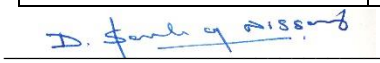
DECLARATION BY CANDIDATE

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contributions (%)
conceived and designed the experiments, performed the experiments, filed work, generated, and analysed the data, led the writing	80%

The following co-authors contributed to the work:

Name	Nature of Contribution	Contributor is also a UC student (Yes/No)
Clare E. Holleley	conceptualized the research, lab work supervised the study, manuscript contribution	No
Laura K Hill	Field work and lab work	Yes
Denis O'Meally	conceived and designed the experiments	No
Janine E. Deakin	manuscript contribution, supervised the study	No
Arthur Georges	conceptualized the research, supervised the study, manuscript contribution	No



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DECLARATION BY CO-AUTHORS

On behalf of the co-authors, and as the primary supervisor of the candidature, I confirm that;

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
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Laura K Hill	3/9/2021
Denis O'Meally	1/9/2021
Janine E. Deakin	29/8/2021
Arthur Georges	1/9/2021

Declaration for Thesis Chapter 3

DECLARATION BY CANDIDATE

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contributions (%)
designed the study, filed work and performed the laboratory work, analysis, modelling, led writing	80%

The following co-authors contributed to the work:

Name	Nature of Contribution	Contributor is also a UC student (Yes/No)
Clare E. Holleley	designed the study, manuscript contribution, supervised the study	No
Janine E. Deakin	designed the study, manuscript contribution, supervised the study	No
Arthur Georges	designed the study, modelling, manuscript contribution, supervised the study	No

Candidate's Signature

16/07/2021
Date

DECLARATION BY CO-AUTHORS

On behalf of the co-authors, and as the primary supervisor of the candidature, I confirm that;

The undersigned hereby certify that:

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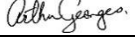
Janine E. Deakin 	29/8/2021
Arthur Georges 	1/9/2021

Declaration for Thesis Chapter 4

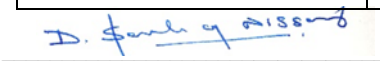
DECLARATION BY CANDIDATE

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contributions (%)
conceived the project and developed the sampling regime and experimental design, fieldwork and all molecular work, data analysis, led writing	80%

The following co-authors contributed to the work:

Name	Nature of Contribution	Contributor is also a UC student (Yes/No)
Clare E. Holleley	conceived the project, manuscript contribution, supervised the study	No
Joanna Sumner	provided input on taxonomic considerations, manuscript contribution	No
Jane Melville	provided input on taxonomic considerations, manuscript contribution	No
Arthur Georges	conceived the project, manuscript contribution, supervised the study	No



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DECLARATION BY CO-AUTHORS

On behalf of the co-authors, and as the primary supervisor of the candidature, I confirm that;

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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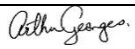
Arthur Georges 	1/9/2021

Declaration for Thesis Chapter 5

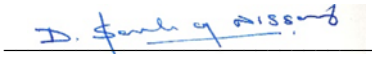
DECLARATION BY CANDIDATE

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contributions (%)
designed the study, monitored field nests and collected eggs from the wild nests and performed the incubation and laboratory work, analysing and modelling, led the writing	80%

The following co-authors contributed to the work:

Name	Nature of Contribution	Contributor is also a UC student (Yes/No)
Clare E. Holleley	designed the study, manuscript contribution, supervised the study	No
Arthur Georges	designed the study, modelling, manuscript contribution, supervised the study	No



Candidate's Signature

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Date

DECLARATION BY CO-AUTHORS

On behalf of the co-authors, and as the primary supervisor of the candidature, I confirm that;

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;
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Clare E. Holleley 	2/9/2021
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Abstract

Global climatic change has significant impacts on several aspects of the biology and ecology of many reptile species on earth, which in some cases has led to population declines or other severe threats. Until now, however, such impacts have not been reviewed for many reptiles, even though these species are often at the critical stage at the conservation priorities. Changing temperature is increasingly being considered one of the major threats for reptiles, especially for species where temperature extremes can influence the outcome of sex determination. In reptiles, sex can be determined genetically (genotypic sex determination or GSD) or environmentally, depending on their incubation temperature (temperature sex determination or TSD). Typically, the two alternatives of sex determination were thought to be incompatible and were never observed in the same species, however sex reversal of some GSD species under extreme environmental conditions, confirmed in wild populations, has revealed that intermediate states are possible.

Sex determination in the endemic Australian skink *Bassiana duperreyi* is influenced by both genetic factors and nest temperature. The discordance between genotypic and phenotypic was observed definitively in the laboratory conditions. Whether sex reversal occurs in natural nests and whether sex-reversed individuals survive to adulthood in wild populations is unknown. Furthermore, it is unclear whether the interaction of sex reversal of male XX individuals under low temperatures occurs at a rate sufficient to trigger loss of the Y chromosome and evolutionary transitions between GSD and TSD. To address these questions in the wild population of *B. duperreyi*, we first developed reliable molecular techniques to identify sex reversal male (XX) from normal male (XY), is known as *in silico* whole-genome subtraction. We used this technique to isolate seven Y chromosome specific markers for B. *duperreyi*. In chapter 3, using these markers, we demonstrated that sex reversal in the wild for the first time in adults of a reptile species with XX/XY sex determination. The highest frequency of sex reversal occurred at the highest coolest elevation location, Mt Ginini (18.46%) and decreased in frequency to zero with decreasing elevation. We model the impact of this under Fisher's frequency-dependent selection to show that, at the highest elevations, populations risk the loss of the Y chromosome and a transition to temperature-dependent sex determination. We also applied our Y chromosome markers to hatchlings, in chapter 5, similar to what we discovered in adults, we discovered that the levels of sex reversal in nests of *B. duperreyi*,

ranged from 28.6% at the highest, coolest locations to zero at the lowest, warmest locations. Further, we observed that the constant temperature equivalent in wild nests dropped below the 20°C during the thermosensitive period, for even for a short time during the incubation period, sex reversal was observed. Therefore, extreme cold temperature events might lead to 100% sex reversal of the XX genotype in the alpine populations.

The variation in the frequency of sex reversal in *B. duperreyi* as a phenotypic response to environmental temperature with altitude, and early work using mtDNA has raised the possibility that underlying phylogenetic history may be confounding interpretation of variation in the frequency of sex reversal with elevation. To test this, in chapter 4, we examined the underlying genetic structure within the geographic wide populations on its distribution used single nucleotide polymorphisms (SNP) generated by reduced representation sequencing. We generated 12,532 reliable polymorphic SNP loci from 60 populations (263 individuals) covering the distribution of *B. duperreyi* to assess geneflow and to define population structure in the form of diagnosable lineages. We identified five well-supported diagnosable operational taxonomic units (OTUs) within *B. duperreyi*. Low levels of divergence of *B. duperreyi* between mainland Australia and Tasmania (no fixed allelic differences) support the notion of episodic exchange of alleles across Bass Strait (*ca* 60 m, 25 Kya) during periods of low sea level during the Upper Pleistocene rather than the much longer period of isolation (1.7 My) indicated by earlier studies using mitochondrial sequence variation. Our study provides foundational work for the detailed taxonomic re-evaluation of this species complex and the need for biodiversity assessment to include an examination of cryptic species and/or cryptic diversity below the level of species.

This thesis contributes to our understanding of the risks of population demography due to climate change in species subject to sex reversal by temperature, and sex reversal in this alpine skink makes it a sensitive indicator of climate change, both in terms of changes in average temperatures, and in terms of climatic variability will provide focus for future research to test on-the-ground management strategies to mitigate the effects of climate in local populations. Further we provide evidence that sex reversal by cool temperatures in *B. duperreyi* occurs in the wild, influences the sexual genotypes-phenotype combinations in adult populations, and is as such a potential means of rapid evolutionary responses to climate change.

Statement of publications and co-authorship

Core manuscripts

Chapter 2: **Dissanayake, D. S. B**., Holleley, C. E., Hill, L. K., O'Meally, D., Deakin, J. E. & Georges, A. (2020). Identification of Y chromosome markers in the eastern three-lined skink (*Bassiana duperreyi*) using in silico whole genome subtraction. BMC genomics 21:1-12. https://doi.org/10.1186/s12864-020-07071-2

Chapter 3: **Dissanayake, D. S. B**., Holleley, C. E., Deakin, J. E. & Georges, A. (2021). High elevation increases the risk of Y chromosome loss in Alpine skink populations with sex reversal. Heredity 126:805–816. https://doi.org/10.1038/s41437-021-00406-z

Chapter 4: **Dissanayake, D. S. B**., Holleley, C. E., Sumner, J., Melville, J. & Georges, A. (In press) (2022). Lineage diversity within a widespread endemic Australian skink to better inform conservation in response to regional-scale disturbance. Ecology and Evolution.

Chapter 5: **Dissanayake, D. S. B.,** Holleley, C. E., & Georges, A. (2021). Effects of natural nest temperatures on sex reversal and sex ratios in an Australian alpine skink. Scientific Reports 11, 20093 (2021). https://doi.org/10.1038/s41598-021-99702-1 .

Other associated manuscripts (Appendix 1)

Whiteley, S. L., Castelli, M. A., **Dissanayake, D. S.B.,** Holleley, C. E. & Georges, A. (2021). Temperature-induced sex reversal in reptiles: prevalence, discovery, and evolutionary implications. Sexual Development. online early view: https://doi.org/10.1159/000515687

*Cornejo-Páramo, P., ***Dissanayake, D. S. B**., *****Lira-Noriega, A., *****Martínez-Pacheco, *M.L., Acosta, A., Ramírez-Suástegui, C., Méndez-de-la-Cruz, F.R., Székely, T., Urrutia, A.O., Georges, A. and Cortez, D., (2020). Viviparous reptile regarded to have temperature-dependent sex determination has old XY chromosomes. Genome biology and evolution, 12:924-930. https://doi.org/10.1093/gbe/evaa104.

***Dissanayake, D. S. B**., *Streeting, L., Georges, A. & Bower, D. (2022). Development of a male-specific sex marker for the endangered western saw-shelled turtle (*Myuchelys bellii*) using in silico whole-genome subtraction. (Manuscript is accepted with minor revision) Conservation Genetic Resources.

*Contributed equally to the study.

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Every person born in this world should at least once in his lifetime have the opportunity to do what they desire to do and I for one had that needed passion, to engage in a dream where I relished fulfilling every bit of it. A Doctor of Philosophy should not be easy or the thoughts of one person working alone. To complete a Ph.D. was a pleasure, spending joyful time with a bunch of people who inspired me and helped me to achieve my goals.

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An adult *Bassiana duperreyi* with a communal nest at Picadilly Circus

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Chapter 1

General Introduction

1.1 Introduction

This introduction draws in part from material published as: Whiteley, S. L., Castelli, M. A., Dissanayake, D. S. B., Holleley, C. E., & Georges, A. (2021). Temperature-induced sex reversal in reptiles: prevalence, discovery, and evolutionary Implications. Sexual Development, *Online Early* https://doi.org/10.1159/000515687 (See Appendix 1).

Evolutionary thought and the study of evolutionary biology go back to the time of the Greek philosophers, such as Anaximander, Thales of Miletus and Empedocles (Kirk et al., 1983). It has been more than one hundred and fifty years since publication of Darwin's *On the Origin of Species*, yet evolutionary biologists still strive to understand evolutionary concepts such as natural selection, common descent, biology, and speciation. We are still far from understanding the complexities of evolutionary biology, however new research approaches and techniques are being developed at an accelerating rate. By using such advances and our improved understanding of evolutionary biology, we can better understand how species can cope with the contemporary issues arising from global environmental change.

Climatic change is one of the primary categories of global environmental change that has a significant impact on several aspects of the biology and ecology of many vertebrate species on earth. It has led to population declines and other severe threats (Brodie et al., 2021; Ceballos et al., 2015; Garcia et al., 2014; Parmesan, 2006; Peñuelas et al., 2013; Sánchez-Bayo & Wyckhuys, 2019). However, such impacts have not been reviewed for many species, including reptiles, until recently (Saha et al., 2018; Tingley et al., 2016), even though these species are often of high conservation priority. Changing temperature is increasingly considered a significant threat, especially for reptiles globally (Gibbons et al., 2000; Whitfield et al., 2007) because reptiles correspond their body temperature and activity patterns (biological and physiological) with seasonal dynamics of environmental temperatures (Garcia-Porta et al., 2019; Huey & Pianka, 1977). The big question is whether these species can adapt rapidly enough to persist under current rates of climate change? Of particular concern are reptile species whose fundamental biology, their phenotype sex, is determined by ambient thermal regimes during embryonic development (Janzen, 1994; Mitchell & Janzen, 2010).

Evolutionary change is often regarded to occur on much slower timescales than ecosystem responses, demographic responses or phenotypic responses (Johansson, 2008), but evidence is emerging to suggest that this is not always the case (Grainger et al., 2021; Hulme, 2017). If the selection pressures are powerful enough, and sufficient variability exists within populations, substantive evolutionary change can happen over relatively short periods coincident with demographic changes. For example, if selection is strong and directional in the presence of high genetic variability and heritability, or if Fisher's frequency-dependent selection is involved, rapid evolution under climate change is possible (Conover & Van- Voorhees, 1990; Dissanayake et al., 2021a, 2021b; Hoffmann & Sgrò, 2011; Holleley et al., 2015; Kopp & Matuszewski, 2014). Some reptile species will change their behaviours in response to the warming trend in global temperatures (e.g., phenology, see chapter 5), and some species change their sex determination mechanism in response to environmental temperatures with as yet unclear adaptive significance (Holleley et al., 2015).

Reptiles are one of the most successful terrestrial vertebrate groups having persisted for more than a quarter of a billion years. Today reptiles occupy most regions of the earth and consist of more than 11,440 species in 92 families (Uetz et al., 2021). However, we are still uncertain about how many extant reptile species exist (Melville et al., 2021). Systematics is central to biology in that new species are described and their evolutionary relationships identified, which then delivers as new objects of research (new evolutionary solutions to past challenges) for biology, ecology, and molecular biology (Costello et al., 2012). Currently, researchers are working on the systematics of Australian squamates use both genetic and morphological approaches to resolve major taxonomic uncertainties (Melville et al., 2021). While some argue about which species concepts describe biological diversity best, there is general agreement that species need to be described and named to assist communication and avoid the confounding effects that arise when studies fail to recognise that not one, but more than one species is the subject of the research (Chambers & Hillis 2020; Dissanayake et al., 2021a; Marshall et al., 2021; see chapters 3 and 5). Hence, much attention is paid to species concepts to ensure comparability across studies and regions when classifying taxa against criteria of endangerment and setting priorities for action (see chapter 4). Accurate identification of species using morphology and advanced bioinformatics tools are crucial to research in all aspects, including reproductive biology in a particular study species. This is because

certain species show a remarkable diversity in their reproduction even within their closely associated species.

The mode of reproduction and mechanism for sex determination in reptiles is remarkably diverse (Bachtrog et al., 2014; Tree of Sex Consortium, 2014) and can be divided into two broad categories - genetic sex determination (GSD) and environmental sex determination (ESD). In GSD, genes inherited from the parents determine the sex of the offspring. These sex-determining genes typically reside on sex chromosomes which fail to recombine in part or in whole, and so diverge from each other both in gene content, repeat content, heterochromatism and overall morphology. For the purpose of this thesis, sex determination refers to the regulatory mechanisms that "decide" which developmental pathway to follow; sex differentiation describes the developmental processes that give effect to that "decision".

The diversity and evolutionary history of reptile sex determination attract many scientists and there remains many questions to be answered, such as: Why such diversity in the mechanisms for sex determination? When did these different systems evolve? What are the proximate mechanisms that produce male and female? Which sex determination mechanisms are ancestral and how have new mechanisms arisen? Which mechanism best delivers stable sex ratios in the wild appropriate to population persistence? Which mechanism is likely to be most resistant to future climatic change? Answers to these and many evolutionary questions are continuously sought by biologists in many disciplines (Bachtrog et al., 2014). Many systems are poorly studied so far; exploring the variation in sex-determining mechanisms is one of the most exciting areas of evolutionary biology (see chapter 3 and 5). Our understanding is far from complete, and reptiles will assuredly continue to serve as excellent models for answering fundamental questions in this field.

Since the discovery of sex chromosomes (Brush, 1978; Stevens, 1905) and the factors responsible for sex determination, advanced cytogenetic and genomic applications have answered many debated questions in sex determination in reptiles (Deakin & Ezaz, 2019). We now know that the sexual phenotypes of many reptile species are determined by either temperature or genetic factors located in sex-specific chromosomes (Bull, 1980, 1981). Temperature dependent sex determination (TSD) and GSD (see section 1.2 and 1.3), were thought to be alternatives, that is, a mutually exclusive dichotomy. Recent discoveries have shown that chromosomal sex and temperature influences can co-exist

within the same species (see section 1.5 and chapter 3 and 5) and imply that sex determination is a continuum of states spanning a spectrum between TSD and GSD as extremes (Sarre et al., 2004, 2011). Despite decades of concerted research effort, the mechanisms by which temperature exerts its influence on reptile sex reversal, or even by which conventional chromosomal sex determination works in reptiles, is poorly understood. No master sex-determining gene has been discovered in any reptile species [though there are a couple of promising candidate TSD genes, such as *CIRBP* and Jumonji family genes (*JARID2*, and *KDM6B*) (Deveson et al., 2017; Ge et al., 2018; Schroeder et al., 2016)], in contrast with other major lineages [fish (Chen et al., 2014; Matsuda et al., 2002), amphibians (Yoshimoto et al., 2008) and, birds (Lambeth et al., 2014; Smith et al., 2003)]. The genetic foundation for transitions between TSD and GSD, and the complex interactions between temperature and genetic sex determination, remain obscure in reptiles.

The extensive work on the emerging reptile model species, central bearded dragon, *Pogona vitticeps*, has led to some astonishing discoveries, such as their ability to produce viable female individuals without W chromosomes in the laboratory and in the wild, and have the potential to rapidly evolve from a genetic system of sex determination to a temperature influenced system of sex determination (Holleley et al., 2015; Quinn et al., 2007, 2011). The consequences of this for the species persistence under climate change are profound (Holleley et al., 2015). It is anticipated that similar notable findings, but substantially different in mode of action and consequences, will emerge from studying the eastern three-lined skink, *Bassiana duperreyi* (Radder et al., 2008; Shine et al., 2002). The discovery of sex reversal by temperature in these two species is likely the tip of the iceberg in terms of diversity of sex determination modes and mechanisms, as relatively few species have been examined. The skink, *B. duperreyi*, has a mechanism of sex determination similar to that of mammals in so far as it has male heterogamety (Donnellan, 1985; Matsubara et al., 2016), but unlike mammals, low incubation temperatures reverse the female XX genotype to a male phenotype (Radder et al., 2008; Shine et al., 2002). The primary aim of this thesis is to determine the ecological and evolutionary implications of sex reversal by low incubation temperatures in adults and hatchlings in the wild *B. duperreyi* in south-eastern Australia. This project addresses our knowledge gap in understanding the ecological consequences of global climate change on reptiles with thermolabile sex.

In the following parts of the General Introduction to the thesis, I will briefly overview the sex determination systems in vertebrates. Second, I will summarize knowledge of reptile sex determination and evolution. Here, I will describe the mode of the sex-determination systems, with a focus on reptiles, discuss the evolution of sex determination, the relationship between TSD and GSD systems, discuss adaptive significance of TSD and transitions between TSD and GSD. Third, I will explain sex reversal and what we know so far. Finally, because it has been one particular focus of my work, I will give a brief overview of genomic techniques used to identify sex-specific molecular markers and summarize their applications in studies to better understand sex determination and sex reversal in reptiles.

I will finish the General Introduction with an account of why I chose *Bassiana duperreyi* as a non-model species to better understand sex reversal in nature, and clearly state the aims and specific objectives of this thesis.

1.2 Vertebrate sex-determining mechanisms

The genetic and regulatory mechanisms that regulate GSD in mammals have been thoroughly studied. The discovery of human sex chromosomes in the 1950s showed that males have a single X and Y; females have two copies of an X chromosome (Figure 1.1) (Graves, 2016; Harper, 2006). The discovery of the *SRY* gene as the mammalian sex determination gene was a landmark in the history of human genetics (Koopman et al., 1991; Sinclair et al., 1990). *SRY* is found only in marsupial and eutherian mammals (Foster et al., 1992; Wallis et al., 2008); monotremes lack *SRY* (Foster et al., 1992; Graves, 2016; Wallis et al., 2008). Mammalian sex determination is unusual in that the mode of sex determination is conserved across most mammal lineages (Graves et al., 1995; Graves & Foster, 1994). What we know of GSD and its evolution comes from studies of taxa as diverse as flies, nematodes, chickens and frogs, where the mechanisms of sex determination and the chromosomes involved are quite different from that of mammals.

Birds have female heterogamety and sex determination is thought to depend on gene dosage (Figure 1.1). Birds have been generally known as good examples of extreme sex chromosome stability, where many species from widely different clades share synteny across the entire Z chromosome (Nanda et al., 2008). The most promising sex-determining gene is the Z chromosome gene *DMRT1* (Doublesex and Mab-3–Related Transcription factor 1) (Hirst et al., 2017; Lambeth et al., 2014).

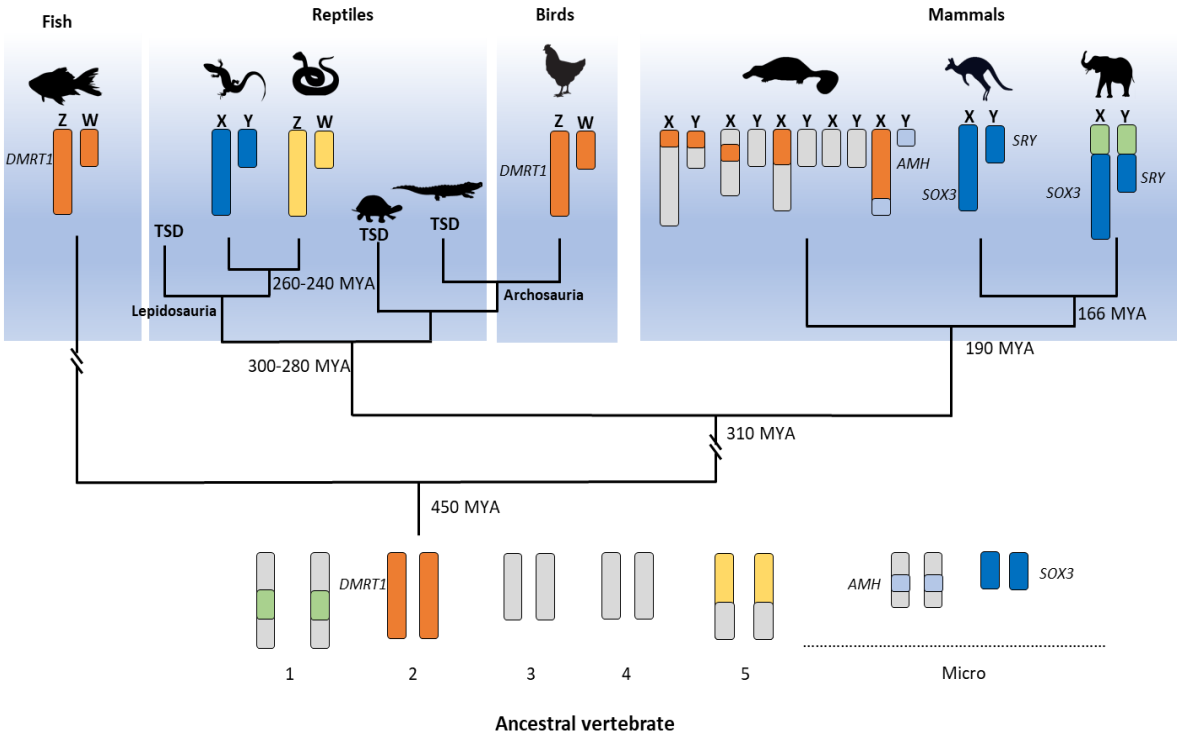

Figure 1-1. Vertebrate sex determination mapped onto the vertebrate phylogeny. The five coloured genome regions became sex chromosomes in different lineages and contain sex-determining genes. XY: male heterogamety; ZW: female heterogamety; TSD: temperature-dependent sex determination; GSD: genotypic sex determination. AMH, anti-Mullerian hormone; DMRT1, doublesex Mab3-related transcription factor 1; Micro, microchromosomes; Mya, million years ago; SOX3, SRY-like HMG-box containing gene 3; SRY, sex-determining region Y; TSD, temperature sex determination. Figure details modified from Graves & Peichel 2010; Graves, 2013.

In fish, both types of sex determination systems exist even in closely related species (Mank et al., 2006). However, in in the Atlantic silverside, *Menidia menidia*, have a latitudinal gradient in sex determination that ranges from primarily TSD in southern populations to GSD in northern environments (Conover & Heins, 1987). There are many varieties of sex-determining genes responsible for sex determination in fish (Devlin & Nagahama, 2002; Heule et al., 2014; Martínez et al., 2014; Piferrer & Anastasiadi, 2021).

There are five genes are involved in sex determination in fish, including *DMRT1*, Y-linked (*DMRT1*) (Matsuda et al., 2002), sexually dimorphic on the Y chromosome (*sdY*), Y-linked anti-Mullerian hormone (*amhy*), the gonadal soma-derived factor (*gsdf*) and *AMH* receptor type-2 (*amhr2*). *DMRT1* homologs and paralogs, such as W-linked *DMRT1* (the DM-domain gene on the W chromosome, *dmW*), and sometimes under external stimuli control (Yoshimoto et al., 2008). These different sex determination genes found either in closely related species such as Japanese rice fish (*Oryzias latipes*); the DM-domain gene on the Y chromosome, *dmY* and *O. luzonensis*; *gsdf* (Hamaguchi et al., 2004; Myosho et al., 2012) or in divergent species such as rainbow trout (*Oncorhynchus mykiss*); *sdY* (Yano et al., 2013), the Tiger Pufferfish (*Takifugu rubripes*); *amhr2* (Kamiya et al., 2012), or pejerrey (*O. hatcheri*); *amhY* (Hattori et al., 2012), or the same genes found in many species such as *O. latipes* and *O. curvinotus*; *dmY* (Matsuda et al., 2002), *T. rubripes*, *T. pardalis*, and *T. poecilonotus*; (*amhr2*) (Kamiya et al., 2012), and *sdY* in most salmonid species studied so far (Yano et al., 2013). Interestingly, some of the same genes are also found in some amphibians and reptiles, such as W-linked *DMRT1*.

Amphibians have GSD as their primary sex determination mode (Flament, 2016; Hayes, 1998; Miura, 2017; Nakamura, 2009; Ruiz-García et al., 2021). Both male and female heterogametic systems occur, and phylogenetic analysis suggests that the ancestral state was male heterogamety (Hillis & Green, 1990). However, their sex chromosomes have been described in only 4-5% of the species (Ruiz-García et al., 2021; Schmid & Steinlein, 2001). Some species (e.g., *Rana rugosa*) show both male and female heterogamety in different populations (Ogata et al., 2002, 2008, 2018; Oike et al., 2017).

There are eight candidate genes proposed as sex-determining genes in amphibians (*DMRT1*, *SOX3*, *CYP19A1*, *CYP17*, *AMH*, *FOXL2*, *SF1*, and *AR*) (review by Miura, 2017). These genes were discovered by using sex-linkage and karyotypic data. The *xdm-w* (DM domain-containing W-link) gene is the only sex-determining gene known for amphibians

with ZZ/ZW sex chromosomes (Yoshimoto et al., 2008). This gene originated by partial duplication of *dmrt1.S* (lacks the transactivation domain) and is on chromosome pair 2L (Bewick et al., 2011; Mawaribuchi et al., 2012; Yoshimoto et al., 2008). There is no confirmed record of the TSD system in amphibians, but some species have sex determined by gene-environment interactions (Dournon et al., 1990; Wallace et al., 1999; Wallace & Wallace, 2004) (see section 1.5).

1.3 Temperature-dependent sex determination in reptiles

Charnier first reported how temperature affected the offspring sex ratio in African agamid lizard, *Agama agama* (Charnier, 1966). TSD is now known to be widespread across reptile lineages, recorded in many turtles, possibly all crocodilians, the tuatara, and many lizards (Cree et al., 1995; Ewert et al., 1994; Janzen & Paukstis, 1991; Lang & Andrews, 1994; Mitchell et al., 2006; Valenzuela, 2021; Viets et al., 1994). TSD is not found in snakes (Bull, 1980; Emerson, 2017; Matsubara et al., 2006; Rovatsos et al., 2015; Viets et al., 1994), which appear to have a conserved system of female heterogamety (ZZ/ZW) (Matsubara et al., 2006; Oguiura et al., 2009; Ohno, 1967; Rovatsos et al., 2015; Vicoso et al., 2013) except for *Python bivittatus* and *Boa imperator* (Gamble et al., 2017).

The response of offspring sex ratio to incubation temperature varies between species. Three patterns of TSD are recognized in reptiles (Bull, 1980; Ewert & Nelson, 1991; Valenzuela & Lance, 2004) (Figure 1.2). In the Type Ia (or MF) pattern, low temperatures produce males and high temperatures produce females (Ewert et al., 1994, 2004). For example, in the pig-nosed turtle (*Carettochelys insculpta*), constant temperatures below 31.5°C produce 100% males, constant temperatures above 32.5°C produce 100% females, and both sexes are produced at 32.0°C (Young et al., 2004). In the Type Ib (or FM) pattern, low temperatures produce females and high temperatures produce males. This pattern is restricted to the tuatara *Sphenodon punctatus* (Cree et al., 1995; Mitchell et al., 2006) where males are produced above and females below a pivotal temperature of 22.0°C (Mitchell et al., 2006). In the Type II (or FMF) pattern, low and high temperatures produce females whereas males are produced only intermediate temperatures. This pattern occurs in some lizards and all crocodilians (Deeming, 2004; Ewert et al., 2004; Harlow, 2004).

These TSD patterns are typically described from sex ratio data obtained under constant temperature conditions in the laboratory. The constant temperature that yields a -

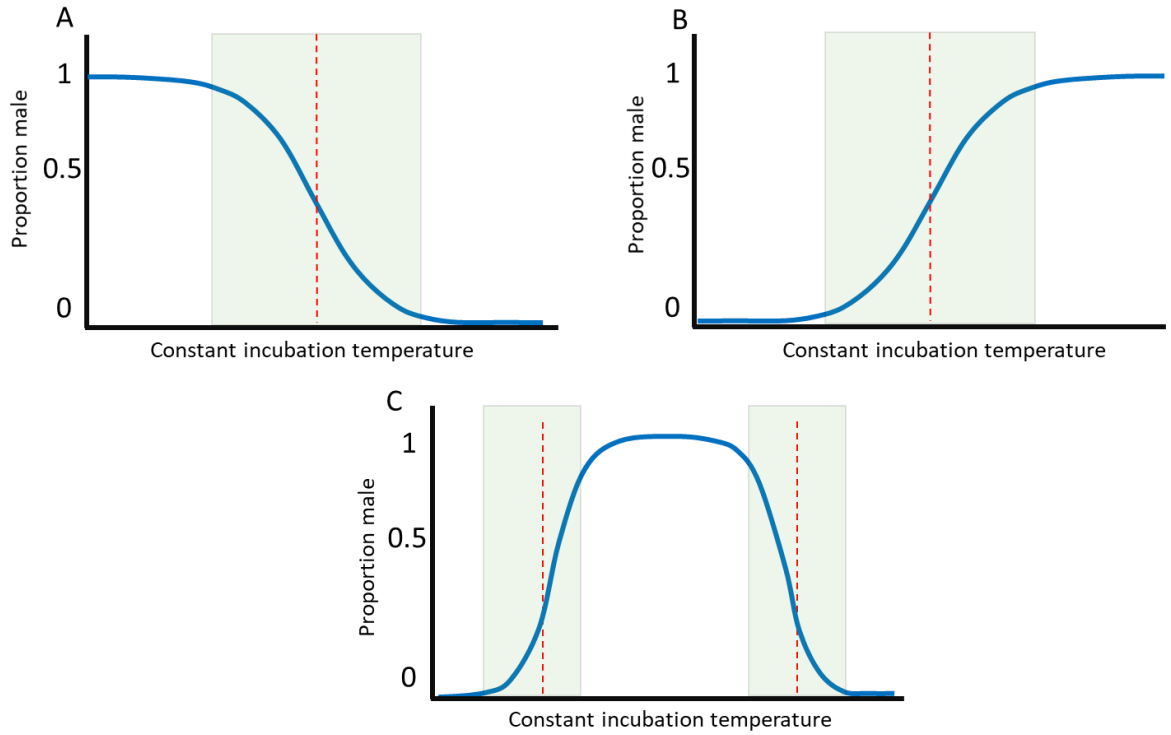

Figure 1-2. Schematic diagram of the three patterns of temperature-dependent sex determination (TSD) found in reptiles. A: Type Ia(or MF) pattern, B: Type Ib (or FM) pattern, C: Type II (or FMF) pattern. Red broken lines denote the pivotal temperature(s) that is, the constant temperatures that produces a 1:1 sex ratio. Shaded area denotes transitional range.

1:1 sex ratio is known as pivotal (or threshold) temperature (Bull et al., 1982). Therefore, the Type II pattern is characterized by two pivotal temperatures, whereas Types Ia and Ib each have a single pivotal temperature (Figure 1-2). When reptile embryos are incubated at the pivotal temperature, they typically develop either testes or ovaries, not intersex gonads, suggesting that stochastic influences or an underlying weak genetic predisposition comes into play when the temperature is equivocal in its effect. The range of temperature that generates mixed sex ratio is termed transitional range (TR) (Pieau & Mrosovsky, 1991) (Figure 1.2) thought to arise because of interindividual variation in the pivotal temperature. Under natural conditions, the reptile TSD species and populations of the same species may respond differently to environmental temperatures. This manifests as variation in pivotal temperatures, widths of the transitional range, and slopes of the transitional range.

The temperature-sensitive period (TSP) (i.e., the portion of the incubation period during which incubation temperature can affect sexual fate of hatchlings) (Bull & Vogt, 1981) is the critical stage of embryonic development in TSD species. As an example, the TSP of the American Alligator (*Alligator mississippiensis*) is found in the middle third of embryonic development (developmental stages 21–24) (Ferguson & Joanen, 1982). TSP in other TSD species also typically encompasses the mid-third to mid-half of embryonic development (Girondot et al., 2018; Pieau, 1975; Pieau & Mrosovsky, 1991; Yntema, 1968, 1979). TSP measured as embryonic stage will not be in the middle third in time when nest temperatures are subjected to wide diel fluctuations and seasonal shifts (Georges, 1989; Georges et al., 1994). Therefore, calculation of TSP in natural nests requires nest temperature traces and knowledge of the response curve of developmental rate against temperature; a process of summation will identify the TSP (Georges et al., 2004; see chapter 5).

The temperature variation within which the offspring sex shifts from one sex to the other is very narrow, often less than 1^{o}C (Ewert et al., 1994; Young et al., 2004). A minor shift in environmental temperature during embryonic development could potentially dramatically alter the offspring sex ratios. Species with TSD are thought to be exceptionally vulnerable to climate change. Rapid current climatic change can potentially lead to local extinction of TSD reptiles by creating populations predominantly made up of just one sex (Boyle et al., 2014; Fuentes et al., 2011; Hawkes et al., 2007; Hays et al., 2017; Janzen, 1994; Jensen et al., 2018). Therefore, understanding the response of sex ratio to the complex environment in nests, how this thermal environment will change over time

under global warming, and identifying options available to species to respond through phenotypic plasticity or evolutionary change, is critical for conserving the future of TSD reptiles.

1.3.1 Adaptive significance of TSD

In recent years, sex-determining mechanisms and the adaptive significance of TSD have received considerable attention from researchers. Nevertheless, current theoretical work and empirical observation leave much to be understood about the adaptive significance of TSD in reptiles (Janzen & Phillips, 2006; Schwanz & Georges, 2021; Warner et al., 2018). The most widely accepted model for the adaptive significance of TSD in reptiles is the Charnov-Bull, (1977) model. This model predicts that TSD is advantageous if one sex is produced at temperatures that lead to a fitness advantage to that sex later in life, but not the other sex. Daughters might be favoured at incubation temperatures that lead to a growth advantage later in life, as fecundity is linked to body size in many reptiles. Alternatively, if large body size is of advantage to males that engage in male-male combat, then males may be favoured at temperatures that lead to a growth advantage later in life. The evolutionary advantages of TSD in reptiles fall into few categories of explanation (reviewed in Shine, 1999).

Empirical evidence in support the Charnov-Bull model for TSD reptiles with long life spans and late sexual maturation is difficult to obtain. However, the Charnov-Bull model was supported by the experiments on a short-lived lizard, the Jacky dragon (*Amphibolurus muricatus*) (Warner & Shine, 2005). This lizard produces female-biased sex ratios at high (30–32°C) and low (23–25°C) temperatures; at intermediate temperatures (27–30°C) approximately 50: 50 sex ratios are produced (Harlow & Taylor, 2000). TSD enhances offspring fitness by promoting early hatchling of females for which incubation temperatures accelerate development (Warner & Shine, 2005). *Amphibolurus muricatus* females produced from either high or low temperatures display higher reproductive success in their lifetime than those incubated at the intermediate temperature (Warner & Shine, 2008).

1.4 TSD and GSD: dichotomy or a continuum?

The classical view of reptilian sex determination mechanisms divides species into two discrete groups: TSD or GSD. This dichotomy is based on the presence or absence of sex -

chromosomes (whether they be heteromorphic or homomorphic) and it follows from this that the mechanisms of TSD and GSD are mutually exclusive (Bull, 1983). Transitions between the two modes of sex determination involve transitionary forms that are thought to be ephemeral, with the passage between the two along neutral pathways (Bull, 1990, 1991; Quinn et al., 2011), pathways supported by Fisher's frequency dependent selection (Dissanayake et al., 2021a, 2021b; Holleley et al. 2015; Pennell et al., 2018; see Schwanz & Georges, 2021 for review, see chapter 3 and 5).

Recent work has suggested that a continuum of states may exist between the endpoints of GSD and TSD (Barske & Capel, 2008; Sarre et al., 2004, 2011) (Figure 1.3). At intermediate points along with the continuum spectrum, it is proposed that sex can be determined by the interaction of genotype and environment. This goes beyond their being a genetic propensity to be one sex or the other varying across individuals and hidden by an over-riding influence of temperature, which is essentially the TSD condition. In at least two species, temperature influences sexual fate in species with known sex chromosomes (Quinn et al., 2007; Radder et al., 2008; Shine et al., 2002), suggesting that the division between GSD and TSD may not be as clear as previously thought (Sarre et al., 2004; Shine et al., 2002). In particular, the *P. vitticeps* has a ZZ/ZW genetic mode of sex determination (Ezaz et al., 2005) which is overridden by the temperatures in higher thermal extremes (Quinn et al., 2007). The W chromosome is not required for female development. In a second case, *Bassiana duperreyi* has XY chromosomes, but XX individuals are reversed to phenotypic males at low incubation temperatures (Quinn et al., 2009; Radder et al., 2008; Shine et al., 2002).

1.5 Sex reversal

The process whereby an offspring develops phenotypic gonadal sex opposite to which would normally occur under the influence of sex chromosomes because of environmental influences is referred to as sex reversal. Individuals with a gonadal phenotype that is the same as that expected from their sex chromosomes are referred to as concordant; those with a gonadal phenotype that is the opposite of that expected from their sex chromosomes are referred to as discordant. The term sex class is used to refer to the combinations of phenotypic and genotypic sex. TSD and sex reversal under the influence of temperature is referred to as thermolabile sex.

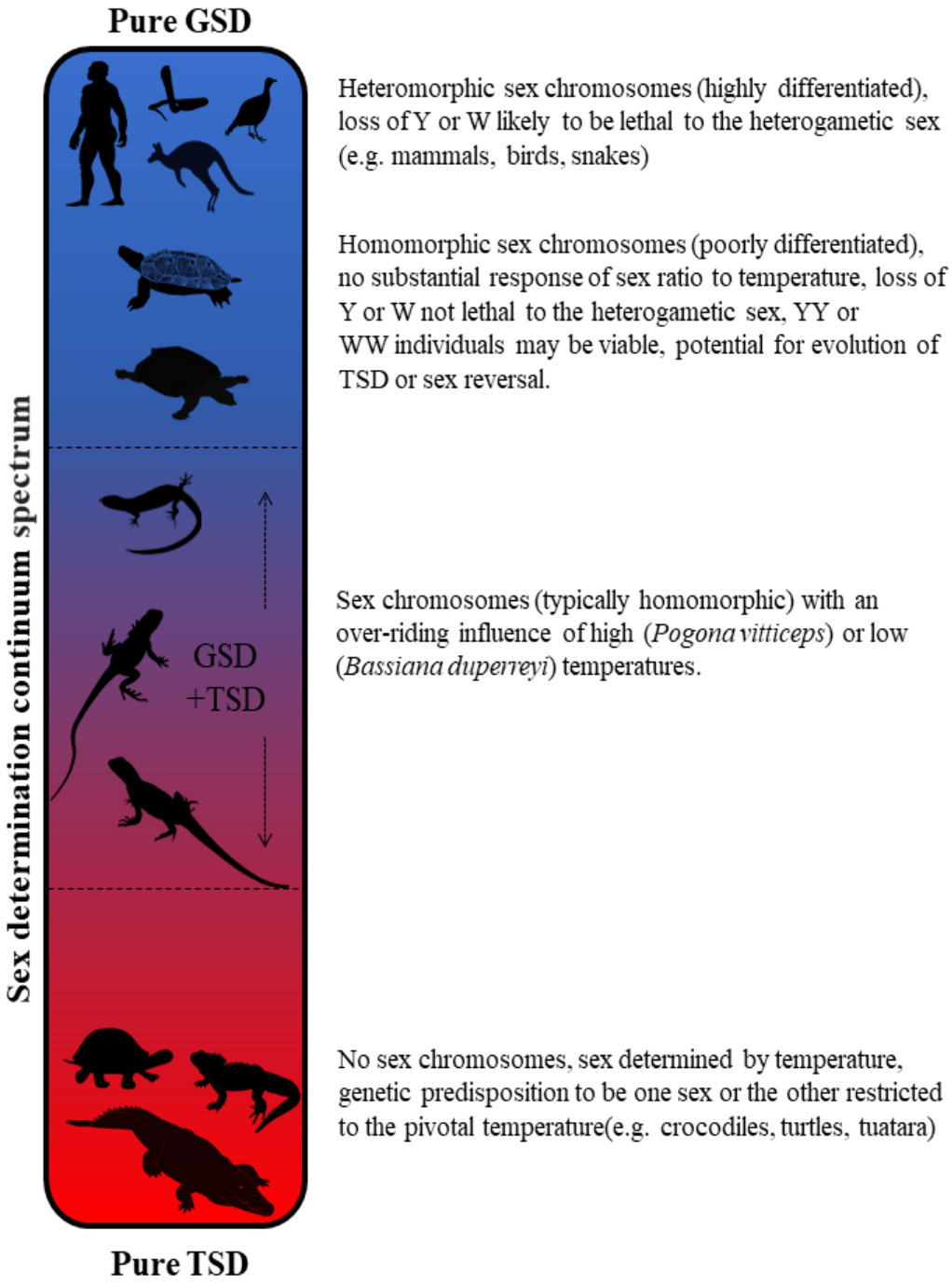

Figure 1-3. GSD+TSD with Sex reversal continuum spectrum modified from Norris & Jones, 2012. TSD and temperature-induced sex reversal are collectively referred to as thermolabile sex.

Sex reversal is a common phenomenon in many vertebrate species, and this could occur due to environmental influences on their GSD system or as a mutational event. For an example, in birds and mammals, sex reversal occurs mostly as point mutations in single genes or mutations in the sex chromosome (Parma et al., 2016). Theoretical and empirical evidence shows that many environmental factors induce sex reversal in many vertebrate species, leading to rapid evolution (Holleley et al., 2016; Holleley et al., 2015; Ogata et al., 2003; Perrin, 2009).

Sex reversal occurs in many fish species responding to temperature, exposure to endocrine-disrupting chemicals causing imbalances in sex ratios, and social status (Brown et al., 2015; Cotton & Wedekind, 2009; Hattori et al., 2018; Shapiro, 1981; Todd et al., 2016). Sex reversed XY females mating with normal XY males can result in YY offspring known as supermales (Cotton & Wedekind, 2009; Wedekind, 2012). These YY supermales may be non-viable or infertile (George & Pandian, 1996; George et al., 2013) or viable and fertile (Chevassus et al., 1988; Hattori et al., 2010; Kavumpurath & Pandian, 1993; Mair et al., 2011; Yamamoto, 1975). Amphibians are recognised as the second most sex reversed group and recent research concluded that amphibians have strict GSD influenced by various environmental factors (Bachtrog et al., 2014; Capel, 2017; Evans et al., 2012; Hayes, 1998; Lambert et al., 2019; Nakamura, 2009). Their sex reversal can occur in response to extreme temperature (Dournon et al., 1990; Hsü et al., 1971; Lambert et al., 2018; Witschi, 1930), anthropogenic chemicals (Hayes et al., 2002; Hermelink et al., 2010; Lambert, 2015; Lambert et al., 2016; Pettersson & Berg, 2007; Tamschick et al., 2016) and by natural causes (Lambert et al., 2019). The emerging studies on sex reversal in amphibians show that this phenomenon may be an important process for amphibian evolution (Perrin, 2009). The presence of YY individuals in a Japanese tree frog (*Hyla japonica*) natural population is an indication of sex reversal in the wild (Kawamura & Nishioka, 1977). No cases of viable YY or ZZ individuals have been reported in reptiles.

1.5.1 Sex reversal in reptiles

Sex reversal was suspected in turtles based on screening for H-Y antigens (Servan et al., 2011; Zaborski et al., 1982), but it was not conclusively demonstrated in reptiles until recently (Holleley et al., 2015; Quinn et al., 2007) and confirmed only in only two Australian reptiles, *Pogona vitticeps* and *Bassiana duperreyi* (Holleley et al., 2015; Quinn et al., 2007; Radder et al., 2008; Shine et al., 2002). Several other species are suspected of

having sex reversal, awaiting confirmation, such as the yellow-bellied water skink (*Eulamprus heatwolei*) (Cornejo-Páramo et al., 2020), the common collared lizard (*Crotaphytus collaris*) (Wiggins et al., 2020), the multi-ocellated racerunner (*Eremias multiocellata*) (Wang et al., 2015), the Japanese gecko (*Gekko japonicus*) (Tokunaga, 1985), and the spotted snow skink (*Niveoscincus ocellatus*) (Hill et al., 2018).

The naturally occurring temperature-sex reversal process has been definitively recognised only in two reptiles: *P. vitticeps* and *B. duperreyi* (Figure 1.4). They possess contrasting systems of thermosensitive sex determination. *Pogona vitticeps* is an agamid dragon lizard with a female heterogametic (ZZ/ZW) GSD system, but high incubation temperatures (> 32°C) result in reversal of the ZZ male genotype to a female phenotype (Holleley et al., 2015; Quinn et al., 2007). In contrast, *B. duperreyi* has a male heterogametic (XX/XY) GSD system in which low incubation temperatures (< 20°C) result in reversal of the XX genotype to a male phenotype (Radder et al., 2008; Shine et al., 2002).

The pioneer modelled species for sex reversal, *P. vitticeps*, sex reversal and the potential loss of the W chromosome is expected to occur well within the range of temperatures experienced in nature (Holleley et al., 2015), and the transition from GSD to TSD appears impeded by local adaptation in the propensity to reverse (Castelli et al., 2020). Sex reversed individuals of *P. vitticeps* are fertile, and ZZ x ZZ crosses can be used to generate viable lines in which the W chromosome is absent. Also at least one population of *P. vitticeps*, in which the sex reversed ZZ female phenotype is fertile, appears to be on the brink of such transition (Holleley et al., 2015).

Furthermore, modelling the consequences of sex reversal has shown that the W chromosome can persist indefinitely at low frequency in the population, particularly if there is immigration from adjacent populations that remain predominantly GSD (Schwanz et al., 2020). So, the transition from GSD to TSD is not a unidirectional process, with the residual and cryptic presence of the W chromosome enabling a rapid halt to the transition and reversion to GSD if and when conditions change. They were existing sex-reversed individuals in colder geographic areas, suggesting that such an arrangement may have previously occurred in the species and remains a multifactorial sex determination system (Castelli et al., 2020).

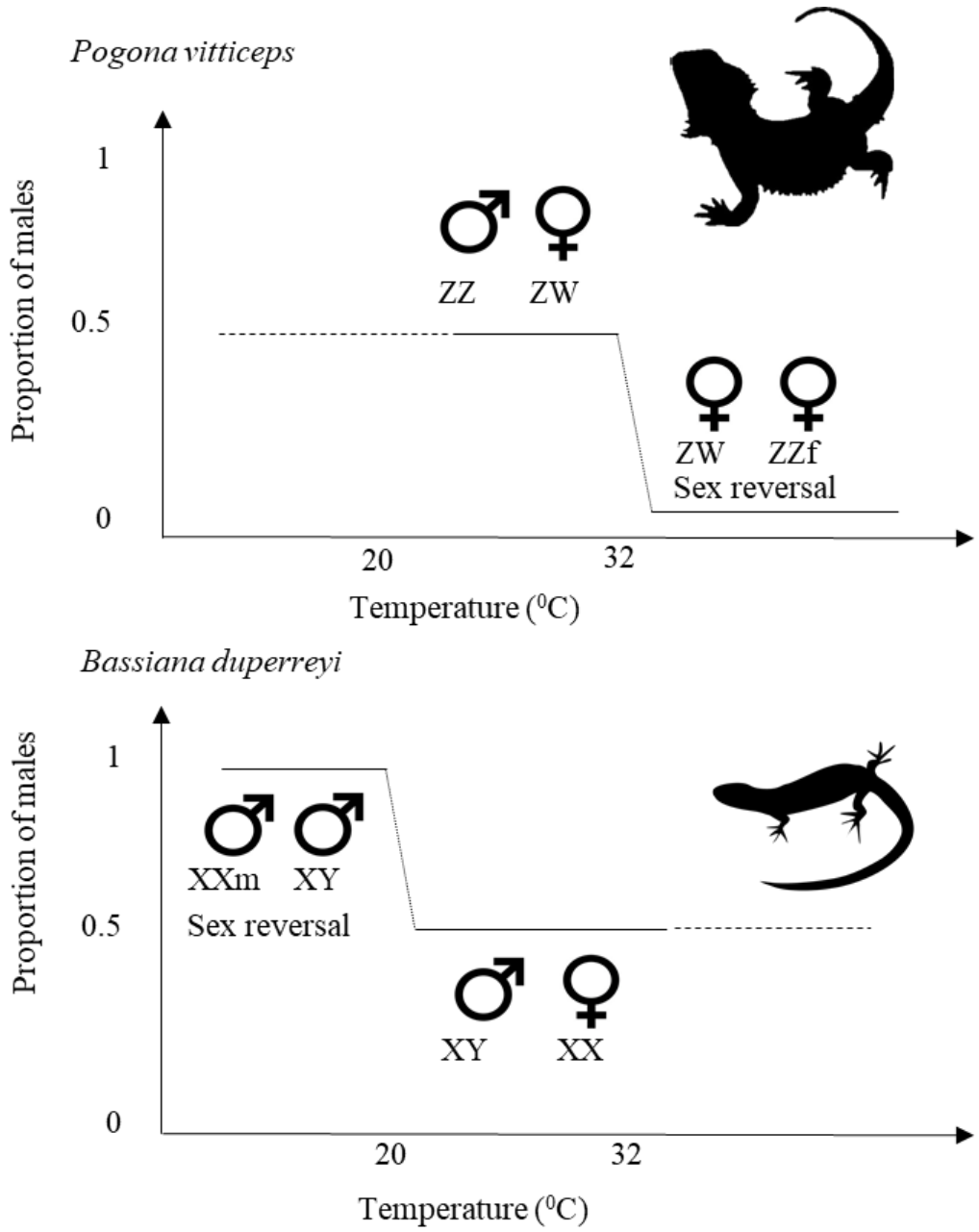

Figure 1-4. Schematic diagram of sex reversal in *Pogona vitticeps* and *Bassiana duperreyi*. In *P. vitticeps* (top) sex reversal occurs when an individual with a male genotype (ZZ) is incubated at temperatures above 32°C, causing it to develop as a phenotypic female. In *B. duperreyi* (bottom) an individual with a female phenotype (XX) incubated at low temperatures will reverse its sex and develop as a phenotypic male.

1.6 Sex-linked sequence and PCR sex tests

Cytogenetic methods (e.g., metaphase chromosome staining or banding, or comparative genomic hybridisation) of sex identification require considerable time, specialized techniques and consumables. In some cases, where the differences between male and female chromosomes are small, or where microchromosomes are the sex chromosomes (Ezaz et al., 2005), these approaches are technically challenging. During the past few decades, various sequencing and bioinformatic approaches have been used to identify sex-linked and sex-specific sequence that can be characterized and used to identify the sexual genotype of individuals. This technology is particularly important in demonstrating sex reversal. Isolating sex specific markers has been of enormous help in understanding sex chromosome systems of many animal and plant species (Charlesworth, 2015) and they can be applied to all life stages, such as embryos or juveniles (Rovatsos & Kratochvíl, 2017). Many species of reptiles lack overt sexual dimorphic characters or become dimorphic for a short period of breeding (Frýdlová et al., 2011; Garland, 1985).

Random amplified polymorphic DNA fingerprinting (RAPD) (Welsh & Mcclelland, 1990; Lind & Welsh, 1994) and the amplified fragment length polymorphism (AFLP) (Chen et al., 2012; Griffiths, 2000; Quinn et al., 2009) were among the first PCR-based DNA fingerprinting techniques. But these techniques have certain drawbacks such as, mismatching between the primer and the template which results in the total absence of PCR product, lack of reproducibility due to mismatch annealing and difficulty in developing locus-specific markers from individual fragments. Also, result interpretation of these methods is difficult.

With the rapid development of next-generation sequencing (NGS) technologies, novel methods have been developed for screening sex-linked or sex-specific DNA fragments. Many of these studies have used Restriction Site-Associated DNA sequencing (RAD-seq), double digest restriction-site associated DNA sequencing (ddRAD-seq) (Gamble & Zarkower, 2014; Gamble et al., 2014; Peterson et al., 2012) and DArT-seq (Lambert et al., 2016; Sopniewski et al., 2019) a combination of genome complexity reduction and next-generation sequencing method (which is similar to ddRAD-seq) (Kilian et al., 2012) to isolate sex specific markers in reptiles to understand the sex chromosome evolution and sex determination (Gamble & Zarkower, 2014; Lambert et al., 2016, 2019; Palaiokostas et al., 2013). But using RAD-seq pipeline to isolate a definite part of the

genome or sex specific region from a non-model organism has a major challenge as it only uses one or two restriction enzymes to cut six or eight base pairs. The technique is representational and can fail to isolate many unique regions from the genome, notably for species with short linkage disequilibrium (Lowry et al., 2017).

Identifying sex reversal species can be difficult, and a lack of promising techniques to identify sex chromosome sequences from autosomal sequences is one of the major drawbacks for the current workflow. Therefore, instead of using above discussed pipelines and the whole reference genome, we developed a simple, promising, and non-representational approach using a whole genome subtraction pipeline. This isolates putative sex-specific marker sequences forms which are embedded in large genomic contexts (see chapter 2). This new method shortens the working time significantly, and when compared to the cost of RAD-seq or ddRAD-seq, this method has a reliable low cost. Also, this in silico method is more straightforward than the Genomic Representational Difference Analysis (gRDA) Hollestelle & Schutte, 2005). The subtractive genomics principles have been successfully used to identify targets in various human bacterial pathogens (Chong et al., 2006; Dutta et al., 2006; Sakharkar et al., 2004), to identify the potential tumour antigen candidates and cancer-specific genes (Kawakami et al., 2004; Mirvish & Shuda, 2016; Nishimura et al., 2015). Further, we used another method (see Appendix 6) on read copy number variation across the genome to identify the half copy number in the XY individuals compared to the XX individuals after screening out repetitive sequence. As such, it is complementary to the genome subtraction approach reported in this thesis.

1.7 *Bassiana duperreyi* as a study species

The eastern three-lined skink, *Bassiana duperreyi* (Gray, 1838), (Figure 1.5.A), is a medium-sized (~70mm, SVL), oviparous scincid lizard, distributed broadly across southern and south-eastern Australia (Cogger, 2018), including areas impacted by the 2020 megafires (Figure 1.5.B). The species occurs at cool high-elevation sites (1050 m a.s.l.) close to the upper elevational limits for oviparous reproduction by Australian lizards (Shine & Harlow, 1996); its distribution extends through an altitudinal gradient to coastal regions of NSW and Victoria. *Bassiana duperreyi* is currently regarded as a single species (but see chapter 4).

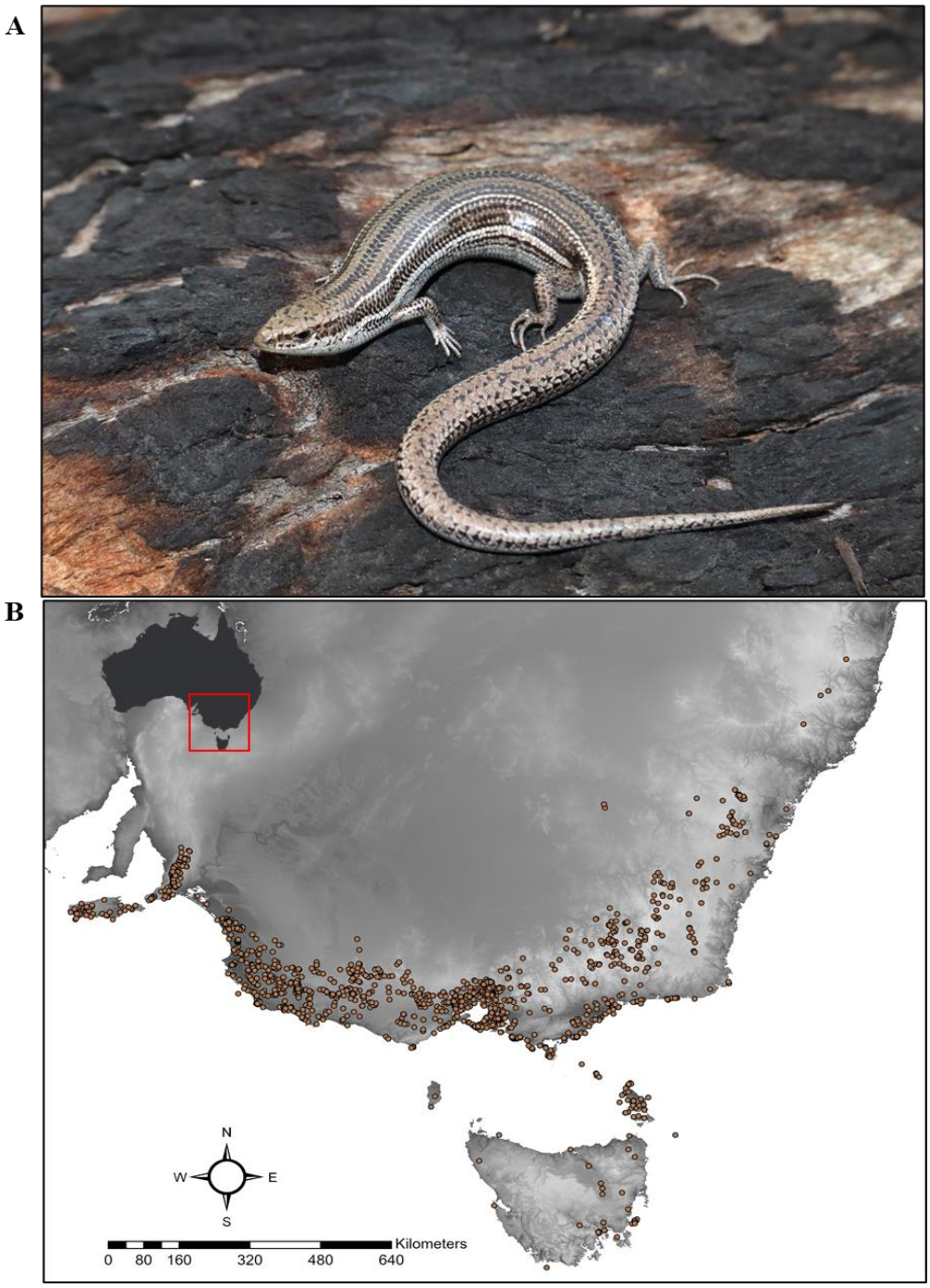

Figure 1-5. Study species and distribution. A. Adult eastern three-lined skink (*Bassiana duperreyi*) from Cape Conran; B. *Bassiana duperreyi* distribution. Recorded locations are indicated by brown dots. Underlying map generated using ArcGIS 10.5.1 (http://www.esri.com) and data from the Digital Elevation Model (Geoscience Australia) made available under Creative Commons Attribution 3.0 Australia (https://creativecommons.org/licenses/by/3.0/au/legalcode, last accessed 9-Jul-20).

Bassiana duperreyi has genotypic sex determination, with male heterogamety (Donnellan, 1985; Matsubara et al., 2016). The XX genotype, which typically yields a female gonadal phenotype, can be reversed to yield a male phenotype by low incubation temperatures (Radder et al., 2008; Shine et al., 2002). Fluctuating laboratory incubation temperatures designed to mimic the conditions experienced in the coolest nests at the highest elevations of its range produced over 70% males. Conversely, hotter incubation temperatures that mimic lower elevation nests produced 50% of offspring sex ratios that did not depart significantly from 1:1, consistent with the Mendelian segregation of sex chromosomes (Shine et al., 2002). In 2008, further work confirmed that in this male-biased sex ratio, genetic XX females' phenotypes are reversed, producing XX phenotypic males (Radder et al., 2008).

The story becomes even more interesting when recording sex reversal in the high elevation nests with 28% XX male (Holleley et al., 2016), but whether this translates to equally high rates of sex-reversed adults in the natural nests in its distribution was poorly studied until this study was initiated. The temporal scale of these combined demographic and evolutionary changes makes these processes challenging to study. However, it is possible to use elevational gradient and (altitudinal or latitudinal) climatic trends as a surrogate for how species might respond to temporal trends (Castelli et al., 2021; Doody et al., 2006). *Bassiana duperreyi* is distributed along an elevation gradient from higher alpine to lower coastal, which usually face drastic climatic changes than the other regions where this species lives. Therefore *B. duperreyi* is one of the best models to understand how natural climatic change influences on sex reversal (GSD and TSD transition) in the wild.. During this project, our published and related data helped to stimulate the development of predictive models that would help predict global climate change on reptile populations in Australia in the future. This project will also serve as a model for species at risk of extinction from climate change to focus on future research to test on-the-ground management strategies to mitigate climate change effects in local populations under various local disturbances, such as bush fires.

1.8 Thesis aim and objectives:

The aim of the study reported in this thesis was to understand sex reversal of *Bassiana duperreyi* under natural environmental conditions and to explore the implications of sex reversal for the species ecology, evolution and conservation.

Specifically, the objectives of the study were:

1. To use subtractive genomics to identify sex specific markers in *Bassiana duperreyi*, to characterize these, and develop a robust PCR sex test for the species.
2. To apply the sex test to adults and hatchlings of *Bassiana duperreyi* in wild populations along an altitudinal and latitudinal gradient (Australian alps to coastal Melbourne) to determine variation in the frequency of sex reversal in determining the primary sex ratio and the level of penetration of sex reversed individuals into the adult population.
3. To develop demographic and evolutionary models to evaluate the ecological and evolutionary consequences of sex reversal in *Bassiana duperreyi* and the impact arising from climate change including changes in the frequency of local disturbance.

1.9 Thesis structure

This thesis is structured and presented as a series of scientific manuscripts, with the exception of the General Introduction (chapter 1) and the Synopsis (chapter 6). The publications or manuscripts for which I led the research and writing of the manuscript, and for which I am the first author, are included as chapters; manuscripts I contributed to substantially and as a co-author are included as Appendices.

Chapter 1 provides a brief overview of sex determination and sex reversal in reptiles, introduces the study species, and states the thesis aim objectives.

Chapter 2 reports a novel technique known as *in silico* whole genome subtraction to identify sex specific markers for *B. duperreyi*. This fulfils the objective 1 of my thesis. We successfully isolated seven Y chromosome specific contigs. Then we developed PCR sex tests to identifying XX male from XY male using these molecular markers. This technique complements an alternative complementary genomic approach used in collaboration with Diego Cortez to develop a PCR sex test for identifying the Y chromosome in *E. heatwolei*, a genus thought to be TSD (Cornejo-Páramo et al., 2020; Appendix 1). The Y chromosomal region is 79–116 Myr old and shared between *B. duperreyi*, water and spotted skinks. This latter work is provided in Appendix 1.

Chapters 3 and 5, report on the application of the sex-specific PCR test to understand sex reversal frequency of *B. duperreyi* adults and hatchlings (nests) in the wild,

respectively, along an altitudinal gradient. These two chapters fulfil my second objective. In particular, Chapter 3, reports modelling of the consequences of sex reversal in the wild population and the potential for loss of Y chromosome owing to sex reversal, and the demographic consequences for this species, which fulfils objective 3 of my thesis.

Chapter 4 reports a population genetic analysis for *B. duperreyi* based on 12,532 reliable polymorphic SNP loci from 60 populations (263 individuals) covering the distribution of Australia's eastern three lined skink, *B. duperreyi*. This chapter provides foundational work for the detailed taxonomic re-evaluation of *B. duperreyi* and its relevance to conservation. More specifically, it allowed me to be sure that the correlations between sex reversal and elevation were interpreted in the context of a single species, and that the effects were direct and not a result of a strong underlying phylogenetic signal. This chapter fulfils the third objective of this thesis.

Chapter 2

Identification of Y chromosome markers in the eastern three-lined skink (*Bassiana duperreyi*) using in silico whole genome subtraction

This chapter was published as: Dissanayake, D. S. B., Holleley, C. E., Hill, L. K., O'Meally, D., Deakin, J. E., & Georges, A. (2020). Identification of Y chromosome markers in the eastern three-lined skink (*Bassiana duperreyi*) using in silico whole genome subtraction. *BMC genomics, 21*(1), 1-12. Apart from formatting, this chapter is identical to the published work.

2.1 Abstract

Homologous sex chromosomes can differentiate over time because recombination is suppressed in the region of the sex-determining locus, leading to the accumulation of repeats, progressive loss of genes that lack differential influence on the sexes and sequence divergence on the hemizygous homolog. Divergence in the non-recombining regions leads to the accumulation of Y or W specific sequence useful for developing sex-linked markers. Here we use *in silico* whole-genome subtraction to identify putative sex-linked sequences in the scincid lizard *Bassiana duperreyi* which has heteromorphic XY sex chromosomes. We generated 96.7 x 10^9 150 bp paired-end genomic sequence reads from a XY male and 81.4 x 10^9 paired-end reads from an XX female for *in silico* whole genome subtraction to yield Y enriched contigs. We identified 7 reliable markers which were validated as Y chromosome specific by polymerase chain reaction (PCR) against a panel of 20 males and 20 females. The sex of *B. duperreyi* can be reversed by low temperatures (XX genotype reversed to a male phenotype). We have developed sex-specific markers to identify the underlying genotypic sex and its concordance or discordance with phenotypic sex in wild populations of *B. duperreyi*. Our pipeline can be applied to isolate Y or W chromosome-specific sequences of any organism and is not restricted to sequence residing within single-copy genes. This study greatly improves our knowledge of the Y chromosome in *B. duperreyi* and will enhance future studies of reptile sex determination and sex chromosome evolution.

Keywords: Sex-specific markers, Sex reversal, Genotypic sex determination, Y chromosome

2.2 Introduction

Most vertebrates reproduce sexually with distinct male and female phenotypes that arise from the complement of chromosomes that are inherited from their parents. These species are said to have their sex determined genotypically (GSD), and the influential genes reside on sex chromosomes that typically assort randomly during meiosis. In the absence of differential investment by the parents in male and female offspring, this system yields an evolutionarily stable 1:1 primary offspring sex ratio (Düsing, 1884; Fisher, 1930; Edwards, 2000).

Sex chromosomes are thought to evolve from autosomes when genes they carry assume the role of determining sex (Ohno, 1967). What follows over time is a chain of mutational events on the hemizygous member of the sex chromosome pair, leading to the accumulation of genes that afford a fitness advantage to the heterogametic sex, a fitness disadvantage to the homogametic sex, suppression of recombination, the accumulation of repetitive sequence, and progressive loss of gene function unrelated to sex (Charlesworth et al., 2005; Abbott et al., 2017). In humans, for example, the non-recombining region of the Y chromosome contains 78 protein coding genes encoding 27 proteins (Skaletsky et al., 2003) compared with the 699 protein-coding genes with known function on the X (Ross et al., 2005); the human Y is smaller than the X and highly heterochromatic.

Unlike mammals, squamates show a remarkable diversity in sex chromosome structure, representing various degrees of differentiation in sex homologs (Olmo, 2008; Ezaz et al., 2009a; O'Meally et al., 2012; Alam et al., 2018; Deakin & Ezaz, 2019). Such heterogeneity is brought about by variation in the evolutionary age of lineages with independently evolved sex chromosomes (O'Meally et al., 2012; Ezaz et al., 2017). In many squamate species with GSD, the sex chromosomes are homomorphic and cannot be distinguished using conventional karyotyping methods such as G or C-banding (Ezaz et al., 2009b; Pokorná & Kratochvíl, 2009). In others, macroscopic differences may exist, but the sex chromosomes are microchromosomes and go undetected until more sensitive techniques, such as comparative genomic hybridisation, are applied (Traut et al., 2001; Ezaz et al., 2005). Suppression of recombination along all or part of the sex chromosome length allows homologous sequences to diverge over time (Wright et al., 2016). Differences between sex chromosome homologues can be substantial as in human and mouse (Guillon & de Massy, 2002; Jeffreys et al., 2001) or very slight, involving even a

single nucleotide polymorphism in an influential gene, as for *Amhr2* in the pufferfish *Takifugu rubripes* (Kikuchi et al., 2007; Kamiya et al., 2012). For these reasons, identifying the sex chromosomes and candidate sex- determining genes can be challenging, particularly for organisms that lack a reference genome. Sex-linked markers provide one important avenue for the identification of sex chromosomes and sequences that may include candidate sex-determining genes (Palaiokostas et al., 2013; Gamble & Zarkower, 2014; Shi et al., 2018).

Various approaches have been used to identify sex-linked markers in non-classical model organisms. Random amplified polymorphic DNA fingerprinting (RAPD) (Welsh & McClelland, 1990; Williams et al., 1990; Martinez et al., 1999) and amplified fragment length polymorphisms (AFLP) (Griffiths et al., 1999; Griffiths, 2000; Quinn et al., 2009) are PCR-based DNA fingerprinting techniques that sample only a fraction of the whole genome. While useful, these techniques have some drawbacks such as poor reproducibility owing to mismatches between primer and template, and difficulty in developing locus-specific markers from individual fragments. Having no knowledge of the genomic context of the typically short markers can also render interpretation difficult.

With the development of next-generation sequencing technologies, new methods have been developed for screening sex linked DNA. For example, assaying for sex-specific expressed genes by RNA-seq (Ayers et al., 2013) or whole genome sequencing based approaches that rely on differences in mapped read depth (Chen et al., 2012; Bidon et al., 2015). Restriction Site-Associated DNA sequencing (RAD-seq) or double digest restriction-site associated DNA sequencing (ddRAD-seq) is increasingly common (Gamble & Zarkower, 2014; Gamble et al., 2014; Peterson et al., 2012; Carmichael et al., 2013; Brown et al., 2016; Fowler & Buonaccorsi, 2016; Gamble et al., 2015; Hime et al., 2019; Luo et al., 2020) as is DArT-seq (Kilian et al., 2012; Lambert et al., 2016; Sopniewski et al., 2019) when searching for sex-linked sequence. These RADseq and reduced representational approaches assess only a limited portion of the genome, and may miss many markers, particularly in species with small sex-specific domains or those with micro-sex chromosomes (Lowry et al., 2017).

Here, we report an *in silico* approach to isolate sex specific markers based on sequence unique to the Y or W chromosome, analogous to genomic representational difference analysis (gRDA) (Hollestelle & Schutte, 2005). Subtractive genomic approaches

have been used to identify targets in various human bacterial pathogens (Sakharkar et al., 2004; Dutta et al., 2006; Chong et al., 2006; Isakov et al., 2011) and identify potential tumour antigen candidates and cancer-specific genes (Kawakami et al., 2004; Nishimura et al., 2015; Kakimi et al., 2017; Mirvish & Shuda, 2016). Our study is the first to apply the subtraction approach for identifying the Y chromosome specific sequence in a reptile, the eastern three-lined skink (*Bassiana duperreyi*). The species has heteromorphic XY sex chromosomes (Donnellan, 1985). Identifying sex-specific markers for this species is of particular interest because XX individuals develop as males at low temperatures (Shine et al., 2002; Radder et al., 2008). Quinn et al., (2009) developed AFLP markers for *B. duperreyi*, however, the fragments are short and difficult to amplify reliably. Here, we use low depth whole genome sequencing of a male and a female *B. duperreyi* to apply an *in silico* whole genome subtraction approach, and develop new practical markers, useful in ongoing studies of this species in the laboratory and the wild.

2.3 Materials and methods

2.3.1 Samples

The eastern three-lined skink, *B. duperreyi*, is a medium-sized (80 mm snout–vent length) lizard widely distributed through south eastern Australia, from the coast to montane cool-climate habitats (Cogger, 2014). Adult individuals (n = 76) were captured by hand at Piccadilly Circus (35°21'37.59"S, 148°48'13.39"E, 1246 m a.s.l.) in Namadgi National Park, 40 km west of Canberra in the Australian Capital Territory, and from Anglesea (38°23'26.76"S, 144°12'52.29"E, 40 m a.s.l.) in Victoria (Figure 2.1, Appendix 2: Table S1). The Anglesea population is a distinct mitochondrial lineage from the Piccadilly Circus lineage (*ca* 3 Myr divergent, Dubey & Shine, 2010).

Snout-vent length was measured with Vernier callipers (+/- 1 mm) and males identified by hemipenal eversion (Harolow, 1996) and breeding colouration. A representative male and female from Piccadilly Circus (focal individuals) were transported to the University of Canberra animal house where each was euthanised by intraperitoneal injection of sodium pentobarbitone (100-150 µg/g body weight), dissected, and phenotypic sex confirmed by examination of the gonads. Tail tips (4-5 mm) were removed with a sterile blade, a portion stored in 95% ethanol at -20˚C, and a portion set aside for cell culture. Tail-snips were removed also from an additional 24 males and 24 females from Piccadilly Circus and 10 males and 10 females from Anglesea and stored in 95% ethanol -

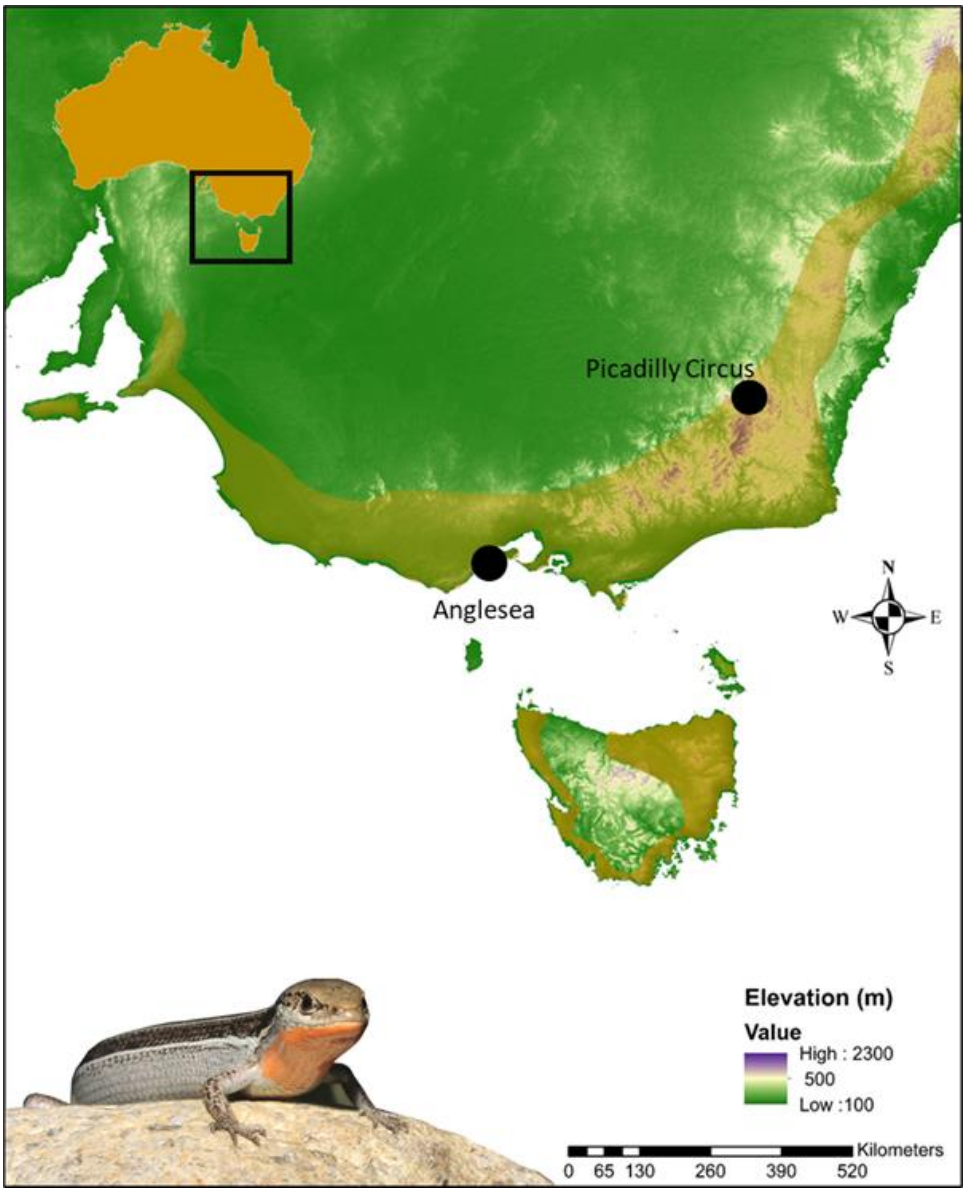

Figure 2-1. *Bassiana duperreyi* sampling localities (black circles) from which the focal and validation individuals in this study were sourced. The species approximate distribution range is indicated by the shaded area. Underlying map generated using ArcGIS 10.5.1 (http://www.esri.com) and data from the Digital Elevation Model (Geoscience Australia) made available under Creative Commons Attribution 3.0 Australia (https://creativecommons.org/licenses/by/3.0/au/legalcode, last accessed 9-Jul-20). The adult male *B. duperreyi* photo was taken by the first author at the Piccadilly Circus, ACT, Australia.

at -20˚C. All animals were released to the capture sites. These are referred to as the validation animals. A portion from three males and three females from Piccadilly Circus were set aside for cell culture and karyotyping.

For cell culture, tail tips were immediately transferred to 10 ml of collection medium (Gibco Dulbecco's Modified Eagle Medium; Thermo Fisher Australia Pty Ltd, Scoresby, Victoria, Australia) with 2.5 µg/ml of Antibiotic Antimycotic Solution (Sigma Chemical Company, St. Louis, USA) and incubated at room temperature for 24 h (Ezaz et al., 2008) before the metaphase chromosomes preparation (see validation of phenotypic sex identification in methods).

2.3.2 DNA extraction, sequencing, and *in silico* whole genome subtraction

DNA was extracted from fresh liver samples of the two focal animals and from the tail snips of the 60 validation animals using the Gentra Puregene Tissue Kit (QIAGEN, Australia) following manufacturer protocols. DNA suspensions were assessed for purity using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, 19810, USA) and quantified using Qubit 2.0 fluorometer (Invitrogen, Life technologies, Sydney, NSW, Australia). Library preparation and sequencing were performed at the Biomolecular Resource Facility at the Australian National University (Canberra, ACT) using the Illumina HiSeq 2000 platform yielding 150bp paired end reads.

Reads from the focal male and the focal female were analysed independently as follows (Figure 2.2). First, overlapping read pairs were combined into fragments then decomposed into k-mers of 27 bp using Jellyfish 2.0 (Marçais & Kingsford, 2011). Unique k-mers were counted, again using Jellyfish 2.0 and k-mers in common between the male and female sets were removed from the male set. This yielded a (subtracted) k-mer set that was enriched for Y chromosome sequence. Strictly, the subtracted k-mer set contains k-mers that are from Y chromosome sequence admixed with k-mers representing polymorphic differences between the female X chromosomes and the male X chromosome. K-mers in the subtraction with a count less than 2 for males and 5 for females were considered to represent sequencing errors and were removed from the analysis. This decision was based on examination of the k-mer spectra, identifying the minima immediately to the right of the peak arising from presumed read errors. This is not a critical decision.

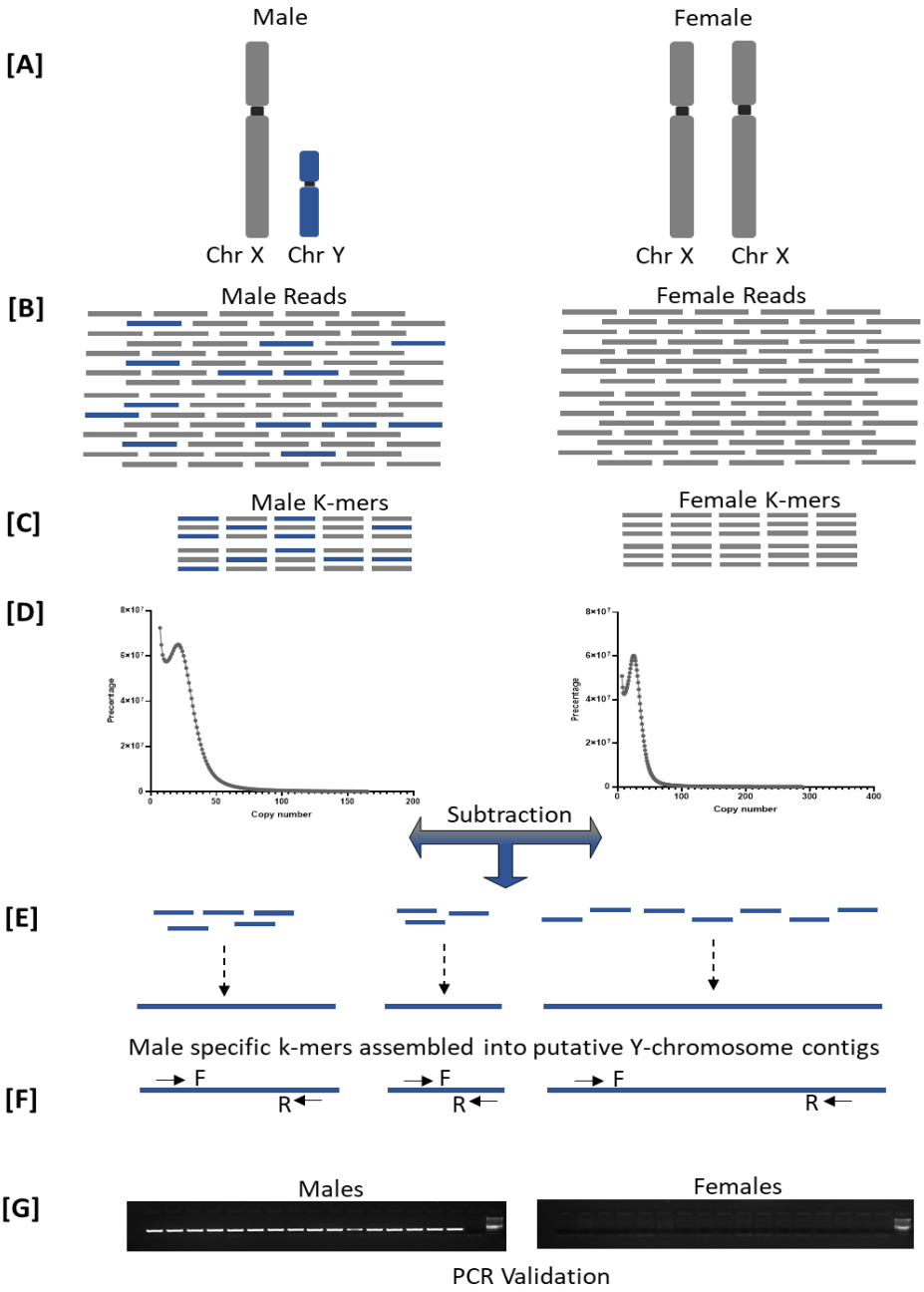

Figure 2-2. Schematic diagram showing methodology of the genome subtraction pipeline a. A hypothetical schematic of the *B. duperreyi* sex chromosomes with the male specific gene region indicated in blue (not to scale); b. Low coverage whole genome sequencing was conducted on an Illumina platform resulting in approximately 8X coverage; c. The raw sequencing reads are decomposed into 27 base pair k-mers; d. The kmer spectrum is plotted and sequences with low counts are removed; e. Female k-mers are subtracted from the male k-mers. Male specific kmers are retained and then assembled into putative Y-chromosome contigs; f. Primers are designed on putative male contigs g. PCR sex test and validation (image shown here is for illustrative purposes only).

Select it too high, and the risk is that some important k-mers will be eliminated from the re-assembly of Y enriched kmers. Select it too low, and the cost is inclusion of low count kmers from reads containing errors and a greater noise to signal ratio. This does not affect the outcome, just the computational resources required for subtraction and reassembly.

The remaining Y enriched k-mers were then reassembled into contigs using an inchworm assembler (kassemble.cgi, https://doi.org/10.5061/dryad.pvmcvdnj1) with stringent extension criteria. Briefly, the assembler initially took a focal k-mer at random and searched for other k-mers that matched exactly k-1 bp of the focal k-mer. If this second k-mer was unique, then the focal k-mer was extended by one bp, and the process was repeated. If the k-mer was not unique, then the extension process was terminated. The extension occurred to both the left and the right, yielding relatively short contigs (up to ca 1400 bp) that contain sequence unique to the male individual.

2.3.3 PCR validation

To validate the sex specificity of each of the contigs and remove false positives derived from autosomal and X chromosome polymorphisms, we designed primers for each contig using Primer 3 Untergasser et al., 2012 implemented in Geneious (Kearse et al., 2012) (version R8). We then applied these presence/absence PCR tests in the validation animals using the following conditions. Each reaction contained 1x My Taq HS Red mix (Bioline), 4 µM each primer and 25 ng of genomic DNA. The PCR cycling conditions used an initial touchdown phase to increase the specificity of amplification: denaturing at 95˚C, annealing temperature stepping down from 70˚C by 0.5˚C for 10 cycles, extension at 72˚C. This was followed by 30 cycles at 65˚C annealing and 72˚C extension.

The PCR screening process was conducted in three stages. To confirm that the subtraction pipeline had successfully identified a presence/absence polymorphism in the two focal individuals, we first screened those two individuals to confirm presence of an amplified fragment in the male and the absence of an amplified fragment in the female. We then screened a panel of an additional 4 male and 4 female individuals for putative sex-linked markers showing a male-specific positive pattern. In a third step, we screened those putative markers on a further 20 males and 20 females from Piccadilly Circus. At each of the stages, the loci that did not appear as sex specific were eliminated as candidate sex markers. The probability of an autosomal or X chromosome polymorphism being present in the focal male, 4 males and 20 additional males, and absent in the focal female, 4

females and 20 additional females, is sufficiently low ($\leq 0.25^{24}$, maximal for autosomal or X allele frequency = 0.5) to eliminate false positives, despite the error rate compounding over multiple markers. Thus, male specific markers that survive the validation process are Y-specific markers.

To confirm the amplification of the desired sequence, PCR products for all 7 putative Y-loci were visually assessed using gel electrophoresis and then Sanger sequenced in a single direction, using the forward primer, on an AB 3730xl DNA Analyzer at the Biomolecular Research Facility, Australian National University, Canberra, Australia. We sequenced 4 male individuals from Piccadilly Circus (Namadgi National Park, ACT) and 4 male individuals from Anglesea (Victoria).

2.3.4 Validation of phenotypic sex identification

The phenotypic sex of each of the karyotyped animals was confirmed by gross examination of gonads followed by histological examination. Dissected gonads were dehydrated through graduations of ethanol (70%, 90%, 100%) and two changes of xylene for 45 min each, before being embedded in paraffin wax, and sectioned 5 to 6 μm using a Leica Rotary Microtome (Leica Microsystems Pty Ltd, Waverley, Australia). Slides were stained with haematoxylin and eosin, with a staining time of 2-3 min in haematoxylin, and 10 dips in 0.25% eosin in 80% ethanol, before being mounted in Depex medium (BDH Laboratory Supplied, England). Gonads were characterized according to standard cellular structures (Doddamani, 1994; 2006).

Karyotyping was carried out by examining metaphase chromosomes prepared from fibroblast cell lines of tail tissues as outlined by Ezaz et al., (2009) with minor modifications. Briefly, three replicate subsamples for each individual were made using sterile scalpel blade. The individual subsamples were transferred to separate T25 culture flasks with 1.5 ml Amnio-Max medium (Thermo Fisher Australia Pty Ltd, Scoresby, Victoria, Australia) and 0.25 μg/ml Antibiotic Antimycotic Solution (Sigma Chemical Company, St. Louis, USA). The cells were allowed to propagate at 28˚C and 5% CO_2. At approximately 80% confluency, cells were split into three T25 flasks for a further 3 to 4 passages before they were harvested by adding colcemid (0.05 μg/mL) for 3.5 hours and treated with hypotonic solution (KCl, 0.075 mM). Slides were fixed with an ice-cold (ca 4˚C) 3:1 mixture of methanol and acetic acid. The cell suspension was dropped on to slides, air dried and frozen at -80°C until use. For DAPI (4',6-diamidino-2-phenylindole)

staining, each slide was mounted with anti-fade medium Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) containing 1.5 mg/ml DAPI.

2.3.5 Contig sequence analysis

To discover homologies of the male-specific contigs and identify any partial gene sequences that may exist, we used BLASTN to search each contig against representative reptilian and avian genomes available in Ensembl, Release 99 (*Anolis carolinensis*, *Crocodylus porosus*, *Gallus gallus* , *Pelodiscus sinensis*, *Podarcis muralis*, *Pogona vitticeps*, *Pseudonaja textilis*, *Notechis scutatus*, *Varanus komodoensis*, *Sphenodon punctatus*) with a minimum E-value of 0.000001 for reported alignments and a filter for low complexity regions. We used the same cut-off and filter to search the non-redundant database at the NCBI (https://blast.ncbi.nlm.nih.gov). The Dfam database (Wheeler et al., 2012) was used to search for known transposable elements.

2.4 Results

2.4.1 *In silico* whole genome subtraction

We generated 96.7 x 10^9 150 bp PE reads from the male and 81.4 x 10^9 PE reads from the female sequencing libraries for the *in silico* whole genome subtraction pipeline. This equates to approximately 8x coverage of the genome estimated from the k-mer analysis. We decomposed these reads into 14,310,783,435 and 36,695,139,446 27-mers for the male and female respectively (Appendix 2: Figures S1 and S2), the difference likely arising from differences in sequence error rates between sequencing runs. To remove k-mers arising from sequence errors, we examined the k-mer spectrum to determine suitable thresholds and eliminated k-mers with counts less than 2 for males and 5 for females to yield 1,431,111,978 and 1,483,106,252 respectively. A total of 1,129,675,305 k-mers were common to both sexes and 301,436,673 k-mers were unique to the male individual. The male-specific k-mers were reassembled to yield 15,280,950 contigs ranging from 80 bp to 1374 bp (Appendix 2: Figure S3). Genome sizes of closely related species are between 1.9 and 2.5 GB.

2.4.2 Verification of phenotypic sex identification

Three karyotyped animals whose sex was identified by hemipenal eversion and presence or absence of breeding coloration had their gonadal sex confirmed by histology and their chromosomal sex confirmed by cytology (Appendix 2: Figures S4 and S5).

2.4.3 PCR validation

We selected the longest 92 contigs from the subtraction for further investigation, because they were of sufficient size to design robust primers and result in a PCR product easily visualised on an agarose gel. The 92 contigs ranged from 623 to 1,374 bases in length (Appendix 2: Figures S6 and S7). As expected, all 92 contigs passed the subtraction validation test where a product of the expected size successfully amplified in the focal male and did not amplify in the focal female. Of these, 52 contigs yielded putative Y-chromosome markers when screened against the panel of 4 male and 4 female individuals, however, only 7 of these putative markers (Table 2.1) ranging in length from 628 bp to 824 bp, were validated as sex-specific when tested in the full panel of an additional 20 males and 20 females (Figure 2.3). We applied the seven Y-chromosome markers to an additional 20 Anglesea animals (10 males and 10 females) and, in each case, the phenotypic sex was concordant with the genotypic sex inferred by the PCR test. Thus the 7 makers were completely concordant with phenotypic sex (present in male absent in female) in a total of 70 animals.

The sequenced PCR products were aligned to the relevant full-length subtraction contig for each of the seven Y loci. When Piccadilly Circus and Anglesea populations were compared, alignment results showed a small number of discrepancies in the nucleotide composition obtained from five of the seven amplicons (Appendix 2: Figures S8 to S14; Table S2). Of those that varied, sequence divergence ranged from 1.7% in the bdM27_79_X5_643 amplicon (Appendix 2: Figure S12) to 0.3% in the bdM27_23_X5_798 amplicon (Appendix 2: Table S2). Both bdM27_74_X11_649 (Appendix 2: Figure S10) and bdM27_87_X6_628 (Appendix 2: Figure S14) amplicons were identical across populations.

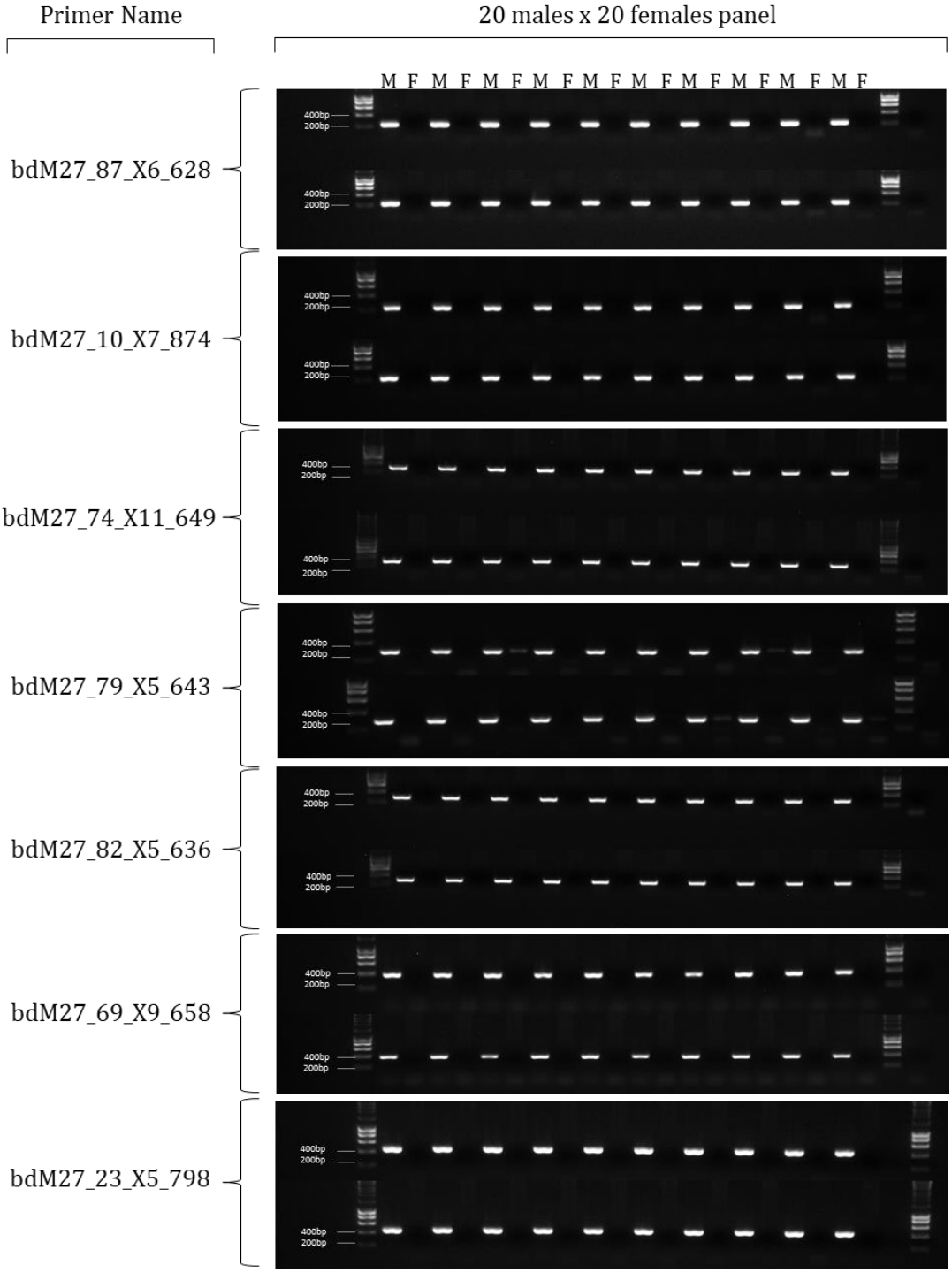

Figure 2-3. Validation of seven male-specific markers in *Bassiana duperreyi* using a panel of 20 male and 20 female individuals of confirmed phenotypic sex. Male specificity was defined as the presence of a distinct amplicon in males and the absence of amplification in females. Raw images are provided in Additional File 2.

Table 2-1. Primers for the amplification of putative Y chromosome markers for *Bassiana duperreyi.*

	Sequence (5′–3′)		
Primer Name	Forward	Reverse	Product size (bp)
bdM27_87_X6_628	TCTGAGGACATTGCAGGAACAA	GGCCTAATGAGACCTAGCAGTC	269
bdM27_10_X7_874	AAGATGGGAACTGCACTGGTAG	CAATATCCCCTGATGCAGCTCT	418
bdM27_74_X11_649	GAGGTCTGACAGAACCCTCTTG	TTTTGGTCCTGGAACAAGGTGA	286
bdM27_79_X5_643	TGTGAGACAATAGTGACCAGGC	TGCTCAGGTCTAGGGATGTGTA	294
bdM27_82_X5_636	TCTTTCTCTTTGCCCCAACCTT	ACTCTTGAATGTCGCAGTAGCA	380
bdM27_69_X9_658	TCAATGGACCTTGCATCATGGA	CCTTGGATTACTGCACTGACCT	390
bdM27_23_X5_798	TGTTCTCCGTACAATCACTGCA	TGACTTTTTGGCCGTGTAATGG	439

2.4.4 Gene and repeat identification

One of the seven Y-chromosome specific contigs, bdM27_23_X5_798, bears the partial sequence of an exon from the gene *UBE2H*, a member of a syntenic block conserved among jawed vertebrates (Nguyen et al., 2018). No other significant hits were found among the 7 sauropsid genomes searched, nor from the non-redundant Genbank database. We expected that the Y-contigs would be enriched for repetitive DNA sequences, coupled with unique flanking regions, so we searched against Dfam (Wheeler et al., 2012), a database of transposable elements. Two contigs, bdM27_79_X5_643 and bdM27_69_X9_658, had partial matches to known murine Class 1 retrotransposon elements, and bdM27_82_X5_636 had a partial match to a DIRS endogenous retrovirus known from the painted turtle (Appendix 2: Table S3 and Table S4).

2.5 Discussion

This study is the first to use an *in silico* whole genome subtraction approach to successfully develop sex chromosome markers without generating a linkage map or a reference genome in a reptile species. We rapidly isolated seven robust Y chromosome markers using a user friendly and cost effective *in silico* whole genome subtraction pipeline. The Y-markers segregated with sex in both the Piccadilly Circus study population and a genetically distinct population of Anglesea *B. duperreyi* which have been isolated from each other since the Late Pliocene, about 3.5 Mya (Dubey & Shine, 2010). This suggests that, all populations retain the ancestral state and that our makers are likely to have broad applicability across the entire species range. That said, the amplified sex specific region revealed some divergence between the Anglesea population and the Piccadilly Circus populations, suggesting that mutations could occur in the primer sites of some populations/taxa, limiting the generality of the sex-linked markers. The identification of sex-specific sequence has important practical value in many contexts, including ecological studies (Taberlet et al., 1999; Ferguson-Smith, 2007; Rovatsos & Kratochvíl, 2017), conservation of threatened or endangered species (Boulanger et al., 2008; Dawson et al., 2015; Literman et al., 2017; Zhang et al., 2012), captive breeding (Sulandart & Zein, 2012), aquaculture (Fang et al., 2020; Zheng et al., 2020), elimination of mortality as a possible explanation for sex ratio bias (Quinn et al., 2011; Zheng et al., 2020), sex forensics (Dash et al., 2020) and identifying genotypic sex (Quinn et al., 2009; Holleley et

al., 2015; Holleley et al., 2016) or in studies of early developmental processes where sex of the developing embryo is important (Whiteley et al., 2017; Whiteley et al., 2018).

Two approaches for identifying sex linked markers using whole genome sequencing seem appropriate, both relying on the divergence of the X and Y homologues in the region of recombination suppression. One technique, championed by Cortez et al., (2014) in exploring variation among mammalian species in the Y chromosome, and recently applied to the yellow-bellied water skink, *Eulamprus heatwolei* (Cornejo-Páramo et al., 2020), is to examine read copy number across the genome and identify the half copy number in the XY individuals compared to the XX individuals after screening out repetitive sequence. This technique identifies regions that have been lost from the non-recombining region of the Y chromosome but, remain on the X chromosome, which can be developed as sex specific markers and validated using PCR (Cornejo-Páramo et al., 2020).

Here we used as an alternative complementary approach, *in silico* whole genome subtraction to identify male-specific markers in the skink *B. duperreyi*, subsequently validated them using a PCR panel with individuals of known sex. Our technique is useful for identifying novel sequences, often repetitive elements, gained by the non-recombining region of the Y chromosome, or lost from the X chromosome. Neither of these approaches requires a reference genome, and so both are applicable to studies of organisms with no or incomplete reference genomes. Our technique does not require substantial read depth and thus avoids the associated high cost. Lower read depth can be a challenge because it reduces the efficiency of the subtraction approach by increasing the number of false positives. Indeed, this may have been a contributing factor to our 8% success rate. However, the ultimate goal was achieved, Y markers were discovered. Thus, PCR validation is effective at eliminating the false positives resulting from autosomal polymorphisms and differential coverage in the male and female.

Our technique decomposes a set of reads from the genome to yield a unique, but highly redundant, representation of the genome as overlapping k-mers. We then select the k-mers found only in the XY (or ZW) individual and reassemble the k-mers to yield Y (or W) enriched contigs that can be validated using PCR on a panel of individuals whose sex is known. In this way, we were able to isolate seven Y chromosome markers. There are several advantages to our *in silico* whole genome subtraction approach for identifying sex specific sequence when compared to AFLP, microsatellite or RAD-seq approaches.

Specifically, our *in silico* subtraction method surveys the entire available genome, assuming adequate read depth, to identify sex specific differences and does not rely on a highly reduced representation of the genome as with RAD and ddRAD approaches, that may miss many putative markers. This is particularly important for species with small sex chromosomes or relatively small differences between the X and the Y (or Z and W) chromosomes. Our method is cost-effective because as demonstrated here, low coverage sequencing (~8x) for a single individual of each sex is sufficient to obtain informative and robust Y-chromosome (or W chromosome) markers.

We have shown that the gene *UBE2H* (Ubiquitin Conjugating Enzyme *E2H*) is present on the Y chromosome in both *B. duperreyi* (this study) and the skink *E. heatwolei* (Cornejo-Páramo et al., 2020). This strongly suggests that the sex chromosomes of these two skinks share a homologous syntenic block and perhaps share homologous sex chromosomes. Ubiquitin-conjugating enzymes are encoded by a family of highly conserved genes involved in post-translational processes targeting abnormal or short-lived proteins for degradation (Seufert et al., 1990). Although various members of the ubiquitin conjugating enzyme family are involved in testes specific processes [e.g. testis-specific UBC4-testis in the rat, (Wing et al., 1996) and an ascidian, (Yokota et al., 2010)] we make no suggestion that UBE2H plays a role in sex determination in these skinks, merely that it is a gene on the sex chromosomes.

Our study paves the way for future work that relies upon successful identification of chromosomal sex in wild populations of *B. duperreyi* subject to sex reversal (Shine et al., 2002; Holleley et al., 2016). Isolating seven novel Y- chromosome markers increase the confidence of chromosomal sex identification in *B. duperreyi* because it reduces the risk of a recombination event being misinterpreted as evidence of sex reversal. Investigating the occurrence of temperature sex reversal will increase our understanding of sex reversal as a driver of sex-chromosome turn-over in the wild (Holleley et al., 2016) and establish links between environmental extremes and reptile sex-determining modes (Schwanz et al., 2020). Also, our Y chromosome markers can be used to identify the chromosomal sex of embryos and so enable developmental studies of sex determination and differentiation. For example, it is unknown whether *B. duperreyi* exhibits the asynchronous gonadal and genital development observed in other species with sex reversal (Whiteley et al., 2018). In addition to identifying sex chromosome markers, this subtraction approach could be leveraged to identify anchor points in a draft assembly to

locate genes on the sex chromosomes in non-model organisms, including candidates for sex-determining genes. Pairing our marker-discovery approach with high quality whole genome assemblies will accelerate our knowledge of sex chromosome evolution.

In this study, we identified a modest number of Y-chromosome markers, numbering 7 of 92 screened (8%). The success rate of future Y-marker discovery via genome subtraction could be improved by implementing efforts to reduce false positives caused by autosomal insertion/deletion polymorphisms in the focal sequenced individuals. This could be achieved through several complementary strategies: 1. subtracting multiple XX individuals from the XY focal individual/s; 2. selecting individuals for sequencing from populations with lower rates of heterozygosity (e.g. small geographically isolated populations or experimentally inbred lines); 3. sequencing siblings or related individuals. These improvements would increase the efficiency of sex chromosome sequence identification using whole genome subtraction.

Here we describe an effective tool for characterising sex chromosomes in non-model organisms. Our approach targets sex-specific insertions and highly differentiated sex chromosome regions that are suitable for developing diagnostic sex-markers. This approach complements existing methods for identifying sex chromosome homologues and aids the classification of sex determination systems in a wide range of species. The ability of our method to provide insights about the evolutionary origins of sex chromosomes is demonstrated here by the discovery of a scincid Y-chromosome gene, common to species separated by *ca* 40 million years of evolution.

Chapter 3

High elevation increases the risk of Y chromosome loss in Alpine skink populations with sex reversal

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3.1 Abstract

The view that has genotypic sex determination and environmental sex determination as mutually exclusive states in fishes and reptiles has been contradicted by the discovery that chromosomal sex and environmental influences can co-exist within the same species, hinting at a continuum of intermediate states. Systems where genes and the environment interact to determine sex present the opportunity for sex reversal to occur, where the phenotypic sex is the opposite of that predicted by their sex chromosome complement. The skink *Bassiana duperreyi* has XX/XY sex chromosomes with sex reversal of the XX genotype to a male phenotype, in laboratory experiments, and in field nests, in response to exposure to cold incubation temperatures. Here we studied the frequency of sex reversal in adult populations of *B. duperreyi* in response to climatic variation, using elevation as a surrogate for environmental temperatures. We demonstrate sex reversal in the wild for the first time in adults of a reptile species with XX/XY sex determination. The highest frequency of sex reversal occurred at the highest coolest elevation location, Mt Ginini (18.46%) and decreased in frequency to zero with decreasing elevation. We model the impact of this under Fisher's frequency dependent selection to show that, at the highest elevations, populations risk the loss of the Y chromosome and a transition to temperature-dependent sex determination. This study contributes to our understanding of the risks of extinction from climate change in species subject to sex reversal by temperature and will provide focus for future research to test on-the-ground management strategies to mitigate the effects of climate in local populations.

Keywords: climate change; thermolabile sex; sex determination; rapid evolution

3.2 Introduction

The primary signal directing embryos to develop phenotypically as either male or female is known as sex determination. The diversity of sex determination systems within vertebrates is remarkable, and has classically been considered to occur via one of two mechanisms – genotypic sex determination (sex determined at the time of fertilization by genetic factors independent of environmental influence, GSD) or environmental sex determination (sex determined by environmental factors that act after fertilization, ESD). In reptiles, these alternatives were thought to be incompatible and to form a mutually exclusive dichotomy. However, reversal of sexual genotype by environmental factors is common in fish (Wang et al., 2019; Hattori et al., 2020; Miyoshi et al., 2020) and amphibians (Flament, 2016; Lambert et al., 2019; Nemesházi et al., 2020). Sex reversal was suspected in turtles based on screening for H-Y antigens (Zaborski et al., 1982; Servan et al., 1989), but it was not conclusively demonstrated in reptiles until recently (Quinn et al., 2007; Holleley et al., 2015; 2016; Wiggins et al., 2020). Sex reversal in reptiles, as with temperature-dependent sex determination or TSD (Mitchell and Janzen, 2010), has profound implications for our understanding of how this diverse group of species can respond to climate change.

Theoretical studies show that as the frequency of sex reversal increases in a population, a likely response is a reduction and possible elimination of the Y or W chromosome under Fisher's frequency-dependent selection (Fisher, 1930; Bull, 1981; Grossen et al., 2011; Holleley et al., 2015; Bókony et al., 2017; Schwanz et al., 2020; Geffroy & Wedekind, 2020). Indeed, at least one population of central bearded dragon (*Pogona vitticeps*, Ahl, 1926), in which the sex reversed ZZ female phenotype is fertile, appears to be on the brink of such a transition (Figure S2 of Holleley et al., 2015). The implications of this work are that species with GSD may not be entirely immune from the demographic destabilization that climate change potentially brings to species with TSD. For some GSD species at least, global warming may well drive a transition to TSD (already demonstrated in the lab in one generation – Holleley et al., 2015); further warming increases the risk of extinction because of insufficient time for them to evolve and optimize the threshold for sex reversal and avoid the adverse demographic consequences of extreme sex ratio bias.

The temporal scale of these combined demographic and evolutionary changes make these processes challenging to study. However, it is possible to use altitudinal or latitudinal

climatic trends as a surrogate for how species might respond to temporal trends (Doody et al., 2006; Pen et al., 2010; Castelli et al., 2020). The widespread dragon lizard, *P. vitticeps*, has a ZZ/ZW system of sex determination (Ezaz et al., 2005) as in birds (Smith et al., 2007) but the ZZ genotype is reversed to present a viable female phenotype by high incubation temperatures (Quinn et al., 2007; Holleley et al., 2015). Furthermore, modelling the consequences of sex reversal has shown that the W chromosome (in this case) can persist indefinitely at low frequency in the population, particularly if there is immigration from adjacent populations that remain predominantly GSD (Schwanz et al., 2020). So the transition from GSD to TSD is not a unidirectional process, with the residual and cryptic presence of the W chromosome enabling a rapid halt to the transition and reversion to GSD if and when conditions change.

The scincid lizard *Bassiana duperreyi* (Gray, 1838) also has genotypic sex determination, but with an XX/XY system (Donnellan, 1985; Matsubara et al., 2016) and reversal of the XX female genotype to a male phenotype by low incubation temperatures (Shine et al., 2002; Radder et al., 2008; Quinn et al., 2009). Despite having sex chromosomes, hatchling sex is influenced by the temperature during incubation, both in the lab and the field (Shine et al., 2002; Radder et al., 2008; Holleley et al., 2016); it is not influenced by hydric variation over the range of soil water potentials recorded in natural nests (Flatt et al., 2001). High rates of sex reversal occurred in nests of an alpine population (36° 5'8.15"S 148°13'1.93"E, Jagumba in Kosciuszko National Park, Australia; 28% XX male – Holleley et al., 2016), but whether this translates to equally high rates of sex-reversed adults, and whether those sex-reversed adults are reproductively viable is not known. Here we use a new and reliable PCR test (Dissanayake et al., 2020) to show that, unlike *P. vitticeps* (Castelli et al., 2020), the frequency of sex reversal in adults of *B. duperreyi* does vary in accordance with expectation along an ambient thermal gradient (elevational gradient) extending from the Australian Alps (1640 m a.s.l.) to the Victorian lowlands (20 m a.s.l.). Our modelling of the impact of sex reversal on the frequency of the XY genotype suggests that, at the upper altitudinal extremes of the distribution of the species, the Y chromosome may be lost, precipitating a transition to TSD.

3.3 Materials and methods

3.3.1 Study species

The eastern three-lined skink, *B. duperreyi*, is a medium-sized (60 to 80 mm snout-vent length) oviparous lizard widely distributed at cool high-elevation sites close to the upper elevational limits for oviparous reproduction by Australian lizards (Shine & Harlow, 1996); its distribution extends through an altitudinal gradient to coastal regions of New South Wales (NSW) and Victoria in south-eastern Australia (Cogger, 2014). *B. duperreyi* appeared in the Upper Miocene and/or Upper Pliocene period (4.7 to 6.6 Mya), during which its distribution would have been strongly influenced by paleoclimatic conditions (which fluctuated between warm-wet and cool-dry) and other biogeographical processes which have prevailed since (Dubey & Shine, 2010). Female *B. duperreyi* lay a single clutch of 3-11 eggs from early Australian summer (late November) to December (Shine & Harlow, 1996).

3.3.2 Study area

Ten sites along an elevational gradient were selected in mainland south-eastern Australia spanning a large portion of the distribution of *B. duperreyi* (Figure 3.1A). Mt Ginini (ACT, 35°31'29.6"S 148°46'58.7"E) was at the highest elevation (1640 m a.s.l.) and Anglesea (Victoria, 38°23'28.0"S 144°12'54.0"E) was at the lowest elevation (40 m a.s.l). Piccadilly Circus (1240 m a.s.l., ACT, 35°21'42.0"S 148°48'12.5"E) and Cooma (960 m a.s.l., NSW, 36°26'48.6"S 149°11'40.6"E) were intermediate in elevation and location. Six additional sites intermediate between Mt Ginini and Anglesea were selected for less intensive sampling – Mt Kosciuszko National Park (1340 m a.s.l., NSW, 35°53'23.9"S 148°25'22.0"E) (*ca* 28 km north east to that reported by Holleley et al. 2016), Coree Flat East (1100 m a.s.l., NSW, 35°16'06.1"S 148°49'54.7"E) and West (1040 m a.s.l., NSW, 35°16'52.0"S 148°48'35.0"E), Shelley (725 m a.s.l., Victoria, 36°10'31.7"S 147°32'58.2"E), Dartmouth (380 m a.s.l, Victoria, 36°31'35.9"S 147°28'53.0"E) and Westernport Bay (20 m a.s.l., Victoria, 38°13'40.0"S 145°18'34.0"E).

The climate in south-eastern Australia is temperate. We compiled climatic data for each study location using the Scientific Information for Land Owners database maintained by the Queensland Department of Natural Resources and Water (SILO, https://www.longpaddock.qld.gov.au/silo/, last accessed 8-Apr-2020) (Jeffrey et al., 2001).

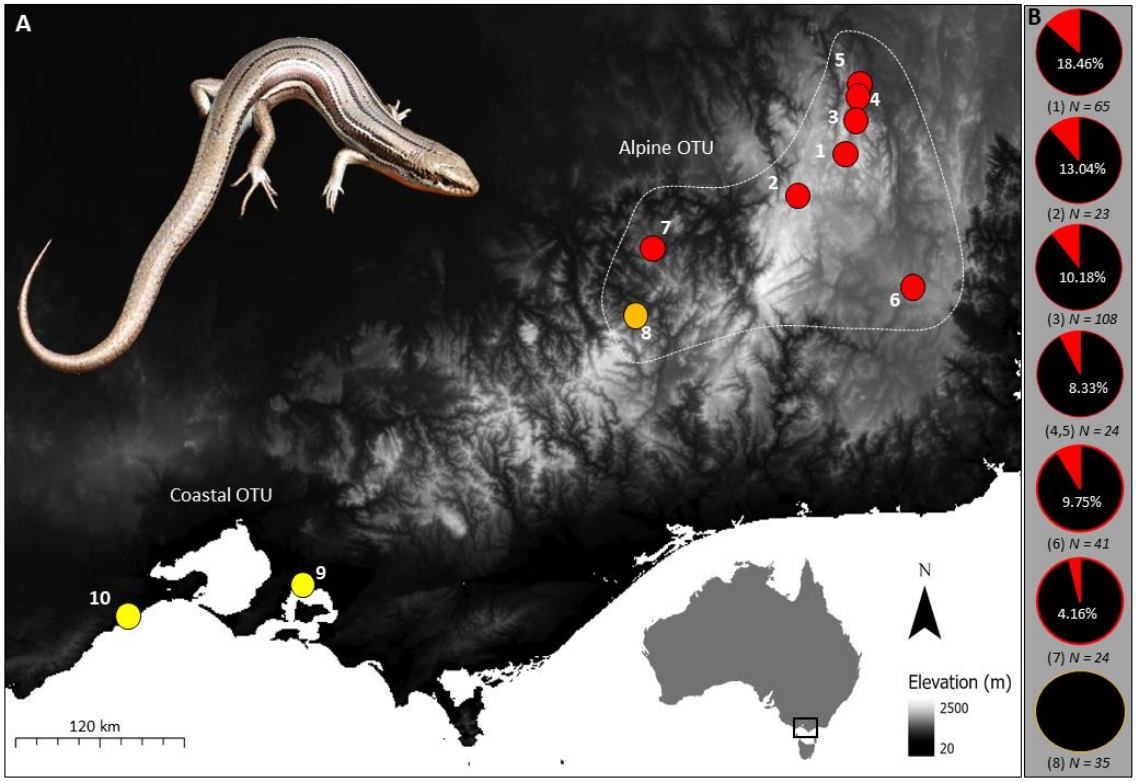

Figure 3-1. The geographic distribution of sex reversal in *Bassiana duperreyi*. (**A**). Sites where sex reversal was detected (the presence of XX males) are indicated with red circles (Populations: 1. Mt Ginini, 2. Mt Kosciuszko, 3. Piccadilly Circus, 4 and 5. Coree Flat East and West (Coree Flat West population assigned to the Coree Flat East population due to low number of samples), 6. Cooma, 7. Shelley). No sex reversal was detected in the lowest elevation within Alpine OTU (8. Dartmouth; orange circle), nor in the genetically distinct Coastal OTU (9. Westernport Bay, 10. Anglesea; yellow circles). (**B**). Pie charts indicate the relative proportion of XY males (black) and sex reversed XX males (red) in each sampling location, *N* = total number of phenotypic males collected in each location. Underlying map generated using ArcGIS Pro 2.6 (http://www.esri.com) and data from the Digital Elevation Model (Geoscience Australia) made available under Creative Commons Attribution 3.0 Australia (https://creativecommons.org/licenses/by/3.0/au/legalcode, last accessed 9-Jul-20). The adult male *B. duperreyi* photo was taken by D.S.B.D. at Cooma, NSW, Australia.

The SILO data drill provides daily temperatures, rainfall and other climate variables for 0.050-degree grids across Australia, interpolated from point measurements made by the Australian Bureau of Meteorology. Historical monthly climate data from January 1895 to January 2019 were downloaded for analysis. The mean annual maximum temperature (Tmax) was 15.4 ± SD 6.02°C and mean annual minimum (Tmin) of 4.5 ± 4.25°C at Mt Ginini (n = 1561). Anglesea Tmax was 18.0 ± 3.95°C and Tmin was 9.6 ± 2.66°C (n = 1561). The highest mean annual rainfall was recorded in the Mt Kosciuszko National Park field location (113.9 ± 70.32 mm) while the highest mean annual solar radiation was recorded in Piccadilly Circus field location (17.3 ± 6.39 MJ/m^2). Detailed climatic data for each location are provided in supplementary materials (Table S1), which we used to analyse the frequency of sex reversal with an elevational gradient.

3.3.3 Sample collection

We conducted our fieldwork in areas where the lizards were most abundant, that is, in natural open areas but also often in areas artificially cleared for overhead hydroelectric power lines, fire banding trails, roads and or tracks inside the forest areas in above-selected field locations. Adults tend to aggregate in these open areas during the nesting season (Radder & Shine, 2007). Adult specimens were captured by hand either when active or when inactive under rocks or logs. We measured each snout–vent length (SVL, tip of snout to the anterior end of cloaca) and tail length (TL, the anterior end of cloaca to tip of the tail) for each lizard. All measurements were taken using digital Vernier callipers on living specimens (nearest 1 mm). The phenotypic sex of males was identified by hemipene extrusion (Harlow, 1996); those for which prominent hemipenes could not be everted were identified as female. Female lizards were checked by abdominal palpation to see if they were gravid (Holmes & Cree, 2006). Breeding coloration was used to corroborate the determination of sex by hemipenile extrusion – males have a prominent reddish-orange throat when in breeding condition, lacked by females. All animals with reddish-orange coloration on the throat extruded hemipenes when probed, so there were no cases of conflict between the two criteria when present. Some animals without breeding coloration also extruded hemipenes, but this was typically outside the breeding season, so it was possible that some individuals who did not extrude hemipenes and did not have breeding coloration were nevertheless males. However, in no cases did individuals presenting as phenotypic females deliver a male outcome (XY) in the sex testing. Indeed, our approach to phenotypic sexing was considered highly reliable because in no case was there a

mismatch in phenotypic sex and gonadal sex (testes or ovary) in dissections for other studies, and no mismatches occurred in the panel of 20 males and 20 females used as the validation panel for our sex-linked marker (Dissanayake et al., 2020). In seven instances, lizards escaped capture before their phenotypic sex could be determined.

Tail tips (4-5 mm) were removed with a sterile blade and the free-flowing blood drop collected onto a labelled Whatman FTA™ Elute Card (WHAWB12-0401, GE Healthcare UK Limited, UK); tail tips were collected into labelled 1.5 ml tubes containing 95% ethanol. All animals were immediately released at their point of capture. All experimental protocols were conducted with the permission of Animal Ethics Committees at the University of Canberra and the CSIRO. All experiments were conducted in accordance with guidelines and regulations established by these committees.

3.3.4 SNP genotyping

Tail tissue samples of a total of 100 *B. duperreyi* from the 10 populations (10 samples per location) were passed to Diversity Arrays Technology Pty Ltd (Canberra) (DArT) for reduced representational sequencing. Genomic DNA (gDNA) was extracted using a NucleoMag® 96 Tissue kit (Macherey- Nagel, Düren, Germany) coupled with NucleoMag SEP (Ref. 744900) to allow automated separation of high-quality DNA on a Freedom Evo robotic liquid handler (TECAN, Männedorf, Switzerland). Four combinations of restriction enzymes were evaluated for *B. duperreyi* (*Pst*I enzyme combined with either *Hpa*II, *Sph*I, *Nsp*I, and *Mse*I) and the restriction enzyme combination of *Pst*I (recognition sequence 5'-CTGCA| G-3') and *Sph*I (5' -GCATG|C-3') was selected for complexity reduction by double digestion (Kilian et al. 2012). A full account of the process of generating SNP genotypes for these samples is given by Georges et al., (2018). The data comprised a matrix of SNP loci by individuals, with the contents stored as 0, homozygote, reference state; 1, heterozygote; and 2, homozygote, alternate state.

3.3.5 Additional SNP filtering and visualization

The SNP data and associated locus metadata were read into a genlight object (R Package adegenet – Jombart, 2008) to facilitate processing with package dartR v.1.5.5 (Gruber et al., 2018). Only loci with > 99% repeatability (repAvg) were chosen for subsequent analysis. Further filtering was undertaken based on the call rate (> 90%) and where multiple SNPs occurred within a single sequence tag, only one was retained at random.

The population sample sizes were small (n = 10), so we could not filter loci for departures from Hardy–Weinberg equilibrium or linkage disequilibrium; the sparse sampling of loci across the genome allows the reasonable assumption of little or no linkage between loci. We regard the data remaining after this additional filtering as highly reliable. Genetic similarities for individuals and populations were visualized using principal coordinates analysis (PCoA) as implemented in the gl.pcoa and gl.pcoa.plot functions of dartR. A scree plot of eigenvalues (Cattell, 1966) guided the number of informative axes to examine, taken in the context of the average percentage variation explained by the original variables (using the diagnostics provided by gl.pcoa function in dartR).

3.3.6 Fixed difference analysis and genetic diversity

To examine the possibility that more than one taxon (Operational Taxonomic Unit, OTU) might be contributing to the altitudinal cline, potentially confounding the comparisons, a fixed-difference analysis was done using the scripts gl.fixed.diff and gl.collapse in dartR. A fixed difference between two populations at a biallelic SNP locus occurs when all individuals in one population are fixed for the reference allele and all individuals in the other population are fixed for the alternate allele. Accumulation of fixed differences between two populations is a clear indication of lack of gene flow (Georges et al., 2018). Expected heterozygosity, a measure of genetic diversity, was obtained for each population from allele frequencies using the gl.report.heterozygosity function of dartR. We used gl.ibd function in dartR to calculate isolation by distance (F_{ST} / 1 - F_{ST} versus Euclidean distance), tested with a Mantel test.

3.3.7 Genotypic sex and sex reversal frequency

For sex testing, DNA was extracted from tail tips using a Gentra Puregene commercial kit (Qiagen Science, Maryland, USA) following manufacturer protocols; DNA was extracted from blood samples (FTA^{TM} Elute Micro Card) following manufacturer protocols. DNA purity was determined using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and quantified using the Qubit 2.0 Fluorometric Quantitation (Invitrogen, Life technologies, Sydney, NSW, Australia). Genotypic sex was identified using a PCR test comprising seven Y-specific markers validated against samples of known sex individuals from across the study sites (i.e. Anglesea to Piccadilly Circus) of *B. duperreyi* (Dissanayake et al., 2020). Briefly, in applying the test we used 1x MyTaq^{TM} HS Red mix (Bioline USA Inc. USA), 4 µM of each primer and 25 ng of genomic DNA.

The PCR cycling conditions used an initial touchdown phase to increase the specificity of amplification: denaturing at 95°C, annealing temperature stepping down from 70°C by 0.5°C per cycle for 10 cycles, extension at 72°C. This was followed by 30 cycles at 65°C annealing and 72°C extension. PCR products were visualized on a 1.5% agarose gel using SYBR Safe (Life Technologies, Carlsbad, USA). The samples that showed an amplified band for each of the seven markers are recognized as XY individuals whereas as the samples for which a band was not amplified in all seven markers were recognized as XX individuals. The seven markers always concurred in their determination in the original study (Dissanayake et al., 2020) and in this study, which renders false negatives highly unlikely (they would present as some but not all markers failing). There were also no feminized individuals. Phenotypic male lizards showing genotype–phenotype discordance were classified as sex-reversed (Dissanayake et al., 2020). All molecular sex tests were conducted blind.

Coree Flat West population was assigned to the Coree Flat East population owing to the low number of samples that we acquired from these two locations. Simple linear regression was used to determine the relationship between the proportion of sex-reversed males (arcsin transformed) in each population with respect to elevation, and to characterise the trends in yearly mean maximum (Tmax) and yearly mean minimum temperature (Tmin) against elevation in each field location. Pearson correlation was used to evaluate the magnitude and direction of the association between the frequency of sex reversal and climatic variables [Tmax, Tmin, total rain (mm), evaporation (mm), radiation (Mj/m^2) and vapor pressure (hPa)] including elevation recorded at each field site. For these correlations, we used only data from the alpine populations.

3.3.8 Modelling the consequences of sex reversal

We modelled the decline of the XY genotype resulting from frequency-dependent selection because of the overproduction of males through sex reversal of the XX genotype with decreasing temperature (Shine et al., 2002; Radder & Shine, 2007) following the logic of Bull (1981). Sex reversed individuals are assumed to be viable and fitness within a sex is considered to be the same for all genotypes. Let the starting frequency of XY among zygotes be *y*, the starting frequency of XX among zygotes be *1-y*, and let a fraction *P[T]* of XX become reversed to a male phenotype. In any one generation *n*, we have the proportion

of male phenotypes (m_n) equal to the sum of the frequencies of normal XY males (y_n) and sex-reversed XX males,

$$m_n = y_n + P[T](1 - y_n)$$

The frequency of the XY genotype in the next generation is given by

$$y_{n+1} = \frac{y_n}{2m_n}$$

We used previously published experimental data (Radder et al., 2008; Shine et al., 2002) and their estimate of 20°C for the threshold below which offspring sex is temperature-dependent and above which offspring sex is solely genetically determined (Telemeco et al., 2009) (not to be confused with the threshold at which a 50:50 sex ratio is observed), to generate the equation for *P*[T] (Figure S1 and Table S2) given by,

$$P[T] = \frac{1}{1 + e^{-15.7152+0.8567*T}}$$

where T is in °C. We iterated for an equilibrium solution for *y* for various values of temperature T. Overlapping generations will delay the rate of convergence to equilibrium but will not affect the equilibrium value for a particular temperature.

3.4 Results

3.4.1 SNP genotyping

A total of 97,417 polymorphic SNP loci were scored for 100 individuals from 10 populations of *B. duperreyi* in south-eastern Australia (Figure 3.1A). After stringent filtering on repeatability (10,161 loci < 0.99) and call rate (61,524 loci < 0.90), and after removing the secondary SNPs (7881), the number of SNP loci in the data set dropped to 17,851. Considerable genetic structure existed in *B. duperreyi* in south-eastern Australia (Figure 3.2). In particular, the lower elevation populations of Anglesea and Westernport Bay were distinctly different from the Australian Alps populations and probably represent a distinct taxon (OTU). One individual (AA080788) was intermediate between the coastal and alpine populations, likely to represent contemporary hybridization, and was removed from the fixed difference analysis. Evidence for the coastal and alpine populations being separate taxa was provided by the fixed difference analysis the Anglesea population differed from the Alpine populations by an average of 280 (256-308) diagnostic fixed -

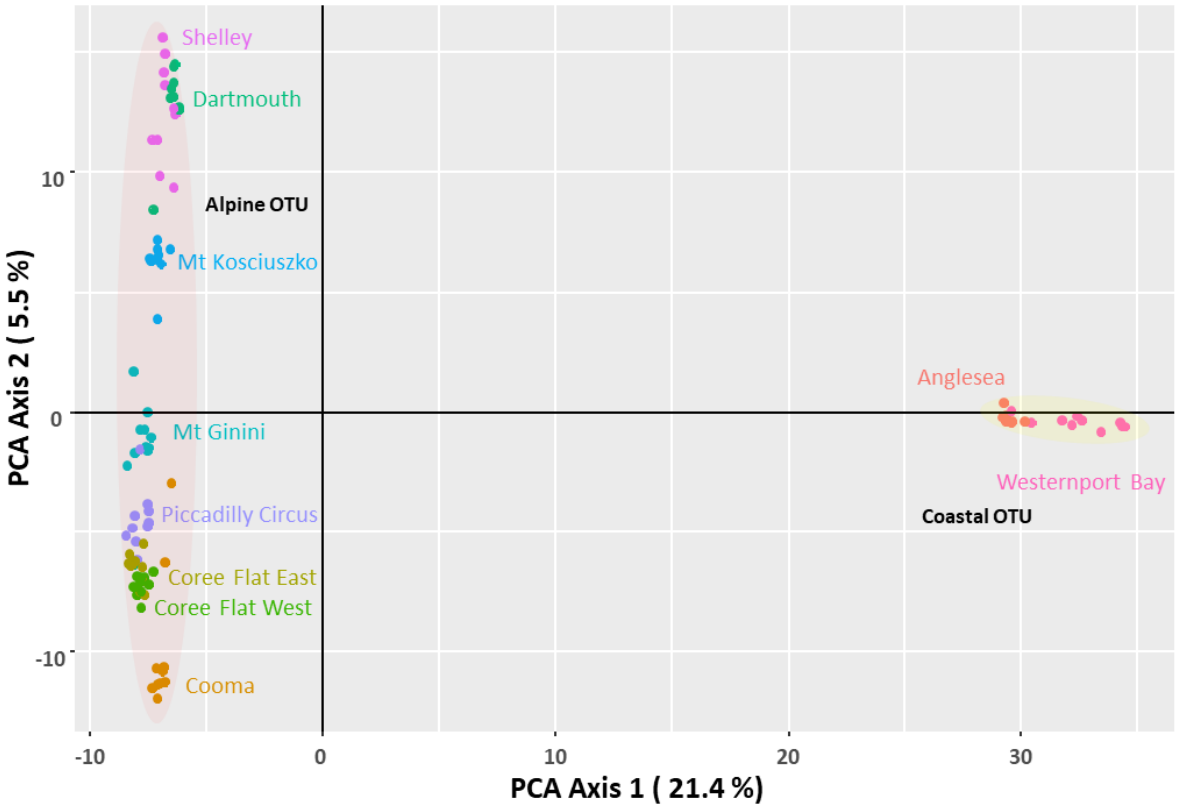

Figure 3-2. Graphical representation of genetic similarity between individuals using principal coordinates analysis. The two primary clusters (Alpine OTU and Coastal OTU) differed by 117 diagnostic fixed allelic differences. There were no significant fixed differences between populations within clusters. Axes not to scale.

differences and the Westernport Bay population differed from the Alpine populations by an average of 336 (308-374) fixed differences (Table 3.1). All the Alpine populations collapsed into a single OTU based on corroborated fixed differences (option tpop=1 in gl.collapse of dartR), after which the Anglesea population differed from the Alpine populations by 160 fixed differences (false positive expectation 76.1, $p < 0.0001$) and the Westernport Bay population differed from the Alpine population by 196 fixed differences (false positive expectation, 79.1, $p < 0.0001$). Anglesea and Westernport Bay differed by only 2 fixed differences (false positive expectation 15.1, $p = 1.0000$, ns) and so collapsed into a single OTU. Hence, two distinct taxa (OTUs) were identified, based on significant fixed differences, that of Anglesea/Westernport Bay (Coastal OTU) and the Alpine populations from Mt Ginini to Dartmouth (Alpine OTU), differing by 117 fixed allelic differences (false positive expectation 37.4) (Table 3.1). The Alpine population showed evidence of isolation by distance (Mantel's test; $r = 0.71$, $p < 0.002$). The average expected heterozygosity varied from 0.10 at Westernport Bay to 0.16 at Piccadilly Circus (mean 0.13). Within the Alpine OTU, average expected heterozygosity varied from 0.19 at Cooma to 0.16 at Piccadilly Circus (mean 0.13).

3.4.2 Genotypic sex and sex reversal frequency

A total of 639 tail snips were collected during the field survey. Of them, 399 were phenotypic males and 233 were phenotypic females, and 7 escaped before they had their phenotypic sex identified, and those samples were removed from the PCR sex test. A total of 33 (5.22%) adult males were sex-reversed in the populations. Zero sex reversal was observed at the low elevation sites ranging from Dartmouth (340 m a.s.l) to Anglesea and Westernport Bay (40 to 20 m a.s.l), despite the markers having been validated for the putative distinct taxon from Anglesea/Westernport Bay (Dissanayake et al., 2020). However, after identifying two OTUs in the *B. duperreyi* distribution within south-eastern Australia, we restricted our attention to the sex reversal frequency in the Alpine OTU (Dartmouth to Mt Ginini). The highest frequency of sex reversal occurred at the highest elevation, Mt Ginini (18.46%) (Figure 3.1B) and a total of 6.58% adult males were sex-reversed across all the sampled Alpine populations (Table S3). Frequency of sex reversal was positively correlated with elevation (Pearson's correlation coefficient $r = 0.97$, $p < 0.001$) (Table S4); Tmax ($R^2 = 0.66$, $p < 0.05$) and Tmin $R^2 = 0.60$, $p < 0.05$) were significantly negatively correlated with sex reversal frequency (Figure 3.3; Table S4).

Table 3-1. Matrix of euclidean genetic distances (below diagonal) and allelic genetic differences (above diagonal) between the ten populations sampled for *Bassiana duperreyi*.

	Anglesea	Cooma	Coree Flat West	Coree Flat East	Dartmouth	Mt Ginini	Mt Kosciuszko	Piccadilly Circus	Shelley	Westernport Bay
Anglesea		308	303	266	256	286	280	256	290	2
Cooma	44.2		12	2	2	7	8	1	7	374
Coree Flat West	42.97	22.22		0	0	31	22	0	22	365
Coree Flat East	42.87	22.45	14.34		0	2	2	0	4	318
Dartmouth	42.67	27.73	23.50	22.99		1	0	0	0	308
Mt Ginini	42.48	22.73	18.27	17.45	20.51		1	0	3	324
Mt Kosciuszko	42.52	25.78	21.86	21.10	18.6	17.61		0	0	334
Piccadilly Circus	42.41	22.59	15.73	13.90	21.23	16.23	19.46		3	312
Shelley	43.19	22.59	24.88	24.25	16.43	21.61	19.12	22.88		353
Westernport Bay	22.63	46.49	45.37	45.22	45.02	44.86	44.83	44.78	45.55	

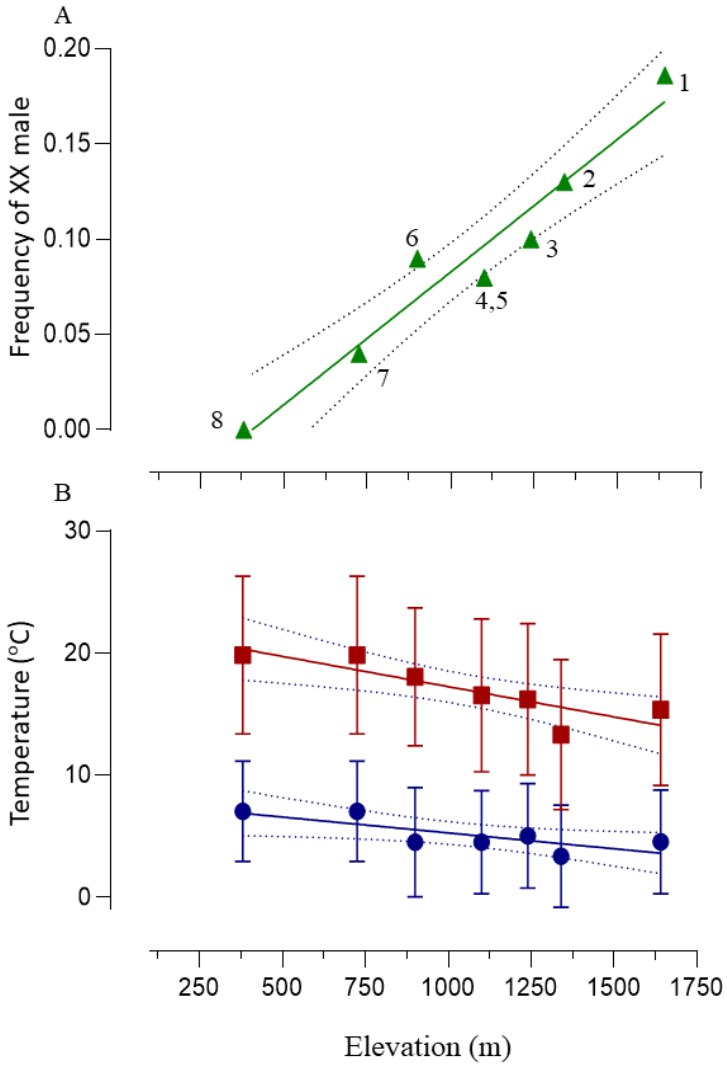

Figure 3-3. (a). The rate of sex reversal in phenotypic males *Bassiana duperreyi* increases with elevation ($F_{(1,5)} = 71.39$, $p < 0.001$; $R^2 = 0.94$) (green triangles). Numbers indicate the field locations, as described in Fig. 1. (**b**). Linear regression of yearly (January 1895 to January 2019) mean Tmax (°C) (red squares) ($F_{(1,5)} = 14.88$, $p < 0.05$; $R^2 = 0.74$) and Tmin (°C) (blue circles) ($F_{(1,5)} = 7.82$, $p < 0.05$; $R^2 = 0.61$) with elevation. Points represent mean ± SD (n = 1561). Temperature data obtained from SILO database (Jeffrey et al., 2001). Broken lines denote the 95% confidence interval.

3.4.3 Modelling the consequences of sex reversal

The frequency of the XY genotype is predicted to decline precipitously with decreasing incubation temperature as the system moves toward a 1:1 sex ratio equilibrium (Figure 3.4). At incubation temperatures of 18°C and below, we would expect the complete loss of the Y chromosome from the population. Our wild populations, using the nest temperature data of Telemeco et al., (2009, 2010), indicate that some high-altitude sites (e.g. Mt Gingera, 1,865 m a.s.l, not sampled in this study) are within the thermal range that would lead to loss of the Y chromosome (Figure 3.4).

3.5 Discussion

The eastern three-lined skink has heteromorphic sex chromosomes, with males as the heterogametic sex (XY) (Donnellan, 1985). Individuals of the species with the XX genotype, normally destined to become female, can be sex reversed to a male phenotype at low incubation temperatures (< 20°C) under laboratory conditions (Radder et al., 2008) and in natural nests (Holleley et al., 2016). Here we have presented a detailed account of naturally occurring sex reversal in adult *B. duperreyi* along an elevational gradient confirming that sex reversed hatchlings are viable and survive to adulthood.

Our combination of seven sex linked markers, concordant in their indication of genotypic sex in all determinations in this study and that of Dissanayake et al., (2020) eliminates the possibility that what we have observed is a gradient in the frequency of recombination. There is no reason to expect our 7 independently derived markers to be tightly linked, so if recombination was varying with elevation, then the concordance of our 7 markers would break down with elevation. There is no evidence of this. Seven novel Y-chromosome markers increases the confidence of chromosomal sex identification in *B. duperreyi* because it dramatically reduces the risk of a recombination event being misinterpreted as evidence of sex reversal.

No sex reversal was observed in the populations at the lowest elevation, on the coast at Anglesea and Westernport Bay. These populations appear to represent a different species from the Alpine populations, which opens the possibility that the coastal taxon does not have thermolabile sex for historical reasons, not as a direct contemporary response to climate. We therefore excluded from consideration the populations from -

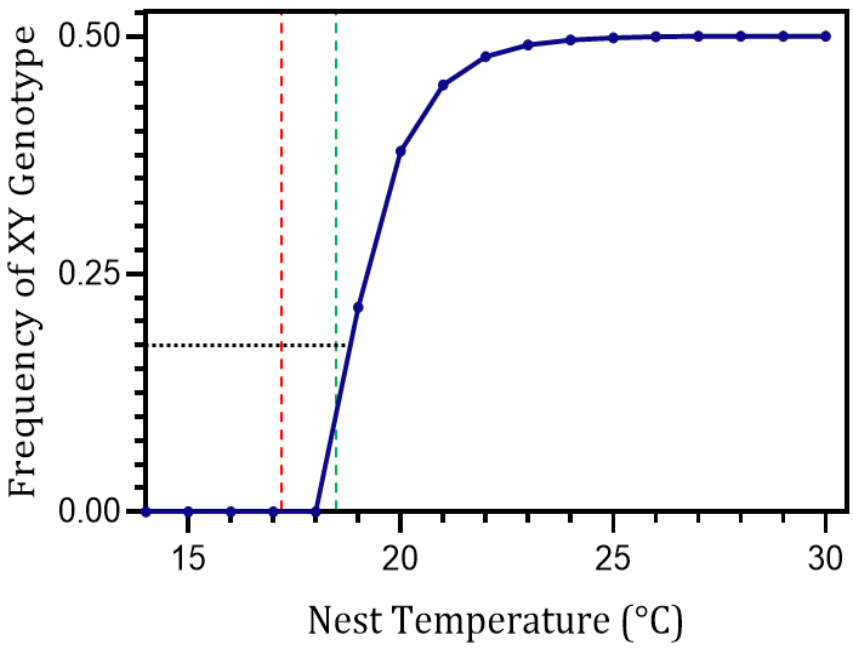

Figure 3-4. Modelling of the decline of the XY genotype resulting from the frequency-dependent selection. Relative frequency of the XY genotype from the entire population declines precipitously with decreasing incubation temperature and requires only a small drop in environmental temperature to precipitate complete loss of the Y chromosome. Nest temperature of the highest altitude wild population sampled in this study is shown in green (Mt Ginini) (Telemeco et al., 2010) and resides on the precipice. One higher elevation population not sampled in this study (Mt Gingera, 1865 m a.s.l) (Telemeco et al., 2010) (red broken line) suggests conditions exist in alpine populations that are suitable for complete loss of the Y chromosome. Black broken line shows the actual recorded frequency of sex reversal in the adult population at the Mt Ginini.

Anglesea and Westernport Bay, and focused our analysis on the single taxon which we refer to as the Alpine OTU. Having done that, there was a significant positive relationship between sex reversal and elevation. The frequency of sex reversal in adults ranged from 18.46% at the highest, coolest location (Mt Ginini, 1640 m a.s.l., mean annual temperature 9.9 ± 7.58°C) to zero at the lowest, warmest location (Dartmouth, 340 m a.s.l., 13.4 ± 8.38°C). These results establish that the frequency of sex reversal varies as expected with prevailing climate.

Our modelling shows that the frequency of the Y chromosome in the population can be expected to decline as the frequency of sex reversal increases. The question arises as to whether temperatures exist within the species range, or are likely to exist under future climate change, that could lead to the complete loss of the Y chromosome driven by overproduction of males through sex reversal. Is a transition to TSD likely? Our modelling of the equilibrium state for the frequency of the XY genotype as nest temperature is decreased shows that under frequency-dependent selection the XY genotype begins to decline in frequency at 23°C and is lost at nest temperatures below 18°C (strictly constant temperature equivalents, CTE (Georges, 1989; Georges et al., 1994). Mean nest temperatures during the middle of incubation (weeks 5-8) were recorded as 17.1°C at Mt Gingera (1855 m a.s.l.), 19.3°C at Mt Ginini (1640 m a.s.l.), 20.6°C at Piccadilly Circus (1240 m a.s.l.) and 20.6°C at Coree Flat (1040 m a.s.l.) (Telemeco et al., 2009; Telemeco et al., 2010; Shine et al., 2003). Although these data have not been corrected for diel fluctuations in the nest, it is clear that at the highest elevations, there is the potential for loss of the Y chromosome and a transition to TSD provided there is no dispersal from adjacent regions where the Y is persistent (Schwanz et al., 2020).

Reduction of the Y chromosome under the influence of climate follows a neutral pathway, in that once the system achieves a 1:1 sex ratio, the frequency of the Y chromosome in the population is evolutionarily stable (Bull, 1981). Many theoretical models, such as genetic drift (Bull & Charnov, 1977) and sex ratio selection (Bulmer & Bull, 1982; Kozielska et al., 2010; Schwanz et al., 2020) emphasise the importance of natural selection for promoting transitions in sex determination systems (Natri et al., 2019). In *P. vitticeps* sex reversal can lead to loss of the W chromosome under Fisher's frequency-dependent selection alone (Holleley et al., 2015), without the need to invoke an advantage under conventional natural selection (see also, Hurley et al., 2004; Grossen et al., 2010; Bókony et al., 2017). The same considerations apply to *B. duperreyi*. Nevertheless, the

transition to a system free of the Y chromosome may be accelerated should a fitness advantage of sex reversal under conventional natural selection exist (Holleley et al., 2015; 2016; Li et al., 2016) or retarded by a fitness cost (Cotton & Wedekind, 2009), depending upon the species and the circumstances. This provides a great opportunity for future research on the evolutionary dynamics of sex reversal in *B. duperreyi*.

Pogona vitticeps differs from *B. duperreyi* in that the direction of the reversal (at high temperatures rather than low) is aligned with the projections for global climate change. In *P. vitticeps*, sex reversal and the potential loss of the W chromosome occurs well within the range of temperatures experienced in nature (Holleley et al., 2015), and is likely to be exacerbated by climate change. Sex reversed individuals of *P. vitticeps* are fertile, and ZZ x ZZ crosses can be used to generate viable lines in which the W chromosome is absent. This raises a second unanswered question – the sex-reversed individuals of *B. duperreyi* survive to adulthood, but are they fertile? Establishing the fertility of sex-reversed *B. duperreyi* is more challenging than for *P. vitticeps* because the reversal is to a male phenotype. The reproductive viability of sex-reversed male *B. duperreyi* is yet to be established. This places a caveat on any interpretations we may place on the evolutionary significance of sex reversal in this species.

A third question arises as to whether the demographic consequences of sex reversal a predominance of males arising before the system can come to a 1:1 sex ratio equilibrium, and consequent reduction in effective population size – is a factor limiting the distribution of *B. duperreyi* at high altitudes, or whether the limitation arises through egg survivorship. If the climate is static, a 1:1 primary sex ratio is achieved locally despite a reduction in the frequency of the Y chromosome. The system comes to a series of equilibria across the landscape with no inherent limitations placed on the species distribution. Under climate change however, the system is pushed to a disequilibrium state and there is the potential for a very substantial sex ratio skew until the populations come to evolutionary equilibrium. Such a skew could deliver a limit to the upper range of the species because of the demographic consequences of the gross overproduction of male phenotypes (Boyle et al., 2014). More detailed work needs to be done at the upper extremes of the distribution of this species (Mt Gingera, 1855 m a.s.l. and Mt Kosciuszko, 2020 m a.s.l.) to identify if these evolutionary and demographic processes are in play. The question of whether sex ratio skew arising from TSD or embryo physiology and viability establishes the -

distributional boundaries to species remains unresolved, complicated by the typical bias of sex ratio to overproduction of females at extreme temperatures in most species of reptile.

For *B. duperreyi*, global warming is likely to alleviate the demographic impact of sex ratio skew, because temperatures that produce a 50:50 sex ratio (23°C; Radder et al., 2008) will extend to higher altitudes leading to range expansion. However, projected changes in future climatic conditions (Benestad, 2003; Tokarska et al., 2020) predict more extreme events, such as elevation of temperatures to greater extremes than have been experienced in the past, or dropping to exceptionally low temperatures during the nesting season with profound impacts on biological processes in many taxa (Easterling, 2000; Hari et al., 2006; Dunham et al., 2011; Scheffer et al., 2015; Gamelon et al., 2017; Valenzuela et al., 2019). Hence, at a finer temporal scale, extremely cold temperatures, even for a short time during the natural incubation period, might have a sex reversing effect in the Alpine populations and disrupt the contribution of a particular breeding year to the future population (see Schwanz et al., 2020 – Figure 3). Nest sites subjected to particularly cold temperatures in one year resulted in 28% of XX hatchlings to be sex-reversed (Holleley et al., 2016).

Not many species have been examined for instances of sex reversal and the widespread occurrence of homomorphic sex chromosomes in reptiles means that instances of sex reversal would not come to notice incidentally. Sex reversal in the wild may also occur in the yellow-bellied water skink (*Eulamprus heatwolei*) and the spotted snow skink (*Niveoscincus ocellatus*), based on thermosensitive sex determination in both species, and on the discovery of XY chromosomes in *E. heatwolei* (Cornejo-Páramo et al., 2020) and sex-linked loci consistent with male heterogamety in *N. ocellatus* (Hill et al., 2018). Clearly, sex reversal under extremes of temperature experienced in natural nests or during gestation is reasonably common in lizards and likely a powerful evolutionary component in generating and maintaining lability and diversity in reptile sex-determining modes generally (Holleley et al., 2015; 2016). This is true also of some amphibians (Rodrigues et al., 2014) and fish species (Baroiller et al., 2009; Honeycutt et al., 2019) in which climatic gradients and environmental temperature strongly correlate with sex chromosome differentiation. In the Atlantic silverside *Menidia menidia*, sex is determined by an -

interaction between genotype and temperature and sex ratio differs among populations from different latitudes in response to temperature (Duffy et al., 2015).

In conclusion, in species with TSD and GSD systems and systems where temperature and genotype interact, predicting evolutionary responses to climate change becomes complex. We have shown a significant correlation between environmental variables and sex reversal in the wild adult population of *B. duperreyi* for the first time in a reptile species with XX/XY sex determination. We provide evidence that sex reversal can be a biologically significant process in Alpine populations and provide an opportunity to reinterpret sex ratio trends observed in other Alpine reptiles (as has been the case with *N. ocellatus* and *E. heatwolii*). Theory suggests that frequency of the Y chromosome in wild populations decreases with higher elevation, and that under extreme cold conditions in Alpine Australia, the likelihood of complete loss of the Y chromosome and a transition of *B. duperreyi* from GSD to TSD is quite high under future climatic cooling. In contrast, lower elevation populations are likely to exist stably as a GSD population utilizing XY sex chromosomes in the absence of sex reversal. Studies of the frequency of sex reversal in the wild nests, reproductive viability of adults, and the fitness consequences of sex reversal in this remarkable species and others like it is a priority, if we are to fully establish the contribution of sex reversal to our understanding of sex ratio evolution and sex chromosome evolution under environmental change in heterothermic vertebrates.

3.6 Acknowledgments

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Chapter 4

Lineage diversity within a widespread endemic Australian skink to better inform conservation in response to regional-scale disturbance

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4.1 Abstract

Much attention is paid in conservation planning to the concept of a species, to ensure comparability across studies and regions when classifying taxa against criteria of endangerment and setting priorities for action. However, various jurisdictions now allow taxonomic ranks below the level of species and non-taxonomic intraspecific divisions to be factored into conservation planning – subspecies, key populations, evolutionarily significant units or designatable units. Understanding phylogeographic patterns is a key component in the identification of species boundaries and determination of substantial geographic structure within species. Here we generated 12,532 reliable polymorphic SNP loci from 60 populations (263 individuals) covering the distribution of Australia's eastern three lined skink, *Bassiana duperreyi* to assess geneflow and to define population structure in the form of diagnosable lineages. We identified five well-supported diagnosable operational taxonomic units (OTUs) within *B. duperreyi*. Low levels of divergence of *B. duperreyi* between mainland Australia and Tasmania (no fixed allelic differences) support the notion of episodic exchange of alleles across Bass Strait (*ca* 60 m, 25 Kya) during periods of low sea level during the Upper Pleistocene rather than the much longer period of isolation (1.7 My) indicated by earlier studies using mitochondrial sequence variation. Our study provides foundational work for the detailed taxonomic re-evaluation of this species complex and the need for biodiversity assessment to include an examination of cryptic species and/or cryptic diversity below the level of species. Such information on lineage diversity within species can be factored into conservation planning regardless of whether a decision is made to formally diagnose new species taxonomically and nomenclaturally.

Keywords: *Bassiana duperreyi*, biogeography, DArTSeq, operational taxonomic units, phylogeny, phylogeography, SNP, 2019–20 Australian bushfires.

4.2 Introduction

Species are often regarded as fundamental units of conservation concern, and correct species delimitation as essential for an unbiased evaluation of biodiversity in a region or a country (Bickford et al., 2007). As a consequence, much attention is paid to species concepts, to ensure comparability across studies and regions when classifying taxa against criteria of endangerment and setting priorities for action. Lack of comparability lies in part in differing opinions on what should be regarded as species and what should be regarded as lineages within species (Sukumaran & Knowles, 2017). Some authors consider all substantive lineages to be species, and that speciation occurs at the point lineages first diverge (Fujita et al., 2012); others follow the biological species concept (Butlin & Stankowski, 2020) and its criterion of reproductive isolation (Mayr, 1942). There are many opinion between these extremes (de Queiroz, 1998), points of view that are often held for operational reasons. In particular, reproductive isolation in allopatry can rarely be definitively demonstrated in context and so presents a fundamental operational challenge to the application of the biological species concept. Regarding lineages as species brings a welcome level of objectivity, and so is favoured by some citing operational imperatives, but this approach admits potentially numerous ephemeral entities as species, that is, those lineages that will rapidly admix should the allopatric populations again come into contact (Kautt et al., 2016; Momigliano et al., 2017; Georges et al., 2018; Matute et al., 2020). In this paper, we take the view most recently espoused by Sukumaran & Knowles (2017) that there is a distinction between species in the biological sense and lineage diversity within species.

Were it not for the focus on species-level taxonomy by legislators, biodiversity assessment could entirely sidestep the issue of species delimitation (Miller et al., 2018). Lineage diversity, whether among or within species, and taking into account phylogenetic weighting (Faith & Baker, 2006), is sufficient as a framework for assessing biodiversity and comparing it through time and across regions. Biodiversity assessment and comparability across geographic and temporal scales has become more prominent as human-induced impacts extend from the local to regional, continental, and global scales (Pecl et al., 2017). Various jurisdictions now allow entities below the level of species to be

factored into conservation planning, as subspecies, key populations, evolutionarily significant units or designatable units (Green, 2005), and policy development continues in this area (Hoban et al., 2020), but the definitions of these entities are often vague, confusing for managers, and often ignored when it comes to implementation of priorities (Coates et al., 2018).

An excellent case in point of the importance of complete and up to date taxonomic assessments in conservation planning is that of the regional catastrophic fires in Australia. In the Austral summer of 2019-2020, approximately 97,000 km2 of vegetation across southern and eastern Australia was scorched by fires of intensity unprecedented in modern times (Ward et al., 2020; Godfree et al., 2021). Over a billion animals perished in the fires (WWF, 2020), and many species, already endangered, were brought closer to the brink of extinction. But what of cryptic diversity, the diversity represented by lineages within currently accepted species? Species-level taxonomy lags behind the demands of data used to set priorities for conservation (the "taxonomic impediment"); diversity of lineages within species is arguably even more poorly documented for many species. Yet many of these lineages within species are deeply divergent and some can be regarded as incipient or undescribed species. They are an essential component of biodiversity and their distribution in the context of regional scale disturbance is an important component of conservation planning. Because their ranges are typically narrower than the species to which they are currently assigned, such substantive lineages are particularly vulnerable to regional-scale catastrophic events, such as widespread bushfires.

Geographical barriers, past palaeoclimatic incidents, climatic/environmental factors, and populations surviving glacial maxima in disconnected refugia have played a vital role in the diversification of Australian reptile fauna (Dubey & Shine, 2010; Chapple et al., 2011a, Chapple., 2011b; Rosauer et al., 2015; Pepper et al., 2018). The family Scincidae represents unsurpassed diversity among vertebrates within the Australian continent (Cogger, 2014; Mitchell et al., 2019). In particular, the forests of eastern Australia are a global biodiversity hotspot and in the top 2.5% of global species richness for lizards (Williams et al., 2011; Roll et al., 2017). Advances in genetic techniques and evolutionary models have accelerated biological studies into isolation and divergence of populations. Current progress in the fields of genomics has opened new opportunities to better to understand relationships among the populations within species and their phylogeography.

The eastern three-lined skink, *Bassiana duperreyi* (Gray, 1838), is a medium-sized, oviparous scincid lizard, distributed broadly across southern and south-eastern Australia (Cogger, 2014), including the areas most impacted by the recent fires (Godfree et al., 2021). The widespread nature of this species across a range of bioregions, including cool Alpine, woodland, and heaths to coastal habitat, provides an excellent model to examine lineage diversity within a single species and relate this to habitat alteration and loss. We generated genome-wide data using the complexity reduction method DArTseq™ (DArT Pty Ltd, Canberra, Australia) which combines next-generation sequencing to generate a genome-wide sample of single-nucleotide polymorphisms (SNPs) (Kilian et al., 2012). This technique has recently become a popular tool for understanding genetic diversity, gene flow, lineage phylogenies, species delimitation, and evolutionary history of a range of organisms for which there is little or no prior genomic information (Melville et al., 2017; Georges et al., 2018).

Our specific goal was to assess lineage diversity within B. duperreyi in south-eastern Australia and make an informed decision on which lineages should be regarded as species and which should be regarded as representing substantial diversity within species. We couple our nuclear DNA data with previously published mtDNA data (Dubey & Shine, 2010) to identify substantial lineage diversity within this species of relevance to management, which will provide a foundation for a comprehensive taxonomic evaluation in the future. We also consider the implications for management in the context of widespread fires of lineage diversification within this species.

4.3 Materials and methods

4.3.1 Sampling

We sampled 286 individuals of *B. duperreyi* from 63 sample localities across the range of the species, including tissues sourced from museum collections (Museums Victoria and South Australian Museum) and collected in the field (Figure 1, see Supporting Information Tables S1). Up to 10 samples per locality were collected when available; for samples obtained from museum collections and field collections, we assigned individuals captured within a 20 km radius to a single locality. We conducted fieldwork in areas where the lizards were most abundant; in natural open areas, fire trails or tracks inside the forest areas. Adult B. duperreyi were captured by hand when actively foraging or under rocks. Tail tips (4-5 mm) were removed with a sterile blade and stored in labelled tubes -

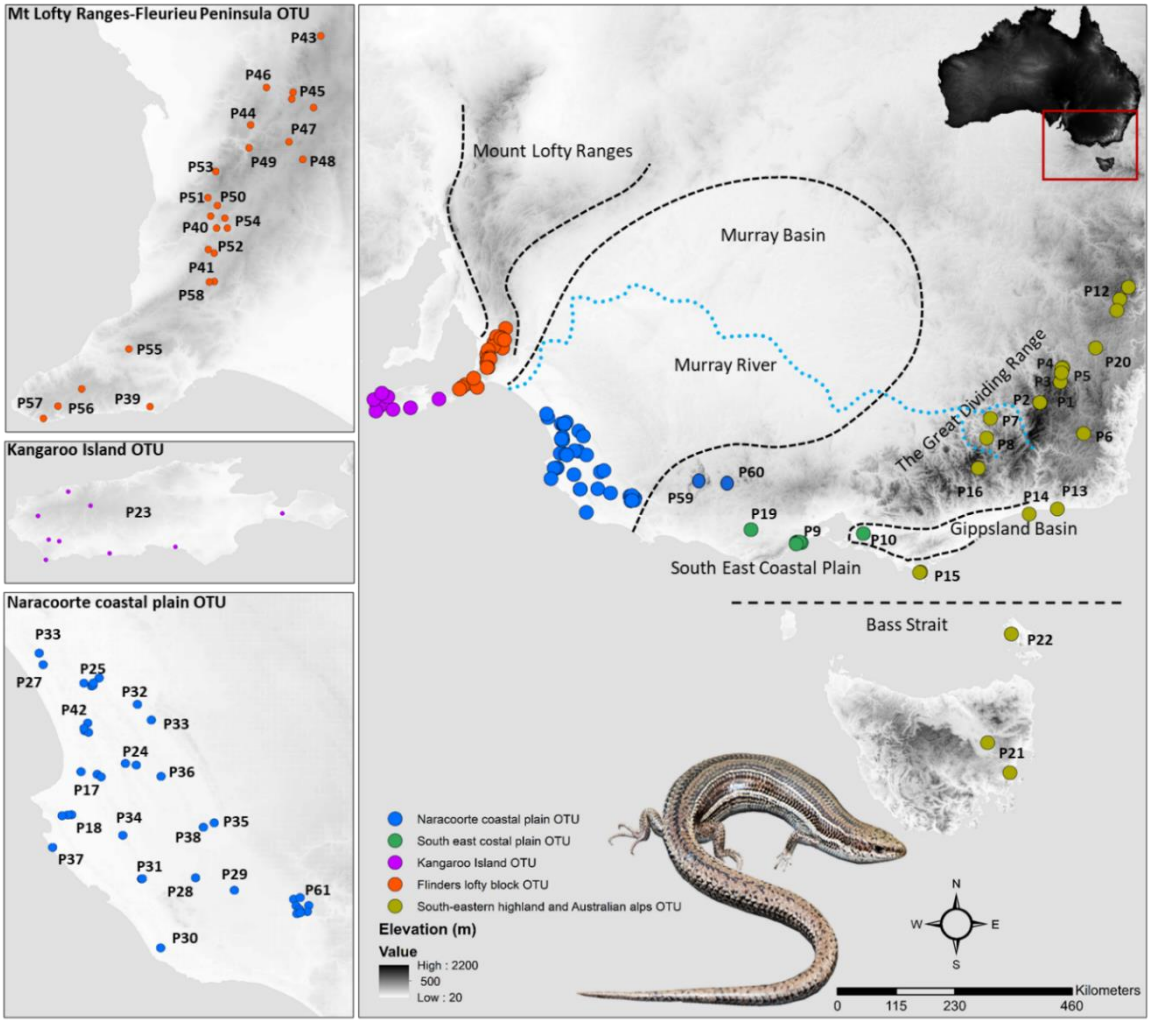

Figure 4-1. Location of *B. duperreyi* populations SNP genotyped in this study from across the range of the species in south-eastern Australia, and including the location of recognised biogeographic barriers. Colour scheme is consistent with other figures and OTUs as described in Figure 4.2. Underlying map generated using ArcGIS 10.5.1 (http://www.esri.com) and data from the Digital Elevation Model (Geoscience Australia) made available under Creative Commons Attribution 3.0 Australia (https://creativecommons.org/licenses/by/3.0/au/legalcode, last accessed 9-Jul-20).

containing 95% ethanol for subsequent DNA extraction. Lizards were released at their point of capture. All collection protocols were conducted with the permission of Animal Ethics Committees at the University of Canberra and the CSIRO. Description of samples of *B. duperreyi* from each locality and their assigned population labels are provided in the Supplemetayr T

Our taxonomic nomenclature follows that of Hutchinson et al., (1990). Outgroup species included were: *Bassiana platynota* (n = 2), *Bassiana trilineatus* (n = 2), *Pseudemoia pagenstecheri* (n = 1) and *Niveoscincus coventryi* (n = 1). Nomenclature for bioregions follow that of Australia's bioregions (IBRA) and the description of the recognized biogeographic barriers in eastern Australia, according to Chapple et al., (2011b).

4.3.2 DNA extraction and SNP genotyping

Tissue samples were sent to Diversity Array Technology Pty Ltd. (DArT), Canberra, Australia, for SNP genotyping. DNA was extracted using the NucleoMag® 96 Tissue kit (Macherey- Nagel, Düren, Germany) coupled with NucleoMag SEP (Ref. 744900) to allow automated separation of high-quality DNA on a Freedom Evo robotic liquid handler (TECAN, Männedorf, Switzerland). Four combinations of restriction enzymes were evaluated for *B. duperreyi* (*Pst*I enzyme combined with either *Hpa*II, *Sph*I, *Nsp*I, and *Mse*I) and the restriction enzyme combination of *Pst*I (recognition sequence 5'-CTGCA|G-3') and *Sph*I (5'-GCATG|C-3') was selected for the complexity reduction by double digestion.

DNA was digested then processed following Kilian et al., (2012), with two different adaptors annealed to the two restriction enzyme overhangs. The library was then subjected to competitive PCR and sequenced using an Illumina Hiseq2500. The sequencing (single-end) was run for 77 cycles. A full account of the DArTseq™ process used to generate sequences for these samples is given by Georges et al., (2018). The data comprised a matrix of SNP loci by individuals, with the contents stored as 0, homozygote, reference state; 1, heterozygote; and 2, homozygote, alternate state.

4.3.3 Additional SNP filtering

The SNP data and associated locus metadata were read into a genlight object (R Package adegenet; Jombart, 2008) to facilitate processing with package dartR v.1.5.533 (Gruber et

al., 2018). Only loci with > 99% repeatability (repAvg) were chosen for subsequent analysis. Further filtering was undertaken based on the call rate (> 95%), and where multiple SNPs occurred within a single sequence tag, only one was retained at random. The population sample sizes were small ($n \leq 11$), so we could not filter loci for departures from Hardy–Weinberg equilibrium (HWE) or linkage disequilibrium; the sparse sampling of loci across the genome allows the reasonable assumption of little or no linkage between loci. We regard the data remaining after filtering as highly reliable.

4.3.4 Visualization and qualitative analysis

Genetic similarities for individuals and populations were visualized using principal coordinates analysis (PCoA) as implemented in the gl.pcoa and gl.pcoa.plot functions of dartR. A scree plot of eigenvalues (Cattell, 1966), taken in the context of the average percentage variation explained by the original variables (using the diagnostics provided by gl.pcoa function in dartR), guided the number of informative axes to examine.

4.3.5 Genetic diversity

Observed heterozygosity was used as a measure of relative genetic diversity. Heterozygosity was obtained for each population from allele frequencies using the gl.report.heterozygosity function of dartR.

4.3.6 Fixed difference analysis

To examine the possibility that more than one taxon (Operational Taxonomic Unit, OTU) might exist within the geographic distribution of *B. duperreyi* sensu lato, a fixed-difference analysis was done using the scripts gl.fixed.diff and gl.collapse in dartR. An OTU is defined here as an aggregation of populations that can be differentiated by other such aggregations by one or more diagnostic characters. A fixed difference between two populations at a locus occurs when the populations share no alleles at that locus. Accumulation of fixed differences between the two populations is a strong indication of a lack of gene flow. Fixed differences were summed over populations taken pairwise, and when two populations had no fixed differences, they were combined, and the process repeated until there was no further reduction. The resultant OTUs are, by definition, putatively diagnosable at one or more SNP loci. The set of putative OTUs arising from the above fixed-difference analysis was then tested for significance (using the test=TRUE option in gl.collapse.recursive.r in dartR, (Georges et al., 2018), and pairs for which the

number of fixed differences was not statistically significant (number less than expected false positives given the sample sizes) were further amalgamated.

4.3.7 Phylogeny

To evaluate the relationships among individuals of *B. duperreyi*, we performed SVDquartets analysis on the individuals grouped by locality (including the individuals from locality: P60 and P59) after further filtration. We selected SVDquartets analysis (Chifman & Kubatko, 2014) because our dataset was composed of short reads with single variable sites per locus (Chou et al., 2015). Heterozygous SNP positions were represented in the dataset by standard ambiguity codes. We used the implementation of SVDquartets in PAUP* v.4.0a165 (Swofford, 2003) with parameters evalQuartets = random, bootstrap = standard, nreps = 10 000 and ambigs = distribute, and designated the *B. platynota* and *B. trilineatus* as the outgroup. We then assessed the relationships concerning their geographic origin and compared the clades identified with their geographic distributions.

4.4 Results

4.4.1 SNP datasets

The full dataset, which includes all samples, comprised 232,230 polymorphic SNP loci from the sample set comprising samples from 63 sampling localities for the ingroup taxa (n individuals per sampling locality = 1-11, N total individuals = 286) and six outgroup taxa. After stringent filtering on repeatability (repAvg = 0.99) and call rate (0.95), the number of SNP loci in the data set dropped to 66,907 and then 58,906, respectively. A total of 1,655 secondary SNPs, multiple SNPs occurring on a single sequence tag, were also filtered leaving one selected at random (see Materials and Methods). Two outgroup specimens (*P. pagenstecheri* and *N. coventryi*) and the sole individuals from the Cooma (P6) and Mount Franklin populations (P11) each had an individual call rate of less than < 0.4 (a threshold set taking into account the presence of the outgroups) and were removed from the data. The resultant data, including outgroup specimens (*B. platynotus and B. trilineatus*), are referred to as the full data set, comprising 14,063 polymorphic SNP loci from 60 ingroup sampling locality (n = 1–11, N = 263) and two sampling localities of the outgroup species (each with n = 4). Finally, the *B. duperreyi* dataset was obtained by sub-setting the ingroup dataset to include only individuals of *B. duperreyi* and removing resultant monomorphic loci to yield 12,532 polymorphic SNP loci from 60 sampling localities.

4.4.2 Visualization and qualitative analysis

Preliminary analysis of the data with PCoA applied to the ingroup data set revealed clear evidence of four distinct clusters within the distribution of *B. duperreyi* in Australia (Figure 4.2). Variation represented in Axis 1 (20.5%) separates populations from the south-eastern highland and Australian alps from populations of the south-east coastal plain of Victoria and to the west. Axis 2 explains 6% of variation, separating the western block of populations into 3 distinct clusters: Kangaroo Island and Flinders/Mt Lofty Ranges, the Naracoorte coastal plain, and south eastern coastal plain.

4.4.3 Fixed difference analysis

Both of the outgroup taxa *B. platynotus* and *B. trilineatus*, emerged as diagnosable in the fixed difference analysis applied to the full data set, differing significantly from the expected false positive rate ($p < 0.0001$). Four individuals Z_60614 (P61), AA80788 (P9), Z_22574 (P60), and Z_22563 (P59) that fell in intermediate positions between major groupings were considered to be examples of contemporary admixture and were removed (following Georges et al.,2018; Unmack et al., 2021). After that, we filtered out 81 monomorphic loci and 12,451 SNPs were retained for further analysis. Five diagnosable OTUs emerged from the fixed difference analysis (Table 1, Figure 4.2). The 16 populations from the south-eastern alpine region to Tasmania and including Wilsons Promontory and Flinders Island, emerged as the single largest diagnosable OTU (South-eastern highland and Australian alps OTUs; Figure 1 and 2). Populations from Westernport bay to Corangamite in southern Victoria formed a second diagnosable OTU (South-east coastal plain OTU), and the genetically distinctive population in the Naracoorte Coastal Plain was defined as a third OTU (Naracoorte Coastal Plain OTU). Populations from the Mt Lofty range extending to southern south Australia formed a fourth diagnosable OTU (Mt Lofty Ranges-Fleurieu Peninsula OTU), and a genetically distinctive population on Kangaroo Island was identified as the fifth OTU (Kangaroo Island OTU). All OTUs differed by 3 – 47 fixed differences (See Table 1). These diagnosable OTUs are in broad agreement with the structure evident in the PCoA plot (Figure 4.2). The fewest fixed differences were observed between Kangaroo Island OTU and Mt Lofty Ranges-Fleurieu Peninsula OTU, whereas the most were observed between South-eastern highland and Australian alps OTU and the Kangaroo Island OTU.

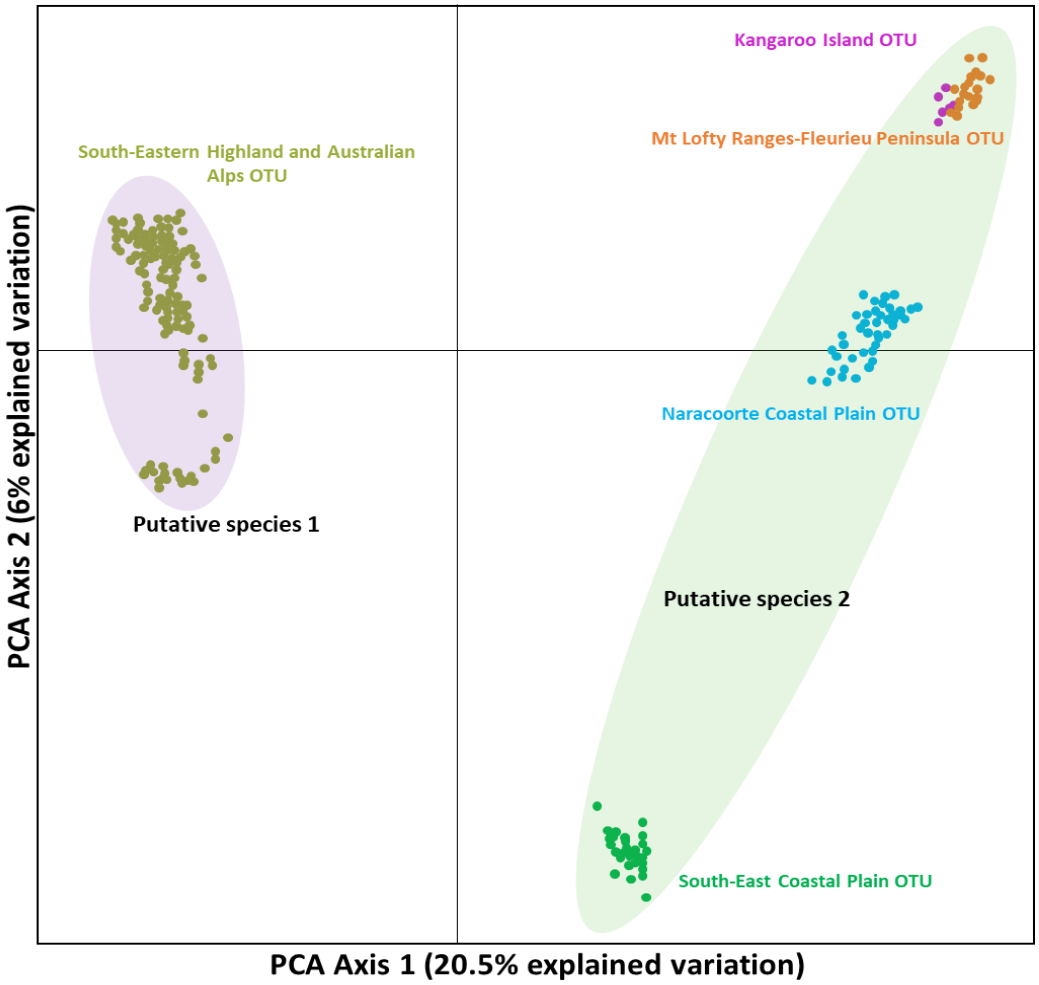

Figure 4-2. Genetic similarity between individuals using principal coordinates analysis of 12,451 SNP (in group analysis only and recalcitrant individual or population does not present here). Five diagnosable OTUs are defined. Axes not to scale. Colour scheme is consistent with figures 4.1 and 4.3.

4.4.4 Phylogenetic inference

The fixed difference analysis directs consideration of diagnosability on to the phylogeny to identify those lineages that are diagnostic (Figure 4.3). The SVDquartets phylogeny of the full phylogeographic dataset had strong bootstrap support across many deeper nodes. Each of the five OTUs identified in the fixed difference analysis emerged as strongly supported clades (> 82% bootstrap support) (Figure 4.3). The South-eastern highland and Australian alps OTU showed two distinct sister lineages with 100% bootstrap support. These two lineages include more eastern distribution from the Australian alps (P1 to P8, P12 and P20) and populations from the south east of the distribution, including Mt Hotham (P16), Cape Conran (P13), Wilsons Promontory (P15), Flinders Island (P22), and south east Tasmania (P21). In general, samples were strongly structured according to their biogeographic locations within the Australian bioregion and with associated geographic barriers, with two exceptions: (1) the sample from the Grampians (P60) exhibited substantial divergence from the neighbouring Naracoorte populations with 98% bootstrap support; (2) the sample from the Black Range Forest (P59) exhibited a substantial divergence from the Naracoorte Coastal Plain OTU with 82% bootstrap support. There were no significant divergence populations within Naracoorte Coastal Plain OTU. The Kangaroo Island and Mt Lofty Ranges-Fleurieu Peninsula OTUs showed a 100% divergence between the population, and there were no significant divergence populations recorded within Mt Lofty Ranges Fleurieu Peninsula OTU.

4.5 Discussion

This study provides quantification of population structure for the eastern three-lined skink *B. duperreyi*, and delineates the distribution of structural elements across the landscape to provide valuable guidance to conservation efforts to protect linage level species diversity in the face of regional scale divergence. Accurate species delimitation of species and structural elements within species are critical for evaluating biodiversity on regional or global scales and for informing effective conservation strategies (Mace, 2004). Failure to appropriately identify species boundaries, structure within species and the distribution of these across the landscape can have significant consequences for conservation management of imperilled species by diverting finite conservation resources (Agapow et al., 2004). Here we studied *B. duperreyi* as a case study for how within-species

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Table 4-1. Matrix of Euclidean genetic distances (above diagonal) and fixed genetic differences (below diagonal) between the final set of operational taxonomic units to arise from a fixed difference analysis applied to the ingroup data set. Comparisons were based on an average of 12,532 loci after filtering for call rate >98%. All fixed differences were significant at $p < 0.0001$.

	South eastern highland & alps	South east coastal plain	Naracoorte coastal plain	Mt Lofty Ranges-Fleurieu Peninsula	Kangaroo Island
South eastern highland & alps	0	15.36	16.19	18.83	19.32
South east coastal plain	8	0	13.9	17.05	17.71
Naracoorte coastal plain	7	6	0	12	13.01
Mt Lofty Ranges-Fleurieu Peninsula	34	5	5	0	8.51
Kangaroo Island	47	37	10	3	0

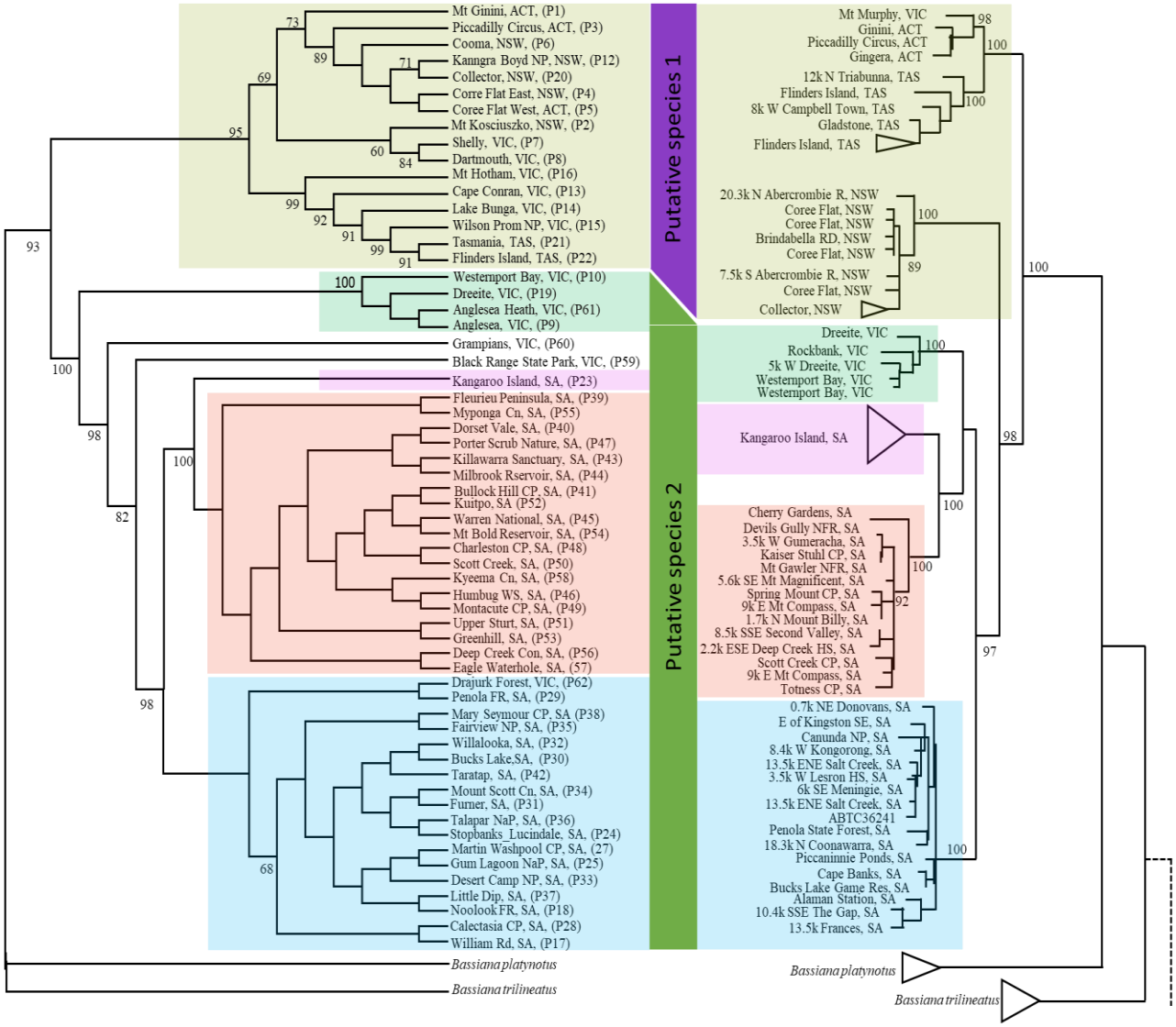

Figure 4-3. Phylogenetic analyses of Dartseq SNPs with SVDquartets (left) compared to a published phylogeny of two partial mitochondrial genes (ND_2 and ND_4) (not to scale) (See Dubey and Shine, 2010). Bootstrap support values are reported for all nodes. Branch lengths are not meaningful for the SVDquartets tree.

diversity and its distribution across the landscape in the context of regional-scale disturbance can substantially alter perspectives and priorities set for conservation.

The eastern three-lined skink *B. duperreyi* is currently regarded as a single species (Hutchinson et al., 1990). Mitochondrial variation within this species (Dubey & Shine, 2010) revealed seven geographically localised mitochondrial lineages within *B. duperreyi* – referred to as Kangaroo Island, western South Australia, eastern South Australia, southern Victoria, Tasmania, northern Victoria and the Australian Capital Territory, and New South Wales. The genetic distances between the mitochondrial lineages of *B. duperreyi* varied from 2.3% (Tasmania cf southern Victoria) to 5.3% (western South Australia cf Tasmania). Given their presumed mitochondrial sequence divergence rate of 1.3% per Myr, Dubey and Shine (2010) concluded that lizards of the genus *Bassiana* evolved in southern Australia over at least 10 Myr (interspecific divergences), and most of the major intraspecific lineages, including those in *B. duperreyi*, diverged between 5.7 to 1.7 Mya. On the basis of these data, they concluded that climatic fluctuations and associated sea level change during the Upper Pleistocene (126,000 to 12,000 years ago, including the Last Glacial Maximum) did not substantially affect the extent of areas within which the lizards could persist, with the result that the genetic signature of ancient divergence events remains clearly expressed in modern-day populations as strong geographically-associated genetic structure (Dubey & Shine, 2010).

Our nuclear data provide additional evidence of strong geographically-associated genetic structure, but with only five well-supported operational taxonomic units (OTUs) within *B. duperreyi* – Kangaroo Island, a Mt Lofty Ranges-Fleurieu Peninsula in South Australia, Naracoorte coastal plain in southwestern Victoria, the south east coastal plain in Victoria, and an eastern highlands and Australian Alps OTU comprising populations from the ACT, NSW, south-eastern Victoria, Flinders Island and Tasmania (Figures 2 and 3). Our five OTUs are broadly consistent with the seven mitochondrial lineages of Dubey and Shine (2010). However, our analysis differs from that of Dubey and Shine (2010) in that they have the Tasmanian, NSW and ACT lineages (and Mt Murphy, Vic) within a paraphyletic assemblage, outside the remaining lineages of *B. duperreyi*, whereas we have the Tasmanian lineage as part of a broader eastern lineage, that is, sister to the remaining lineages to the west (Figure 3). Our largest OTU from the fixed difference analysis combines the 16 populations from the south-eastern Alpine region (see Table 1) with Tasmania as a single diagnosable OTU, including Wilsons Promontory and Flinders island.

This raises the question as to how this series of populations could have maintained common allelic composition (no significant fixed allelic differences) at all loci over the > 1.7 Myr of hypothesized isolation (Dubey & Shine, 2010). A more likely explanation is that the divergence of the mitochondria and the divergence of the lizard lineages do not concur temporally, and that there has been episodic exchange of alleles across Bass Strait (ca 60 m, 25 Kya, Harris et al., 2005) during periods of low sea level during the Upper Pleistocene. The distinction in mitochondrial sequence between Tasmania and the eastern populations of Victoria, NSW and the ACT may have arisen through incomplete lineage sorting, preferential dispersal by males or localized selection for what is a highly constrained mitochondrial genome. Hence the dates of separation of the Tasmanian and mainland populations, estimated to be 1.7 Myr by Dubey and Shine (2010), may apply to the mitochondria but not reflect the history of isolation of the species between Tasmania (incl. Flinders Island) and the mainland, a history that admits exchange during their last terrestrial interconnection in the Upper Pliestocene.In contrast, both the mitochondrial and nuclear data support isolation of the Kangaroo Island populations from the adjacent mainland that dates back beyond the last terrestrial interconnection between the two (36 m, ca 1 Kya). The reasons for this remain speculative, but it is possible that the intervening land bridge between the two areas, when sea levels were lower, contained habitat that was not conducive to the dispersal of *B. duperreyi*.

Volcanic activity in western Victoria during the late Pliocene to Holocene and Murray basin (see Schodde & Mason, 1999) could have led to a deep divergence between South-east coastal plain OTU (Anglesea) and Grampians populations and Naracoorte Coastal Plain OTU of *B. duperreyi*. This observation is similar to that made for several other species, including lizards (Chapple et al., 2005; Chapple et al., 2011a; Ng et al., 2014; Ansari et al., 2019), amphibians (Schäuble & Moritz, 2001; Symula et al., 2008), marsupial dunnarts (Cooper et al., 2000) and grasshoppers (Kawakami et al., 2009). It is a concordant pattern that is believed to be the result of repeated marine inundation of the area since the Miocene, coupled with the abovementioned volcanic activity. We highly recommend further work on these populations with a larger sample size to understand gene flow between the populations and effect of volcanic isolation. Deep divergence was observed between Kangaroo Island OTU and Mt Lofty Ranges-Fleurieu Peninsula OTU. The two-populations are believed to have split during the Upper Pliocene-Lower

Pleistocene. This agrees with the sea-level-driven dispersal opportunities identified by Dubey and Shine (2010).

The level of structure we observed across the range of *B. duperreyi* is comparable to other widely distributed species within a geographical range of similar size (Symula et al., 2008; Chapple et al., 2011b; Smissen et al., 2013). This presumably arises as a response to common ecological or geomorphological barriers – such as the Great Dividing Range, the Murray River or habitat structure across the landscape and, as we have discussed, episodic isolation from the mainland of Tasmania, Flinders Island, and Kangaroo Island by rising sea levels. The Murray River appears to be a barrier to dispersal for a range of species (reviewed by Ansari et al., 2019), including lizards, since it extensively dries and breaks into discrete pools nearly once every century (Close, 1990). *Bassiana duperreyi* fits this pattern, and dating studies for this (Dubey & Shine, 2010) and other species suggest a Plio-Pleistocene diversification in response to barriers afforded by Lake Bungunnia which formed ca 2.5 Mya and persisted to ca 700 Kya when the modern Murray River was established (Stephenson, 1986; McLaren et al., 2011). It appears that attributes of the modern Murray River and the habitats supported in its basin have been sufficient to maintain signatures of the more ancient divergences in both nuclear and mitochondrial genes of *B. duperreyi* (present study) and *Tiliqua rugosa* (Ansari et al., 2019), though the haplotype distribution of the 11 nuclear genes in *T. rugosa* were less definitive than the SNP markers were for *B. duperreyi*.

Does *B. duperreyi* comprise more than one species? We have shown the species to comprise five lineages that have diverged to the point of being diagnosable by one or more corroborated allelic fixed differences. Under some species concepts, diagnosability of a lineage is sufficient to warrant recognition as a species. We take the view, however, that genetic diagnosability, while necessary for a lineage to be considered as a named taxon, is not sufficient (Georges et al., 2018). That is, we admit the possibility of substantial structure within species and take a conservative approach to which diagnosable lineages should be regarded as species as opposed to recognized entities within species (Evolutionarily Significant Units, Management Units or other Designatable Units). Although there are five diagnosable OTUs, one in particular stands out as particularly distinct, and we have thus identified two putative species within what is currently regarded as *Bassiana duperreyi*. That is, results of both SNPs and the mitochondrial gene tree analysis suggest that the current taxonomy of *B. duperreyi* which has it as a single species,

is not supported and will hopefully prompt revision supported by a combined genetic and morphological analysis. A formal description of the species is beyond the scope of our current study.

Without adequate morphological data, we cannot resolve the taxonomic issues here. However, we propose that there are two putative species within *B. duperreyi*. The first is distributed in south-eastern highland and Australian alpine region (including Wilsons Promontory, Flinders Island, and Tasmanian) and the second is an aggregation of diagnosable lineages (ESUs) occupying the lower elevation regions and coastal regions (including Kangaroo Island, the type locality) (Figure 4.3). They have broadly parapatric distributions (Figure 1). The south-eastern highland and alpine taxon is of particular interest, because it has a system of sex determination that involves both differentiated sex chromosomes and sex reversal of the XX genotype to a male phenotype at a frequency that aries predictably with elevation (Dissanayake et al., 2020; Dissanayake et al., 2021a, 2021b).

Our study has clear management implications in the context of regional catastrophic events and progressive habitat fragmentation and modification at regional levels. The recent fires in Australia were restricted to the eastern mainland portion of the range of *B. duperreyi* as currently defined (and Kangaroo Island) (Figure 4.4). Concern for the impact of the fires is ameliorated somewhat by the existence of substantial populations of *B. duperreyi* to the west that were unimpacted by fire. When we consider biodiversity within the species, and in particular the existence of five diagnosable lineages, two of which could be considered putative species, then concerns are reignited. The mainland habitat of the distinctive eastern highlands and Australian Alps OTU (putative species 1 and potentially unnamed) has been severely impacted by fire, which raises concerns for its conservation. Tasmania and Flinders Island provide insurance, but the widespread impact of fire on the habitat of this eastern highlands and Australian Alps lineage is now of serious management concern.

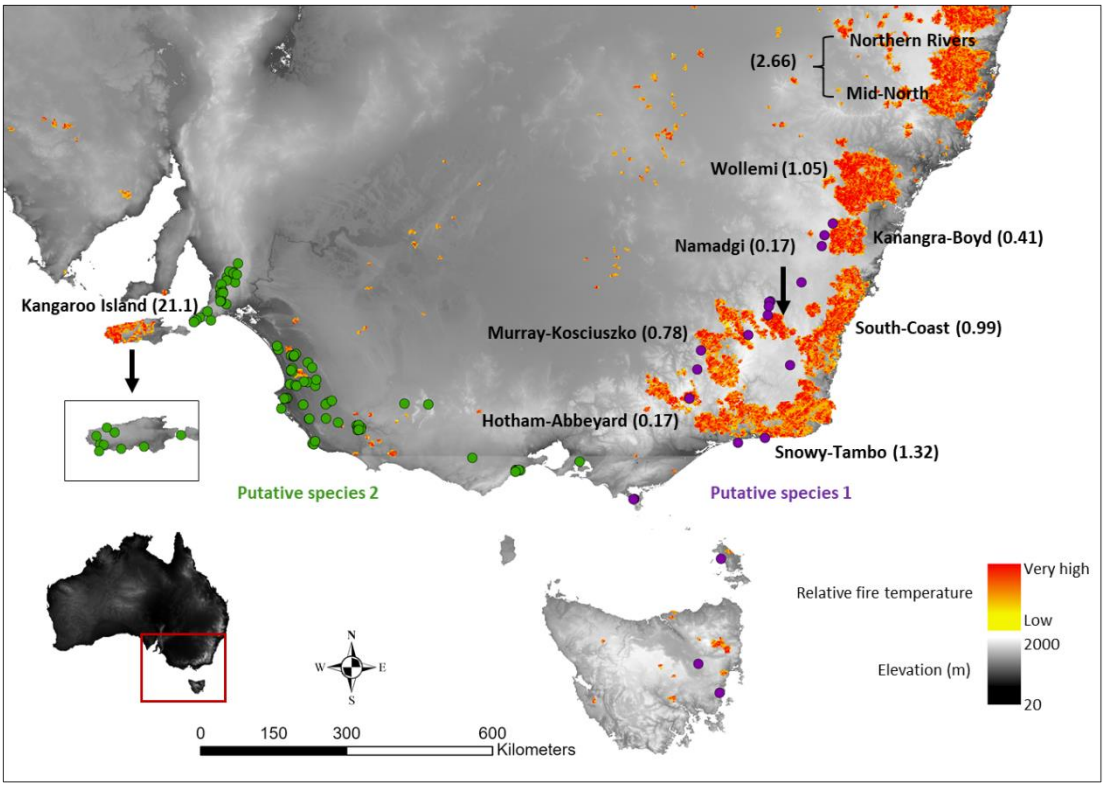

Figure 4-4. The distribution of *Bassiana duperreyi* in relation to the intensity and extent of the Australian megafire event, which occurred from 1st July 2019 - 11th February 2020. Refer to Supporting Information Tables S1 and Figures 1 and 3 for the corresponding population details. The fire intensity and distribution data were obtained from Godfree et al. (2021). The name of the each megafire bracketed value after each megafire is the fire area in millions of hectares. Underlying map generated using ArcGIS 10.5.1 (http://www.esri.com) and data from the Digital Elevation Model (Geoscience Australia) made available under Creative Commons Attribution 3.0 Australia (https://creativecommons.org/licenses/by/3.0/au/legalcode, last accessed 9-Jul-20).

Similarly, our demonstration of the distinctive lineage of *B. duperreyi* on Kangaroo Island, also seriously impacted by the recent fires, raises its priority for its assessment and management. Our genome-wide SNP analysis of structure within a widespread species highlights the need for biodiversity assessment at the regional scale to include an examination of cryptic diversity below the level of species, and perhaps also a re-evaluation of species delimitation. Such information on lineage diversity within species and the distribution of those lineages across the landscape can be factored into conservation planning regardless of whether a decision is made to define new species with formal taxonomic decisions.

4.6 Acknowledgments

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Chapter 5

Skewed sex ratios in an alpine lizard: understanding the effects of natural nest temperature on sex reversal in the Eastern three-lined skink.

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5.1 Abstract

Altered climate regimes have the capacity to affect the physiology, development, ecology and behaviour of organisms dramatically, with consequential changes in individual fitness and so the ability of populations to persist under climatic change. More directly, extreme temperatures can directly skew the population sex ratio in some species, with substantial demographic consequences that influence the rate of population decline and recovery rates. In contrast, this is particularly true for species whose sex is determined entirely by temperature (TSD). The recent discovery of sex reversal in species with genotypic sex determination (GSD) due to extreme environmental temperatures in the wild broadens the range of species vulnerable to changing environmental temperatures through an influence on primary sex ratio. Here we document the levels of sex reversal in nests of the Australian alpine three-lined skink (*Bassiana duperreyi*), a species with sex chromosomes and sex reversal at temperatures below 20°C and variation in rates of sex reversal with elevation. The frequency of sex reversal in nests of *B. duperreyi* ranged from 28.6% at the highest, coolest locations to zero at the lowest, warmest locations. Sex reversal in this alpine skink makes it a sensitive indicator of climate change, both in terms of changes in average temperatures and in terms of climatic variability.

Keywords: Climatic change, Sex ratio, Y chromosome, Nest temperature

5.2 Introduction

Climate change has one of the most widespread effects on organisms across diverse ecosystems (Parmesan, 2006; Peñuelas et al., 2013). The big question is whether living species can adapt quickly enough to persist given current rates of climate change? Of particular concern are species whose fundamental biology is directly affected by ambient thermal regimes, particularly those whose sex is determined by temperature. Evolution is typically thought to occur slowly in comparison with ecological and demographic processes. Phenotypic plasticity not an evolutionary response, at least proximally, and is generally invoked to explain or predict species responses to rapid climate change (Chevin et al., 2010; Oostra et al., 2018; Parmesan, 2006). Still, if the selection is particularly strong in the context of high genetic variability and heritability, or if Fisher's frequency-dependent selection is involved, rapid evolution under climate change is possible (Conover & Voorhees, 1990; Hoffmann & Sgrò, 2011; Holleley et al., 2015; Kopp & Matuszewski, 2014).

While the process of sex determination is relatively conserved in mammals (Graves et al., 1995) and birds (Smith et al., 2007), reptiles exhibit many different sex-determining systems, some involving nest temperatures as the sex-determining factor (Bull, 1980; Deeming et al.,1988; Janzen & Paukstis, 1991). In some other species, sex is determined by an interaction between genotype (chromosomal sex) and environment (Holleley et al., 2015; Quinn et al., 2007; Shine et al., 2002; Wiggins et al., 2020). In particular, sex reversal under the influence of high or low developmental temperatures has been demonstrated in two squamates. The Australian dragon lizard, *Pogona vitticeps*, has a female heterogametic ZZ/ZW system of chromosomal sex determination whereby the ZZ genotype is reversed to a female phenotype at high incubation temperatures in both the laboratory (Quinn et al., 2007) and the field (Holleley et al., 2015). The Australian skink, *Bassiana duperreyi* (Our taxonomic nomenclature follows that of Hutchinson et al., 1990), has a male heterogametic XX/XY system of chromosomal sex determination whereby the XX genotype is reversed to a male phenotype at low incubation temperatures, again both in the laboratory and the field (Dissanayake et al., 2021a; Holleley et al., 2016; Quinn et al., 2009; Radder et al., 2008; Shine et al., 2002). Other reptile species appear to show an underlying genetic predisposition that is over-ridden by temperature (Capel, 2017; Whiteley et al., 2021), suggesting that the phenomenon of sex reversal could be quite widespread.

Understanding sex reversal in reptiles is essential because it has profound demographic and evolutionary consequences. Alpine *B. duperreyi* is distributed from *ca* 300 m a.s.l. to, *ca* 2020 m a.s.l in the Australian Alps of south-eastern Australia (Cogger, 2018; Dissanayake et al., 2021a). As an oviparous lizard, the developing embryos experience a wide range of environmental conditions, particularly high daily, seasonal, and stochastic temperature variation during their incubation period. The question arises as to what effect this has on the frequency of sex reversal and the sex ratio? What are the likely consequences of sex reversal under changing climate, and what scope does the species have to moderate climate change effects through phenotypic plasticity in the timing of nesting, nest site selection, and nest construction? To address these questions, we provide data on the relationship between fluctuating temperatures in natural nests, the frequency of sex reversal in those nests and the impact on offspring sex ratios.

5.3 Materials and methods

5.3.1 Study sites and field season

Four sites along an elevational gradient were selected within the alpine region of the range of *B. duperreyi* in mainland south-eastern Australia (Figure 5.1a). This series of populations is within a single substantive evolutionary lineage of the species (Dissanayake et al., 2021a). Mount Ginini (ACT, 35°31’29.6"S 148°46’58.7"E) has the highest elevation (1640 m a.s.l.), followed by Piccadilly Circus (1240 m a.s.l., ACT, 35°21’42.0"S 148°48’12.5"E), Cooma (960 m a.s.l., NSW, 36°26’48.6"S 149°11’40.6"E) (Figure 5.1b) and Dartmouth (380 m a.s.l, Victoria, 36°31’35.9"S 147°28’53.0"E) with the lowest elevation.

Due to a limited time frame and logistical constraints (significant bush fires in the field locations and adjacent areas (Godfree et al., 2021), we conducted fieldwork at Piccadilly Circus only during 2017/18 (first season). During the subsequent season of 2018/19, we completed fieldwork at all four sites. For this reason, nest temperatures with the season and associated sex reversal frequency were analysed only for Piccadilly Circus.

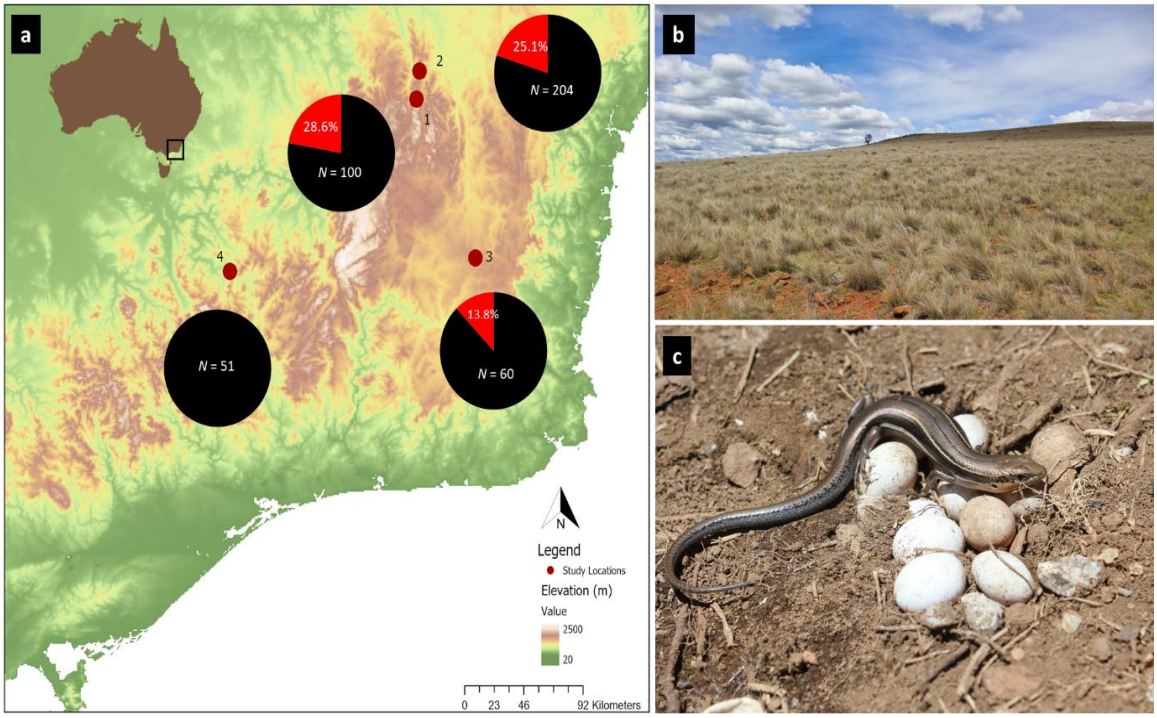

Figure 5-1. (a). Study locations and relative proportion of phenotypic males (black) and sex- reversed XX males (red); (Populations: 1. Mt Ginini, 2. Piccadilly Circus, 3. Cooma and 4. Dartmouth. N = total number of eggs collected during the study period. Underlying map generated using ArcGIS 10.5.1 (http://www.esri.com) and data from the Digital Elevation Model (Geoscience Australia) made available under Creative Commons Attribution 3.0 Australia (https://creativecommons.org/licenses/by/3.0/au/legalcode, last accessed 21-Dec-20). (b). Typical habitat of *B. duperreyi* distribution (Cooma field location). (c). A typical nest of *B. duperreyi* (Location: Piccadilly Circus). All photos were taken by the first author.

5.3.2 Climatic data

Thermal data-loggers (iButton® model DS1921G, Maxim Integrated, San Jose, CA, USA; accuracy ±1°C from -30°C to +70°C; dia 17 mm, height 6.2 mm, mass 3.2 g) were placed to measure the soil temperature at the surface, 10 cm and 20 cm depth at each field locations.

For each location, daily projections of climatic data were obtained from a publicly available spatial data repository (SILO, Scientific Information for Landowners (Jeffrey et al., 2001) constructed from records provided by the Australian Bureau of Meteorology, Canberra. The data were for the period 1889 to present at a grid resolution of 0.05° latitude by 0.05° longitude (approximately 5 km × 5 km) (Jeffrey et al., 2001).

5.3.3 Timing of breeding season and fieldwork

Female *B. duperreyi* lay clutches of eggs in communal nests (Figure 5-1c). in late November to early January each year depending upon seasonal conditions. Nests are typically found under rocks in exposed areas subject to high solar radiation (Pengilley, 1972; Shine, 1995; Shine & Harlow, 1996). Therefore, we started our fieldwork in the first week of November to locate nests as soon as possible after laying (typically 3-7 days incubation before discovery) in both open and forested areas in both seasons. We searched thoroughly for nests from November to mid-January in each year.

5.3.4 Nest characteristics and nest temperature

Each nest was marked with a plastic flag and had the GPS locations recorded. The flag was temporarily removed, and we recorded minimum nest depth (to top of the shallowest egg) and maximum nest depth (to the bottom of the deepest egg). If nest depth was less than 10 cm, one thermal data-logger (iButton® model DS1921G, Maxim Integrated, San Jose, CA, USA; accuracy ±1°C from -30°C to +70°C; diameter 17 mm, height 6.2 mm, mass 3.2 g) was placed in the core of the nest; if nest depth was greater than 10 cm, two iButtons were placed immediately above and below the egg mass of each nest. The iButtons were factory calibrated and were set to record the temperature at hourly intervals throughout the incubation period. We monitored nests weekly (9 weeks) for their condition, except at Dartmouth (7 weeks). We compared the data from 1997–1998 through 2006– 2007 and 2005 – 2006 summer seasons nest temperature and nest depth data published by Telemeco et al. 2009 using a regression model for *B. duperreyi* at the Piccadilly Circus.

5.3.5 Egg collection, incubation and sample collection

After 9-10 weeks of development in the field (approximately 90% of the incubation period), we removed 415 eggs from 42 randomly selected nests and transferred them to plastic boxes in which they were buried in moist vermiculite (4 parts water to 5 parts vermiculate by weight). Each egg was separated by plastic partitions. The eggs were transported in a portable incubator set to 23°C with high but unmeasured humidity. All experimental protocols were conducted with permission and in accordance with the procedures of Animal Ethics Committees at the University of Canberra and the CSIRO. Eggs were weighed with an electronic balance ($\pm$0.01 g), and egg lengths and widths were measured using digital vernier callipers ($\pm$0.01 mm). Eggs were incubated 23°C (+ 0.5°C), which typically produces a balanced sex ratio (Shine et al., 2002); this was a precaution only, because the eggs were harvested after the temperature is likely to exert an influence on offspring sex (Shine et al., 2002). The boxes were gently rotated inside the incubator every couple of days, and hatchlings were removed as soon as they emerged from the egg. Sex was identified by manually everting the hemipenes of males (Harlow, 1996; Shine et al., 2002). Tail tips (4–5 mm) were removed with a sterile blade and the free-flowing blood drop collected onto a labeled Whatman FTA™ Elute Card (WHAWB12-0401, GE Healthcare UK Limited, UK); tail tips were collected into labelled 1.5 ml tubes containing 90% ethanol.

5.3.6 Molecular detection of sex reversal

DNA was extracted from tail tips using a Gentra Puregene commercial kit (Qiagen Science, Maryland, U.S.A.) following manufacturer protocols; DNA was extracted from blood samples following manufacturer protocols. DNA purity was determined using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and quantified using the Qubit 2.0 Fluorometric Quantitation (Invitrogen, Life technologies, Sydney, N.S.W., Australia). The genotypic sex was identified using a PCR test based on seven Y-specific markers (Dissanayake et al., 2020). Briefly, in applying the test we used 1x MyTaq^{TM} HS Red mix (Bioline U.S.A. Inc. USA), 4 µM of each primer, and 25 ng of genomic DNA. The PCR cycling conditions used an initial touchdown phase to increase the specificity of amplification: denaturing at 95°C, annealing temperature stepping down from 70°C by 0.5°C per cycle. This was followed by 30 cycles of 95°C denaturing (20 sec), 65°C annealing and 72°C extension (10 min). PCR products were

visualised on a 1.5% agarose gel using SYBR Safe (Life Technologies, Carlsbad, USA). The samples that showed an amplified band for each of the seven markers are recognised as XY individuals, whereas as the samples for which a band was not amplified in all seven markers were recognised as XX individuals. The seven markers always concurred in their identification of genotypic sex, as did they in the original study published by Dissanayake et al. 2020. False negatives arising from recombination events are thus highly unlikely as they would present as some but not all markers detecting the presence of a Y chromosome. No XY females were observed, another indication that recombination and/or mutation involving these loci is negligible and has not affected the accuracy of genotypic sex assignment. Phenotypic male lizards showing genotype-phenotype discordance were classified as sex-reversed (Dissanayake et al., 2020, 2021). All molecular sex tests were conducted blind to the phenotypic sex of the individuals.

5.3.7 Analysis

Results are presented as means ± standard errors unless otherwise indicated. Correlation and regression analysis were used to describe relationships between variables, and the Student's t-test used to compare mean weekly air and nest temperatures. To analyse the relationship between natural thermal regimes and sex ratio of *B. duperreyi*, we corrected nest temperatures to constant temperature equivalents (CTE) (Georges, 1989; Georges et al., 1994) using the reaction norm for development rate against temperature estimated using the developmental model developed (Dallwitz & Higgins, 1978) (Figure 5.2; Figure S1A). We estimated the middle third of development by summing development as a function of temperature in small but finite increments, back from the point of hatching. The likelihood of reversal was based on two criteria: raw temperature and temperature corrected for developmental rate (CTE). Data analyses were performed in R (R Core Team, 2017) and GraphPad Prism version 9 for Windows (GraphPad Software, La Jolla California USA).

Simple linear regression was used to determine the relationship between the frequency of sex-reversed hatchlings in each population with respect to elevation and to characterise the trends in mean nests temperature, yearly mean maximum (Tmax), and yearly mean minimum temperature (Tmin) against elevation in each field location. Pearson correlation was used to evaluate the magnitude and direction of the association between the frequency of sex reversal and climatic variables [Tmax, Tmin, total rain (mm), -

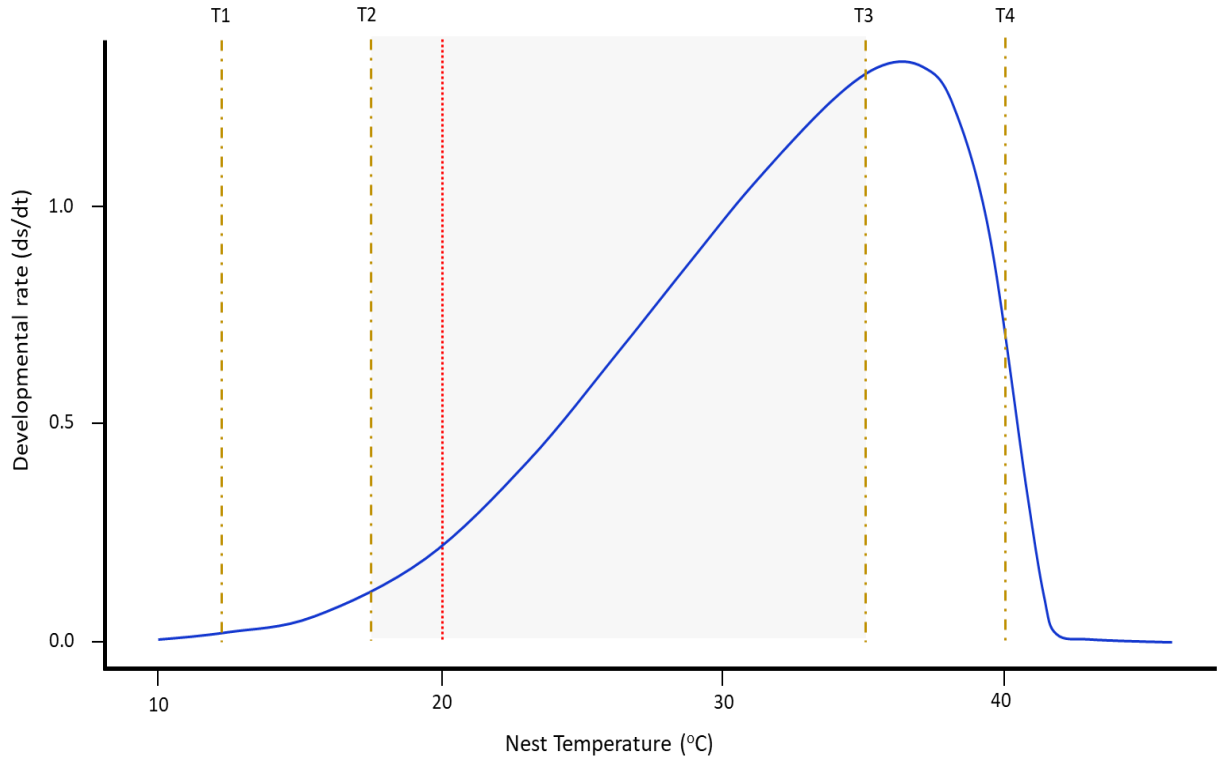

Figure 5-2. The Dallwitz-Higgins model (Dallwitz & Higgins, 1978) applied to *Bassiana duperreyi* nest data. T1 and T4 are the lower and upper absolute lethal limits, outside which even brief exposure causes embryo death. T2 and T3 are the constant-temperature lethal limits, outside which a temperature held constant throughout incubation will cause embryo death or gross abnormality. Temperatures in the sublethal ranges T1 –T2 and T3 – T4 will support embryonic development (Optimal Thermal Range indicating the shaded area), provided exposure is for a part of each day only, but the duration of vulnerability that can be tolerated will decline as one move to extremes. Red broken line denotes the sex reversal threshold for *B. duperreyi* according to Shine et al., 2002.

evaporation (mm), radiation (Mj/m^2) and vapor pressure (hPa)] including elevation recorded at each field site. We used a t-test to compare the sex reversal frequency of adult males (Dissanayake et al. 2021) with the sex reversal frequency our nests.

5.4 Results

5.4.1 Climate Data

Thermal profiles showed considerable diel variation at each field location (Figure 5-3). Soil temperatures on the ground surface fluctuated the most, reaching maximum temperature (Mt Ginini, 45.5 °C; Piccadilly Circus first season, 46.5 °C; Piccadilly Circus second season, 46.0 °C; Cooma, 42.1 °C; Dartmouth, 47.5 °C) during the day (1330–1730 h) and dropping to low level (Mt Ginini, 4.0 °C; Piccadilly Circus first season, 8.5 °C; Piccadilly Circus second season, 6.5 °C; Cooma, 7 °C; Dartmouth 12.5 °C) at night (2330–0400 h) (ANOVA, $F_{4, 6521} = 279.4$, $P < 0.0001$). The soil temperature at 20 cm, the deepest we monitored, showed the least fluctuation at all locations (see Figure 5-3).

Nests intermediate in depth between these two extremes (surface to 20 cm) showed intermediate diel fluctuations. At all the field locations, lower mean and lower minimum temperatures and less diel thermal variation occurred at 20 cm depth than the soil surface. Monthly mean air temperatures were averaged over the skink active months (i.e. early November to late February, a 16 week period) in each year to reveal a warming trend between 1889 to 2019 (Figure S2 -Mt Ginini: $F_{1,517} = 523.9$, $P < 0.0001$, $R^2 = 0.50$; Piccadilly Circus: $F_{1,517} = 539.6$, $P < 0.0001$, $R^2 = 0.51$; Cooma: $F_{1,517} = 537.9$, $P < 0.0001$, $R^2 = 0.50$; Dartmouth: $F_{1,517} = 627.5$, $P < 0.0001$, $R^2 = 0.54$). Air temperatures were consistently cooler at higher elevational locations than the lower elevations, which inversely correlated with elevation when *B. duperreyi* eggs were incubation ($F_{1,22} = 4.795$, $P < 0.039$, $R^2 = 0.17$). The weekly mean Tmax and Tmin showed that temperatures fluctuated substantially during the 9-week eggs incubation period (Figure 5-4). The highest mean rainfall events (17.0 ± 23.87 mm) were recorded at the Piccadilly Circus in the first week of egg incubation period in the first season. The highest mean rainfall events (5 ± 11.85 mm) were recorded at Mt Ginini during the third week of *B. duperreyi* egg incubation. The highest rainfalls were recorded during the fourth week of the egg incubation period at Cooma (5 ± 6.3 mm) and Dartmouth (1 ± 0.99 mm). Rainfall among the four sample locations were statistically different (Kruskal–Wallis; $H = 16.83$, $P < 0.05$).

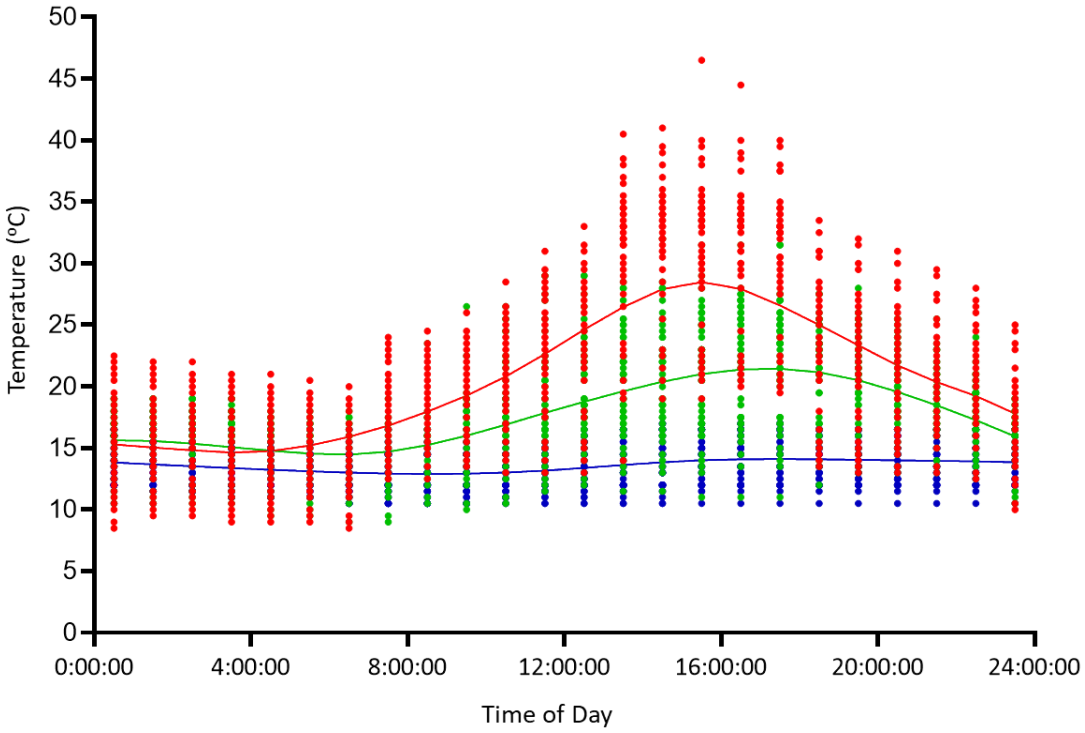

Figure 5-3. The diurnal variation of hourly soil temperature at the Picadilly circus field station. LOWESS curve fitted (solid lines) with a smooth curve to aid visual interpretation, (red): soil surface, green: at the depth of 10 cm and blue: at depth of 20 cm (blue). The minimum amplitude of the soil temperature variation approached in 20 cm depth. The daily variation of soil temperature showed a sinusoidal pattern, and the soil temperature decreased with the increase of the soil depth at all field stations. The order of the measured soil temperatures from high to low is: T0 cm > T10 cm > T20 cm.

5.4.2 Timing of breeding season and nest search protocols

In Piccadilly Circus during first season, female *B. duperreyi* laid their first eggs in the first week of December and 39 nests were found (only 35 monitored). In the second season (2018/19) females laid their eggs in early January and 26 nests were found (only 14 monitored). In the second season, in early January we found 11 nests at Mt Ginini (only nine were monitored) and nine nests at Cooma (only eight monitored). In the second week of December four nests were found (all monitored) at Dartmouth. Overall, we observed 1335 eggs in 89 nests at the four locations (Figure S3). A total of 84 nests survived the natural incubation period; five nests (3 from Piccadilly Circus and 2 from Mt Ginini) loss their eggs for unknown reasons.

5.4.3 Nest characteristics and nest temperature

Nests were typically constructed beneath rocks (98%), though some were found associated with logs (2%). All nests were deposited in open grassland and received direct sunlight at the surface for a large proportion of each day. Once the rock was removed, nests were typically partially buried in the soil (a few eggs were visible without disturbance) (91%) with a vertical nest chamber; some nests were completely buried (eggs well covered by soil) (7%) and few were found on top of the soil (not buried) (2%). Lengths of the rocks used for nesting averaged 28.8 ± 9.5 cm for Mt Ginini, 32.8 ± 20.7 cm for Piccadilly Circus, 38.6 ± 16.8 cm for Cooma, and 32.00 ± 9 cm for Dartmouth. Rock length differed significantly with location ($F_{3,87} = 5.71$, $p < 0.0001$), decreasing with elevation ($F_{1,87} = 11.94$, $p < 0.001$, $R^2 = 0.12$). Nests were shallower in the first season compared to the second season in Piccadilly Circus ($t = 3.7$, $df = 61$, $p < 0.05$). At Piccadilly Circus, females constructed progressively deeper nests over past 22 years when combining our data (2017 to 2019) an those of Telemeco et al 2009 (1997 to 2007) ($F_{1,8} = 11.36$, $p < 0.05$, $R^2 = 0.58$; Figure 5.5).

Of the 89 nests we located, 69 (71.91%) nests were communal i.e., contained more than nine eggs which according to Radder and Shine 2007 is the maximum clutch size, Radder and Shine 2007). Nest depth varied from 1 mm to 85 mm. The deepest nests were recorded at the lowest elevational location of Dartmouth (79 ± 6.1 mm). The shallowest nests (i.e., 15 mm) were found at the highest elevational of Mt Ginini (28.8 ± 11.7 mm) (Figure 5.4). Nest depth was inversely related to elevation ($F_{1,86} = 39.80$, $p < 0.0001$, $R^2 = 0.32$; Figure S4).

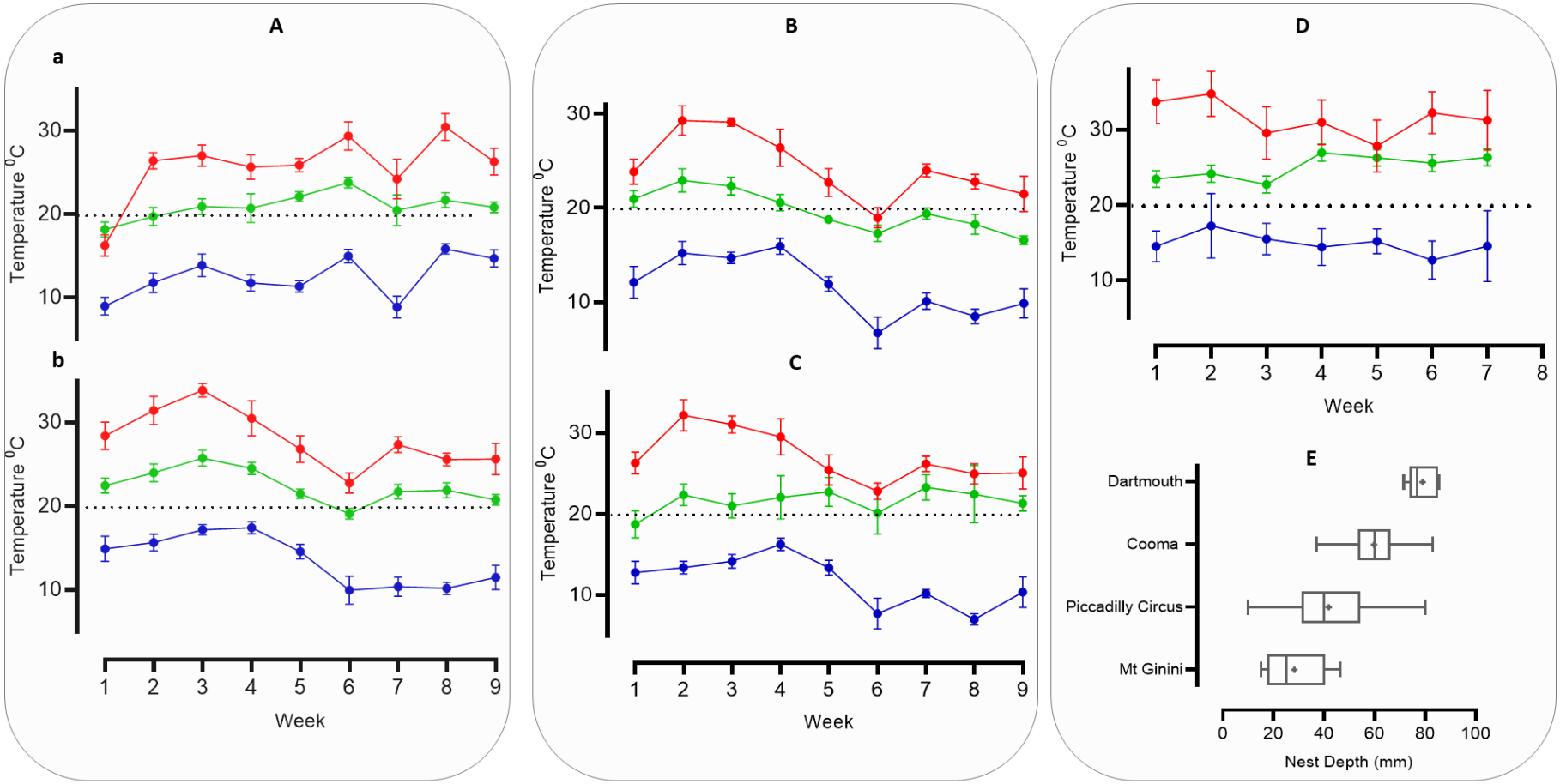

Figure 5-4. Weekly mean temperatures in the core of *Bassiana duperreyi* nests in each field locations (A). (a) Picadilly Circus first season (2017/18), (b) Picadilly Circus second season (2018/19); (B). Mt Ginini (2018/19); (C). Cooma (2018/19); (D). Dartmouth 2018/19 season; (E). Nest depth in each field location. Red denotes Tmax and Blue denotes Tmin.

Table 5-1. Descriptors of nest temperatures of *B. duperreyi* in four locations. One-factor ANOVA, with nest number as the factor and each day's data as a replicate. Nest temperature data were continuous temperatures taken by data loggers at one-hour intervals throughout incubation (9 weeks in Mt Ginini, Piccadilly Circus, Cooma) (7 weeks in Dartmouth).

Location	**Mean nest temperature**	**Mean daily nest temperature range °C**	**Mean nest temperature maxima**		**Mean nest temperature minima**	
			Temperature range °C	ANOVA	Temperature range °C	ANOVA
Mt Ginini	19.67 ± 0.63°C	18.76 – 20.57	27.8 – 35.8	$F_{8,549} = 15.18$, $P < 0.0001$	10.6 – 13.5	$F_{8,558} = 7.69$, $P < 0.0001$
Piccadilly Circus I season	21.61 ± 1.41°C	16.98 – 23.56	22.38 – 33.18	$F_{34,1834} = 19.71$, $P < 0.0001$	12.25 – 17.99	$F_{34,1832} = 14.25$, $P < 0.0001$
Piccadilly Circus II season	22.20 ± 0.22°C	19.18 – 23.76	26.71 – 35.09	$F_{13,770} = 19.30$, $P < 0.0001$	15.61 – 17.72	$F_{13,756} = 2.89$, $P < 0.0001$
Cooma	22.1 ± 1.56	19.58 – 23.59	25.1 – 32.9	$F_{7,432} = 13.02$, $P < 0.0001$	14.5 – 18	$F_{7,440} = 14.27$, $P < 0.0001$
Dartmouth	25.59 ± 1.25°C	23.89 – 27.39	34.67 - 38.67	$F_{4,225} = 2.63$, $P = 0.035$	15.25 – 17.75	$F_{4,230} = 9.52$, $P < 0.0001$

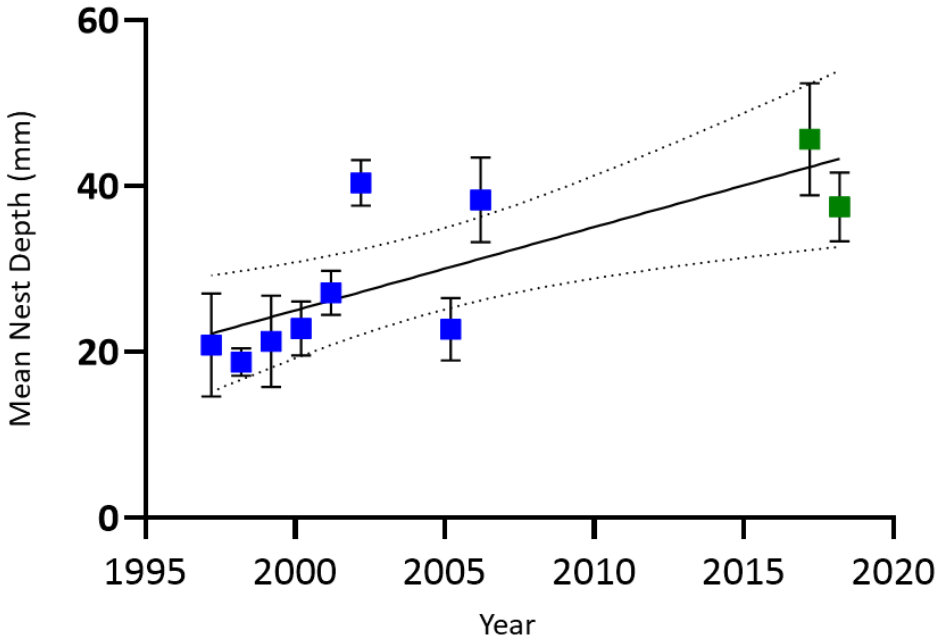

Figure 5-5. Long-term variation of mean nest depth of *Bassiana duperreyi*. The data from 1997–1998 through 2006– 2007 and 2005–2006 seasons nest depth data (blue) (Telemeco et al.30) and current study (green) (2017/18 and 2018/2019).

The lowest (19.67 ± 0.63 °C) and the highest (25.59 ± 1.25 °C) mean nest temperatures were recorded at Mt Ginini and Dartmouth, respectively. During nest incubation at Piccadilly Circus, mean nest temperature was lower (21.6 ± 1.41 °C) in the first season than in the second (22.2 ± 0.22 °C) ($F_{8, 410} = 88.59$, df = 8, $P < 0.0001$). The highest nest temperature (47.5 °C) and the highest mean daily range temperature (19.1 ± 2.30 °C) were recorded at the highest elevational location, Mt Ginini.

Most nests experienced high mean temperatures and a considerable diel range of temperatures in all field locations. Mean daily temperatures experienced by the eggs differed among nests in highest elevation, Mt Ginini (18.76–20.57 °C) to lowest elevation Dartmouth (23.89–27.39 °C), as did mean maxima in Mt Ginini (35.8–27.8 °C) and minima (10.6–13.5 °C), and mean maxima in Dartmouth (34.67–38.67 °C) and minima (15.25–17.75 °C). The nests showed significant differences in mean nest temperature maxima in all locations except Dartmouth, but mean nest temperature minima shows a significant difference in all locations (Table 5.1).

Mean weekly nest temperature was correlated with mean weekly air Tmax ($R^2 = 0.42 - 0.74$, $p < 0.05$) and Tmin ($R^2 = 0.70 - 0.93$, $p < 0001$) at all field locations. The significant warming trend was recorded during the incubation weeks in first season at the Piccadilly Circus ($F_{1,300} = 31.74$, $p < 0.001$, $R^2 = 0.09$), but a significant cooling trend was recorded in the second season ($F_{1,124} = 52.63$, $p < 0.001$, $R^2 = 0.29$). In the second season a significant cooling trend was also recorded at Mt Ginini ($F_{1,79} = 132.3$, $p < 0.001$, $R^2 = 0.62$). Whereas at Cooma and Dartmouth showed no significant trend as the season progressed.

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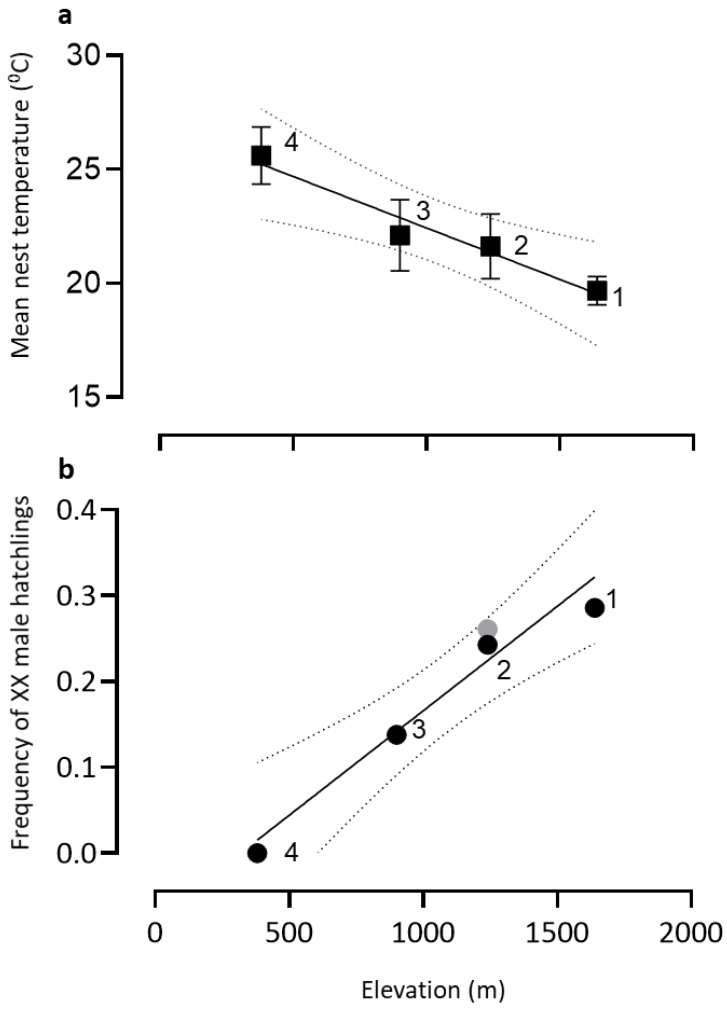

Figure 5-6. The frequency of sex reversal in nests *Bassiana duperreyi*. (**a**) Linear regression of mean nest temperature in each field location ($F_{1,2} = 41.71$, $P = 0.023$; $R^2 = 0.95$). (**b**). The trend in sex reversal frequency of *B. duperreyi* with the elevation ($F_{1,3} = 41.71$, $P < 0.05$; $R^2 = 0.95$). The number indicates field locations, as indicated in Figure 5.1. Grey circles indicate first season data (2017/18) and black circles indicate second season data (2018/19). Broken lines denote the 95% confidence interval, and significance was assumed if $P < 0.05$.

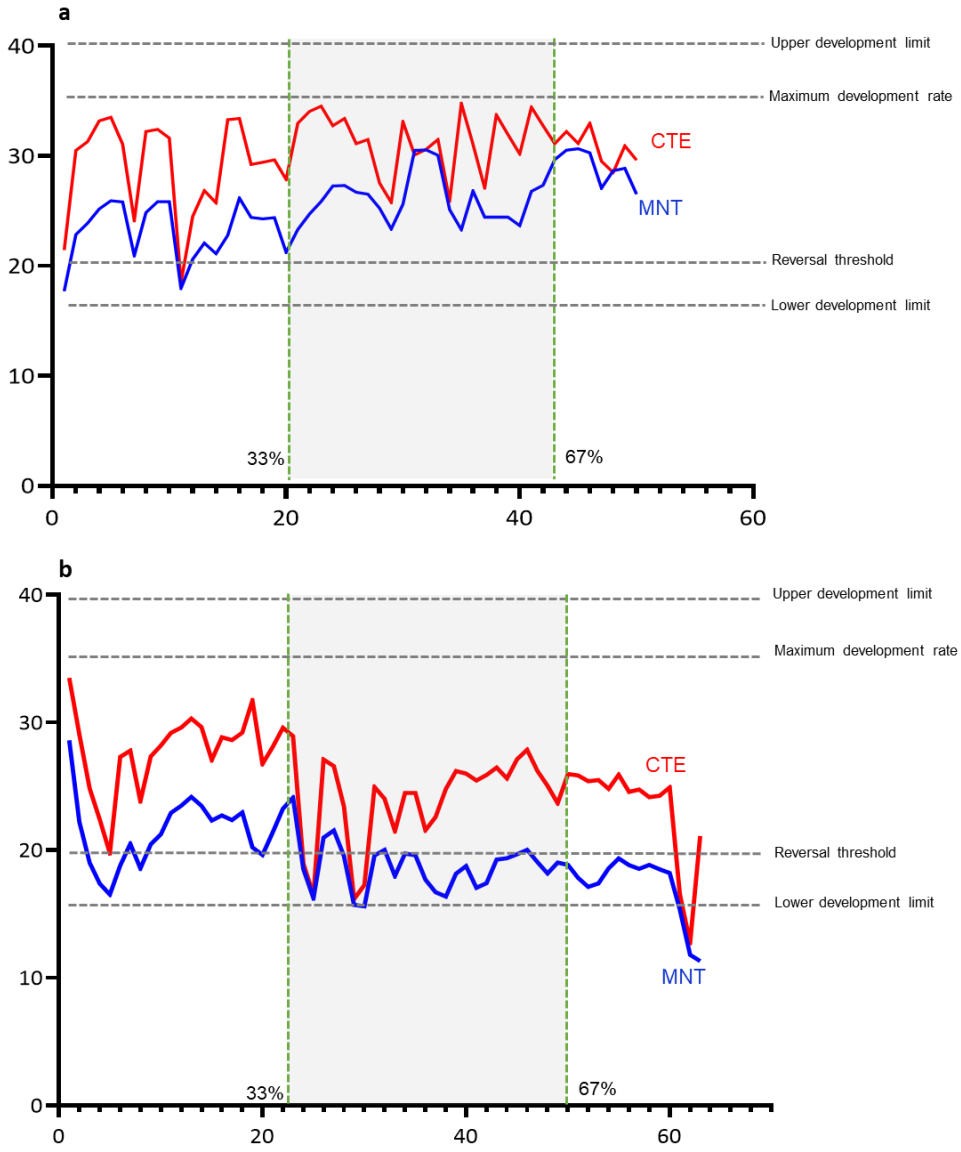

Figure 5-7. Temperature trace for the core of a nest of *Bassiana duperreyi* showing traces for the mean and the constant-temperature equivalent (CTE) for the Dallwitz-Higgins model (Dallwitz & Higgins, 1978; Georges et al., 1994). (a). The nest produced only XYmale and XXfemale offspring, nest location: Dartmouth. (b). The nest produced XYmale, XXmale and XXfemale offspring, nest location: Mt Ginini. Shaded area: expecting to sex reversal happening during the incubation period. The threshold for sex determination (20˚C; Shine et al., 2002) and the thermosensitive period lies between lower and upper limit of development. Note that the thermosensitive period does not correspond to the middle third of incubation, either in position or duration, owing to the nonstationary trend in temperatures with season.

Mean nest temperatures during the incubation period were inversely correlated with elevation ($F_{1,2} = 41.71$, $P < 0.05$, $R^2 = 0.95$) (Figure 5-6a). The highest and lowest mean daily CTE were recorded at Dartmouth (30.35 ± 0.12 °C) and Mt Ginini (26.2 ± 1.98 °C), respectively. The mean daily CTE was significantly inversely correlated with elevation ($F_{1,74} = 11.39$, $P < 0.001$, $R^2 = 0.17$). When the CTE dropped below the 20 °C (the threshold for reversal, Shine et al., 2002) during the thermosensitive period, for even for a short time during the incubation period, sex reversal was observed (Figure 5.7 and Figure S1 B-F).

5.4.4 Genotypic sex identification and the frequency of sex reversal

During the study period, we collected a total of 415 eggs from the natural nests. The hatching success rate was 95.2%. Therefore, a total of 395 hatchlings were able to be phenotypically identified. Of them, 262 (66.3%) were phenotypic males and 133 (33.6%) were phenotypic females. The sex ratio (Phenotypic male: female) of each location is as follows; Mt Ginini (2018/19)= 2.3 : 1; Picadilly Circus (2017/18 and 2018/19) = 2.27 : 1; Cooma (2018/19) = 1.38 : 1 and Dartmouth (2018/19) = 1.14 : 1, yielding a male-biased sex ratio in the high elevation sites, where adult sex reversal has been previously identified by Dissanayake et al. 2021 (chi-squared test with Yates correction, $\chi 2 = 4.05$, df=1, $p < 0.05$). A total of 59 (7.03%) phenotypically male hatchlings were sex-reversed. The highest frequency of sex reversal in hatchlings was recorded at the highest elevation site (28.6%, Mt Ginini, 1,640 m a.s.l.), and zero sex reversal was observed at the lowest elevation (Dartmouth, 380 m a.s.l) (Figure 5.1a). This observation concorded with the previous study has been contacted for adult individuals (see Dissanayake et al. 2021). The frequency of sex reversal was positively correlated with elevation ($F_{1,3} = 41.71$, $p < 0.05$; $R^2 = 0.95$) (Figure 5.6b) and each population showed a negative correlation with mean nest temperature (Pearson's correlation coefficient $r = - 0.95$, $p < 0.05$); Tmax ($R2 = 0.99$, $p < 0.05$) was significantly negatively correlated with sex reversal frequency (Table S2). When we compared the current study with Dissanayake et al. 2021 for adult sex reversal frequency, the frequency of sex reversal in the nest was higher than the sex-reversed adult in the same field locations, but not significantly ($p = 0.36$). However, both hatchlings and adults (Dissanayake et al., 2021) rates of sex reversal frequency positively correlate with their respective elevation ($F_{1,2} = 15.17$, $p < 0.005$; $R^2 = 0.77$) (Figure S5) (see also Dissanayake et al., 2021a).

5.5 Discussion

Over the last two decades, many field studies and laboratory experiments have been conducted to understand *Bassiana duperreyi* nesting ecology and phenotypic plasticity (Du & Shine, 2010; Elphick & Shine, 1998; Shine, 1999b; Shine et al., 1997; Shine & Harlow, 1996; Telemeco et al., 2009). However, the consequences of cold nest temperatures on sex reversal in *B. duperreyi* have been little studied in the wild (but see Holleley et al., 2016). We show that sex reversal in natural nests of this species is common at elevations above 900 m a.s.l. The frequency of sex reversal in nests ranges from 28.6% at the highest, coolest location (Mt Ginini, 1,640 m a.s.l., mean nest temperature 19.7 ± 0.62°C) to zero percent at the lowest, warmest mean nest temperature recorded location (Dartmouth, 380 m a.s.l., 25.6 ± 1.25°C). Thus, both hatchlings (this study) and adults as observed by Dissanayake et al., 2021a, exhibit a comparable relationship between the frequency of sex reversal and elevational variation in ambient temperatures. These substantial levels of sex reversal in nests and the adult population, derived from the rates of sex reversal we have observed in the nest, indicate that the sex-reversed phenotype is an important component of the demography of this species.

How sex reversal will come to influence demographic processes that govern the persistence of local populations in the Australian high country requires additional information on the fertility of sex-reversed individuals. The presence of sex-reversed adults (Dissanayake et al., 2021a) at a similar rate that observed in the nest (this study) establishes the viability of sex-reversed individuals. But are they fertile? If the *B. duperreyi* sex reversal yields infertile male individuals, then males of the species are subject to latent mortality – a significant component of the population could comprise viable but infertile males. As these are generated by reversal of XX individuals otherwise destined to be females, the effective population size, which depends on reproductive female number, is drawn down numerically. It is also drawn down because many of the remaining XX females will potentially mate with the infertile males to no effect. and this proportion of females will not contribute to effective population size. Together, this could lead to a rapid population decline in effective population size and local extinction should there be a sequence of episodic cold seasons or cold spells at a time when the embryos in the nests are thermosensitive. In contrast, if the sex-reversed males are both viable and fertile, then sex reversal under cooler temperatures will lead to an overproduction of males and, in the context of other evolutionary and phenotypic responses, Fisher's frequency-dependent selection (Düsing, 1884; Fisher, 1930), may be invoked in

support of traits that bring the population sex ratio back to equilibrium. This will manifest as a reduction in the sensitivity of sex to temperature or reduction of the frequency of the Y chromosome in the population or both and, potentially, its loss (Bull, 1981; Dissanayake et al., 2021a; Holleley et al., 2015; Schwanz et al., 2020). Suppose for example, a future change in climate or annual weather is sudden, such that the species is subject to substantial sex reversal but unable to evolutionarily adjust the male-biased sex ratio. In that case, effective population size will decline, again putting local populations at greater risk of extinction. Clearly, under either scenario, sex reversal has profound implications for the demography of *B. duperreyi* at the high elevation distributional limits of its range whether or not sex-reversed males are fertile.

The above impacts of sex reversal can be ameliorated if the frequency of sex reversal can be constrained by phenotypic responses. In particular, nest site choice and nesting phenology are potentially both important in ameliorating the frequency of sex reversal. We recorded that *B. duperreyi* selected open grassland and 98% of rock substrate at all field locations for laying their eggs, similar to the findings of previous studies (Shine & Harlow, 1996; Telemeco et al., 2009). We show that the consequence of egg laying in exposed sites is a high diel variance in nest temperature. *B. duperreyi* typically select nesting sites under rocks compared to other alpine skinks that live at the same field location that typically nest in logs and rotting organic material (Shine & Harlow, 1996). Furthermore, *B. duperreyi* appear to select nest sites on both thermal averages and high thermal variability both of which have measurable effects on hatchling phenotype (Shine et al., 1997). Adjusting for diel fluctuations, we found that when the CTE dropped below the 20°C (the threshold for reversal, Shine et al., 2002) during the thermosensitive period, for even for a short time during the incubation period, sex reversal occurred (Figure 5.7). Therefore, despite the observed temperature fluctuations in nests, extreme cold temperature events could lead to 100% sex reversal of the XX genotype in the alpine populations at the highest elevations.

When considering the alpine populations, *B. duperreyi* embryo development occurred under high thermal fluctuations and some nests reached beyond the embryo physiological limits for brief periods. The high level of diel fluctuation of nest temperature leads some nests reaching both upper and lower limits in development temperatures. At Mt Ginini (i.e., highest elevational location for this study), four nests show that their temperature exceeded both the maximum and minimum temperature for embryo survival during the incubation period (see

Table 5.1). However, we observed that these nests survived and successfully completed their natural embryonic development to hatchling. Therefore, a short period of exposure to extreme temperature events did not affect egg survivorship but may well have influenced the frequency of sex reversal.

The embryonic survivorship under the current increasing global temperature trend is a significant challenge for oviparous species globally. The current trend in global warming inescapably will increase the nest temperatures of many oviparous reptiles (Du et al., 2019; Janzen, 1994; Telemeco et al., 2009). Therefore, natural selection should favour potential nesting sites, nest phenology and other maternal behaviors that enhance embryonic survivorship. Egg laying of *B. duperreyi* shifted temporally, with the nesting season starting from mid-November to early December, confirming a similar observation by Telemeco et al.[30]. However, in our second season (2018/19), females initiated egg laying in early January, as they did in 1968 (Pengilley, 1972). *B. duperreyi* appear to shift their egg laying from year to year, presumably in response to variation in natural conditions conducive to successful incubation and emergence (see also Pengilley, 1972). Incubation conditions, and so timing or egg laying, also have strong effects on offspring phenotypes (Shine, 2004; Shine et al., 2002) and sex determination, including sex-reversal. In addition to altering the timing of nesting, *B. duperreyi* has progressively been digging deeper nests over the last two decades (Figure 5.5 and see Telemeco et al., 2009), likely to reduce exposure to extreme daily temperatures and have a complex impact on average nest temperatures depending upon soil composition and structure.

We have shown a significant relationship between the frequency of sex reversal in hatchlings and nest temperatures during their natural incubation period. Our work establishes and quantifies current elevational trends in the frequency of sex reversal in response to cold temperatures both in the nests and in the adult population (Dissanayake et al., 2021a). This forms a baseline for examining the effects of climate change, and in particular climate warming. On the basis of the trends in primary (egg) and operational (adult) sex ratio with elevation, presumably driven by associated variation in thermal conditions, we expect to see populations without sex reversal, that is, governed by GSD, to increase in elevation as the climate warms. At the highest altitudes, if climate change is accompanied by increased variability, this will potentially disrupt the demographic processes on a local scale, leading to local population crashes and local extinction events, driven by fluctuating frequency of sex

reversal. Temperature induced sex reversal in *B. duperreyi* and its demographic consequences likely make this species a very sensitive indicator of climate change. Monitoring changes in the frequency of sex reversal through time along our elevational gradient and at the species highest distributional limits is recommended as one option for assessing the impacts of climate change on the biota of the Australian high country.

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Chapter 6

Synopsis

Why study sex determination in reptiles? One reason is simply an interest in better understanding one of the most fundamental developmental processes of profound importance in the life of the individual. As a branch of developmental biology, sex determination mechanisms and their implications for the life history of organisms is particularly fascinating because of the astonishing diversity within and between species. It is not enough to study sex determination in its many forms in the laboratory, but equally important to study it *in situ*, that is, in the context in which the organisms have evolved, continue to evolve, and interact with their environment at an ecological level.

This Ph.D. thesis is an account of how a species with temperature-induced sex reversal responds to the natural environment. For this, I chose the three-lined skink, *Bassiana duperreyi*, as a study species with sex reversal of the XX genotype at low incubation temperatures within the range experienced in nature. I developed a robust PCR test based on 7 sex linked markers to replace the existing test (Quinn et al., 2009), which was a single marker and somewhat difficult to reliably apply (Chapter 2). I used this new test to examine the frequency of sex reversal in adults and hatchlings of populations on an elevational gradient, after having confirmed the taxon subject to study (Chapter 3 and 5). The data generated against elevation will serve as a foundation for future monitoring of the rates of sex reversal with change in climate, as a potentially very sensitive indicator of the biotic impacts of global warming.

In chapter 2, I report on the success of an *in silico* approach to enriching whole genome sequence for Y specific sequence, that can subsequently be validated as sex specific by applying PCR to the putative markers across a panel of known male and female individuals not likely, by virtue of their incubation conditions, to be sex reversed. The generation of seven such markers, eliminates the risk of recombination events delivering misleading results provided, as I have reported, all seven markers are concordant in their determination across all individuals. Recombination would manifest as conflict among the markers, assuming they are not extremely tightly linked. The approach is cost effective because it can be applied to low coverage short-read sequencing. I have used this tool also to

isolate a sex-specific marker in the endangered western saw-shelled turtle (*Myuchelys bellii*), to enables sex identification of embryos, hatchlings, and small juveniles that cannot otherwise be sexed (Dissanayake et al., unpublished; Appendix 1). This technique will be of wide applicability in conservation and research more generally to understanding sex determination processes. As I have shown, the development of robust sex-linked markers is essential for demonstrating sex reversal and evaluating trends in sex reversal through time or along elevational gradients or with latitude.

The efficiency of future Y-marker discovery via genome subtraction can be improved by efforts to reduce false positives caused by autosomal insertion/deletion polymorphisms in the focal sequenced individuals. This can be achieved through several complementary strategies: (a) subtracting multiple XX individuals from the XY focal individual/s; (b) selecting individuals for sequencing from populations with lower rates of heterozygosity (e.g. small geographically isolated populations or experimentally inbred lines); or (c) sequencing siblings or related individuals or individuals from inbred lines. These improvements would increase the efficiency of the enrichment of Y or W specific sequence and reduce the effort in applying PCR to develop useable sex-specific markers.

A second technique for developing sex linked markers applied to reptiles for the first time, was collaboratively published (Cornejo-Páramo et al., 2020; Appendix 1). This technique relies on read copy number variation across the genome to identify the half copy number in the XY individuals compared to the XX individuals after screening out repetitive sequence. As such, it is complementary to the genome subtraction approach reported in this thesis. We applied this approach to the yellow-bellied water skink, *Eulamprus heatwolei*, isolated four sex-specific markers which I characterized, validated against a panel of individuals of known sex and developed into a PCR test. Both techniques rely on the divergence of the X and Y homologous sequencing in the region of recombination suppression. This research, included as an appendix to the thesis, allowed us to demonstrate that *E. heatwolei* had XY chromosomes including 14 gametologs with regulatory functions. We estimated the date of divergence of the Y chromosomal region from its X chromosome counterpart to 79–116 Myr. This work demonstrated that *E. heatwolei* formerly thought to be the first viviparous species with TSD, had sex chromosomes. We showed that the sex chromosomes were homologous between *E. heatwolei, B. duperreyi* and the spotted skink *Niveoscincus ocellatus.* This suggests that sex reversal may be quite common in reptiles, and

that the two species in which it has been conclusively identified, *Pogona vitticeps* and *Bassiana duperreyi,* are the tip of the iceberg. If so, the implications for the extent of impact of climate change on reptile populations could be grossly underestimated.

Climatic change and in particular its current rapidity, has significant impacts on the biology, ecology of many reptile species, and can lead to catastrophic population declines. Changing environmental temperature is increasingly considered a significant threat for reptiles with TSD. An interesting recent extension of this is that GSD species with temperature-induced sex reversal may also be vulnerable to shifts in climate. *Bassiana duperreyi* is reversed by low temperatures during incubation and so the frequency of sex reversal is likely to reduce under global warming. However, increased frequency of cold spells in a more unpredictable environment could still lead to impacts and local extinctions, as I have argued in Chapter 3. Whether or not *B. duperreyi* is adversely impacted by climate change, the species is likely to be a very sensitive indicator of climate change via frequencies of sex reversal in the wild. The elevational trends I have reported in Chapter 3 for adults and Chapter 5 for nests could form a firm quantitative basis for the evaluation of the biotic response to climate change through time.

Elaborating on these findings from chapter 3 and 5, I showed that the elevational gradient correlates with average temperature conditions and presumably through that, to the frequency of sex reversal. Cooler alpine locations had the highest rates of sex reversal which decreased progressively with decreasing elevation and associated increases in mean air temperatures and the mean nest temperatures. Our study was concordant with controlled laboratory experiments, incubating eggs at temperature regimes (Shine et al., 2002; Radder et al., 2008). Our proposed models for *B. duperreyi* show that the frequency of the XY genotype is predicted to decline with decreasing incubation temperature as the system strives, under frequency-dependent selection, to maintain a 1:1 sex ratio at equilibrium. In fact, under current climate regimes within the species range, some with averages below 18°C, we expect the complete loss of the Y chromosome at above around 2000 m elevation sites where *B. duperreyi* is currently distributed. Unfortunately, we did not have the opportunity to examine these populations. However, sex reversal clearly has the potential to impact the evolution of sex determination in this species under the pressure of future climate change, where climate cooling occurs. The populations on independent evolutionary trajectories (chapter 4) may

each have their own particular responses to incubation temperature, and so vulnerability to climate change.

In Chapter 4, I demonstrated that dates of divergence of *B. duperreyi* based on mitochondrial sequence was not consistent with the lack of accumulation of fixed allelic differences between the lineages of Tasmania and the mainland. The mitochondrial sequences suggest a divergence of 1.7 My whereas the SNP data suggest an episodic exchange of alleles across the Bass Strait during more recent periods of low sea level in the Upper Pleistocene. The distinction in mitochondrial sequence between Tasmania and the eastern populations of Victoria, NSW, and the ACT might have arisen through an incomplete lineage sorting or through a localized selection for a highly constrained mitochondrial genome. Hence the dates of separation of the Tasmanian and mainland populations, estimated to be 1.7 Myr by Dubey and Shine (2010), may apply to the mitochondria but does not reflect the history of isolation of the species between Tasmania (incl. Flinders Island) and the mainland. In addition to this insight, the analysis of "species" boundaries indicates how important it is to examine taxonomy before placing evolutionary interpretations on spatial trends. As was shown in chapter 5, it was critically important to eliminate the populations in the vicinity of Melbourne (South east coastal plain OTU) before placing interpretation on the elevational trends in sex reversal frequency. The absence of sex reversal in the South east coastal plain OTU populations may not have been a response to lower environmental temperatures, but rather differences in thermolability between two "species".

The impact of sex reversal on populations of *B. duperreyi* is uncertain because of lack of critical information. Two scenarios present themselves.

(a) If the sex-reversed males have compromised fertility or are infertile, then they introduce to the demography cryptic (or latent) mortality. As the frequency of sex reversal increases, as it might under cooler climates or bouts of cold weather, the effective population size is driven down, increasing the risk of local episodic extinctions. There is anecdotal evidence of unexplained local population crashes in the *B. duperreyi* in Brindabellas, where they have been most intensively studied.

(b) If the sex-reversed males are fertile, then as the frequency of sex reversal increases, the frequency of the Y chromosome in the population is expected to fall (XY individuals are more likely to yield less of the rarer sex, invoking Fishers Frequency-

Dependent Selection. Ultimately the Y chromosome will be lost altogether, and the population will move to temperature-dependent sex determination (TSD). Time will be required to evolve a response norm of sex ratio to incubation temperature that delivers an appropriate balance between males and females and maintains population viability. In the meantime, the overproduction of males will again drive down the effective population size, increasing the risk of local episodic extinctions.

Sex reversal in a reptile is a fascinating evolutionary mechanism, which is likely to be primarily driven by temperatures that directly influence their sex determination pathways during embryonic development. However, so long as sex reversal is validated only in two reptiles species, it will remain unclear whether sex reversal is widespread throughout the reptile phylogeny or if it occurs only in a certain extent of evolutionary mechanisms that support to adaptive evolution via phenotypic plasticity. During my thesis, I suggested that there is presumably an evolutionary mechanism that exists mainly in the face of a rapidly changing climate. Future research on *B. duperreyi* will greatly benefit from field and lab-based research to better understand all aspects of sex reversal in reptiles more generally. This could include:

1. Blast the Y chromosome contigs that we isolated using the whole genome subtraction pipeline against the current ongoing *B. duperreyi* genome assembly to develop probes to physically map and identify Y chromosome gene content of *B. duperreyi*. This will also create extended Y chromosome contigs.
2. The extended Y chromosome contigs from *B. duperreyi* could be physically mapped to related GSD and TSD skinks. This will help to reveal conservation, or variation, in sex chromosome pairs in family Scincidae, and may identify homologous chromosomes in GSD and TSD species.
3. The Y chromosome loss model that we proposed in Chapter 3 can be tested by collecting samples from the highest elevation locations in the Australian high country where *B. duperreyi* is distributed.
4. Examine responses of *B. duperreyi* to interannual climatic variation and long-term climatic trends, with special focus on the demographic implications of sex reversal on local population viability as a sensitive indicator of climatic change.

5. Use transcriptomics data to understand the temporal dynamics of sex reversal during embryonic development of *B. duperreyi*, at constant temperatures in the laboratory specially on 18°C, 20°C and 23°C, and in field nests to compare both lab and field.

It is interesting, then, if temperature induced sex determination and sex reversal in reptiles is accepted as a natural mechanism underlying evolutionary transitions in sex determining modes. My thesis and related publications will ideally begin to encourage scientist in many fields to consider whether sex reversal is an evolutionarily process important to the phenotypic plasticity of reptiles.

References

Abbott, J. K., Nordén, A. K., & Hansson, B. (2017). Sex chromosome evolution: historical insights and future perspectives. *Proceedings of the Royal Society B: Biological Sciences*, *284*(1854), 20162806.

Alam, S. M. I., Sarre, S. D., Gleeson, D., Georges, A., & Ezaz, T. (2018). Did lizards follow unique pathways in sex chromosome evolution? *Genes*, *9*(5), 239.

Ansari, M. H., Cooper, S. J., Schwarz, M. P., Ebrahimi, M., Dolman, G., Reinberger, L., ... & Gardner, M. G. (2019). Plio-Pleistocene diversification and biogeographic barriers in southern Australia reflected in the phylogeography of a widespread and common lizard species. *Molecular Phylogenetics and Evolution*, *133*, 107-119.

Ayers, K. L., Davidson, N. M., Demiyah, D., Roeszler, K. N., Grützner, F., Sinclair, A. H., ... & Smith, C. A. (2013). RNA sequencing reveals sexually dimorphic gene expression before gonadal differentiation in chicken and allows comprehensive annotation of the W-chromosome. *Genome Biology*, *14*(3), 1-17.

Bachtrog, D., Mank, J. E., Peichel, C. L., Kirkpatrick, M., Otto, S. P., Ashman, T.-L., Hahn, M. W., Kitano, J., Mayrose, I., Ming, R., Perrin, N., Ross, L., Valenzuela, N., & Vamosi, J. C. (2014). Sex determination: why so many ways of doing it? *PLOS Biology*, *12*(7).

Baroiller, J. F., D'Cotta, H., Bezault, E., Wessels, S., & Hoerstgen-Schwark, G. (2009). Tilapia sex determination: where temperature and genetics meet. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *153*(1), 30-38.

Barske, L. A., & Capel, B. (2008). Blurring the edges in vertebrate sex determination. *Current Opinion in Genetics & Development*, *18*(6), 499–505.

Benestad, R. E. (2003). What can present climate models tell us about climate change? *Climatic Change*, *59*(3), 311-331.

Bewick, A. J., Anderson, D. W., & Evans, B. J. (2011). Evolution of the Closely Related, Sex-Related Genes Dm-W and Dmrt1 in African Clawed Frogs (*Xenopus*). *Evolution*, *65*(3), 698–712.

Bickford, D., Lohman, D. J., Sodhi, N. S., Ng, P. K., Meier, R., Winker, K., ... & Das, I. (2007). Cryptic species as a window on diversity and conservation. *Trends in Ecology and Evolution*, *22*, 148-155.

Bidon, T., Schreck, N., Hailer, F., Nilsson, M. A., & Janke, A. (2015). Genome-wide search identifies 1.9 Mb from the polar bear Y chromosome for evolutionary analyses. *Genome Biology and Evolution*, *7*(7), 2010-2022.

Bókony, V., Kövér, S., Nemesházi, E., Liker, A., & Székely, T. (2017). Climate-driven shifts in adult sex ratios via sex reversals: The type of sex determination matters. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *372*(1729).

Boulanger, J., White, G. C., Proctor, M., Stenhouse, G., Machutchon, G., & Himmer, S. (2008). Use of occupancy models to estimate the influence of previous live captures on DNA-based detection probabilities of grizzly bears. *The Journal of Wildlife Management*, *72*(3), 589-595.

Boyle, M., Hone, J., Schwanz, L. E., & Georges, A. (2014). Under what conditions do climate-driven sex ratios enhance versus diminish population persistence? *Ecology and Evolution*, *4*(23), 4522–4533.

Brodie, J. F., Williams, S., & Garner, B. (2021). The decline of mammal functional and evolutionary diversity worldwide. *Proceedings of the National Academy of Sciences*, *118*(3) e1921849118.

Brown, A. R., Owen, S. F., Peters, J., Zhang, Y., Soffker, M., Paull, G. C., Hosken, D. J., Wahab, M. A., & Tyler, C. R. (2015). Climate change and pollution speed declines in zebrafish populations. *Proceedings of the National Academy of Sciences*, *112*(11), E1237–E1246.

Brown, J. K., Taggart, J. B., Bekaert, M., Wehner, S., Palaiokostas, C., Setiawan, A. N., ... & Penman, D. J. (2016). Mapping the sex determination locus in the hāpuku (*Polyprion oxygeneios*) using ddRAD sequencing. *BMC Genomics*, *17*(1), 1-12.

Brush, S. G. (1978). Nettie M. Stevens and the discovery of sex determination by chromosomes. *Isis*, *69*(2), 163-172.

Bull, J. J. (1980). Sex determination in reptiles. *The Quarterly Review of Biology*, *55*, 3–21.

Bull, J. J. (1981). Evolution of environmental sex determination from genotypic sex determination. *Heredity*, *47*(2), 173–184.

Bull, J. J. (1983). *Evolution of sex determining mechanisms.* The Benjamin/Cummings Publishing Company, Inc.

Bull, J. J., & Charnov, E. L. (1977). Changes in the heterogametic mechanism of sex determination. *Heredity*, *39*(1), 1-14.

Bull, J. J., & Vogt, R. C. (1981). Temperature-sensitive periods of sex determination in emydid turtles. *Journal of Experimental Zoology*, *218*(3), 435-440.

Bull, J. J., Vogt, R. C., & McCoy, C. J. (1982). Sex determining temperatures in turtles: a geographic comparison. *Evolution*, 326-332.

Bulmer, M. G., & Bull, J. J. (1982). Models of polygenic sex determination and sex ratio control. *Evolution*, 13-26.

Butlin, R. K., & Stankowski, S. (2020). Is it time to abandon the biological species concept? No. *National Science Review*, *7*, 1400-1401.

Capel, B. (2017). Vertebrate sex determination: Evolutionary plasticity of a fundamental switch. *Nature Reviews Genetics*, *18*(11), 675–689.

Carmichael, S. N., Bekaert, M., Taggart, J. B., Christie, H. R., Bassett, D. I., Bron, J. E., ... & Sturm, A. (2013). Identification of a sex-linked SNP marker in the salmon louse (*Lepeophtheirus salmonis*) using RAD sequencing. *PLOS One*, *8*(10), e77832.

Castelli, M. A., Georges, A., Cherryh, C., Rosauer, D. F., Sarre, S. D., Contador-Kelsall, I., & Holleley, C. E. (2021). Evolving thermal thresholds explain the distribution of temperature sex reversal in an Australian dragon lizard. *Diversity and Distributions*, *27*(3), 427–438.

Castelli, M. A., Whiteley, S. L., Georges, A., & Holleley, C. E. (2020). Cellular calcium and redox regulation: The mediator of vertebrate environmental sex determination? *Biological Reviews*, *95*(3), 680–695.

Cattell, R. B. (1966). The scree test for the number of factors. *Multivariate Behavioral Research*, *1*(2), 245-276.

Ceballos, G., Ehrlich, P. R., Barnosky, A. D., García, A., Pringle, R. M., & Palmer, T. M. (2015). Accelerated modern human–induced species losses: entering the sixth mass extinction. *Science Advances*, *1*(5), e1400253.

Chambers, E. A., & Hillis, D. M. (2020). The multispecies coalescent over-splits species in the case of geographically widespread taxa. *Systematic Biology*, *69*(1), 184-193.

Chapple, D. G., Chapple, S. N., & Thompson, M. B. (2011a). Biogeographic barriers in south-eastern Australia drive phylogeographic divergence in the garden skink, *Lampropholis guichenoti*. *Journal of Biogeography*, *38*, 1761-1775.

Chapple, D. G., Hoskin, C. J., Chapple, S. N., & Thompson, M. B. (2011b). Phylogeographic divergence in the widespread delicate skink (*Lampropholis delicata*) corresponds to dry habitat barriers in eastern Australia. *BMC Evolutionary Biology*, *11*, 1-18.

Chapple, D. G., Keogh, J. S., & Hutchinson, M. N. (2005). Substantial genetic substructuring in southeastern and alpine Australia revealed by molecular phylogeography of the *Egernia whitii* (Lacertilia: Scincidae) species group. *Molecular Ecology*, *14*, 1279-1292.

Charlesworth, D. (2015). The status of supergenes in the 21st century: Recombination suppression in Batesian mimicry and sex chromosomes and other complex adaptations. *Evolutionary Applications*, *9*(1), 74–90.

Charlesworth, D., Charlesworth, B., & Marais, G. (2005). Steps in the evolution of heteromorphic sex chromosomes. *Heredity*, *95*(2), 118–128.

Charnier, M. (1966). Action of temperature on the sex ratio in the *Agama agama* (Agamidae, Lacertilia) embryo. *Comptes Rendus Des Seances De La Societe De Biologie Et De Ses Filiales*, *160*(3), 620–622.

Chen, N., Bellott, D. W., Page, D. C., & Clark, A. G. (2012). Identification of avian W-linked contigs by short-read sequencing. *BMC Genomics*, *13*, 183.

Chen, S., Zhang, G., Shao, C., Huang, Q., Liu, G., Zhang, P., Song, W., An, N., Chalopin, D., Volff, J.-N., Hong, Y., Li, Q., Sha, Z., Zhou, H., Xie, M., Yu, Q., Liu, Y., Xiang, H., Wang, N., … Wang, J. (2014). Whole-genome sequence of a flatfish provides insights into ZW sex chromosome evolution and adaptation to a benthic lifestyle. *Nature Genetics*, *46*(3), 253–260.

Chevassus, B., Devaux, A., Chourrout, D., & Jalabert, B. (1988). Production of YY Rainbow Trout Males by Self-Fertilization of Induced Hermaphrodites. *Journal of Heredity*, *79*(2), 89–92.

Chevin, L. M., Lande, R., & Mace, G. M. (2010). Adaptation, plasticity, and extinction in a changing environment: towards a predictive theory. *PLOS Biology*, *8*(4), e1000357.

Chifman, J., & Kubatko, L. (2014). Quartet inference from SNP data under the coalescent model. *Bioinformatics*, *30*, 3317-3324.

Chong, C. E., Lim, B. S., Nathan, S., & Mohamed, R. (2006). In silico analysis of *Burkholderia pseudomallei* genome sequence for potential drug targets. *In silico Biology*, *6*(4), 341-346.

Chou, J., Gupta, A., Yaduvanshi, S., Davidson, R., Nute, M., Mirarab, S., & Warnow, T. (2015). A comparative study of SVDquartets and other coalescent-based species tree estimation methods. *BMC Genomics*, *16*(10), 1-11.

Close, A. (1990). River salinity. In N. Mackay & D. Eastburn (Eds.), *The Murray* (pp. 127–144). Murray-Darling Basin Commission, Canberra.

Coates, D. J., Byrne, M., & Moritz, C. (2018). Genetic diversity and conservation units: dealing with the species-population continuum in the age of genomics. *Frontiers in Ecology and Evolution*, *6*, 165.

Cogger, H. (2018). *Reptiles and Amphibians* (7th ed.). CSIRO Publishing.

Conover, D. O., & Heins, S. W. (1987). Adaptive variation in environmental and genetic sex determination in a fish. *Nature*, *326*(6112), 496-498.

Conover, D. O., & Van Voorhees, D. A. (1990). Evolution of a balanced sex ratio by frequency-dependent selection in a fish. *Science*, *250*(4987), 1556-1558.

Cooper, S. J. B., Adams, M., & Labrinidis, A. (2000). Phylogeography of the Australian dunnart *Sminthopsis crassicaudata* (Marsupialia: Dasyuridae). *Australian Journal of Zoology*, *48*, 461-473.

Cornejo-Páramo, P., Dissanayake, D. S. B., Lira-Noriega, A., Martínez-Pacheco, M. L., Acosta, A., Ramírez-Suástegui, C., Méndez-de-la-Cruz, F. R., Székely, T., Urrutia, A. O., Georges, A., & Cortez, D. (2020). Viviparous reptile regarded to have temperature-dependent sex determination has old XY chromosomes. *Genome Biology and Evolution*, *12*(6), 924–930.

Cortez, D., Marin, R., Toledo-Flores, D., Froidevaux, L., Liechti, A., Waters, P. D., ... & Kaessmann, H. (2014). Origins and functional evolution of Y chromosomes across mammals. *Nature*, *508*(7497), 488-493.

Costello, M. J., Wilson, S., & Houlding, B. (2012). Predicting total global species richness using rates of species description and estimates of taxonomic effort. *Systematic Biology*, *61*(5), 871.

Cotton, S., & Wedekind, C. (2009). Population consequences of environmental sex reversal. *Conservation Biology*, *23*(1), 196–206.

Cree, A., Thompson, M. B., & Daugherty, C. H. (1995). Tuatara sex determination. *Nature*, *375*(6532), 543–543.

Dallwitz, M. J., & Higgins, J. P. (1978). *User's Guide to DEVAR. A computer program for estimating development rate as a function of temperature*. Canberra, Division of Entomology, CSIRO.

Dash, H. R., Rawat, N., & Das, S. (2020). Alternatives to amelogenin markers for sex determination in humans and their forensic relevance. *Molecular Biology Reports*, *47*(3), 2347-2360.

Dawson, D. A., Bird, S., Horsburgh, G. J., & Ball, A. D. (2015). Autosomal and Z-linked microsatellite markers enhanced for cross-species utility and assessed in a range of

birds, including species of conservation concern. *Conservation Genetics Resources*, *7*(4), 881-886.

De Queiroz, K. (1998). The general lineage concept of species, species criteria, and the process of speciation, In D. J. Howard & S. H. Berlocher (Eds.), *Endless forms: Species and Speciation* (pp. 57-75). Oxford Univ. Press, Oxford, U.K.

Deakin, J. E., & Ezaz, T. (2014). Tracing the evolution of amniote chromosomes. *Chromosoma*, *123*(3), 201–216.

Deakin, J. E., & Ezaz, T. (2019). Understanding the evolution of reptile chromosomes through applications of combined cytogenetics and genomics approaches. *Cytogenetic and Genome Research*, *157*(1–2), 7–20.

Deeming, D. C. (2004). Prevalence of TSD in crocodilians. In *Temperature-dependent sex determination in vertebrates* (Nicole Valenzuela and Valentine Lance, pp. 33–41). Smithsonian Books.

Deeming, D. C., & Ferguson, M. W. J. (1988). Environmental regulation of sex determination in reptiles. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, *322*(1208), 19-39.

Deveson, I. W., Holleley, C. E., Blackburn, J., Graves, J. A. M., Mattick, J. S., Waters, P. D., & Georges, A. (2017). Differential intron retention in *Jumonji* chromatin modifier genes is implicated in reptile temperature-dependent sex determination. *Science advances*, *3*(6), e1700731.

Devlin, R. H., & Nagahama, Y. (2002). Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. *Aquaculture*, *208*(3), 191–364.

Dissanayake, D. S. B., Holleley, C. E., Hill, L. K., O'Meally, D., Deakin, J. E., & Georges, A. (2020). Identification of Y chromosome markers in the eastern three-lined skink (*Bassiana duperreyi*) using in silico whole genome subtraction. *BMC Genomics*, *21*(1), 1-12.

Dissanayake, D. S., Holleley, C. E., Deakin, J. E., & Georges, A. (2021a). High elevation increases the risk of Y chromosome loss in alpine skink populations with sex reversal. *Heredity*, *126*(5), 805-816.

Dissanayake, D. S. B., Holleley, C. E., & Georges, A. (2021b). Effects of natural nest temperatures on sex reversal and sex ratios in an Australian alpine skink. *Scientific Reports*, 11, 20093.

Doddamani, L. S. (1994). Histoenzymological studies on embryonic and posthatching development of the ovary in the tropical oviparous lizard, *Calotes versicolor*. *Journal of Morphology*, *222*(1), 1-10.

Doddamani, L. S. (2006). Differentiation and development of testis in the oviparous lizard, Calotes versicolor (Daud.). *Journal of Experimental Zoology Part A: Comparative Experimental Biology*, *305*(3), 299-308.

Donnellan, S. (1985). *The evolution of sex chromosomes in scincid lizards* [PhD thesis]. Macquarie University.

Doody, J. S., Guarino, E., Georges, A., Corey, B., Murray, G., & Ewert, M. (2006). Nest site choice compensates for climate effects on sex ratios in a lizard with environmental sex determination. *Evolutionary Ecology*, *20*(4), 307-330.

Dournon, C., Houillon, C., & Pieau, C. (1990). Temperature sex-reversal in amphibians and reptiles. *The International Journal of Developmental Biology*, *34*(1), 81–92.

Du, W. G., & Shine, R. (2010). Why do the eggs of lizards (*Bassiana duperreyi*: Scincidae) hatch sooner if incubated at fluctuating rather than constant temperatures? *Biological Journal of the Linnean Society*, *101*(3), 642-650.

Du, W. G., Shine, R., Ma, L., & Sun, B. J. (2019). Adaptive responses of the embryos of birds and reptiles to spatial and temporal variations in nest temperatures. *Proceedings of the Royal Society B*, *286*(1915), 20192078.

Dubey, S., & Shine, R. (2010). Evolutionary Diversification of the Lizard Genus *Bassiana* (Scincidae) across Southern Australia. *PLOS One*, *5*(9), e12982.

Duffy, T. A., Hice, L. A., & Conover, D. O. (2015). Pattern and scale of geographic variation in environmental sex determination in the Atlantic silverside, *Menidia menidia*. *Evolution; International Journal of Organic Evolution*, *69*(8), 2187–2195.

Dunham, A. E., Erhart, E. M., & Wright, P. C. (2011). Global climate cycles and cyclones: Consequences for rainfall patterns and lemur reproduction in southeastern Madagascar. *Global Change Biology*, *17*(1), 219–227.

Düsing, K. (1884). Die Regulierung des geschlechtsverhä ltnisses bei der vermehrung der menschen, tiere und pflanzen. *Jena Zschr Naturwise*. *17*:593–940.

Dutta, A., Singh, S. K., Ghosh, P., Mukherjee, R., Mitter, S., & Bandyopadhyay, D. (2006). In silico identification of potential therapeutic targets in the human pathogen Helicobacter pylori. *In Silico Biology*, *6*(1–2), 43–47.

Easterling, D. R., Meehl, G. A., Parmesan, C., Changnon, S. A., Karl, T. R., & Mearns, L. O. (2000). Climate Extremes: Observations, Modeling, and Impacts. *Science*, *289*(5487), 2068–2074.

Edwards, A. W. F. (2000). Carl Düsing (1884) on the regulation of the sex-ratio. *Theoretical Population Biology*, *58*(3), 255-257.

Elphick, M. J., & Shine, R. (1998). Longterm effects of incubation temperatures on the morphology and locomotor performance of hatchling lizards (*Bassiana duperreyi*, Scincidae). *Biological Journal of the Linnean Society*, *63*(3), 429-447.

Emerson, J. J. (2017). Evolution: A paradigm shift in snake sex chromosome genetics. *Current Biology*, *27*(16), R800–R803.

Evans, B. J., Alexander Pyron, R., & Wiens, J. J. (2012). Polyploidization and sex chromosome evolution in amphibians. In P. S. Soltis & D. E. Soltis (Eds.), *Polyploidy and Genome Evolution* (pp. 385–410). Springer.

Ewert, M. A., & Nelson, C. E. (1991). Sex determination in turtles: Diverse patterns and some possible adaptive values. *Copeia*, *1991*(1), 50–69.

Ewert, M. A., Jackson, D. R., & Nelson, C. E. (1994). Patterns of temperature-dependent sex determination in turtles. *Journal of Experimental Zoology*, *270*(1), 3–15.

Ewert, M., Etchberger, C., & Nelson, C. (2004). Turtle sex-determination modes and TSD patterns, and some TSD pattern correlates. In *Temperature-dependent sex determination in vertebrates* (Valenzuela N, and Lance VA, pp. 21–32). Smithsonian Books.

Ezaz, T., O'Meally, D., Quinn, A. E., Sarre, S. D., Georges, A., & Graves, J. A. M. (2008). A simple non-invasive protocol to establish primary cell lines from tail and toe explants for cytogenetic studies in Australian dragon lizards (Squamata: Agamidae). *Cytotechnology, 58*(3), 135-139.

Ezaz, T., Quinn, A. E., Miura, I., Sarre, S. D., Georges, A., & Graves, J. A. M. (2005). The dragon lizard *Pogona vitticeps* has ZZ/ZW micro-sex chromosomes. *Chromosome Research, 13*(8), 763-776.

Ezaz, T., Quinn, A. E., Sarre, S. D., O'Meally, D., Georges, A., & Marshall Graves, J. A. (2009). Molecular marker suggests rapid changes of sex-determining mechanisms in Australian dragon lizards. *Chromosome Research, 17*(1), 91–98.

Ezaz, T., Sarre, S. D., O'Meally, D., Graves, J. A. M., & Georges, A. (2009b). Sex Chromosome Evolution in Lizards: Independent origins and rapid transitions. *Cytogenetic and Genome Research, 127*(2–4), 249–260.

Ezaz, T., Srikulnath, K., & Graves, J. A. M. (2017). Origin of amniote sex chromosomes: an ancestral super-sex chromosome, or common requirements? *Journal of Heredity, 108*(1), 94-105.

Ezaz, T., Stiglec, R., Veyrunes, F., & Marshall Graves, J. A. (2006). Relationships between vertebrate ZW and XY sex chromosome systems. *Current Biology: CB, 16*(17), R736-743.

Ezaz, T., Valenzuela, N., Grützner, F., Miura, I., Georges, A., Burke, R. L., & Marshall Graves, J. A. (2006). An XX/XY sex microchromosome system in a freshwater turtle, *Chelodina longicollis* (Testudines: Chelidae) with genetic sex determination. *Chromosome Research, 14*(2), 139–150.

Faith, D. P., & Baker, A. M. (2006). Phylogenetic diversity (PD) and biodiversity conservation: some bioinformatics challenges. *Evolutionary Bioinformatics, 2*, 117693430600200007.

Fang, S., Zhang, Y., Shi, X., Zheng, H., Li, S., Zhang, Y., Fazhan, H., Waiho, K., Tan, H., Ikhwanuddin, M. and Ma, H. (2020). Identification of male-specific SNP markers and development of PCR-based genetic sex identification technique in crucifix crab (*Charybdis feriatus*) with implication of an XX/XY sex determination system. *Genomics*, *112*(1), 404-411.

Ferguson, M. W. J., & Joanen, T. (1982). Temperature of egg incubation determines sex in *Alligator mississippiensis*. *Nature*, *296*(5860), 850–853.

Ferguson-Smith, M. (2007). The evolution of sex chromosomes and sex determination in vertebrates and the key role of DMRT1. *Sexual Development*, *1*(1), 2-11.

Fisher, R. A. (1930). *The genetical theory of natural selection* (pp. xiv, 272). Clarendon Press.

Flament, S. (2016). Sex Reversal in Amphibians. *Sexual Development*, *10*(5–6), 267–278.

Flatt, T., Shine, R., Borges-Landaez, P. A., & Downes, S. J. (2001). Phenotypic variation in an oviparous montane lizard (*Bassiana duperreyi*): the effects of thermal and hydric incubation environments. *Biological Journal of the Linnean Society*, *74*(3), 339-350.

Foster, J. W., Brennan, F. E., Hampikian, G. K., Goodfellow, P. N., Sinclair, A. H., Lovell-Badge, R., Selwood, L., Renfree, M. B., Cooper, D. W., & Marshall Graves, J. A. (1992). Evolution of sex determination and the Y chromosome: SRY -related sequences in marsupials. *Nature*, *359*(6395), 531–533.

Fowler, B. L., & Buonaccorsi, V. P. (2016). Genomic characterization of sex-identification markers in *Sebastes carnatus* and *Sebastes chrysomelas* rockfishes. *Molecular Ecology*, *25*(10), 2165-2175.

Frýdlová, P., Velenský, P., Šimková, O., Cikánová, V., Hnízdo, J., Rehák, I., & Frynta, D. (2011). Is body shape of mangrove-dwelling monitor lizards (*Varanus indicus*; Varanidae) sexually dimorphic? *Amphibia-Reptilia*, *32*(1), 27–37.

Fuentes, M. M. P. B., Limpus, C. J., & Hamann, M. (2011). Vulnerability of sea turtle nesting grounds to climate change. *Global Change Biology*, *17*(1), 140–153.

Fujita, M. K., Leaché, A. D., Burbrink, F. T., McGuire, J. A., & Moritz, C. (2012). Coalescent-based species delimitation in an integrative taxonomy. *Trends in Ecology and Evolution*, *27*, 480–488.

Gamble, T., & Zarkower, D. (2014). Identification of sex-specific molecular markers using restriction site-associated DNA sequencing. *Molecular Ecology Resources*, *14*(5), 902–913.

Gamble, T., Castoe, T. A., Nielsen, S. V., Banks, J. L., Card, D. C., Schield, D. R., ... & Booth, W. (2017). The discovery of XY sex chromosomes in a Boa and *Python*. *Current Biology*, *27*(14), 2148-2153.

Gamble, T., Coryell, J., Ezaz, T., Lynch, J., Scantlebury, D. P., & Zarkower, D. (2015). Restriction site-associated DNA sequencing (RAD-seq) reveals an extraordinary number of transitions among gecko sex-determining systems. *Molecular Biology and Evolution*, *32*(5), 1296-1309.

Gamble, T., Geneva, A. J., Glor, R. E., & Zarkower, D. (2014). Anolis sex chromosomes are derived from a single ancestral pair. *Evolution*, *68*(4), 1027-1041.

Gamelon, M., Grøtan, V., Nilsson, A.L., Engen, S., Hurrell, J.W., Jerstad, K., Phillips, A.S., Røstad, O.W., Slagsvold, T., Walseng, B. and Stenseth, N.C. (2017). Interactions between demography and environmental effects are important determinants of population dynamics. *Science Advances*, *3*(2), e1602298.

Garcia, R. A., Cabeza, M., Rahbek, C., & Araújo, M. B. (2014). Multiple dimensions of climate change and their implications for biodiversity. *Science*, *344*(6183), 1247579.

Garcia-Porta, J., Irisarri, I., Kirchner, M., Rodríguez, A., Kirchhof, S., Brown, J. L., ... & Valero, K. C. W. (2019). Environmental temperatures shape thermal physiology as well as diversification and genome-wide substitution rates in lizards. *Nature communications*, *10*(1), 1-12.

Garland, T. (1985). Ontogenetic and individual variation in size, shape and speed in the Australian agamid lizard *Amphibolurus nuchalis*. *Journal of Zoology*, *207*(3), 425–439.

Gauthier, J., Kluge, A. G., & Rowe, T. (1988). Amniote phylogeny and the importance of fossils. *Cladistics*, *4*(2), 105–209.

Ge, C., Ye, J., Weber, C., Sun, W., Zhang, H., Zhou, Y., Cai, C., Qian, G., & Capel, B. (2018). The histone demethylase *KDM6B* regulates temperature-dependent sex determination in a turtle species. *Science*, *360*(6389), 645–648.

Geffroy, B., & Wedekind, C. (2020). Effects of global warming on sex ratios in fishes. *Journal of Fish Biology*, *97*(3), 596-606.

George, T., & Pandian, T. J. (1996). Hormonal induction of sex reversal and progeny testing in the zebra cichlid *Cichlasoma nigrofasciatum*. *Journal of Experimental Zoology*, *275*(5), 374–382.

George, T., Pandian, T., & Kavumphuath, S. (2013). Inviability of YY zygotes of the fighting fish, betta-splendens. *Israeli journal of aquaculture-bamidgeh*, *46*(1), 3–8.

Georges, A. (1989). Female turtles from hot nests: Is it duration of incubation or proportion of development at high temperatures that matters? *Oecologia*, *81*(3), 323–328.

Georges, A. (1992). Thermal-Characteristics and Sex Determination in Field Nests of the Pig-Nosed Turtle, *Carettochelys insculpta* (Chelonia, Carettochelydidae), From Northern Australia. *Australian Journal of Zoology*, *40*(5), 511-521.

Georges, A., & Holleley, C. E. (2018). How does temperature determine sex? *Science*, *360*(6389), 601–602.

Georges, A., Doody, S. E. A. N., Beggs, K., & Young, J. E. A. N. N. E. (2004). Thermal models of TSD under laboratory and field conditions. *Temperature-dependent sex determination in vertebrates*, 79-89.

Georges, A., Gruber, B., Pauly, G. B., White, D., Adams, M., Young, M. J., ... & Unmack, P. J. (2018). Genome wide SNP markers breathe new life into phylogeography and species delimitation for the problematic short-necked turtles (Chelidae: *Emydura*) of eastern Australia. *Molecular Ecology*, *27*, 5195-5213.

Georges, A., Limpus, C., & Stoutjesdijk, R. (1994). Hatchling sex in the marine turtle Caretta caretta is determined by proportion of development at a temperature, not daily duration of exposure. *Journal of Experimental Zoology*, *270*(5), 432–444.

Georges, A., Limpus, C., & Stoutjesdijk, R. (1994). Hatchling sex in the marine turtle *Caretta caretta* is determined by proportion of development at a temperature, not daily duration of exposure. *Journal of Experimental Zoology*, *270*(5), 432-444.

Gibbons, J. W., Scott, D. E., Ryan, T. J., Buhlmann, K. A., Tuberville, T. D., Metts, B. S., ... & Winne, C. T. (2000). The Global Decline of Reptiles, Déjà Vu Amphibians: Reptile species are declining on a global scale. Six significant threats to reptile populations are habitat loss and degradation, introduced invasive species, environmental pollution, disease, unsustainable use, and global climate change. *BioScience*, *50*(8), 653-666.

Girondot, M., Monsinjon, J., & Guillon, J.-M. (2018). Delimitation of the embryonic thermosensitive period for sex determination using an embryo growth model reveals a potential bias for sex ratio prediction in turtles. *Journal of Thermal Biology*, *73*, 32–40.

Godfree, R. C., Knerr, N., Encinas-Viso, F., Albrecht, D., Bush, D., Cargill, D. C., ... & Broadhurst, L. M. (2021). Implications of the 2019–2020 megafires for the biogeography and conservation of Australian vegetation. *Nature Communications*, *12*, 1023.

Grainger, T. N., Rudman, S. M., Schmidt, P., & Levine, J. M. (2021). Competitive history shapes rapid evolution in a seasonal climate. *Proceedings of the National Academy of Sciences*, *118*(6).

Graves, J. A. M. (1995). The evolution of mammalian sex chromosomes and the origin of sex determining genes. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *350*(1333), 305-312.

Graves, J. A. M. (2013). How to evolve new vertebrate sex determining genes. *Developmental Dynamics*, *242*(4), 354-359.

Graves, J. A. M. (2016). How Australian mammals contributed to our understanding of sex determination and sex chromosomes. *Australian Journal of Zoology*, *64*(4), 267–276.

Graves, J. A. M., & Foster, J. W. (1994). Evolution of Mammalian Sex Chromosomes and Sex-Determining Genes. In K. W. Jeon & J. Jarvik (Eds.), *International Review of Cytology* (Vol. 154, pp. 191–259). Academic Press.

Graves, J. A. M., & Peichel, C. L. (2010). Are homologies in vertebrate sex determination due to shared ancestry or to limited options? *Genome biology*, *11*(4), 1-12.

Graves, J. A. M., & Shetty, S. (2001). Sex from W to Z: Evolution of vertebrate sex chromosomes and sex determining genes. *Journal of Experimental Zoology*, *290*(5), 449–462.

Graves, J. A. M., Ferguson-Smith, M. A., McLaren, A., Mittwoch, U., Renfree, M. B., Burgoyne, P., Ferguson-Smith, M. A., Lovell-Badge, R. H., & McLaren, A. (1995). The evolution of mammalian sex chromosomes and the origin of sex determining genes. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *350*(1333), 305–312.

Gray, J. E. (1838). Catalogue of the slender-tongued saurians, with descriptions of many new genera and species. Part 3. *Annals of Natural History.*, *1*, 388–394.

Green, D. M. (2005). Designatable units for status assessment of endangered species. *Conservation Biology*, *19*, 1813-1820.

Griffiths, R. (2000). Sex identification using DNA markers. In *Molecular methods in ecology* (Baker AJ, pp. 295–322). Blackwell.

Griffiths, R. I. C. H. A. R. D., & Orr, K. A. T. E. (1999). The use of amplified fragment length polymorphism (AFLP) in the isolation of sex-specific markers. *Molecular Ecology*, *8*(4), 671-674.

Grossen, C., Neuenschwander, S., & Perrin, N. (2011). Temperature-dependent turnovers in sex-determination mechanisms: A quantitative model. *Evolution; International Journal of Organic Evolution*, *65*(1), 64–78.

Gruber, B., Unmack, P. J., Berry, O. F., & Georges, A. (2018). dartr: An r package to facilitate analysis of SNP data generated from reduced representation genome sequencing. *Molecular Ecology Resources*, *18*(3), 691-699.

Guillon, H., & de Massy, B. (2002). An initiation site for meiotic crossing-over and gene conversion in the mouse. *Nature genetics*, *32*(2), 296-299.

Hamaguchi, S., Toyazaki, Y., Shinomiya, A., & Sakaizumi, M. (2004). The XX-XY Sex-determination System in *Oryzias luzonensis* and *O. mekongensis* revealed by the sex ratio of the progeny of sex-reversed fish. *Zoological Science*, *21*(10), 1015–1018.

Hari, R. E., Livingstone, D. M., Siber, R., Burkhardt-holm, P. A. T. R. I. C. I. A., & Guettinger, H. (2006). Consequences of climatic change for water temperature and brown trout populations in Alpine rivers and streams. *Global Change Biology*, *12*(1), 10-26.

Harlow, P. S., & Taylor, J. E. (2000). Reproductive ecology of the Jacky dragon (*Amphibolurus muricatus*): An agamid lizard with temperature-dependent sex determination. *Austral Ecology*, *25*(6), 640–652.

Harlow, P., S. (2004). TSD in lizards. In *Temperature-Dependent Sex Determination in Vertebrates* (N. Valenzuela and V.A. Lance). Smithsonian Books.

Harolow, P. S. (1996). A harmless technique for sexing hatchiling lizards. *Herpetological Review*, *27*(2), 71-71.

Harper, P. S. (2006). The discovery of the human chromosome number in Lund, 1955-1956. *Human Genetics*, *119*(1–2), 226–232.

Harris, P., Heap, A., Passlow, V., Sbaffi, L., Fellows, M., Porter-Smith, R., ... & Daniell, J. (2003). *Geomorphic features of the continental margin of Australia*. Geoscience Australia, Canberra.

Hattori, R. S., Castañeda-Cortés, D. C., Padilla, L. A., Strobl-Mazzulla, P. H., & Fernandino, J. I. (2020). Activation of stress response axis as a key process in environment-induced sex plasticity in fish. *Cellular and Molecular Life Sciences*, 1-14.

Hattori, R. S., Murai, Y., Oura, M., Masuda, S., Majhi, S. K., Sakamoto, T., Fernandino, J. I., Somoza, G. M., Yokota, M., & Strüssmann, C. A. (2012). A Y-linked anti-Müllerian hormone duplication takes over a critical role in sex determination. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(8), 2955–2959.

Hattori, R. S., Oura, M., Sakamoto, T., Yokota, M., Watanabe, S., & Strüssmann, C. A. (2010). Establishment of a strain inheriting a sex-linked SNP marker in Patagonian

pejerrey (*Odontesthes hatcheri*), a species with both genotypic and temperature-dependent sex determination. *Animal Genetics*, *41*(1), 81–84.

Hattori, R. S., Tashiro, S., Zhang, Y., Kakuta, N., Yokota, M., Strüssmann, C. A., & Yamamoto, Y. (2018). Demonstration of viability and fertility and development of a molecular tool to identify YY supermales in a fish with both genotypic and environmental sex determination. *Ecology and Evolution*, *8*(15), 7522–7528.

Hawkes, L. A., Broderick, A. C., Godfrey, M. H., & Godley, B. J. (2007). Investigating the potential impacts of climate change on a marine turtle population. *Global Change Biology*, *13*(5), 923–932.

Hayes, T. B. (1998). Sex determination and primary sex differentiation in amphibians: Genetic and developmental mechanisms. *Journal of Experimental Zoology*, *281*(5), 373–399.

Hayes, T. B., Collins, A., Lee, M., Mendoza, M., Noriega, N., Stuart, A. A., & Vonk, A. (2002). Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(8), 5476–5480.

Hays, G. C., Mazaris, A. D., Schofield, G., & Laloë, J.-O. (2017). Population viability at extreme sex-ratio skews produced by temperature-dependent sex determination. *Proceedings of the Royal Society B: Biological Sciences*, *284*(1848), 20162576.

Hermelink, B., Urbatzka, R., Wiegand, C., Pflugmacher, S., Lutz, I., & Kloas, W. (2010). Aqueous leaf extracts display endocrine activities in vitro and disrupt sexual differentiation of male Xenopus laevis tadpoles in vivo. *General and Comparative Endocrinology*, *168*(2), 245–255.

Heule, C., Salzburger, W., & Böhne, A. (2014). Genetics of Sexual Development: An Evolutionary Playground for Fish. *Genetics*, *196*(3), 579–591.

Hill, P. L., Burridge, C. P., Ezaz, T., & Wapstra, E. (2018). Conservation of sex-linked markers among conspecific populations of a viviparous skink, *Niveoscincus ocellatus*, exhibiting genetic and temperature-dependent sex determination. *Genome Biology and Evolution*, *10*(4), 1079-1087.

Hillis, D. M., & Green, D. M. (1990). Evolutionary changes of heterogametic sex in the phylogenetic history of amphibians. *Journal of Evolutionary Biology*, *3*(1–2), 49–64.

Hime, P. M., Briggler, J. T., Reece, J. S., & Weisrock, D. W. (2019). Genomic data reveal conserved female heterogamety in giant salamanders with gigantic nuclear genomes. *G3: Genes, Genomes, Genetics*, *9*(10), 3467-3476.

Hirst, C. E., Major, A. T., Ayers, K. L., Brown, R. J., Mariette, M., Sackton, T. B., & Smith, C. A. (2017). Sex Reversal and Comparative Data Undermine the W Chromosome and Support Z-linked DMRT1 as the Regulator of Gonadal Sex Differentiation in Birds. *Endocrinology*, *158*(9), 2970–2987.

Hoban, S., Bruford, M., Jackson, J. D. U., Lopes-Fernandes, M., Heuertz, M., Hohenlohe, P. A., ... & Laikre, L. (2020). Genetic diversity targets and indicators in the CBD post-2020 Global Biodiversity Framework must be improved. *Biological Conservation*, *248*, 108654.

Hoffmann, A. A., & Sgro, C. M. (2011). Climate change and evolutionary adaptation. *Nature*, *470*(7335), 479-485.

Holleley, C. E., O'Meally, D., Sarre, S. D., Marshall Graves, J. A., Ezaz, T., Matsubara, K., Azad, B., Zhang, X., & Georges, A. (2015). Sex reversal triggers the rapid transition from genetic to temperature-dependent sex. *Nature*, *523*(7558), 79–82.

Holleley, C. E., Sarre, S. D., O'Meally, D., & Georges, A. (2016). Sex reversal in reptiles: Reproductive oddity or powerful driver of evolutionary change? *Sexual Development*, *10*(5-6), 279-287.

Hollestelle, A., & Schutte, M. (2005). Representational difference analysis as a tool in the search for new tumor suppressor genes. *Pancreatic Cancer*, 143-159.

Holmes, K. M., & Cree, A. (2006). Annual reproduction in females of a viviparous skink (*Oligosoma maccanni*) in a subalpine environment. *Journal of Herpetology*, *40*(2), 141-151.

Honeycutt, J. L., Deck, C. A., Miller, S. C., Severance, M. E., Atkins, E. B., Luckenbach, J. A., ... & Godwin, J. (2019). Warmer waters masculinize wild populations of a fish with temperature-dependent sex determination. *Scientific Reports*, *9*(1), 1-13.

Hsü, C. Y., Yü, N. W., & Liang, H. M. (1971). Induction of sex reversal in female tadpoles of Rana catesbeiana by temperature treatment. *Endocrinologia Japonica*, *18*(3), 243–251.

Huey, R. B., & Pianka, E. R. (1977). Seasonal variation in thermoregulatory behavior and body temperature of diurnal Kalahari lizards. *Ecology*, *58*(5), 1066-1075.

Hulme, P. E. (2017). Climate change and biological invasions: evidence, expectations, and response options. *Biological Reviews*, *92*(3), 1297-1313.

Hurley, M. A., Matthiessen, P., & Pickering, A. D. (2004). A model for environmental sex reversal in fish. *Journal of Theoretical Biology*, *227*(2), 159-165.

Hutchinson, M. N., Donnellan, S. C., Baverstock, P. R., Krieg, M., Simms, S., & Burgin, S. (1990). Immunological relationships and generic revision of the Australian lizards assigned to the genus *Leiolopisma* (Scincidae, Lygosominae). *Australian Journal of Zoology*, *38*, 535-554.

Isakov, O., Modai, S., & Shomron, N. (2011). Pathogen detection using short-RNA deep sequencing subtraction and assembly. *Bioinformatics*, *27*(15), 2027-2030.

Janzen, F. J. (1994). Climate change and temperature-dependent sex determination in reptiles. *Proceedings of the National Academy of Sciences*, *91*(16), 7487–7490.

Janzen, F. J., & Paukstis, G. L. (1991). Environmental sex determination in reptiles: ecology, evolution, and experimental design. *The Quarterly review of biology*, *66*(2), 149-179.

Janzen, F. J., & Phillips, P. C. (2006). Exploring the evolution of environmental sex determination, especially in reptiles. *Journal of Evolutionary Biology*, *19*(6), 1775–1784.

Jeffrey, S. J., Carter, J. O., Moodie, K. B., & Beswick, A. R. (2001). Using spatial interpolation to construct a comprehensive archive of Australian climate data. *Environmental Modelling & Software*, *16*(4), 309-330.

Jeffreys, A. J., Kauppi, L., & Neumann, R. (2001). Intensely punctate meiotic recombination in the class II region of the major histocompatibility complex. *Nature genetics*, *29*(2), 217-222.

Jensen, M. P., Allen, C. D., Eguchi, T., Bell, I. P., La Casella, E. L., Hilton, W. A., Hof, C. A. M., & Dutton, P. H. (2018). Environmental Warming and Feminization of One of the Largest Sea Turtle Populations in the World. *Current Biology, 28*(1), 154-159.e4.

Johansson, J. (2008). Evolutionary responses to environmental changes: how does competition affect adaptation? *Evolution: International Journal of Organic Evolution*, *62*(2), 421-435

Jombart, T. (2008). adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics*, *24*(11), 1403-1405.

Kakimi, K., Karasaki, T., Matsushita, H., & Sugie, T. (2017). Advances in personalized cancer immunotherapy. *Breast Cancer*, *24*(1), 16-24.

Kamiya, T., Kai, W., Tasumi, S., Oka, A., Matsunaga, T., Mizuno, N., Fujita, M., Suetake, H., Suzuki, S., Hosoya, S., Tohari, S., Brenner, S., Miyadai, T., Venkatesh, B., Suzuki, Y., & Kikuchi, K. (2012). A Trans-Species Missense SNP in Amhr2 Is Associated with Sex Determination in the Tiger Pufferfish, *Takifugu rubripes* (Fugu). *PLOS Genetics*, *8*(7), e1002798.

Kautt, A. F., Machado-Schiaffino, G., & Meyer, A. (2016). Multispecies outcomes of sympatric speciation after admixture with the source population in two radiations of Nicaraguan crater lake cichlids. *PLOS Genetics*, *12*(6), e1006157.

Kavumpurath, S., & Pandian, T. J. (1993). Production of a YY female guppy, *Poecilia reticulata*, by endocrine sex reversal and progeny testing. *Aquaculture, 118*(3), 183–189.

Kawakami, T., Butlin, R. K., Adams, M., Saint, K. M., Paull, D. J., & Cooper, S. J. (2009). Re-examination of a proposed case of stasipatric speciation: phylogeography of the Australian morabine grasshoppers (*Vandiemenella viatica* species group). *Molecular Ecology*, *18*, 3429-3442.

Kawakami, Y., Fujita, T., Matsuzaki, Y., Sakurai, T., Tsukamoto, M., Toda, M., & Sumimoto, H. (2004). Identification of human tumor antigens and its implications for diagnosis and treatment of cancer. *Cancer Science*, *95*(10), 784–791.

Kawamura, T., & Nishioka, M. (1977). Aspects of the reproductive biology of Japanese anurans. In *The reproductive biology of amphibians* (pp. 103-139). Springer, Boston, MA.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C. and Thierer, T., (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, *28*(12), 1647-1649.

Kikuchi, K., Kai, W., Hosokawa, A., Mizuno, N., Suetake, H., Asahina, K., & Suzuki, Y. (2007). The sex-determining locus in the tiger pufferfish, *Takifugu rubripes*. *Genetics*, *175*(4), 2039-2042.

Kilian, A., Wenzl, P., Huttner, E., Carling, J., Xia, L., Blois, H., ... & Uszynski, G. (2012). Diversity arrays technology: a generic genome profiling technology on open platforms. In *Data production and analysis in population genomics* (pp. 67-89). Humana Press, Totowa, NJ.

Kirk, G. S., Raven, J. E., & Malcolm, SchofieldJ. E. (1983). *The Presocratic Philosophers: A Critical History with a Selection of Texts*. Cambridge University Press.

Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., & Lovell-Badge, R. (1991). Male development of chromosomally female mice transgenic for *SRY*. *Nature*, *351*(6322), 117–121.

Kopp, M., & Matuszewski, S. (2014). Rapid evolution of quantitative traits: Theoretical perspectives. *Evolutionary Applications*, *7*(1), 169–191.

Kozielska, M., Weissing, F. J., Beukeboom, L. W., & Pen, I. (2010). Segregation distortion and the evolution of sex-determining mechanisms. *Heredity*, *104*(1), 100-112.

Lambert, M. R. (2015). Clover root exudate produces male-biased sex ratios and accelerates male metamorphic timing in wood frogs. *Royal Society Open Science*, *2*(12), 150433.

Lambert, M. R., Skelly, D. K., & Ezaz, T. (2016). Sex-linked markers in the North American green frog (*Rana clamitans*) developed using DArTseq provide early insight into sex chromosome evolution. *BMC Genomics*, *17*(1), 844.

Lambert, M. R., Smylie, M. S., Roman, A. J., Freidenburg, L. K., & Skelly, D. K. (2018). Sexual and somatic development of wood frog tadpoles along a thermal gradient. *Journal of Experimental Zoology. Part A, Ecological and Integrative Physiology*, *329*(2), 72–79.

Lambert, M. R., Stoler, A. B., Smylie, M. S., Relyea, R. A., & Skelly, D. K. (2016). Interactive effects of road salt and leaf litter on wood frog sex ratios and sexual size dimorphism. *Canadian Journal of Fisheries and Aquatic Sciences*.

Lambert, M. R., Tran, T., Kilian, A., Ezaz, T., & Skelly, D. K. (2019). Molecular evidence for sex reversal in wild populations of green frogs (*Rana clamitans*). *PeerJ*, *7*, e6449.

Lambeth, L. S., Raymond, C. S., Roeszler, K. N., Kuroiwa, A., Nakata, T., Zarkower, D., & Smith, C. A. (2014). Over-expression of *DMRT1* induces the male pathway in embryonic chicken gonads. *Developmental Biology*, *389*(2), 160–172.

Lang, J. W., & Andrews, H. V. (1994). Temperature-dependent sex determination in crocodilians. *Journal of Experimental Zoology*, *270*(1), 28–44.

Li, H., Holleley, C. E., Elphick, M., Georges, A., & Shine, R. (2016). The behavioural consequences of sex reversal in dragons. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1832), 20160217.

Literman, R., Radhakrishnan, S., Tamplin, J., Burke, R., Dresser, C., & Valenzuela, N. (2017). Development of sexing primers in *Glyptemys insculpta* and *Apalone spinifera* turtles uncovers an XX/XY sex-determining system in the critically endangered bog turtle *Glyptemys muhlenbergii*. Conservation Genetics Resources, 9(4), 651-658.

Lowry, D. B., Hoban, S., Kelley, J. L., Lotterhos, K. E., Reed, L. K., Antolin, M. F., & Storfer, A. (2017). Breaking RAD: An evaluation of the utility of restriction site-associated DNA sequencing for genome scans of adaptation. *Molecular Ecology Resources*, *17*(2), 142–152.

Luo, W., Xia, Y., Yue, B., & Zeng, X. (2020). Assigning the sex-specific markers via genotyping-by-sequencing onto the Y chromosome for a torrent frog *Amolops mantzorum*. *Genes*, *11*(7), 727.

Mair, G. C., Abucay, J. S., Abella, T. A., Beardmore, J. A., & Skibinski, D. O. F. (2011). Genetic manipulation of sex ratio for the large-scale production of all-male tilapia Oreochromis niloticus. *Canadian Journal of Fisheries and Aquatic Sciences*.

Mank, J. E., Promislow, D. E. L., & Avise, J. C. (2006). Evolution of alternative sex-determining mechanisms in teleost fishes. *Biological Journal of the Linnean Society*, *87*(1), 83–93.

Marçais, G., & Kingsford, C. (2011). A fast, lock-free approach for efficient parallel counting of occurrences of k-mers. *Bioinformatics*, *27*(6), 764-770.

Marshall, T. L., Chambers, E. A., Matz, M. V., & Hillis, D. M. (2021). How mitonuclear discordance and geographic variation have confounded species boundaries in a widely studied snake. *Molecular Phylogenetics and Evolution*, *162*, 107194.

Martinez, E. A., Destombe, C., Quillet, M. C., & Valero, M. (1999). Identification of random amplified polymorphic DNA (RAPD) markers highly linked to sex determination in the red alga *Gracilaria gracilis*. *Molecular Ecology*, *8*(9), 1533-1538.

Martínez, P., Viñas, A. M., Sánchez, L., Díaz, N., Ribas, L., & Piferrer, F. (2014). Genetic architecture of sex determination in fish: Applications to sex ratio control in aquaculture. *Frontiers in Genetics, 5*.

Matsubara, K., O'Meally, D., Azad, B., Georges, A., Sarre, S. D., Graves, J. A. M., Matsuda, Y., & Ezaz, T. (2016). Amplification of microsatellite repeat motifs is associated with the evolutionary differentiation and heterochromatinization of sex chromosomes in Sauropsida. *Chromosoma*, *125*(1), 111–123.

Matsubara, K., Tarui, H., Toriba, M., Yamada, K., Nishida-Umehara, C., Agata, K., & Matsuda, Y. (2006). Evidence for different origin of sex chromosomes in snakes, birds, and mammals and stepwise differentiation of snake sex chromosomes. *Proceedings of the National Academy of Sciences*, *103*(48), 18190–18195.

Matsuda, M., Nagahama, Y., Shinomiya, A., Sato, T., Matsuda, C., Kobayashi, T., Morrey, C. E., Shibata, N., Asakawa, S., Shimizu, N., Hori, H., Hamaguchi, S., & Sakaizumi, M. (2002). DMY is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature*, *417*(6888), 559–563.

Matute, D. R., Comeault, A. A., Earley, E., Serrato-Capuchina, A., Peede, D., Monroy-Eklund, A., ... & Coyne, J. A. (2020). Rapid and predictable evolution of admixed populations between two Drosophila species pairs. *Genetics*, *214*, 211-230.

Mawaribuchi, S., Yoshimoto, S., Ohashi, S., Takamatsu, N., & Ito, M. (2012). Molecular evolution of vertebrate sex-determining genes. *Chromosome Research, 20*(1), 139–151.

Mayr, E. (1942). *Systematics and the Origin of Species*, Columbia University Press, New York.

McLaren, S., Wallace, M. W., Gallagher, S. J., Miranda, J. A., Holdgate, G. R., Gow, L. J., ... & Sandgren, P. (2011). Palaeogeographic, climatic and tectonic change in southeastern Australia: the Late Neogene evolution of the Murray Basin. *Quaternary Science Reviews*, *30*, 1086-1111.

Melville, J., Chapple, D. G., Keogh, J. S., Sumner, J., Amey, A., Bowles, P., Brennan, I. G., Couper, P., Donnellan, S. C., Doughty, P., Edwards, D. L., Ellis, R. J., Esquerré, D., Fenker, J., Gardner, M. G., Georges, A., Haines, M. L., Hoskin, C. J., Hutchinson, M., … Tingley, R. (2021). A return-on-investment approach for prioritization of rigorous taxonomic research needed to inform responses to the biodiversity crisis. *PLOS Biology, 19*(6), e3001210.

Melville, J., Haines, M. L., Boysen, K., Hodkinson, L., Kilian, A., Smith Date, K. L., ... & Parris, K. M. (2017). Identifying hybridization and admixture using SNPs: application of the DArTseq platform in phylogeographic research on vertebrates. *Royal Society Open Science*, *4*, 161061.

Miller, J. T., Jolley-Rogers, G., Mishler, B. D., & Thornhill, A. H. (2018). Phylogenetic diversity is a better measure of biodiversity than taxon counting. *Journal of Systematics and Evolution*, *56*, 663-667.

Mirvish, E. D., & Shuda, M. (2016). Strategies for Human Tumor Virus Discoveries: From Microscopic Observation to Digital Transcriptome Subtraction. *Frontiers in Microbiology, 7*.

Mitchell, N. J., & Janzen, F. J. (2010). Temperature-dependent sex determination and contemporary climate change. *Sexual Development*, *4*(1-2), 129-140.

Mitchell, N. J., Nelson, N. J., Cree, A., Pledger, S., Keall, S. N., & Daugherty, C. H. (2006). Support for a rare pattern of temperature-dependent sex determination in archaic reptiles: Evidence from two species of tuatara (*Sphenodon*). *Frontiers in Zoology*, *3*(1), 9.

Mitchell, N., Cox, N., Bowles, P., Tingley, R., Macdonald, S., Shea, G., ... & Chapple, D. (2019). *The Action Plan for Australian Lizards and Snakes 2017*. CSIRO Publishing.

Miura, I. (2017). Sex Determination and Sex Chromosomes in Amphibia. *Sexual Development*, *11*(5–6), 298–306.

Miyoshi, K., Hattori, R. S., Strüssmann, C. A., Yokota, M., & Yamamoto, Y. (2020). Phenotypic/genotypic sex mismatches and temperature-dependent sex determination in a wild population of an Old World atherinid, the cobaltcap silverside *Hypoatherina tsurugae*. *Molecular Ecology*, *29*(13), 2349-2358.

Momigliano, P., Jokinen, H., Fraimout, A., Florin, A. B., Norkko, A., & Merilä, J. (2017). Extraordinarily rapid speciation in a marine fish. *Proceedings of the National Academy of Sciences*, *114*, 6074-6079.

Myosho, T., Otake, H., Masuyama, H., Matsuda, M., Kuroki, Y., Fujiyama, A., Naruse, K., Hamaguchi, S., & Sakaizumi, M. (2012). Tracing the Emergence of a Novel Sex-Determining Gene in Medaka, Oryzias luzonensis. *Genetics*, *191*(1), 163–170.

Nakamura, M. (2009). Sex determination in amphibians. *Seminars in Cell & Developmental Biology*, *20*(3), 271–282.

Nanda I, Schlegelmilch K, Haaf T, Schartl M, Schmid M. (2008). Synteny conservation of the Z chromosome in 14 avian species (11 families) supports a role for Z dosage in avian sex determination. *Cytogenetic and Genome Research 122*, 150-156.

Natri, H. M., Merilä, J., & Shikano, T. (2019). The evolution of sex determination associated with a chromosomal inversion. *Nature communications*, *10*(1), 1-13.

Nemesházi, E., Gál, Z., Ujhegyi, N., Verebélyi, V., Mikó, Z., Üveges, B., Lefler, K.K., Jeffries, D.L., Hoffmann, O.I. and Bókony, V. (2020). Novel genetic sex markers reveal

high frequency of sex reversal in wild populations of the agile frog (*Rana dalmatina*) associated with anthropogenic land use. *Molecular Ecology, 29*(19), 3607-3621.

Ng, J., Clemann, N., Chapple, S. N., & Melville, J. (2014). Phylogeographic evidence links the threatened 'Grampians' Mountain Dragon (*Rankinia diemensis* Grampians) with Tasmanian populations: conservation implications in south-eastern Australia. *Conservation Genetics, 15*, 363-373.

Nguyen, N. T. T., Vincens, P., Roest Crollius, H., & Louis, A. (2018). Genomicus 2018: karyotype evolutionary trees and on-the-fly synteny computing. *Nucleic Acids Research, 46*(D1), D816-D822.

Nishimura, Y., Tomita, Y., Yuno, A., Yoshitake, Y., & Shinohara, M. (2015). Cancer immunotherapy using novel tumor-associated antigenic peptides identified by genome-wide cDNA microarray analyses. *Cancer Science, 106*(5), 505-511.

Norris, D. O., & Jones, R. E. (Eds.). (2012). *Hormones and reproduction in fishes, amphibians, and reptiles*. Springer Science & Business Media.

O'Meally, D., Ezaz, T., Georges, A., Sarre, S. D., & Graves, J. A. M. (2012). Are some chromosomes particularly good at sex? Insights from amniotes. *Chromosome Research*, *20*(1), 7-19.

Ogata, M., Hasegawa, Y., Ohtani, H., Mineyama, M., & Miura, I. (2008). The ZZ/ZW sex-determining mechanism originated twice and independently during evolution of the frog, Rana rugosa. *Heredity*, *100*(1), 92–99.

Ogata, M., Lambert, M., Ezaz, T., & Miura, I. (2018). Reconstruction of female heterogamety from admixture of XX-XY and ZZ-ZW sex-chromosome systems within a frog species. *Molecular Ecology, 27*(20), 4078–4089.

Ogata, M., Lee, J.-Y., Kim, S., Ohtani, H., Sekiya, K., Igarashi, T., Hasegawa, Y., Ichikawa, Y., & Miura, I. (2002). The prototype of sex chromosomes found in Korean populations of Rana rugosa. *Cytogenetic and Genome Research*, *99*(1–4), 185–193.

Ogata, M., Ohtani, H., Igarashi, T., Hasegawa, Y., Ichikawa, Y., & Miura, I. (2003). Change of the heterogametic sex from male to female in the frog. *Genetics*, *164*(2), 613–620.

Oguiura, N., Ferrarezzi, H., & Batistic, R. F. (2009). Cytogenetics and Molecular Data in Snakes: A Phylogenetic Approach. *Cytogenetic and Genome Research*, *127*(2–4), 128–142.

Ohno S. Sex chromosomes and sex-linked genes. Berlin: Springer; 1967.

Ohno, S. (1967). *Sex Chromosomes and Sex-Linked Genes*. Springer Science & Business Media.

Oike, A., Watanabe, K., Min, M.-S., Tojo, K., Kumagai, M., Kimoto, Y., Yamashiro, T., Matsuo, T., Kodama, M., Nakamura, Y., Notsu, M., Tochimoto, T., Fujita, H., Ota, M., Ito, E., Yasumasu, S., & Nakamura, M. (2017). Origin of sex chromosomes in six groups of Rana rugosa frogs inferred from a sex-linked DNA marker. *Journal of Experimental Zoology. Part A, Ecological and Integrative Physiology*, *327*(7), 444–452.

Olmo, E. (2008). Trends in the evolution of reptilian chromosomes. *Integrative and Comparative Biology*, *48*(4), 486-493.

Oostra, V., Saastamoinen, M., Zwaan, B. J., & Wheat, C. W. (2018). Strong phenotypic plasticity limits potential for evolutionary responses to climate change. *Nature communications*, *9*(1), 1-11.

Palaiokostas, C., Bekaert, M., Khan, M. G. Q., Taggart, J. B., Gharbi, K., McAndrew, B. J., & Penman, D. J. (2013). Mapping and Validation of the Major Sex-Determining Region in Nile Tilapia (*Oreochromis niloticus L.*) Using RAD Sequencing. *PLOS One*, *8*(7).

Parma, P., Veyrunes, F., & Pailhoux, E. (2016). Sex Reversal in Non-Human Placental Mammals. *Sexual Development*, *10*(5–6), 326–344.

Parmesan, C. (2006). Ecological and Evolutionary Responses to Recent Climate Change. *Annual Review of Ecology, Evolution, and Systematics*, *37*(1), 637–669.

Pecl, G. T., Araújo, M. B., Bell, J. D., Blanchard, J., Bonebrake, T. C., Chen, I. C., ... & Williams, S. E. (2017). Biodiversity redistribution under climate change: Impacts on ecosystems and human well-being. *Science*, *355* eaai9214

Pen, I., Uller, T., Feldmeyer, B., Harts, A., While, G. M., & Wapstra, E. (2010). Climate-driven population divergence in sex-determining systems. *Nature*, *468*(7322), 436-438.

Pengilley, R. Systematic relationships and ecology of some lygosomine lizards from southeastern Australia. (PhD Thesis, Australian National University, 1972).

Pennell, M. W., Mank, J. E., & Peichel, C. L. (2018). Transitions in sex determination and sex chromosomes across vertebrate species. *Molecular Ecology*, *27*(19), 3950-3963.

Peñuelas, J., Sardans, J., Estiarte, M., Ogaya, R., Carnicer, J., Coll, M., Barbeta, A., Rivas-Ubach, A., Llusià, J., Garbulsky, M., Filella, I., & Jump, A. S. (2013). Evidence of current impact of climate change on life: A walk from genes to the biosphere. *Global Change Biology*, *19*(8), 2303–2338.

Pepper, M., Sumner, J., Brennan, I. G., Hodges, K., Lemmon, A. R., Lemmon, E. M., ... & Keogh, J. S. (2018). Speciation in the mountains and dispersal by rivers: Molecular phylogeny of *Eulamprus* water skinks and the biogeography of Eastern Australia. *Journal of Biogeography*, *45*, 2040-2052.

Perrin, N. (2009). Sex Reversal: A Fountain of Youth for Sex Chromosomes? *Evolution*, *63*(12), 3043–3049.

Peterson, B. K., Weber, J. N., Kay, E. H., Fisher, H. S., & Hoekstra, H. E. (2012). Double digest RADseq: an inexpensive method for de novo SNP discovery and genotyping in model and non-model species. *PLOS One*, *7*(5), e37135.

Pettersson, I., & Berg, C. (2007). Environmentally relevant concentrations of ethynylestradiol cause female-biased sex ratios in *Xenopus tropicalis* and *Rana temporaria*. *Environmental Toxicology and Chemistry*, *26*(5), 1005–1009.

Pickrell, J., & Pritchard, J. (2012). Inference of population splits and mixtures from genome-wide allele frequency data. *PLOS Genetics*, *8*(11), e1002967.

Pieau, C. (1975). Temperature and Sex Differentiation in Embryos of Two Chelonians, Emys orbicularis L and Testudo graeca L. In R. Reinboth (Ed.), *Intersexuality in the Animal Kingdom* (pp. 332–339). Springer.

Pieau, C., & Mrosovsky, N. (1991). Transitional range of temperature, pivotal temperatures and thermosensitive stages for sex determination in reptiles. *Amphibia-Reptilia*, *12*(2), 169–179.

Piferrer, F., & Anastasiadi, D. (2021). Do the offspring of sex reversals have higher sensitivity to environmental perturbations? *Sexual Development*, 1–14.

Pokorna, M., & Kratochvíl, L. (2009). Phylogeny of sex-determining mechanisms in squamate reptiles: are sex chromosomes an evolutionary trap? *Zoological Journal of the Linnean Society*, *156*(1), 168-183.

Quinn, A. E., Georges, A., Sarre, S. D., Guarino, F., Ezaz, T., & Graves, J. A. M. (2007). Temperature sex reversal implies sex gene dosage in a reptile. *Science*, *316*(5823), 411.

Quinn, A. E., Radder, R. S., Sarre, S. D., Georges, A., Ezaz, T., & Shine, R. (2009). Isolation and development of a molecular sex marker for *Bassiana duperreyi*, a lizard with XX/XY sex chromosomes and temperature-induced sex reversal. *Molecular Genetics and Genomics*, *281*(6), 665–672.

Quinn, A. E., Sarre, S. D., Ezaz, T., Marshall Graves, J. A., & Georges, A. (2011). Evolutionary transitions between mechanisms of sex determination in vertebrates. *Biology Letters*, *7*(3), 443–448.

Radder, R. S., & Shine, R. (2007). Sex-based hatching asynchrony in an oviparous lizard (*Bassiana duperreyi*, Scincidae). *Austral Ecology*, *32*(5), 502-508.

Radder, R. S., Quinn, A. E., Georges, A., Sarre, S. D., & Shine, R. (2008). Genetic evidence for co-occurrence of chromosomal and thermal sex-determining systems in a lizard. *Biology Letters*, *4*(2), 176-178.

Rodrigues, N., Merilä, J., Patrelle, C., & Perrin, N. (2014). Geographic variation in sex-chromosome differentiation in the common frog (*Rana temporaria*). *Molecular Ecology*, *23*(14), 3409-3418.

Roll, U., Feldman, A., Novosolov, M., Allison, A., Bauer, A. M., Bernard, R., ... & Meiri, S. (2017). The global distribution of tetrapods reveals a need for targeted reptile conservation. *Nature Ecology & Evolution*, *1*, 1677-1682.

Rosauer, D. F., Catullo, R. A., VanDerWal, J., Moussalli, A., & Moritz, C. (2015). Lineage range estimation method reveals fine-scale endemism linked to Pleistocene stability in Australian rainforest herpetofauna. *PLOS One*, *10*(5), e0126274.

Ross, M.T., Grafham, D.V., Coffey, A.J., Scherer, S., McLay, K., Muzny, D., Platzer, M., Howell, G.R., Burrows, C., Bird, C.P. and Frankish, A., (2005). The DNA sequence of the human X chromosome. *Nature*, *434*(7031), 325.

Rovatsos, M., & Kratochvíl, L. (2017). Molecular sexing applicable in 4000 species of lizards and snakes? From dream to real possibility. *Methods in Ecology and Evolution*, *8*(8), 902–906.

Rovatsos, M., Vukić, J., Lymberakis, P., & Kratochvíl, L. (2015). Evolutionary stability of sex chromosomes in snakes. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1821), 20151992.

Ruiz-García, A., Roco, Á. S., & Bullejos, M. (2021). Sex differentiation in amphibians: effect of temperature and its influence on sex reversal. *Sexual Development*, 1–11.

Saha, A., McRae, L., Dodd Jr, C. K., Gadsden, H., Hare, K. M., Lukoschek, V., & Böhm, M. (2018). Tracking global population trends: Population time-series data and a living planet index for reptiles. *Journal of Herpetology*, *52*(3), 259-268.

Sakharkar, K. R., Sakharkar, M. K., & Chow, V. T. (2004). A novel genomics approach for the identification of drug targets in pathogens, with special reference to Pseudomonas aeruginosa. *In Silico Biology*, *4*(3), 355-360.

Sánchez-Bayo, F., & Wyckhuys, K. A. G. (2019). Worldwide decline of the entomofauna: A review of its drivers. *Biological Conservation*, *232*, 8–27.

Sarre, S. D., Ezaz, T., & Georges, A. (2011). Transitions between sex-determining systems in reptiles and amphibians. *Annual Review of Genomics and Human Genetics*, *12*(1), 391–406.

Sarre, S. D., Georges, A., & Quinn, A. (2004). The ends of a continuum: Genetic and temperature-dependent sex determination in reptiles. *Bio Essays: News and Reviews in Molecular, Cellular and Developmental Biology*, *26*(6), 639–645.

Schäuble, C. S., & Moritz, C. (2001). Comparative phylogeography of two open forest frogs from eastern Australia. *Biological Journal of the Linnean Society*, *74*, 157-170.

Scheffer, M., Barrett, S., Carpenter, S.R., Folke, C., Green, A.J., Holmgren, M., Hughes, T.P., Kosten, S., Van de Leemput, I.A., Nepstad, D.C. and van Nes, E.H. (2015). Creating a safe operating space for iconic ecosystems. *Science*, *347*(6228), 1317-1319.

Schmid, M., & Steinlein, C. (2001). Sex chromosomes, sex-linked genes, and sex determination in the vertebrate class Amphibia. In G. Scherer & M. Schmid (Eds.), *Genes and Mechanisms in Vertebrate Sex Determination* (pp. 143–176). Birkhäuser.

Schodde, R., & Mason, I. J. (1999). *The Directory of Australian Birds: Passerines.* CSIRO Publishing.

Schroeder, A. L., Metzger, K. J., Miller, A., & Rhen, T. (2016). A novel candidate gene for temperature-dependent sex determination in the common snapping turtle. *Genetics*, *203*(1), 557-571.

Schwanz, L. E., & Georges, A. (2021). Sexual development and the environment: conclusions from 40 years of theory. *Sexual Development,* 1-16. Online early view: https://doi.org/10.1159/000515221

Schwanz, L. E., Georges, A., Holleley, C. E., & Sarre, S. D. (2020). Climate change, sex reversal and lability of sex-determining systems. *Journal of Evolutionary Biology*, *33*(3), 270–281.

Servan, J., Zaborski, P., Dorizzi, M., & Pieau, C. (1989). Female-biased sex ratio in adults of the turtle *Emys orbicularis* at the northern limit of its distribution in France: a probable consequence of interaction of temperature with genotypic sex determination. *Canadian Journal of Zoology*, *67*(5), 1279-1284.

Seufert, W., & Jentsch, S. (1990). Ubiquitin-conjugating enzymes UBC4 and UBC5 mediate selective degradation of short-lived and abnormal proteins. *The EMBO Journal*, *9*(2), 543-550.

Shapiro, D. Y. (1981). Size, maturation and the social control of sex reversal in the coral reef fish *Anthais squamipinnis*. *Journal of Zoology*, *193*(1), 105-128.

Shi, X., Waiho, K., Li, X., Ikhwanuddin, M., Miao, G., Lin, F., Zhang, Y., Li, S., Zheng, H., Liu, W. and Aweya, J.J. (2018). Female-specific SNP markers provide insights into a WZ/ZZ sex determination system for mud crabs *Scylla paramamosain*, *S.*

tranquebarica and *S. serrata* with a rapid method for genetic sex identification. *BMC Genomics*, *19*(1), 981.

Shine, R. (1995). A new hypothesis for the evolution of viviparity in reptiles. *The American Naturalist*, *145*(5), 809-823.

Shine, R. (1999). Why is sex determined by nest temperature in many reptiles? *Trends in Ecology & Evolution*, *14*(5), 186-189.

Shine, R. (1999b). Egg-laying reptiles in cold climates: determinants and consequences of nest temperatures in montane lizards. *Journal of evolutionary biology*, *12*(5), 918-926.

Shine, R. (2004). Seasonal shifts in nest temperature can modify the phenotypes of hatchling lizards, regardless of overall mean incubation temperature. *Functional Ecology*, 43-49.

Shine, R., & Harlow, P. S. (1996). Maternal Manipulation of Offspring Phenotypes via Nest-Site Selection in an Oviparous Lizard. *Ecology*, *77*(6), 1808–1817.

Shine, R., Elphick, M. J., & Barrott, E. G. (2003). Sunny side up: lethally high, not low, nest temperatures may prevent oviparous reptiles from reproducing at high elevations. *Biological Journal of the Linnean Society*, *78*(3), 325-334.

Shine, R., Elphick, M. J., & Donnellan, S. (2002). Co-occurrence of multiple, supposedly incompatible modes of sex determination in a lizard population. *Ecology Letters*, *5*(4), 486–489.

Shine, R., Elphick, M. J., & Harlow, P. S. (1997). The influence of natural incubation environments on the phenotypic traits of hatchling lizards. *Ecology*, *78*(8), 2559-2568.

Sinclair, A. H., Berta, P., Palmer, M. S., Hawkins, J. R., Griffiths, B. L., Smith, M. J., Foster, J. W., Frischauf, A.-M., Lovell-Badge, R., & Goodfellow, P. N. (1990). A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature*, *346*(6281), 240–244.

Skaletsky, H., Kuroda-Kawaguchi, T., Minx, P.J., Cordum, H.S., Hillier, L., Brown, L.G., Repping, S., Pyntikova, T., Ali, J., Bieri, T. and Chinwalla, A., (2003). The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature*, *423*(6942), 825-837.

Smissen, P. J., Melville, J., Sumner, J., & Jessop, T. S. (2013). Mountain barriers and river conduits: phylogeographical structure in a large, mobile lizard (Varanidae: *Varanus varius*) from eastern Australia. *Journal of Biogeography*, *40*, 1729-1740.

Smith, C. A., Katz, M., & Sinclair, A. H. (2003). *DMRT1* is upregulated in the gonads during female-to-male sex reversal in ZW chicken embryos. *Biology of Reproduction*, *68*(2), 560–570.

Smith, C. A., Roeszler, K. N., Hudson, Q. J., & Sinclair, A. H. (2007). Avian sex determination: what, when and where? *Cytogenetic and Genome Research*, *117*(1-4), 165-173.

Sopniewski, J., Shams, F., Scheele, B. C., Kefford, B. J., & Ezaz, T. (2019). Identifying sex-linked markers in Litoria aurea: a novel approach to understanding sex chromosome evolution in an amphibian. *Scientific Reports*, *9*(1), 1-10.

Stephenson, A. E. (1986). Lake Bungunnia—a plio-pleistocene megalake in southern Australia. *Palaeogeography, Palaeoclimatology, Palaeoecology*, *57*, 137-156.

Stevens, N. M. (1905). *Studies in Spermatogenesis.* (Vol. 36). Carnegie institution of Washington.

Sukumaran, J., & Knowles, L. L. (2017). Multispecies coalescent delimits structure, not species. *Proceedings of the National Academy of Sciences, USA*, *114*(7), 1607–1612.

Sulandart, S. R. I., & Zein, M. S. A. (2012). Application of two molecular sexing methods for Indonesian bird species: implication for captive breeding programs in Indonesia. *HAYATI Journal of Biosciences*, *19*(4), 183-190.

Swofford, D. L. (2002). PAUP*: phylogenetic analysis using parsimony. v. 4.0 b10. *Sinauer, Sunderland, MA*.

Symula, R., Keogh, J. S., & Cannatella, D. C. (2008). Ancient phylogeographic divergence in southeastern Australia among populations of the widespread common froglet, *Crinia signifera*. *Molecular Phylogenetics and Evolution*, *47*, 569-580.

Taberlet, P., Waits, L. P., & Luikart, G. (1999). Noninvasive genetic sampling: look before you leap. *Trends in ecology & evolution*, *14*(8), 323-327.

Tamschick, S., Rozenblut-Kościsty, B., Ogielska, M., Kekenj, D., Gajewski, F., Krüger, A., Kloas, W., & Stöck, M. (2016). The plasticizer bisphenol a affects somatic and sexual development, but differently in pipid, hylid and bufonid anurans. *Environmental Pollution* (*Barking, Essex: 1987*), *216*, 282–291.

Telemeco, R. S., Elphick, M. J., & Shine, R. (2009). Nesting lizards (*Bassiana duperreyi*) compensate partly, but not completely, for climate change. *Ecology*, *90*(1), 17–22.

Telemeco, R. S., Radder, R. S., Baird, T. A., & Shine, R. (2010). Thermal effects on reptile reproduction: adaptation and phenotypic plasticity in a montane lizard. *Biological Journal of the Linnean Society*, *100*(3), 642-655.

Tingley, R., Meiri, S., & Chapple, D. G. (2016). Addressing knowledge gaps in reptile conservation. *Biological Conservation, 204*, 1-5.

Todd, E. V., Liu, H., Muncaster, S., & Gemmell, N. J. (2016). Bending genders: the biology of natural sex change in fish. *Sexual Development*, *10*(5-6), 223-241.

Tokarska, K. B., Stolpe, M. B., Sippel, S., Fischer, E. M., Smith, C. J., Lehner, F., & Knutti, R. (2020). Past warming trend constrains future warming in CMIP6 models. *Science Advances*, *6*(12), eaaz9549.

Tokunaga, S. (1985). Temperature dependent sex determination in *Gekko japonicus* (Gekkonidae, Reptilia). *Development, Growth & Differentiation*, *27*(2), 117–120.

Traut, W., Eickhoff, U., & Schorch, J. C. (2001). Identification and analysis of sex chromosomes by comparative genomic hybridization (CGH). In Chromosome Painting (pp. 155-161). Springer, Dordrecht.

Tree of Sex Consortium. (2014). Tree of Sex: A database of sexual systems. *Scientific Data, 1*, 140015.

Uetz, P., Freed, P, Aguilar, R. & Hošek, J. (eds.) (2021). The Reptile Database, http://www.reptile-database.org, accessed (27/06/2021).

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., & Rozen, S. G. (2012). Primer3—new capabilities and interfaces. *Nucleic acids research*, *40*(15), e115-e115.

Valenzuela, N. (2021). Podocnemis expansa Turtles Hint to a Unifying Explanation for the Evolution of Temperature-Dependent Sex Determination in Long-Lived and Short-Lived Vertebrates. *Sexual Development*, 1–15.

Valenzuela, N., & Lance, V. (Eds.). (2004). *Temperature-dependent sex determination in vertebrates*. Smithsonian Books.

Valenzuela, N., Literman, R., Neuwald, J. L., Mizoguchi, B., Iverson, J. B., Riley, J. L., & Litzgus, J. D. (2019). Extreme thermal fluctuations from climate change unexpectedly accelerate demographic collapse of vertebrates with temperature-dependent sex determination. *Scientific Reports*, *9*(1), 4254.

Vicoso, B., Emerson, J. J., Zektser, Y., Mahajan, S., & Bachtrog, D. (2013). Comparative sex chromosome genomics in snakes: differentiation, evolutionary strata, and lack of global dosage compensation. *PLOS Biology*, *11*(8), e1001643.

Viets, B. E., Ewert, M. A., Talent, L. G., & Nelson, C. E. (1994). Sex-determining mechanisms in squamate reptiles. *Journal of Experimental Zoology*, *270*(1), 45–56.

Wallace, H. U. G. H., & Wallace, B. M. (2004). Sex reversal of the newt *Triturus cristatus* reared at extreme temperatures. *International Journal of Developmental Biology*, *44*(7), 807-810.

Wallace, H., Badawy, G. M., & Wallace, B. M. (1999). Amphibian sex determination and sex reversal. *Cellular and Molecular Life Sciences: CMLS*, *55*(6–7), 901–909.

Wallis, M. C., Waters, P. D., Delbridge, M. L., Kirby, P. J., Pask, A. J., Grützner, F., Rens, W., Ferguson-Smith, M. A., & Graves, J. A. M. (2008). Sex determination in platypus and echidna: Autosomal location of SOX3 confirms the absence of *SRY* from monotremes. *Chromosome Research*, *15*(8), 949.

Wang, C., Tang, X., Xin, Y., Yue, F., Yan, X., Liu, B., An, B., Wang, X., & Chen, Q. (2015). Identification of sex chromosomes by means of comparative genomic hybridization in a lizard, *Eremias multiocellata*. *Zoological Science*, *32*(2), 151–156.

Wang, Q., Liu, K., Feng, B., Zhang, Z., Wang, R., Tang, L., ... & Shao, C. (2019). Transcriptome of gonads from high temperature induced sex reversal during sex

determination and differentiation in Chinese tongue sole, *Cynoglossus semilaevis*. *Frontiers in Genetics*, *10*, 1128.

Ward, M., Tulloch, A. I., Radford, J. Q., Williams, B. A., Reside, A. E., Macdonald, S. L., ... & Watson, J. E. (2020). Impact of 2019–2020 mega-fires on Australian fauna habitat. *Nature Ecology and Evolution*, *4*, 1321-1326.

Warner, D. A., & Shine, R. (2005). The adaptive significance of temperature-dependent sex determination: experimental tests with a short-lived lizard. *Evolution*, *59*(10), 2209–2221.

Warner, D. A., & Shine, R. (2008). The adaptive significance of temperature-dependent sex determination in a reptile. *Nature*, *451*(7178), 566–568.

Warner, D. A., Du, W.-G., & Georges, A. (2018). Introduction to the special issue—Developmental plasticity in reptiles: Physiological mechanisms and ecological consequences. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, *329*(4–5), 153–161.

Wedekind, C. (2012). Managing population sex ratios in conservation practice: How and Why? In *Topics in Conservation Biology*. IntechOpen.

Welsh, J., & McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research*, *18*(24), 7213-7218.

Wheeler, T.J., Clements, J., Eddy, S.R., Hubley, R., Jones, T.A., Jurka, J., Smit, A.F. and Finn, R.D (2012). Dfam: a database of repetitive DNA based on profile hidden Markov models. *Nucleic Acids Research*, *41*(D1), D70-D82.

Whiteley, S. L., Georges, A., Weisbecker, V., Schwanz, L. E., & Holleley, C. E. (2021). Ovotestes suggest cryptic genetic influence in a reptile model for temperature-dependent sex determination. *Proceedings of the Royal Society B: Biological Sciences*, *288*(1943), 20202819.

Whiteley, S. L., Holleley, C. E., Wagner, S., Blackburn, J., Deveson, I. W., Graves, J. A. M., & Georges, A. (2021). Two transcriptionally distinct pathways drive female development in a reptile with both genetic and temperature dependent sex determination. *PLOS Genetics*, *17*(4), e1009465.

Whiteley, S. L., Weisbecker, V., Georges, A., Gauthier, A. R. G., Whitehead, D. L., & Holleley, C. E. (2018). Developmental asynchrony and antagonism of sex determination pathways in a lizard with temperature-induced sex reversal. *Scientific Reports*, *8*(1), 1-9.

Whiteley, S.L., Holleley, C.E., Ruscoe, W.A., Castelli, M., Whitehead, D.L., Lei, J., Georges, A. and Weisbecker, V. (2017). Sex determination mode does not affect body or genital development of the central bearded dragon (*Pogona vitticeps*). *EvoDevo*, *8*(1), 1-15.

Whitfield, S. M., Bell, K. E., Philippi, T., Sasa, M., Bolaños, F., Chaves, G., ... & Donnelly, M. A. (2007). Amphibian and reptile declines over 35 years at La Selva, Costa Rica. *Proceedings of the National Academy of Sciences*, *104*(20), 8352-8356.

Wiggins, J. M., Santoyo-Brito, E., Scales, J. B., & Fox, S. F. (2020). Gene Dose Indicates Presence of Sex Chromosomes in Collared Lizards (*Crotaphytus collaris*), a Species with Temperature-Influenced Sex Determination. *Herpetologica*, *76*(1), 27–30.

Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A., & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, *18*(22), 6531-6535.

Williams, K. J., Ford, A., Rosauer, D. F., De Silva, N., Mittermeier, R., Bruce, C., ... & Margules, C. (2011). Forests of East Australia: the 35th biodiversity hotspot. In *Biodiversity Hotspots* (pp. 295-310). Springer, Berlin, Heidelberg.

Wing, S. S., Bedard, N., Morales, C., Hingamp, P., & Trasler, J. (1996). A novel rat homolog of the Saccharomyces cerevisiae ubiquitin-conjugating enzymes UBC4 and UBC5 with distinct biochemical features is induced during spermatogenesis. *Molecular and Cellular Biology*, *16*(8), 4064-4072.

Witschi, E. (1930). Studies on sex differentiation and sex determination in amphibians. IV. The geographical distribution of the sex races of the European grass frog (*Rana temporaria*, L.). A contribution to the problem of the evolution of sex. *Journal of Experimental Zoology*, *56*(2), 149–165.

Wright, A. E., Dean, R., Zimmer, F., & Mank, J. E. (2016). How to make a sex chromosome. *Nature communications*, *7*(1), 1-8.

Yamamoto, T. O. (1975). A YY male goldfish from mating estrone-induced XY female and normal male. *The Journal of Heredity*, *66*(1), 2–4.

Yano, A., Nicol, B., Jouanno, E., Quillet, E., Fostier, A., Guyomard, R., & Guiguen, Y. (2013). The sexually dimorphic on the Y-chromosome gene (sdY) is a conserved male-specific Y-chromosome sequence in many salmonids. *Evolutionary Applications*, *6*(3), 486–496.

Yntema, C. L. (1968). A series of stages in the embryonic development of Chelydra serpentina. *Journal of Morphology*, *125*(2), 219–251.

Yntema, C. L. (1979). Temperature levels and periods of sex determination during incubation of eggs of Chelydra serpentina. *Journal of Morphology*, *159*(1), 17–27.

Yokota, N., Harada, Y., & Sawada, H. (2010). Identification of testis-specific ubiquitin-conjugating enzyme in the ascidian *Ciona intestinalis*. *Molecular Reproduction and Development*, *77*(7), 640-647.

Yoshimoto, S., Okada, E., Umemoto, H., Tamura, K., Uno, Y., Nishida-Umehara, C., Matsuda, Y., Takamatsu, N., Shiba, T., & Ito, M. (2008). A W-linked DM-domain gene, DM-W, participates in primary ovary development in Xenopus laevis. *Proceedings of the National Academy of Sciences*, *105*(7), 2469–2474.

Young, J. E., Georges, A., Doody, J. S., West, P. B., & Alderman, R. L. (2004). Pivotal range and thermosensitive period of the pig-nosed turtle, *Carettochelys insculpta* (Testudines: Carettochelydidae), from northern Australia. *Canadian Journal of Zoology*, *82*(8), 1251-1257.

Zaborski, P., Dorizzi, M., & Pieau, C. (1982). H-Y antigen expression in temperature sex-reversed turtles (*Emys orbicularis*). *Differentiation; Research in Biological Diversity*, *22*(2), 73–78.

Zhang, L., Ma, X., Jiang, J., & Lu, X. (2012). Stronger condition dependence in female size explains altitudinal variation in sexual size dimorphism of a Tibetan frog. *Biological Journal of the Linnean Society*, *107*(3), 558-565.

Zheng, S., Wang, X., Zhang, S., Long, J., Tao, W., Li, M., & Wang, D. (2020). Screening and characterization of sex-linked DNA markers and marker-assisted selection in the southern catfish (*Silurus meridionalis*). *Aquaculture, 517*, 734783.

Appendices

Appendix 1

Publications to which I contributed during my candidature, but which do not form part of the examinable material.

1. Whiteley, S. L., Castelli, M. A., **Dissanayake, D. S.B.,** Holleley, C. E. & Georges, A. (2021). Temperature-induced sex reversal in reptiles: prevalence, discovery, and evolutionary implications. Sexual Development. online early view: https://doi.org/10.1159/000515687

2. *Cornejo-Páramo, P., ***Dissanayake, D. S. B**., *****Lira-Noriega, A., *****Martínez-Pacheco, *M.L., Acosta, A., Ramírez-Suástegui, C., Méndez-de-la-Cruz, F.R., Székely, T., Urrutia, A.O., Georges, A. and Cortez, D., (2020). Viviparous reptile regarded to have temperature-dependent sex determination has old XY chromosomes. Genome biology and evolution, 12:924-930. https://doi.org/10.1093/gbe/evaa104.

   Supplementary Data: https://academic.oup.com/gbe/article/12/6/924/5841216?login=true#supplementary-data

3. ***Dissanayake, D. S. B**., *Streeting, L., Georges, A. & Bower, D. (2021). Development of a male-specific sex marker for the endangered western saw-shelled turtle (*Myuchelys bellii*) using in silico whole-genome subtraction. (Manuscript is accepted with minor revision Conservation Genetic Resources).

*Contributed equally to the study.

Appendix 2

Supplementary Materials for Chapter 2

Table S1. Specimen data, sex, locality, and measurements for the *Bassiana duperreyi* specimens used in this study. Specimen numbers refer to the University of Canberra Wildlife Tissue Collection (GenBank UC <Aus>).

Field No	Specimen No.	Sex	Sex Basis	Maturity	Population	Latitude	Longitude	SVL (mm)	Specimen status
65DM	AA064225	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.35775	148.8051444	64	Focal animal
64DM	AA064226	F	Hemipenal extrusion (ovary)	Adult	Piccadilly Circus	-35.35775	148.8051444	71	Focal animal
DDBD_3	AA064426	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36165833	148.8034583	59	Validation animal
DDBD_4	AA064427	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36165833	148.8034583	70	Validation animal
DDBD_5	AA064428	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36165833	148.8034583	59	Validation animal
DDBD_7	AA064430	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36165833	148.8034583	64	Validation animal
DDBD_8	AA064431	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36165833	148.8034583	67	Validation animal
DDBD_9	AA064432	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36165833	148.8034583	64	Validation animal
DDBD_12	AA064435	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36165833	148.8034583	64	Validation animal
DDBD_13	AA064436	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.35775	148.8051444	74	Validation animal
DDBD_14	AA064437	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.35775	148.8051444	60	Validation animal
DDBD_16	AA064439	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.35775	148.8051444	64	Validation animal
DDBD_18	AA064441	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.35775	148.8051444	73	Validation animal
DDBD_19	AA064442	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.35775	148.8051444	74	Validation animal

DDBD_21	AA064444	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.35775	148.8051444	77	Validation animal
DDBD_22	AA064445	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.35775	148.8051444	66	Validation animal
DDBD_26	AA064449	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36165833	148.8034583	73	Validation animal
DDBD_28	AA064451	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36240556	148.8023111	61	Validation animal
DDBD_29	AA064047	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36240556	148.8023111	63	Validation animal
DDBD_31	AA064049	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36240556	148.8023111	71	Validation animal
DDBD_32	AA064050	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36240556	148.8023111	70	Validation animal
DDBD_33	AA064051	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36240556	148.8023111	65	Validation animal
DDBD_42	AA064060	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36240556	148.8023111	64	Validation animal
DDBD_43	AA064061	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36240556	148.8023111	62	Validation animal
DDBD_44	AA064062	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36240556	148.8023111	69	Validation animal
DDBD_45	AA064063	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36240556	148.8023111	59	Validation animal
DDBD_10	AA064433	F	Hemipenal extrusion (Gravid)	Adult	Piccadilly Circus	-35.35775	148.8051444	60	Validation animal
DDBD_15	AA064438	F	Hemipenal extrusion (Gravid)	Adult	Piccadilly Circus	-35.35775	148.8051444	72	Validation animal
DDBD_17	AA064440	F	Hemipenal extrusion	Adult	Piccadilly Circus	-35.35775	148.8051444	58	Validation animal
DDBD_20	AA064443	F	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36165833	148.8034583	66	Validation animal
DDBD_23	AA064446	F	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36240556	148.8023111	69	Validation animal
DDBD_24	AA064447	F	Hemipenal extrusion (Gravid)	Adult	Piccadilly Circus	-35.36240556	148.8023111	66	Validation animal
DDBD_25	AA064448	F	Hemipenal extrusion (Gravid)	Adult	Piccadilly Circus	-35.36240556	148.8023111	66	Validation animal

DDBD_27	AA064450	F	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36240556	148.8023111	76	Validation animal
DDBD_30	AA064048	F	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36240556	148.8023111	62	Validation animal
DDBD_35	AA064053	F	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36240556	148.8023111	66	Validation animal
DDBD_36	AA064054	F	Hemipenal extrusion (Gravid)	Adult	Piccadilly Circus	-35.36240556	148.8023111	75	Validation animal
DDBD_39	AA064057	F	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36240556	148.8023111	75	Validation animal
DDBD_40	AA064058	F	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36240556	148.8023111	79	Validation animal
DDBD_41	AA064059	F	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36240556	148.8023111	68	Validation animal
DDBD_47	AA064065	F	Hemipenal extrusion	Adult	Piccadilly Circus	-35.35775	148.8051444	75	Validation animal
DDBD_56	AA064074	F	Hemipenal extrusion (Gravid)	Adult	Piccadilly Circus	-35.36050833	148.8005528	63	Validation animal
DDBD_57	AA064075	F	Hemipenal extrusion (Gravid)	Adult	Piccadilly Circus	-35.36050833	148.8005528	67	Validation animal
DDBD_59	AA064077	F	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36050833	148.8005528	73	Validation animal
DDBD_60	AA064078	F	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36050833	148.8005528	72	Validation animal
DDBD_62	AA064080	F	Hemipenal extrusion (Gravid)	Adult	Piccadilly Circus	-35.36050833	148.8005528	66	Validation animal
DDBD_100	AA064118	F	Hemipenal extrusion (Gravid)	Adult	Piccadilly Circus	-35.36050833	148.8005528	71	Validation animal
DDBD_287	AA094000	F	Hemipenal extrusion (Gravid)	Adult	Piccadilly Circus	-35.36050833	148.8005528	73	Validation animal
DDBD_288	AA094001	F	Hemipenal extrusion (Gravid)	Adult	Piccadilly Circus	-35.36050833	148.8005528	69	Validation animal
DDBD_289	AA094002	F	Hemipenal extrusion (Gravid)	Adult	Piccadilly Circus	-35.36050833	148.8005528	67	Validation animal
DDBD_111	AA064130	M	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	59	Validation animal
DDBD_115	AA084795	M	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	62	Validation animal
DDBD_116	AA084796	M	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	66	Validation animal
DDBD_117	AA084798	M	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	73	Validation animal

DDBD_120	AA084805	M	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	67	Validation animal
DDBD_121	AA084806	M	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	67	Validation animal
DDBD_122	AA084808	M	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	58	Validation animal
DDBD_125	AA084813	M	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	60	Validation animal
DDBD_126	AA084815	M	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	68	Validation animal
DDBD_129	AA084373	M	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	59	Validation animal
DDBD_110	AA064126	F	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	63	Validation animal
DDBD_112	AA064135	F	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	62	Validation animal
DDBD_113	AA064134	F	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	70	Validation animal
DDBD_114	AA084791	F	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	66	Validation animal
DDBD_118	AA084800	F	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	61	Validation animal
DDBD_119	AA084802	F	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	62	Validation animal
DDBD_123	AA084810	F	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	74	Validation animal
DDBD_124	AA084811	F	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	73	Validation animal
DDBD_128	AA084370	F	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	75	Validation animal
DDBD_130	AA084374	F	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	70	Validation animal
DDBD_49	AA064067	M	Hemipenal extrusion	Adult	Piccadilly Circus	-35.36050833	148.8005528	65	Validation animal
DDBD_50	AA064068	M	Hemipenal extrusion	Adult	Piccadilly Circus	35.36050833	148.8005528	69	Validation animal
DDBD_58	AA064076	M	Hemipenal extrusion	Adult	Piccadilly Circus	35.36050833	148.8005528	71	Validation animal
DDBD_364	AA094425	M	Hemipenal extrusion	Adult	Piccadilly Circus	35.36050833	148.8005528	64	Validation animal
DDBD_G	AA094426	M	Hemipenal extrusion	Adult	Piccadilly Circus	35.36050833	148.8005528	62	Validation animal
DDBD_165	AA080794	M	Hemipenal extrusion	Adult	Anglesea	-38.39111111	144.215	58	Validation animal

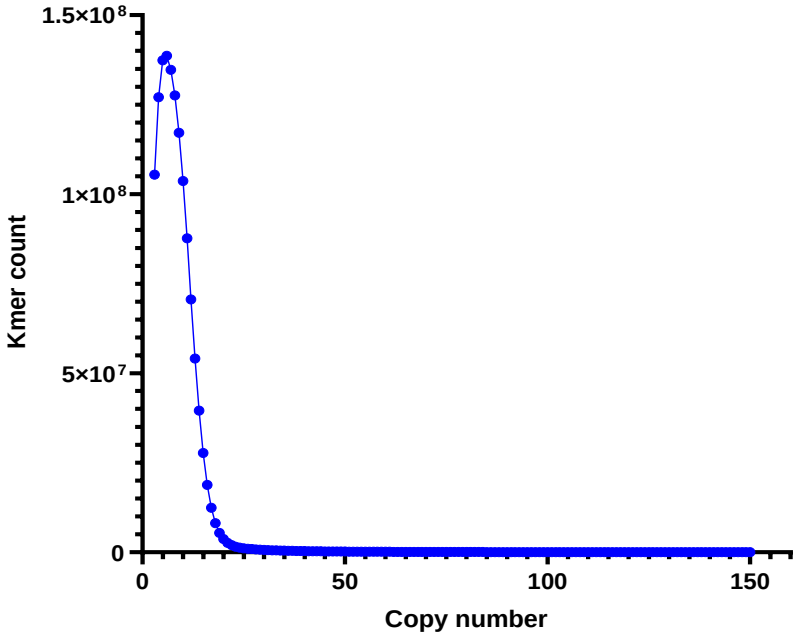

Figure S1. K-mer spectrum for the genome sequence of a male *Bassiana duperreyi*. The Illumina run yielded 96.7 Gb of clean data, which generated 14,310,783,435 K-mer sequences.

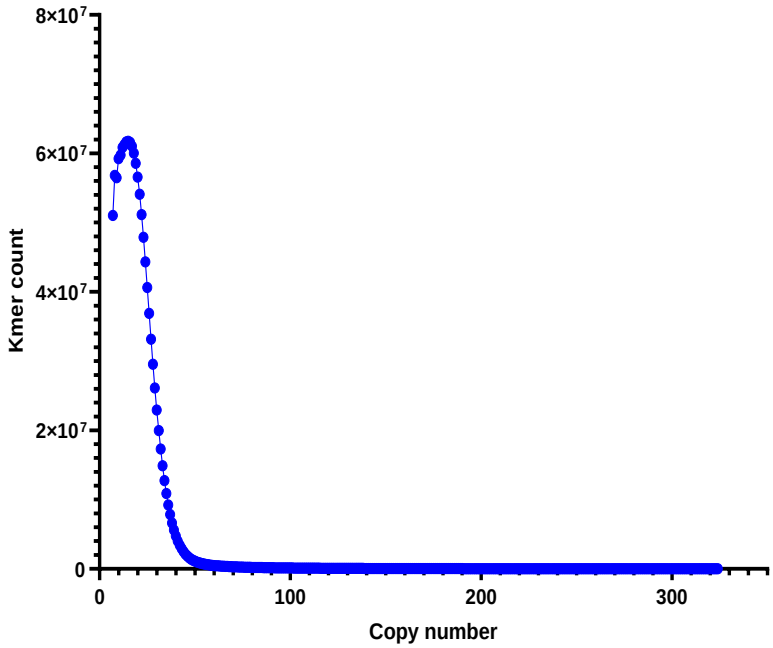

Figure S2. K-mer spectrum for the genome sequence of a female *Bassiana duperreyi*. The Illumina run yielded 81.41 Gb of clean data, which generated 36,695,139,446 k-mer sequences.

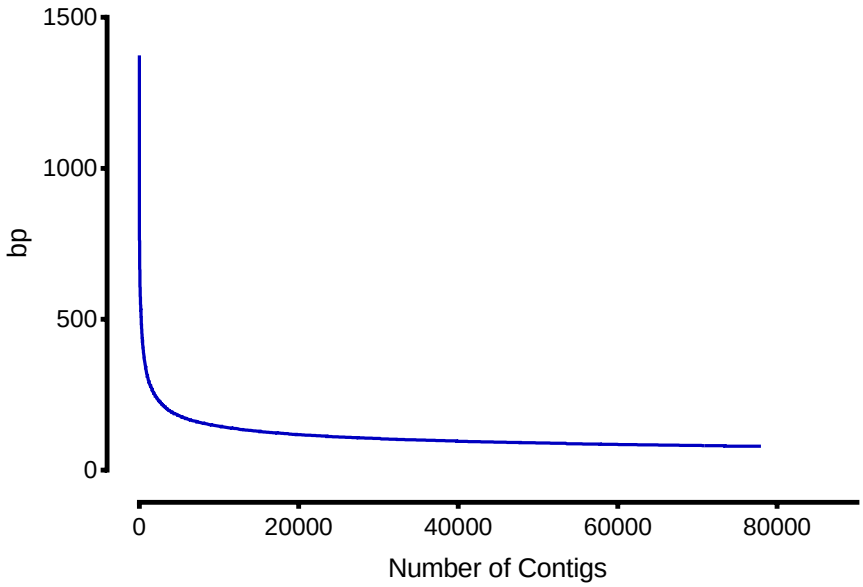

Figure S3. Number of Y enriched contigs ranging from 80 bp to 1374 bp resulting from the inchworm assembler.

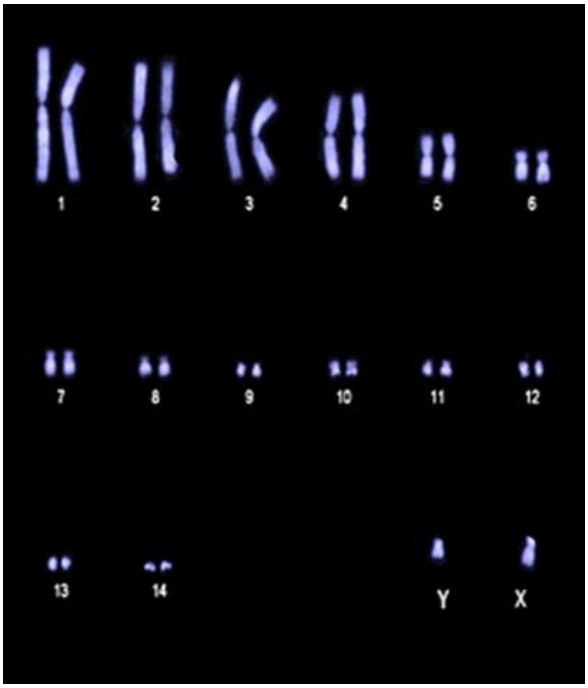

Figure S4. External and histological views of a) ovary b) testis in adult individuals of *Bassiana duperreyi*.

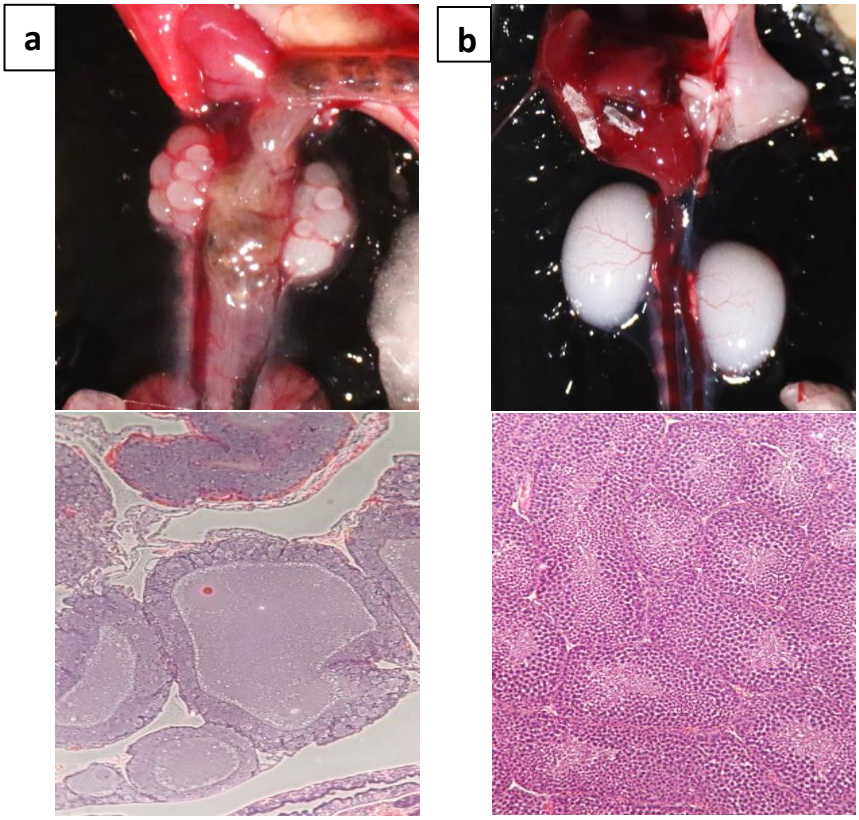

Figure S5. External and histological views of a) ovary b) testis in adult individuals of *Bassiana duperreyi*.

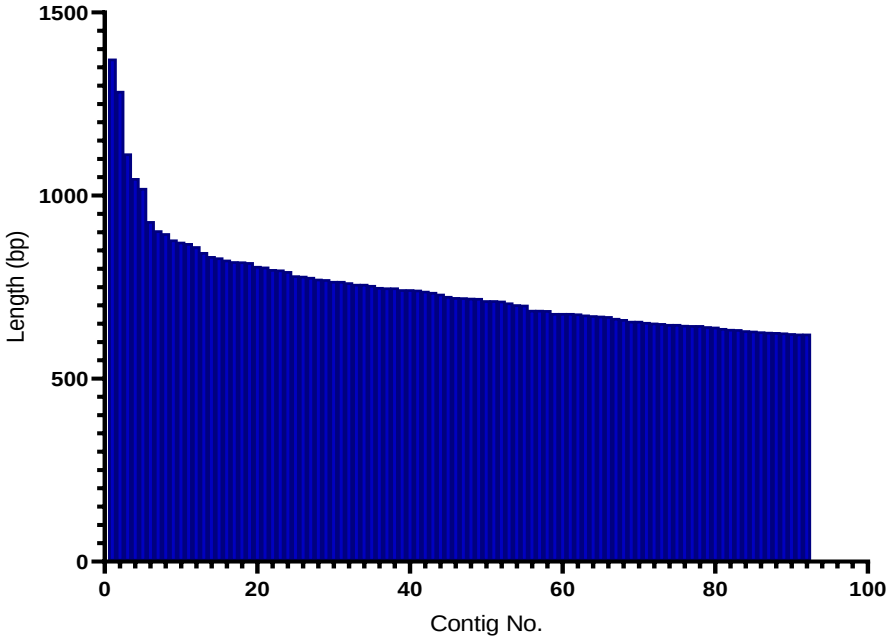

Figure S6. Contig length (bp) for the 92 subtraction contigs selected for PCR-based screening.

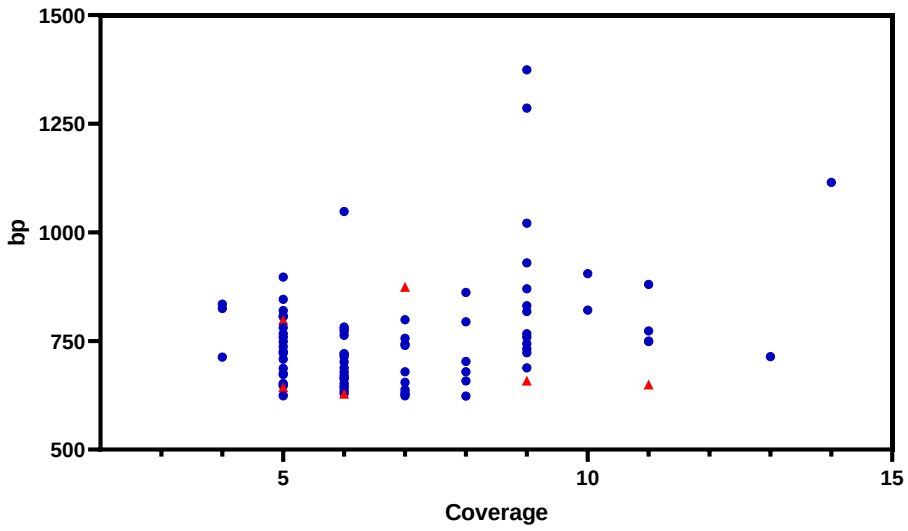

Figure S7. Sequencing coverage for the 92 subtraction contigs selected for PCR-based screening. Red triangles indicate the seven confirmed Y-chromosome subtraction contigs. Blue circles indicate contigs that did not pass PCR validation with a sex-specific pattern of amplification.

a)

b)

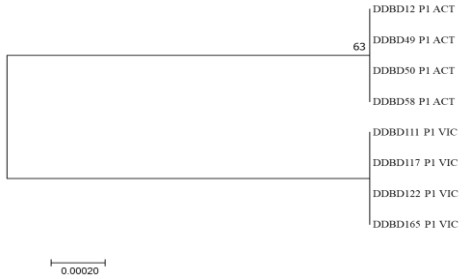

Figure S8. Sequence alignment (a) and phylogeny (b) of bdM27_23_X5_798 contigs (top blue color highlight) with amplified 4 males (Piccadilly Circus_ACT) and 4 males (Anglesea _VIC). Sequences were aligned with Geneious R10.2.6. The results show differences in nucleotide sites, suggesting that Y chromosome region is divergent in each population. Dash indicates gaps and sequences highlighted in colors denote mismatches. Tree is a phylogenetic analysis of sequence variation between ACT and VIC populations. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. Bootstrap support values the number of substitutions per site. All position with gaps and missing data have been eliminated. The analysis involved 8 nucleotide sequences. There was a total of 377 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

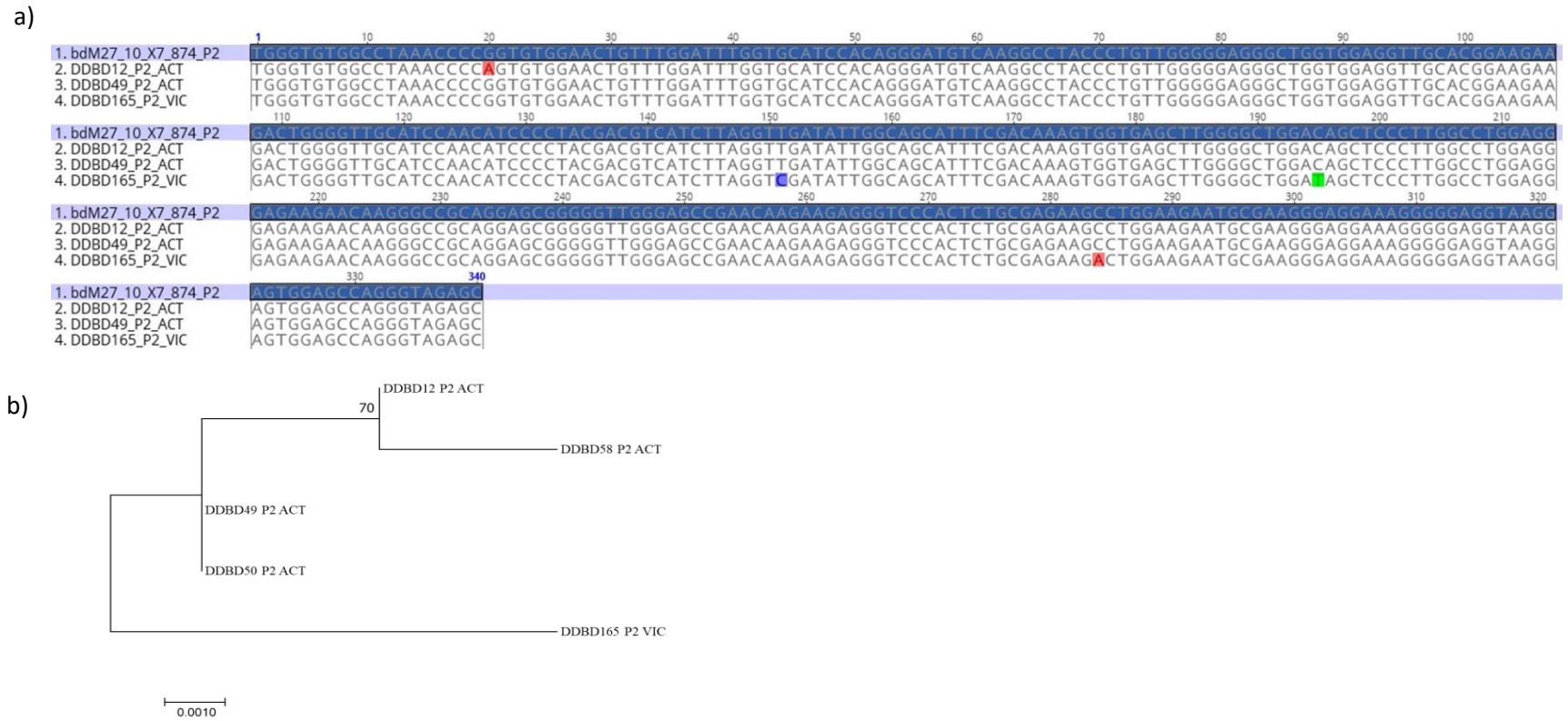

Figure S9. Sequence alignment (a) and phylogeny (b) of bdM27_10_X7_874 contigs (top blue color highlight) with amplified 2 males (Piccadilly Circus_ACT) and a male (Anglesea _VIC). Sequences were aligned with Geneious R10.2.6. The results show differences in nucleotide sites, suggesting that Y chromosome region is divergent in each population. Dash indicates gaps and sequences highlighted in colors denote mismatches. Tree is a phylogenetic analysis of sequence variation between ACT and VIC populations. The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano mode. Bootstrap support values the number of substitutions per site. All position with gaps and missing data have been eliminated. The analysis involved 5 nucleotide sequences. There was a total of 340 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

Figure S10. Sequence alignment of bdM27_74_X11_649 contigs (top blue color highlight) with amplified 3 males (Piccadilly Circus_ACT) and 4 males (Anglesea _VIC). Sequences were aligned with Geneious R10.2.6. No differences found in sequences.

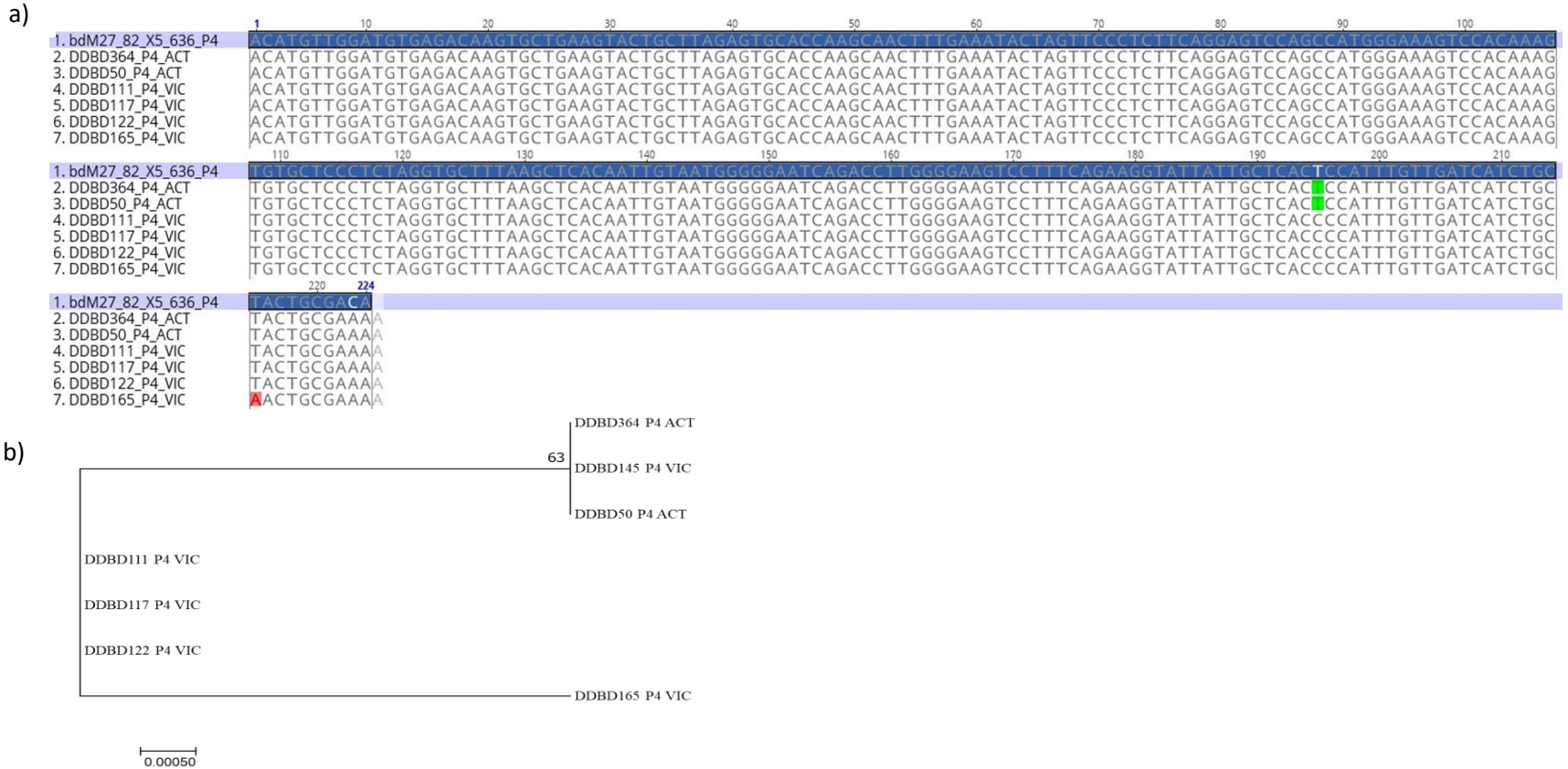

Figure S11. Sequence alignment (a) and phylogeny (b) of bdM27_82_X5_636 contigs (top blue color highlight) with amplified 2 males (Piccadilly Circus_ACT) and 4 males (Anglesea _VIC). Sequences were aligned with Geneious R10.2.6. The results show differences in nucleotide sites, suggesting that Y chromosome region is divergent in each population. Dash indicates gaps and sequences highlighted in colors denote mismatches. Tree is a phylogenetic analysis of sequence variation between ACT and VIC populations. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model [4]. Bootstrap support values the number of substitutions per site. All position with gaps and missing data have been eliminated. The analysis involved 7 nucleotide sequences. There was a total of 251 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

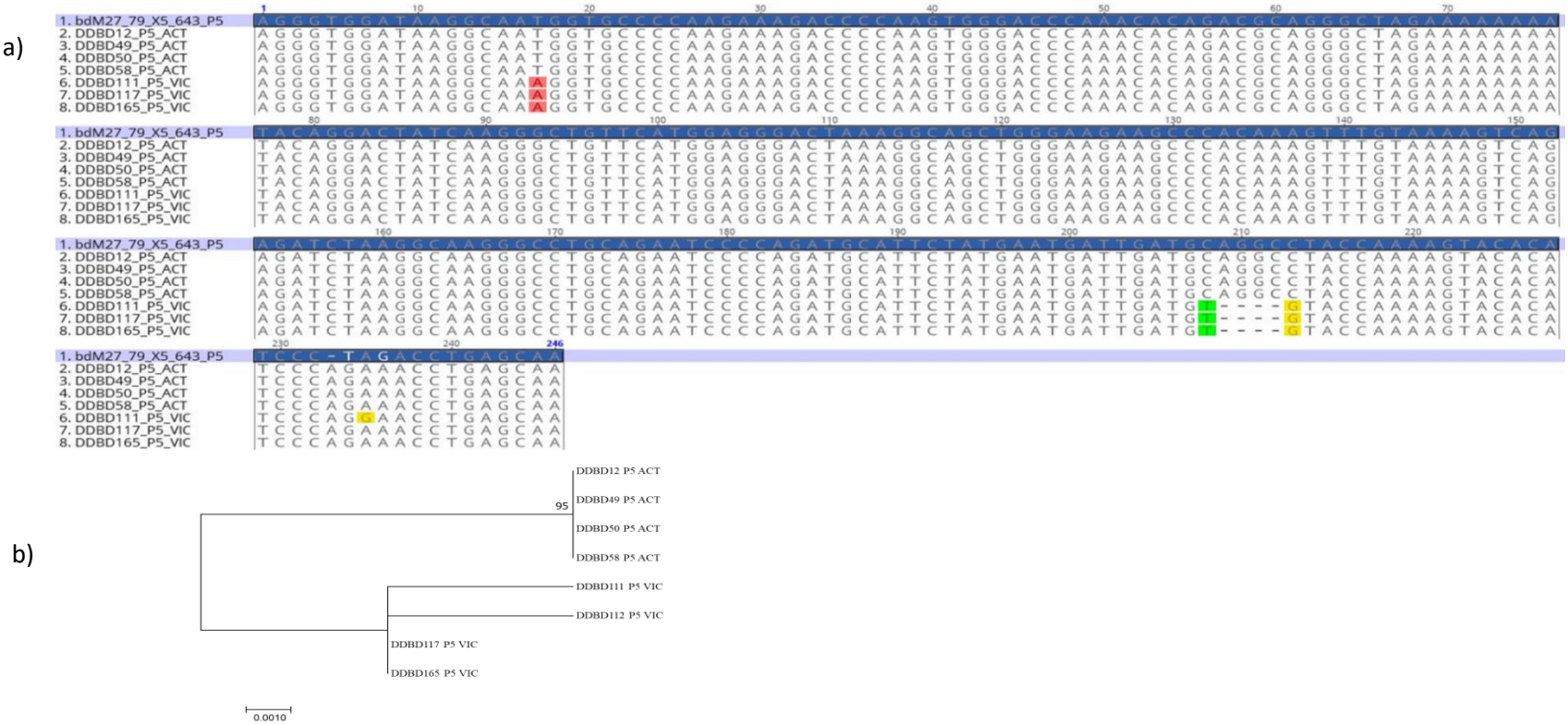

Figure S12. Sequence alignment (a) and phylogeny (b) of bdM27_79_X5_643 contigs (top blue color highlight) with amplified 4 males (Piccadilly Circus_ACT) and 4 males (Anglesea _VIC). Sequences were aligned with Geneious R10.2.6. The results show differences in nucleotide sites, suggesting that Y chromosome region is divergent in each population. Dash indicates gaps and sequences highlighted in colors denote mismatches. Tree is a phylogenetic analysis of sequence variation between ACT and VIC populations. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model. Bootstrap support values the number of substitutions per site. All position with gaps and missing data have been eliminated. The analysis involved 8 nucleotide sequences. There was a total of 266 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

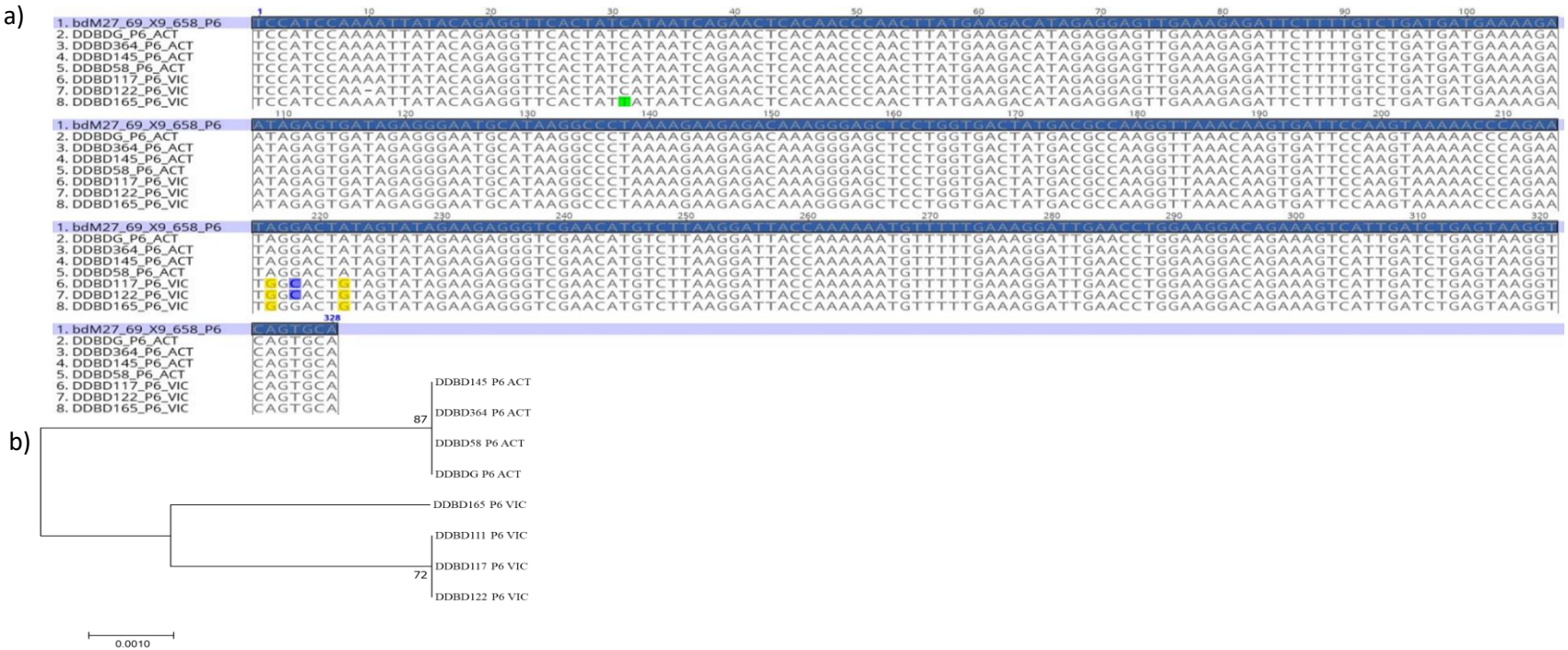

Figure S13. Sequence alignment (a) and phylogeny of bdM27_69_X9_658 contigs (top blue color highlight) with amplified 4 males (Piccadilly Circus_ACT) and 4 males (Anglesea _VIC). Sequences were aligned with Geneious R10.2.6. The results show differences in nucleotide sites, suggesting that Y chromosome region is divergent in each population. Dash indicates gaps and sequences highlighted in colors denote mismatches. Tree is a phylogenetic analysis of sequence variation between ACT and VIC populations using bdM27_69_X9_658. The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model. Bootstrap support values the number of substitutions per site. All position with gaps and missing data have been eliminated. The analysis involved 8 nucleotide sequences. There was a total of 367 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

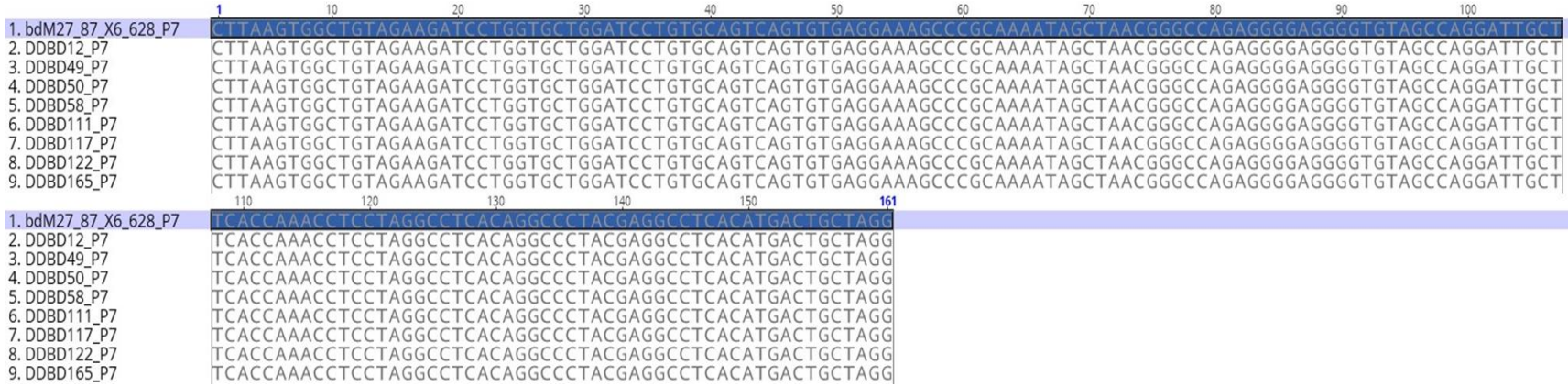

Figure S14. Sequence alignment of bdM27_87_X6_628 contigs (top blue color highlight) with amplified 4 males (Piccadilly Circus_ACT) and 4 males (Anglesea _VIC). Sequences were aligned with Geneious R10.2.6. No differences found in sequences.

Table S2. Estimates of evolutionary divergence between Piccadilly Circus and Anglesea individuals of *Bassiana duperreyi*. The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7.

Amplified contigs name	Piccadilly Circus Population	Anglesea Population	Genetic Distance
bdM27_23_X5_798	DDBD12 P1 ACT	DDBD111 P1 VIC	0.003
	DDBD49 P1 ACT	DDBD111 P1 VIC	0.003
	DDBD50 P1 ACT	DDBD111 P1 VIC	0.003
	DDBD58 P1 ACT	DDBD111 P1 VIC	0.003
	DDBD12 P1 ACT	DDBD117 P1 VIC	0.003
	DDBD49 P1 ACT	DDBD117 P1 VIC	0.003
	DDBD50 P1 ACT	DDBD117 P1 VIC	0.003
	DDBD58 P1 ACT	DDBD117 P1 VIC	0.003
	DDBD12 P1 ACT	DDBD122 P1 VIC	0.003
	DDBD49 P1 ACT	DDBD122 P1 VIC	0.003
	DDBD50 P1 ACT	DDBD122 P1 VIC	0.003
	DDBD58 P1 ACT	DDBD122 P1 VIC	0.003
	DDBD12 P1 ACT	DDBD165 P1 VIC	0.003
	DDBD49 P1 ACT	DDBD165 P1 VIC	0.003
	DDBD50 P1 ACT	DDBD165 P1 VIC	0.003
	DDBD58 P1 ACT	DDBD165 P1 VIC	0.003
bdM27_10_X7_874	DDBD12 P2 ACT	DDBD49 P2 ACT	0.001
	DDBD12 P2 ACT	DDBD50 P2 ACT	0.001
	DDBD12 P2 ACT	DDBD58 P2 ACT	0.001
	DDBD49 P2 ACT	DDBD58 P2 ACT	0.002
	DDBD50 P2 ACT	DDBD58 P2 ACT	0.002
	DDBD12 P2 ACT	DDBD165 P2 VIC	0.005
	DDBD49 P2 ACT	DDBD165 P2 VIC	0.004
	DDBD50 P2 ACT	DDBD165 P2 VIC	0.004
	DDBD58 P2 ACT	DDBD165 P2 VIC	0.006
of bdM27_82_X5_636	DDBD50 P4 ACT	DDBD111 P4 VIC	0.004
	DDBD364 P4 ACT	DDBD111 P4 VIC	0.004
	DDBD50 P4 ACT	DDBD117 P4 VIC	0.004
	DDBD364 P4 ACT	DDBD117 P4 VIC	0.004
	DDBD50 P4 ACT	DDBD122 P4 VIC	0.004
	DDBD364 P4 ACT	DDBD122 P4 VIC	0.004
	DDBD111 P4 VIC	DDBD145 P4 VIC	0.004
	DDBD117 P4 VIC	DDBD145 P4 VIC	0.004
	DDBD122 P4 VIC	DDBD145 P4 VIC	0.004
	DDBD50 P4 ACT	DDBD165 P4 VIC	0.009
	DDBD364 P4 ACT	DDBD165 P4 VIC	0.009

	DDBD111 P4 VIC	DDBD165 P4 VIC	0.004
	DDBD117 P4 VIC	DDBD165 P4 VIC	0.004
	DDBD122 P4 VIC	DDBD165 P4 VIC	0.004
	DDBD145 P4 VIC	DDBD165 P4 VIC	0.009
bdM27_79_X5_643	DDBD12 P5 ACT	DDBD111 P5 VIC	0.017
	DDBD49 P5 ACT	DDBD111 P5 VIC	0.017
	DDBD50 P5 ACT	DDBD111 P5 VIC	0.017
	DDBD58 P5 ACT	DDBD111 P5 VIC	0.017
	DDBD12 P5 ACT	DDBD112 P5 VIC	0.017
	DDBD49 P5 ACT	DDBD112 P5 VIC	0.017
	DDBD50 P5 ACT	DDBD112 P5 VIC	0.017
	DDBD58 P5 ACT	DDBD112 P5 VIC	0.017
	DDBD111 P5 VIC	DDBD112 P5 VIC	0.008
	DDBD12 P5 ACT	DDBD117 P5 VIC	0.013
	DDBD49 P5 ACT	DDBD117 P5 VIC	0.013
	DDBD50 P5 ACT	DDBD117 P5 VIC	0.013
	DDBD58 P5 ACT	DDBD117 P5 VIC	0.013
	DDBD111 P5 VIC	DDBD117 P5 VIC	0.004
	DDBD112 P5 VIC	DDBD117 P5 VIC	0.004
	DDBD12 P5 ACT	DDBD165 P5 VIC	0.013
	DDBD49 P5 ACT	DDBD165 P5 VIC	0.013
	DDBD50 P5 ACT	DDBD165 P5 VIC	0.013
	DDBD58 P5 ACT	DDBD165 P5 VIC	0.013
	DDBD111 P5 VIC	DDBD165 P5 VIC	0.004
	DDBD112 P5 VIC	DDBD165 P5 VIC	0.004
bdM27_69_X9_658	DDBD58 P6 ACT	DDBD111 P6 VIC	0.009
	DDBD58 P6 ACT	DDBD117 P6 VIC	0.009
	DDBD58 P6 ACT	DDBD122 P6 VIC	0.009
	DDBD111 P6 VIC	DDBD145 P6 ACT	0.009
	DDBD117 P6 VIC	DDBD145 P6 ACT	0.009
	DDBD122 P6 VIC	DDBD145 P6 ACT	0.009
	DDBD58 P6 ACT	DDBD165 P6 VIC	0.009
	DDBD111 P6 VIC	DDBD165 P6 VIC	0.006
	DDBD117 P6 VIC	DDBD165 P6 VIC	0.006
	DDBD122 P6 VIC	DDBD165 P6 VIC	0.006
	DDBD145 P6 ACT	DDBD165 P6 VIC	0.009
	DDBD111 P6 VIC	DDBD364 P6 ACT	0.009
	DDBD117 P6 VIC	DDBD364 P6 ACT	0.009
	DDBD122 P6 VIC	DDBD364 P6 ACT	0.009
	DDBD165 P6 VIC	DDBD364 P6 ACT	0.009
	DDBD111 P6 VIC	DDBDG P6 ACT	0.009
	DDBD117 P6 VIC	DDBDG P6 ACT	0.009
	DDBD122 P6 VIC	DDBDG P6 ACT	0.009
	DDBD165 P6 VIC	DDBDG P6 ACT	0.009

Table S3. BLAST results for Y-specific contigs queried against representative reptile genomes, indicating hits for the protein coding gene UBEH2 from contig bdM27_23_X5_798; no other significant hits were found. We also searched against representative bird genomes (see main text) but recovered no significant hits. Here we report only matches with an E-value less than 10^{-5}.

Genome assembly	Scaffold	Score	E-value	Identities	Gaps	Strand
Pseudonaja textilis EBS10Xv2-PRI	ULFR01000216.1	83.5	9.00E-14	48/50 (96%)	0/50 (0%)	Plus/Minus
Pogona vitticeps pvi1.1	CEMB01027918.1	95.4	3.00E-17	51/52 (98%)	0/52 (0%)	Plus/Plus
Notechis scutatus TS10Xv2	ULFQ01011403.1	83.5	9.00E-14	48/50 (96%)	0/50 (0%)	Plus/Minus
Sphenodon punctatus ASM311381v1	QEPC01000343.1	95.4	6.00E-17	51/52 (98%)	0/52 (0%)	Plus/Plus
Varanus komodoensis ASM479886v1	SJPD01000001.1	107	6.00E-21	54/54 (100%)	0/54 (0%)	Plus/Minus
Anolis carolinensis AnoCar2.0	GL343708.1	91.4	4.00E-16	55/58 (95%)	0/58 (0%)	Plus/Plus
Crocodylus porosus CroPor_comp1	MDVP01000050.1	85.5	3.00E-14	56/60 (93%)	1/60 (2%)	Plus/Minus

Table S4. Hits to known repeats in the Dfam database. Each row indicates a hit of a *B. duperreyi* contig to an entry in the Dfam database, showing the repeat model's ID, name, match score and E-value and the position of the hit in that model.

***B. duperreyi* contig name**	**model accession**	**model name**	**bit score**	**e-value**	**model start**	**model end**	**strand**	**alignment start**	**alignment end**	**envelope start**	**envelope end**
bdM27_82_X5_636	DF0006275	DIRS-1e_Amnio	34.9	9.80E-11	4421	4871	+	23	481	10	507
bdM27_79_X5_643	DF0004176	RLTR6-int	92.6	2.40E-28	1409	1969	+	6	560	1	581
bdM27_69_X9_658	DF0004173	RLTR4_MM-int	44.2	9.40E-14	1200	1693	+	161	657	140	658

Appendix 3

Supplementary material for chapter 3

Table S1. Mean annual climatic data at the field locations (January 1895 to January 2019). Abbreviations: Tmax, maximum temperature (°C); Tmin, minimum temperature (°C), Tot. Rain, total rain (mm); Evap, synthetic estimate of evaporation (mm). Means are given with standard deviations.

Field Location	Elevation (m)	Tmax (^{o}C)	Tmin (^{o}C)	Tot. Rain (mm)	Evap (mm)	Radiation (MJ/m^2)	Vapor Pressure (hPa)
Mt Ginini	1640	15.35 ± 6.20	4.53 ± 4.25	96.62 ± 71.26	89.16 ± 58.31	17.10 ± 6.41	8.78 ± 2.50
Mt Kosciuszko	1340	13.29 ± 6.14	3.33 ± 4.20	113.90 ± 70.32	75.84 ± 54.40	16.96 ± 6.60	8.13 ± 2.33
Piccadilly Circus	1240	16.20 ± 6.21	5.00 ± 4.28	94.50 ± 69.76	93.02 ± 59.12	17.25 ± 6.39	9.12 ± 2.57
Coree Flat East	1100	16.51 ± 6.26	4.50 ± 4.22	107.98 ± 82.61	84.61 ± 56.45	16.70 ± 6.06	9.00 ± 2.53
Coree Flat West	1040	16.51 ± 6.26	4.50 ± 4.22	107.98 ± 82.61	84.61 ± 56.45	16.70 ± 6.06	9.00 ± 2.53
Cooma	960	18.04 ± 5.65	4.49 ± 4.48	42.91 ± 37.39	97.04 ± 53.58	16.78 ± 6.05	9.62 ± 2.82
Shelley	725	19.83 ± 6.45	7.03 ± 4.10	85.67 ± 57.25	98.24 ± 60.61	16.82 ± 6.59	10.95 ± 2.69
Dartmouth	380	19.83 ± 6.45	7.03 ± 4.10	85.67 ± 57.25	98.24 ± 60.61	16.82 ± 6.59	10.95 ± 2.69
Anglesea	40	18.02 ± 3.95	9.55 ± 2.66	53.95 ± 32.05	104.50 ± 51.69	14.65 ± 5.79	12.06 ± 2.13
Westernport Bay	20	19.18 ± 4.37	9.78 ± 2.95	63.79 ± 34.22	100.05 ± 48.49	14.90 ± 5.80	12.47 ± 1.95

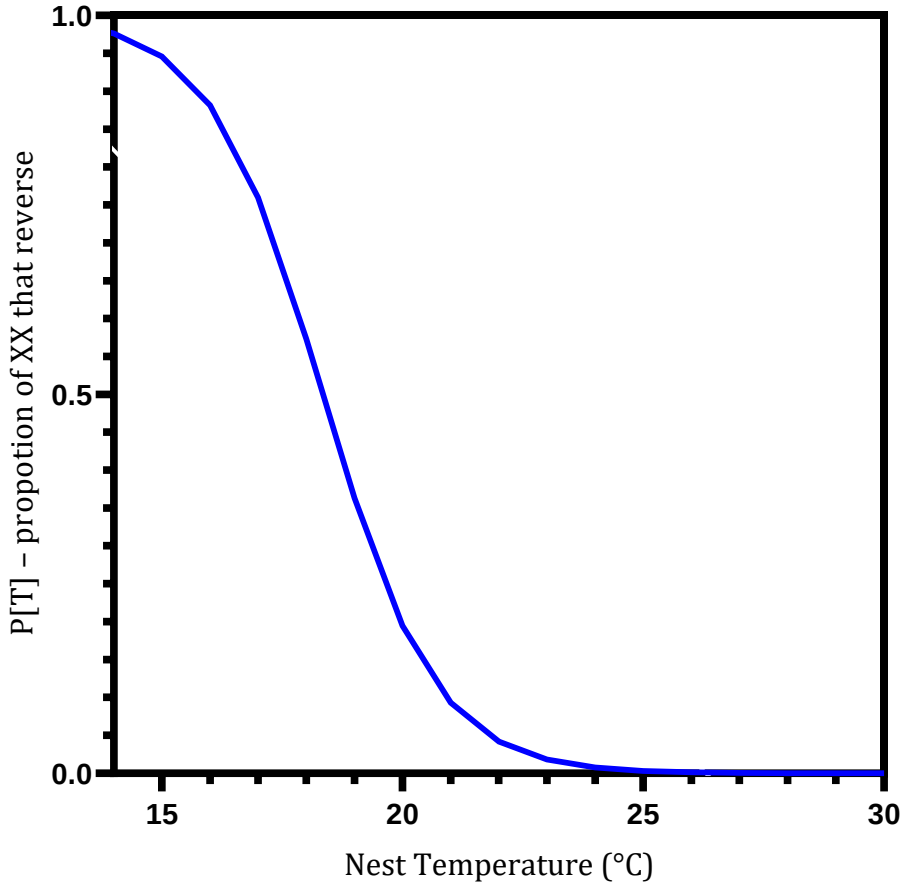

Figure S1. Logistic regression of best fit describing the relationship between individual sex and incubation temperature. Data are taken from Rader et al., (2008); Shine et al., (2002); Telemeco et al., (2009). See Table S2 for statistics. Note that Telemenco et al., (2009) report the temperature above which no sex reversal occurs as 20^{o}C.

Table S2. Simple logistic regression statistics for generating *P*[T], the probability of sex reversal as a function of temperature T. See figure S1 for the logistic regression curve.

Best-fit values	
β0	15.7
β1	-0.857
X at 50%	18.3
Std. Error	
β0	1.51
β1	0.0809
X at 50%	0.161
95% CI (profile likelihood)	
β0	12.9 to 18.8
β1	-1.02 to -0.705
X at 50%	18.0 to 18.7
Odds ratios	
β0	6683948
β1	0.425
95% CI (profile likelihood) for odds ratios	
β0	392504 to 146804421
β1	0.360 to 0.494
Is slope significantly non-zero?	
|Z|	10.6
P value	<0.0001
Deviation from zero?	Significant
Likelihood ratio test	
Log-likelihood ratio (G squared)	180
P value	<0.0001
Reject Null Hypothesis?	Yes
P value summary	****
Area under the ROC curve	
Area	0.862
Std. Error	0.0188
95% confidence interval	0.825 to 0.899
P value	<0.0001
Goodness of Fit	
Tjur's R squared	0.401
Cox-Snell's R squared	0.373
Model deviance, G squared	336
Equation	log odds = 15.7-0.857*X

Table S3. Number of individuals sampled (tail snips) from the Alpine OTU and their phenotypes and genotypes.

Locations (Alpine OTU)	Phenotypic males	Male (XY)	Male (XX)	Phenotypic females (XX)
Mt Ginini	65	53	12	25
Mt Kosciuszko	23	20	3	18
Piccadilly Circus	108	97	11	56
Coree Flat East and West	24	22	2	24
Cooma	41	37	4	15
Shelley	24	23	1	19
Dartmouth	35	35	0	20

Table S4. *P* value for Pearson correlation (below diagonal) and Pearson r (above diagonal) for the association of frequency of sex reversal and elevation and climatic variables. Significance: * < 0.05; ** < 0.01; *** < 0.001.

	Frequency of Sex Reversal	Elevation (m)	Tmax (^{0}C)	Tmin (^{0}C)	Rain Total (mm)	Evaporation (mm)	Radiation (MJ/m^2)	Vapor pressure (hPa)
Frequency of Sex Reversal		0.97137	-0.81843	-0.77907	0.24732	-0.56888	0.61742	-0.83676
Elevation (m)	0.0003***		-0.86515	-0.78107	0.41282	-0.64543	0.64803	-0.87834
Tmax (^{0}C)	0.0244*	0.01191*		0.90763	-0.55695	0.89886	-0.48429	0.97551
Tmin (^{0}C)	0.0389*	0.03813*	0.00474**		-0.23412	0.79873	-0.24972	0.96231
Rain Total (mm)	0.59285	0.35733	0.19403	0.61336		-0.71320	0.26451	-0.44469
Evaporation (mm)	0.18260	0.11743	0.00591**	0.03122*	0.07198		-0.12903	0.85436
Radiation (MJ/m^2)	0.13961	0.11548	0.27076	0.58915	0.56649	0.78277		-0.42197
Vapor pressure (hPa)	0.01890*	0.00928**	0.00018***	0.00052***	0.31744	0.01435*	0.34568	

Appendix 4

Supplementary material for chapter 4

Table S1. Specimen information for tissue samples SNP genotyped for this study, including species, population, Bioregion, number on figures, collection codes, and location. The database reference for samples is UC<Aus> University of Canberra Wildlife Tissue Collection.

Species	Population Name	Bio Region (IBR7)	Number on Figure 1 and 2	Database Reference	Latitude	Longitude
Pseudomia pagenstecheri	Cooma_NSW	Australian Alps	P6	DDAUS_1	-36.44683	149.19461
Niveoscincus coventryi	Mt_Franklin_ACT	Australian Alps	P11	DDAUS_34	-35.440969	148.776979
Bassiana duperreyi	Westernport Bay_VIC	South East Coastal Plain	P10	DDBD_104	-38.22777778	145.3094444
Bassiana duperreyi	Westernport Bay_VIC	South East Coastal Plain	P10	DDBD_108	-38.22777778	145.3094444
Bassiana duperreyi	Westernport Bay_VIC	South East Coastal Plain	P10	DDBD_146	-38.22777778	145.3094444
Bassiana duperreyi	Westernport Bay_VIC	South East Coastal Plain	P10	DDBD_147	-38.22777778	145.3094444
Bassiana duperreyi	Westernport Bay_VIC	South East Coastal Plain	P10	DDBD_148	-38.22777778	145.3094444
Bassiana duperreyi	Westernport Bay_VIC	South East Coastal Plain	P10	DDBD_149	-38.22777778	145.3094444
Bassiana duperreyi	Anglesea_VIC	South East Coastal Plain	P9	DDBD_150	-38.39111111	144.215
Bassiana duperreyi	Anglesea_VIC	South East Coastal Plain	P9	DDBD_152	-38.39111111	144.215
Bassiana duperreyi	Anglesea_VIC	South East Coastal Plain	P9	DDBD_153	-38.39111111	144.215
Bassiana duperreyi	Anglesea_VIC	South East Coastal Plain	P9	DDBD_155	-38.39111111	144.215
Bassiana duperreyi	Anglesea_VIC	South East Coastal Plain	P9	DDBD_156	-38.39111111	144.215
Bassiana duperreyi	Anglesea_VIC	South East Coastal Plain	P9	DDBD_157	-38.39111111	144.215
Bassiana duperreyi	Anglesea_VIC	South East Coastal Plain	P9	DDBD_159	-38.39111111	144.215
Bassiana duperreyi	Anglesea_VIC	South East Coastal Plain	P9	DDBD_161	-38.39111111	144.215
Bassiana duperreyi	Anglesea_VIC	South East Coastal Plain	P9	DDBD_170	-38.39111111	144.215
Bassiana duperreyi	Anglesea_VIC	South East Coastal Plain	P9	DDBD_178	-38.39111111	144.215
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBD_21	-35.36165833	148.8034583

Bassiana duperreyi	Westernport Bay_VIC	South East Coastal Plain	P10	DDBD_210	-38.22777778	145.3094444
Bassiana duperreyi	Westernport Bay_VIC	South East Coastal Plain	P10	DDBD_211	-38.22777778	145.3094444
Bassiana duperreyi	Westernport Bay_VIC	South East Coastal Plain	P10	DDBD_212	-38.22777778	145.3094444
Bassiana duperreyi	Westernport Bay_VIC	South East Coastal Plain	P10	DDBD_213	-38.22777778	145.3094444
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBD_22	-35.36165833	148.8034583
Bassiana duperreyi	Corre Flat East_ACT	Australian Alps	P4	DDBD_221	-35.26836	148.83186
Bassiana duperreyi	Corre Flat East_ACT	Australian Alps	P4	DDBD_222	-35.26836	148.83186
Bassiana duperreyi	Corre Flat East_ACT	Australian Alps	P4	DDBD_223	-35.26836	148.83186
Bassiana duperreyi	Corre Flat East_ACT	Australian Alps	P4	DDBD_224	-35.26836	148.83186
Bassiana duperreyi	Corre Flat East_ACT	Australian Alps	P4	DDBD_225	-35.26836	148.83186
Bassiana duperreyi	Corre Flat East_ACT	Australian Alps	P4	DDBD_226	-35.26836	148.83186
Bassiana duperreyi	Corre Flat East_ACT	Australian Alps	P4	DDBD_227	-35.26836	148.83186
Bassiana duperreyi	Corre Flat East_ACT	Australian Alps	P4	DDBD_228	-35.26836	148.83186
Bassiana duperreyi	Corre Flat East_ACT	Australian Alps	P4	DDBD_229	-35.26836	148.83186
Bassiana duperreyi	Corre Flat East_ACT	Australian Alps	P4	DDBD_232	-35.26836	148.83186
Bassiana duperreyi	Coree Flat West_ACT	Australian Alps	P5	DDBD_234	-35.28111111	148.8097222
Bassiana duperreyi	Coree Flat West_ACT	Australian Alps	P5	DDBD_235	-35.28111111	148.8097222
Bassiana duperreyi	Coree Flat West_ACT	Australian Alps	P5	DDBD_236	-35.28111111	148.8097222
Bassiana duperreyi	Coree Flat West_ACT	Australian Alps	P5	DDBD_237	-35.28111111	148.8097222
Bassiana duperreyi	Coree Flat West_ACT	Australian Alps	P5	DDBD_238	-35.28111111	148.8097222
Bassiana duperreyi	Coree Flat West_ACT	Australian Alps	P5	DDBD_239	-35.28111111	148.8097222
Bassiana duperreyi	Coree Flat West_ACT	Australian Alps	P5	DDBD_240	-35.28111111	148.8097222
Bassiana duperreyi	Coree Flat West_ACT	Australian Alps	P5	DDBD_241	-35.28111111	148.8097222
Bassiana duperreyi	Coree Flat West_ACT	Australian Alps	P5	DDBD_242	-35.28111111	148.8097222
Bassiana duperreyi	Coree Flat West_ACT	Australian Alps	P5	DDBD_244	-35.28111111	148.8097222

Bassiana duperreyi	Cooma_NSW	Australian Alps	P6	DDBD_247	-36.44683	149.19461
Bassiana duperreyi	Cooma_NSW	Australian Alps	P6	DDBD_249	-36.44683	149.19461
Bassiana duperreyi	Cooma_NSW	Australian Alps	P6	DDBD_250	-36.44683	149.19461
Bassiana duperreyi	Cooma_NSW	Australian Alps	P6	DDBD_251	-36.44683	149.19461
Bassiana duperreyi	Cooma_NSW	Australian Alps	P6	DDBD_252	-36.44683	149.19461
Bassiana duperreyi	Cooma_NSW	Australian Alps	P6	DDBD_253	-36.44683	149.19461
Bassiana duperreyi	Cooma_NSW	Australian Alps	P6	DDBD_254	-36.44683	149.19461
Bassiana duperreyi	Cooma_NSW	Australian Alps	P6	DDBD_257	-36.44683	149.19461
Bassiana duperreyi	Cooma_NSW	Australian Alps	P6	DDBD_259	-36.44683	149.19461
Bassiana duperreyi	Cooma_NSW	Australian Alps	P6	DDBD_260	-36.44683	149.19461
Bassiana duperreyi	Mt_Ginini_ACT	Australian Alps	P1	DDBD_271	-35.52487778	148.7829583
Bassiana duperreyi	Mt_Ginini_ACT	Australian Alps	P1	DDBD_272	-35.52487778	148.7829583
Bassiana duperreyi	Mt_Ginini_ACT	Australian Alps	P1	DDBD_273	-35.52487778	148.7829583
Bassiana duperreyi	Mt_Ginini_ACT	Australian Alps	P1	DDBD_274	-35.52487778	148.7829583
Bassiana duperreyi	Mt_Ginini_ACT	Australian Alps	P1	DDBD_275	-35.52487778	148.7829583
Bassiana duperreyi	Mt_Ginini_ACT	Australian Alps	P1	DDBD_276	-35.52487778	148.7829583
Bassiana duperreyi	Mt_Ginini_ACT	Australian Alps	P1	DDBD_277	-35.52487778	148.7829583
Bassiana duperreyi	Mt_Ginini_ACT	Australian Alps	P1	DDBD_279	-35.52487778	148.7829583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBD_28	-35.36165833	148.8034583
Bassiana duperreyi	Mt_Ginini_ACT	Australian Alps	P1	DDBD_283	-35.52487778	148.7829583
Bassiana duperreyi	Mt_Ginini_ACT	Australian Alps	P1	DDBD_285	-35.52487778	148.7829583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBD_287	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBD_288	-35.36165833	148.8034583
Bassiana duperreyi	Dartmouth_VIC	Australian Alps	P8	DDBD_298	-36.52665	147.4814
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBD_290	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBD_291	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBD_292	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBD_293	-35.36165833	148.8034583
Bassiana duperreyi	Dartmouth_VIC	Australian Alps	P8	DDBD_296	-36.52665	147.4814
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBD_289	-35.36165833	148.8034583

Bassiana duperreyi	Dartmouth_VIC	Australian Alps	P8	DDBD_300	-36.52665	147.4814
Bassiana duperreyi	Dartmouth_VIC	Australian Alps	P8	DDBD_301	-36.52665	147.4814
Bassiana duperreyi	Dartmouth_VIC	Australian Alps	P8	DDBD_302	-36.52665	147.4814
Bassiana duperreyi	Dartmouth_VIC	Australian Alps	P8	DDBD_303	-36.52665	147.4814
Bassiana duperreyi	Dartmouth_VIC	Australian Alps	P8	DDBD_304	-36.52665	147.4814
Bassiana duperreyi	Dartmouth_VIC	Australian Alps	P8	DDBD_305	-36.52665	147.4814
Bassiana duperreyi	Dartmouth_VIC	Australian Alps	P8	DDBD_306	-36.52665	147.4814
Bassiana duperreyi	Dartmouth_VIC	Australian Alps	P8	DDBD_309	-36.52665	147.4814
Bassiana duperreyi	Shelly_VIC	Australian Alps	P7	DDBD_315	-36.17547	147.54951
Bassiana duperreyi	Shelly_VIC	Australian Alps	P7	DDBD_316	-36.17547	147.54951
Bassiana duperreyi	Shelly_VIC	Australian Alps	P7	DDBD_318	-36.17547	147.54951
Bassiana duperreyi	Shelly_VIC	Australian Alps	P7	DDBD_320	-36.17547	147.54951
Bassiana duperreyi	Shelly_VIC	Australian Alps	P7	DDBD_321	-36.17547	147.54951
Bassiana duperreyi	Shelly_VIC	Australian Alps	P7	DDBD_323	-36.17547	147.54951
Bassiana duperreyi	Shelly_VIC	Australian Alps	P7	DDBD_324	-36.17547	147.54951
Bassiana duperreyi	Shelly_VIC	Australian Alps	P7	DDBD_325	-36.17547	147.54951
Bassiana duperreyi	Shelly_VIC	Australian Alps	P7	DDBD_330	-36.17547	147.54951
Bassiana duperreyi	Shelly_VIC	Australian Alps	P7	DDBD_332	-36.17547	147.54951
Bassiana duperreyi	Mt_Kosciuszko _NSW	Australian Alps	P2	DDBD_338	-35.88998	148.42279
Bassiana duperreyi	Mt_Kosciuszko _NSW	Australian Alps	P2	DDBD_339	-35.88998	148.42279
Bassiana duperreyi	Mt_Kosciuszko _NSW	Australian Alps	P2	DDBD_340	-35.88998	148.42279
Bassiana duperreyi	Mt_Kosciuszko _NSW	Australian Alps	P2	DDBD_341	-35.88998	148.42279
Bassiana duperreyi	Mt_Kosciuszko _NSW	Australian Alps	P2	DDBD_342	-35.88998	148.42279
Bassiana duperreyi	Mt_Kosciuszko _NSW	Australian Alps	P2	DDBD_344	-35.88998	148.42279
Bassiana duperreyi	Mt_Kosciuszko _NSW	Australian Alps	P2	DDBD_345	-35.88998	148.42279
Bassiana duperreyi	Mt_Kosciuszko _NSW	Australian Alps	P2	DDBD_346	-35.88998	148.42279
Bassiana duperreyi	Mt_Kosciuszko _NSW	Australian Alps	P2	DDBD_347	-35.88998	148.42279
Bassiana duperreyi	Mt_Kosciuszko _NSW	Australian Alps	P2	DDBD_348	-35.88998	148.42279
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN27S1	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN27S10	-35.36165833	148.8034583

Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN27S11	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN27S12	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN28S15	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN27S14	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN28S16	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN28S17	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN28S18	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN28S19	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN27S2	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN28S21	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN28S22	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBD_S23	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN28S24	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN28S25	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN28S26	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN28S29	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN28S28	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN28S27	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN27S3	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN28S30	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN28S31	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN28S32	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN29S33	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN29S34	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN29S35	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN29S36	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN29S37	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN2S4	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN29S39	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN27S4	-35.36165833	148.8034583

Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN29S40	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN29S41	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN2S42	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN2S43	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN2S44	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN27S5	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN27S6	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN27S7	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN27S8	-35.36165833	148.8034583
Bassiana duperreyi	Piccadilly Circus_ACT	Australian Alps	P3	DDBDN27S9	-35.36165833	148.8034583
Bassiana duperreyi	Kanngra Boyd NP_NSW	South Eastern Highlands	P12	DDBD_512	-33.8327	149.984444
Bassiana duperreyi	Kanngra Boyd NP_NSW	South Eastern Highlands	P12	DDBD_513	-33.8327	149.984444
Bassiana duperreyi	Kanngra Boyd NP_NSW	South Eastern Highlands	P12	DDBD_514	-33.8327	149.984444
Bassiana duperreyi	Kanngra Boyd NP_NSW	South Eastern Highlands	P12	DDBD_515	-33.8327	149.984444
Bassiana duperreyi	Kanngra Boyd NP_NSW	South Eastern Highlands	P12	DDBD_516	-33.8327	149.984444
Bassiana duperreyi	Cape Conran_VIC	South East Corner	P13	DDBD_517	-37.96777778	148.8983333
Bassiana duperreyi	Cape Conran_VIC	South East Corner	P13	DDBD_518	-37.96777778	148.8983333
Bassiana duperreyi	Cape Conran_VIC	South East Corner	P13	DDBD_519	-37.96777778	148.8983333
Bassiana duperreyi	Cape Conran_VIC	South East Corner	P13	DDBD_520	-37.96777778	148.8983333
Bassiana duperreyi	Lake Bunga_VIC	South East Coastal Plain	P14	DDBD_521	-37.87916667	148.2327778
Bassiana duperreyi	Wilson Prom NP_VIC	Furneaux	P15	DDBD_522	-38.9158333	146.3177778
Bassiana duperreyi	Wilson Prom NP_VIC	Furneaux	P15	DDBD_523	-38.9158333	146.3177778
Bassiana duperreyi	Wilson Prom NP_VIC	Furneaux	P15	DDBD_524	-38.9158333	146.3177778
Bassiana duperreyi	Wilson Prom NP_VIC	Furneaux	P15	DDBD_525	-38.9158333	146.3177778
Bassiana duperreyi	Wilson Prom NP_VIC	Furneaux	P15	DDBD_526	-38.9158333	146.3177778
Bassiana duperreyi	Wilson Prom NP_VIC	Furneaux	P15	DDBD_527	-38.9158333	146.3177778
Bassiana duperreyi	Wilson Prom NP_VIC	Furneaux	P15	DDBD_528	-38.9158333	146.3177778
Bassiana duperreyi	Wilson Prom NP_VIC	Furneaux	P15	DDBD_529	-38.9158333	146.3177778
Bassiana duperreyi	Wilson Prom NP_VIC	Furneaux	P15	DDBD_530	-38.9158333	146.3177778
Bassiana duperreyi	Wilson Prom NP_VIC	Furneaux	P15	DDBD_531	-38.9158333	146.3177778

Bassiana duperreyi	Mt_Hotham_VIC	Australian Alps	P16	DDBD_532	-37.0627777	147.3352777
Bassiana duperreyi	Mt_Hotham_VIC	Australian Alps	P16	DDBD_533	-37.0627777	147.3352777
Bassiana duperreyi	Mt_Hotham_VIC	Australian Alps	P16	DDBD_534	-37.0627777	147.3352777
Bassiana duperreyi	Mt_Hotham_VIC	Australian Alps	P16	DDBD_535	-37.0627777	147.3352777
Bassiana duperreyi	William Rd_SA	Naracoorte Coastal Plain	P17	ABTC106109	-36.7994	139.9639
Bassiana duperreyi	Noolook Forest Reserve_SA	Naracoorte Coastal Plain	P18	ABTC106112	-37.0558	139.91
Bassiana duperreyi	Noolook Forest Reserve_SA	Naracoorte Coastal Plain	P18	ABTC106113	-37.0567	139.8886
Bassiana duperreyi	Dreeite_VIC	Southern Volcanic Plain	P19	ABTC11242	-38.1575	143.3272222
Bassiana duperreyi	Collector_NSW	South Eastern Highlands	P20	ABTC11243	-34.918888	149.405277
Bassiana duperreyi	Collector_NSW	South Eastern Highlands	P20	ABTC11244	-34.918888	149.405277
Bassiana duperreyi	Collector_NSW	South Eastern Highlands	P20	ABTC11245	-34.918888	149.405277
Bassiana duperreyi	Tasmania_TAS	Tasmanian South East	P21	ABTC23542	-42.4952777	147.893611
Bassiana duperreyi	Tasmania_TAS	Tasmanian South East	P21	ABTC23543	-41.959722	147.500277
Bassiana duperreyi	Flinders Island_TAS	Furneaux	P22	ABTC23589	-40.021111	147.91966
Bassiana duperreyi	Flinders Island_TAS	Furneaux	P22	ABTC23592	-40.021111	147.91966
Bassiana duperreyi	Flinders Island_TAS	Furneaux	P22	ABTC23598	-40.021111	147.91966
Bassiana duperreyi	Flinders Island_TAS	Furneaux	P22	ABTC23606	-40.021111	147.91966
Bassiana duperreyi	Flinders Island_TAS	Furneaux	P22	ABTC23610	-40.021111	147.91966
Bassiana duperreyi	Kangaroo Is_SA	Kanmantoo	P23	ABTC33362	-36.015	137.0142
Bassiana duperreyi	Kangaroo Is_SA	Kanmantoo	P23	ABTC33478	-35.9508	136.7286
Bassiana duperreyi	Kangaroo Is_SA	Kanmantoo	P23	ABTC33498	-35.9583	136.7778
Bassiana duperreyi	Kangaroo Is_SA	Kanmantoo	P23	ABTC33499	-35.7917	136.925
Bassiana duperreyi	Kangaroo Is_SA	Kanmantoo	P23	ABTC33512	-36.0458	136.7153
Bassiana duperreyi	Kangaroo Is_SA	Kanmantoo	P23	ABTC33514	-35.8397	136.6786
Bassiana duperreyi	Kangaroo Is_SA	Kanmantoo	P23	ABTC33533	-35.7256	136.82
Bassiana duperreyi	Kangaroo Is_SA	Kanmantoo	P23	ABTC33624	-35.8278	137.8244
Bassiana duperreyi	Kangaroo Is_SA	Kanmantoo	P23	ABTC33733	-35.985	137.3228
Bassiana duperreyi	Stopbanks F	Naracoorte Coastal Plain	P24	ABTC36241	-36.7597	140.2828

Bassiana duperreyi	Gum Lagoon Nature Preserve	Naracoorte Coastal Plain	P25	ABTC37080	-36.2403	140.0686
Bassiana duperreyi	Gum Lagoon Nature Preserve	Naracoorte Coastal Plain	P25	ABTC37083	-36.2714	139.9808
Bassiana duperreyi	Gum Lagoon Nature Preserve	Naracoorte Coastal Plain	P25	ABTC37089	-36.2886	140.0256
Bassiana duperreyi	Gum Lagoon Nature Preserve	Naracoorte Coastal Plain	P25	ABTC37090	-36.2886	140.0256
Bassiana duperreyi	Gum Lagoon Nature Preserve	Naracoorte Coastal Plain	P25	ABTC37093	-36.2886	140.0256
Bassiana duperreyi	Martin Washpool Conservation Park	Naracoorte Coastal Plain	P27	ABTC37395	-36.1611	139.7453
Bassiana duperreyi	Calectasia Conservation Park	Naracoorte Coastal Plain	P28	ABTC37463	-37.4314	140.6253
Bassiana duperreyi	Penola Forest Reserve	Naracoorte Coastal Plain	P29	ABTC37467	-37.5058	140.8492
Bassiana duperreyi	Bucks Lake	Naracoorte Coastal Plain	P30	ABTC37505	-37.8933	140.3744
Bassiana duperreyi	Bucks Lake	Naracoorte Coastal Plain	P30	ABTC37522	-37.9108	140.4
Bassiana duperreyi	Furner	Naracoorte Coastal Plain	P31	ABTC37538	-37.4378	140.3136
Bassiana duperreyi	Furner	Naracoorte Coastal Plain	P31	ABTC37541	-37.4367	140.3175
Bassiana duperreyi	Willalooka	Naracoorte Coastal Plain	P32	ABTC37635	-36.3981	140.2892
Bassiana duperreyi	Desert Camp National Park	Naracoorte Coastal Plain	P33	ABTC37642	-36.4911	140.37
Bassiana duperreyi	Conmurra	Naracoorte Coastal Plain	P34	ABTC37672	-37.1778	140.2047
Bassiana duperreyi	Fairview National Park	Naracoorte Coastal Plain	P35	ABTC37713	-36.8269	140.4261
Bassiana duperreyi	Talapar Nature preserve	Naracoorte Coastal Plain	P36	ABTC37714	-36.7178	140.4442
Bassiana duperreyi	Furner	Naracoorte Coastal Plain	P31	ABTC37721	-37.4367	140.3175
Bassiana duperreyi	German Creek	Naracoorte Coastal Plain	P32	ABTC37729	-37.8494	140.4236
Bassiana duperreyi	Messent Conservation	Naracoorte Coastal Plain	P33	ABTC37730	-36.0928	139.7225
Bassiana duperreyi	Noolook Forest	Naracoorte Coastal Plain	P18	ABTC37752	-37.0617	139.8547
Bassiana duperreyi	Mount Scott Conservation	Naracoorte Coastal Plain	P34	ABTC37753	-36.815	140.0575
Bassiana duperreyi	Dreeite_VIC	Southern Volcanic Plain	P19	ABTC4110	-38.1575	143.3272222
Bassiana duperreyi	Bool Lagoon	Naracoorte Coastal Plain	P35	ABTC53643	-37.13	140.67
Bassiana duperreyi	Little Dip	Naracoorte Coastal Plain	P37	ABTC53679	-37.25	139.8

Bassiana duperreyi	Mary Seymour Conservation Park	Naracoorte Coastal Plain	P38	ABTC53718	-37.17	140.62
Bassiana duperreyi	Mary Seymour Conservation Park	Naracoorte Coastal Plain	P38	ABTC53719	-37.17	140.62
Bassiana duperreyi	Mary Seymour Conservation Park	Naracoorte Coastal Plain	P38	ABTC53720	-37.17	140.62
Bassiana duperreyi	Stopbanks F	Naracoorte Coastal Plain	P24	ABTC53794	-36.75	140.22
Bassiana duperreyi	Kanngra Boyd NP_NSW	South Eastern Highlands	P12	ABTC57497	-34.05	149.83
Bassiana duperreyi	Kanngra Boyd NP_NSW	South Eastern Highlands	P12	ABTC57498	-34.05	149.83
Bassiana duperreyi	Kanngra Boyd NP_NSW	South Eastern Highlands	P12	ABTC57507	-34.25	149.78
Bassiana duperreyi	Mount Scott Conservation	Naracoorte Coastal Plain	P34	ABTC58204	-36.83	140.08
Bassiana duperreyi	Mount Scott Conservation	Naracoorte Coastal Plain	P34	ABTC58205	-36.83	140.08
Bassiana duperreyi	Mount Scott Conservation	Naracoorte Coastal Plain	P34	ABTC58206	-36.83	140.08
Bassiana duperreyi	Gum Lagoon Nature Preserve	Naracoorte Coastal Plain	P25	ABTC58301	-36.2844	140.0333
Bassiana duperreyi	Gum Lagoon Nature Preserve	Naracoorte Coastal Plain	P25	ABTC58326	-36.27	140.0353
Bassiana duperreyi	Fleurieu Peninsula_SA	Kanmantoo	P39	ABTC58418	-35.57	138.3
Bassiana duperreyi	Fleurieu Peninsula_SA	Kanmantoo	P39	ABTC58697	-35.62	138.5
Bassiana duperreyi	Bool Lagoon	Naracoorte Coastal Plain	P35	ABTC68784	-37.1039	140.7328
Bassiana duperreyi	Dorset Vale	Kanmantoo	P40	ABTC68894	-35.0808	138.6775
Bassiana duperreyi	Bullock Hill Conservation Park	Kanmantoo	P41	ABTC68909	-35.3172	138.7811
Bassiana duperreyi	Taratap	Naracoorte Coastal Plain	P42	ABTC70605	-36.5561	139.9853
Bassiana duperreyi	Taratap	Naracoorte Coastal Plain	P42	ABTC70606	-36.5092	140.0028
Bassiana duperreyi	Taratap	Naracoorte Coastal Plain	P42	ABTC70613	-36.5647	140.0069
Bassiana duperreyi	Taratap	Naracoorte Coastal Plain	P42	ABTC70614	-36.5092	140.0028
Bassiana duperreyi	Taratap	Naracoorte Coastal Plain	P42	ABTC70615	-36.5406	139.9811
Bassiana duperreyi	Killawarra Sanctuary	Flinders Lofty Block	P43	ABTC73290	-34.5706	138.9997
Bassiana duperreyi	Milbrook Rservoir	Flinders Lofty Block	P44	ABTC74255	-34.8228	138.7947
Bassiana duperreyi	Warren National	Flinders Lofty Block	P45	ABTC74307	-34.7303	138.9186
Bassiana duperreyi	Humbug Wildlife Sanctuary	Flinders Lofty Block	P46	ABTC74316	-34.7164	138.8408

Bassiana duperreyi	Porter Scrub Nature Charleston	Flinders Lofty Block	P47	ABTC74323	-34.8708	138.9069
Bassiana duperreyi	Conservation Park Montacute	Flinders Lofty Block	P48	ABTC74328	-34.9203	138.9469
Bassiana duperreyi	Conservation Park	Flinders Lofty Block	P49	ABTC74352	-34.8875	138.7897
Bassiana duperreyi	Scott Creek	Flinders Lofty Block	P50	ABTC74364	-35.0508	138.6975
Bassiana duperreyi	Upper Sturt	Flinders Lofty Block	P51	ABTC74386	-35.0289	138.6694
Bassiana duperreyi	Kuitpo	Flinders Lofty Block	P52	ABTC74400	-35.175	138.6706
Bassiana duperreyi	Kuitpo	Flinders Lofty Block	P52	ABTC74403	-35.1853	138.6878
Bassiana duperreyi	Greenhill	Flinders Lofty Block	P53	ABTC74460	-34.9542	138.6928
Bassiana duperreyi	Mt_Bold Reservoir	Flinders Lofty Block	P54	ABTC74483	-35.0864	138.7192
Bassiana duperreyi	Mt_Bold Reservoir	Flinders Lofty Block	P54	ABTC74489	-35.1147	138.695
Bassiana duperreyi	Mt_Bold Reservoir	Flinders Lofty Block	P54	ABTC74490	-35.1144	138.7264
Bassiana duperreyi	Warren National	Flinders Lofty Block	P45	ABTC79669	-34.7492	138.9147
Bassiana duperreyi	Warren National	Flinders Lofty Block	P45	ABTC79674	-34.7736	138.9786
Bassiana duperreyi	Myponga Conservation	Kanmantoo	P55	ABTC94851	-35.4569	138.4381
Bassiana duperreyi	Deep Creek Conservation	Kanmantoo	P56	ABTC94990	-35.6186	138.2306
Bassiana duperreyi	Eagle Waterhole	Kanmantoo	P57	ABTC95031	-35.6533	138.1881
Bassiana duperreyi	Kyeema Conservation	Kanmantoo	P58	ABTC95051	-35.2658	138.6886
Bassiana duperreyi	Kyeema Conservation	Kanmantoo	P58	ABTC95052	-35.2672	138.6736
Bassiana duperreyi	Wilson Prom NP_VIC	Furneaux	P15	Z_21485	-38.92228	146.30161
Bassiana duperreyi	Black Range State Park_VIC	Southern Volcanic Plain	P59	Z_22563	-37.15755	142.07426
Bassiana duperreyi	Grampians_NP_VIC	Southern Volcanic Plain	P60	Z_22574	-37.17234	142.5145
Bassiana duperreyi	Anglesea Heath_VIC	South East Coastal Plain	P61	Z_60612	-38.3796	144.18525
Bassiana duperreyi	Anglesea Heath_VIC	South East Coastal Plain	P61	Z_60614	-38.3826333	144.1580833
Bassiana duperreyi	Anglesea Heath_VIC	South East Coastal Plain	P61	Z_60616	-38.38255	144.1582833
Bassiana duperreyi	Anglesea Heath_VIC	South East Coastal Plain	P61	Z_60626	-38.3810833	144.1283
Bassiana duperreyi	Anglesea Heath_VIC	South East Coastal Plain	P61	Z_60636	-38.3810833	144.1283
Bassiana duperreyi	Anglesea Heath_VIC	South East Coastal Plain	P61	Z_60649	-38.38255	144.1582833
Bassiana duperreyi	Anglesea Heath_VIC	South East Coastal Plain	P61	Z_62663	-38.3826333	144.1580833

Bassiana duperreyi	Anglesea Heath_VIC	South East Coastal Plain	P61	Z_62666	-38.4150333	144.1242
Bassiana duperreyi	Anglesea Heath_VIC	South East Coastal Plain	P61	Z_62679	-38.3796	144.18525
Bassiana duperreyi	Anglesea Heath_VIC	South East Coastal Plain	P61	Z_62691	-38.4150333	144.1242
Bassiana duperreyi	Anglesea Heath_VIC	South East Coastal Plain	P61	Z_62696	-38.4150333	144.1242
Bassiana duperreyi	Anglesea Heath_VIC	South East Coastal Plain	P61	Z_62702	-38.41535	144.12415
Bassiana duperreyi	Drajurk Forest_VIC	Naracoorte Coastal Plain	P62	Z_7339	-37.5597	141.1914
Bassiana duperreyi	Drajurk Forest_VIC	Naracoorte Coastal Plain	P62	Z_7340	-37.5597	141.1914
Bassiana duperreyi	Drajurk Forest_VIC	Naracoorte Coastal Plain	P62	Z_7342	-37.5969	141.2786
Bassiana duperreyi	Drajurk Forest_VIC	Naracoorte Coastal Plain	P62	Z_7347	-37.6192	141.2233
Bassiana duperreyi	Drajurk Forest_VIC	Naracoorte Coastal Plain	P62	Z_7353	-37.5992	141.2058
Bassiana duperreyi	Drajurk Forest_VIC	Naracoorte Coastal Plain	P62	Z_7360	-37.6208	141.2233
Bassiana duperreyi	Drajurk Forest_VIC	Naracoorte Coastal Plain	P62	Z_7366	-37.6333	141.2703
Bassiana duperreyi	Drajurk Forest_VIC	Naracoorte Coastal Plain	P62	Z_7389	-37.55	141.2289
Bassiana duperreyi	Drajurk Forest_VIC	Naracoorte Coastal Plain	P62	Z_7394	-37.6458	141.2103
Bassiana duperreyi	Drajurk Forest_VIC	Naracoorte Coastal Plain	P62	Z_7395	-37.6458	141.2103
Bassiana duperreyi	Drajurk Forest_VIC	Naracoorte Coastal Plain	P62	Z_7400	-37.6397	141.2303
Bassiana duperreyi	Mt Murphy_VIC	Australian Alps	P63	Z_7615	-36.7422	148.0056
Bassiana platynotus	Outgroup_BP	BP	Outgroup_BP	ABTC3999	NA	NA
Bassiana platynotus	Outgroup_BP	BP	Outgroup_BP	ABTC_000	NA	NA
Bassiana trilineatus	Outgroup_BT	BT	Outgroup_BT	ABTC106108	NA	NA
Bassiana trilineatus	Outgroup_BT	BT	Outgroup_BT	ABTC106110	NA	NA

Appendix 5

Supplementary material for chapter 5

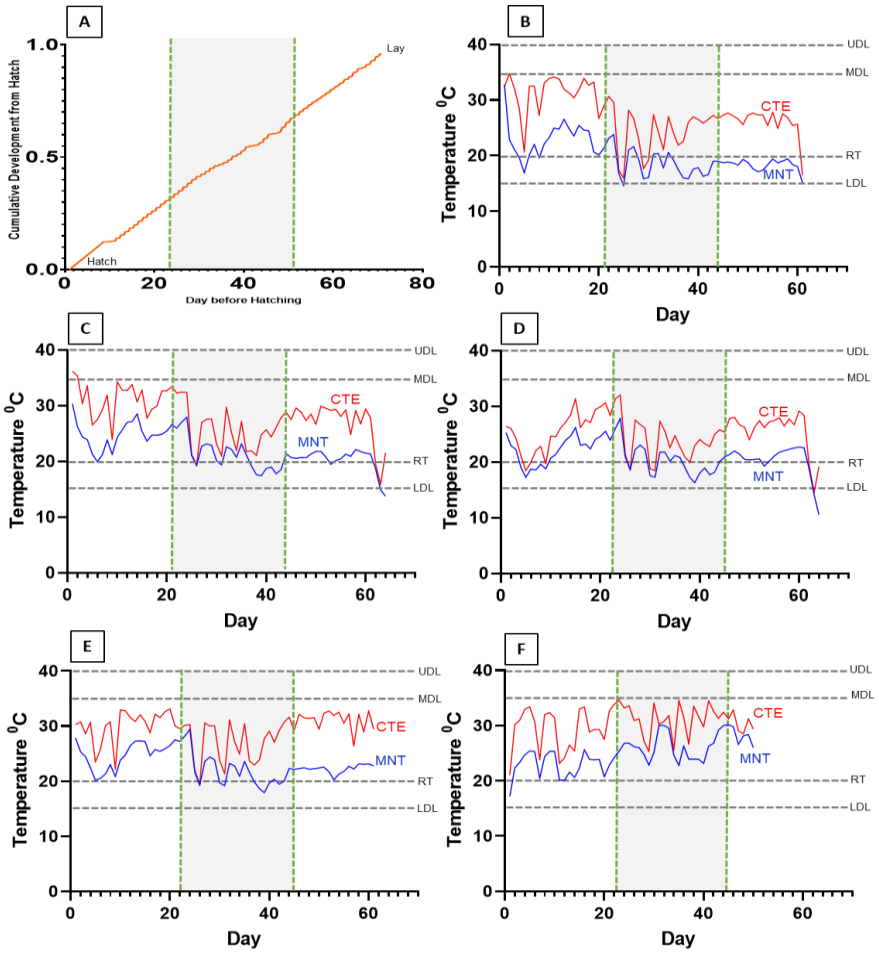

Figure S1. (**A)** Development of *Bassiana duperreyi* embryos under a temperature regime at the natural incubation environment; **B** to **F**: Temperature trace for the core of a nest of *B. duperreyi* showing traces for the mean nest temperature (MNT) and the constant-temperature equivalent (CTE) for the Dallwitz-Higgins model. The threshold for sex determination (20˚C; Shine et al., 2002) (RT) and the thermosensitive period lies between lower development limit (LDL) and upper limit of development (UDL). Note that the thermosensitive period does not correspond to the middle third of incubation, either in position or duration, owing to the nonstationary trend in temperatures with season. Shaded area showing 33% to 67% of the thermosensitive period of each nest; **B**. Mt Ginini nest (Nest 66) with XXmale produced nest; **C**. Piccadilly circus nest (Nest 52) XXmale not recorded nest; **D**. Piccadilly circus nest (Nest 47) XXmale recorded nest; **E**. Cooma (Nest 76) nest XXmale recorded; **F**. Dartmouth nest (Nest 86) XXmale not recorded.

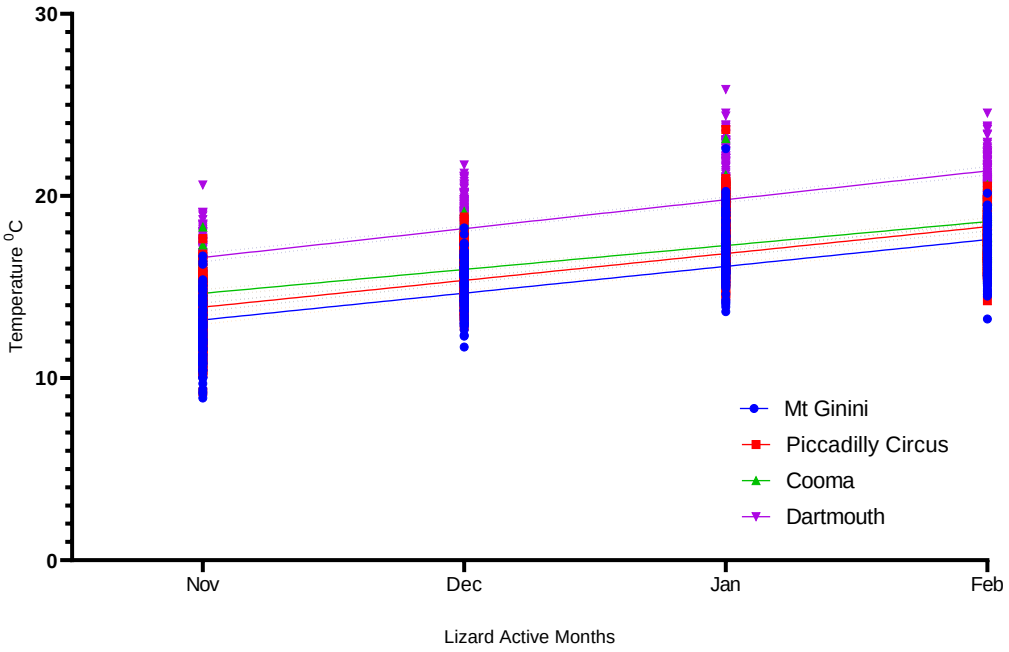

Figure S2. Mean air temperature during the skink active months (i.e., usually early November to late February, a 16-week period). Mean air temperature revealed a warming trend between 1889 to 2019.

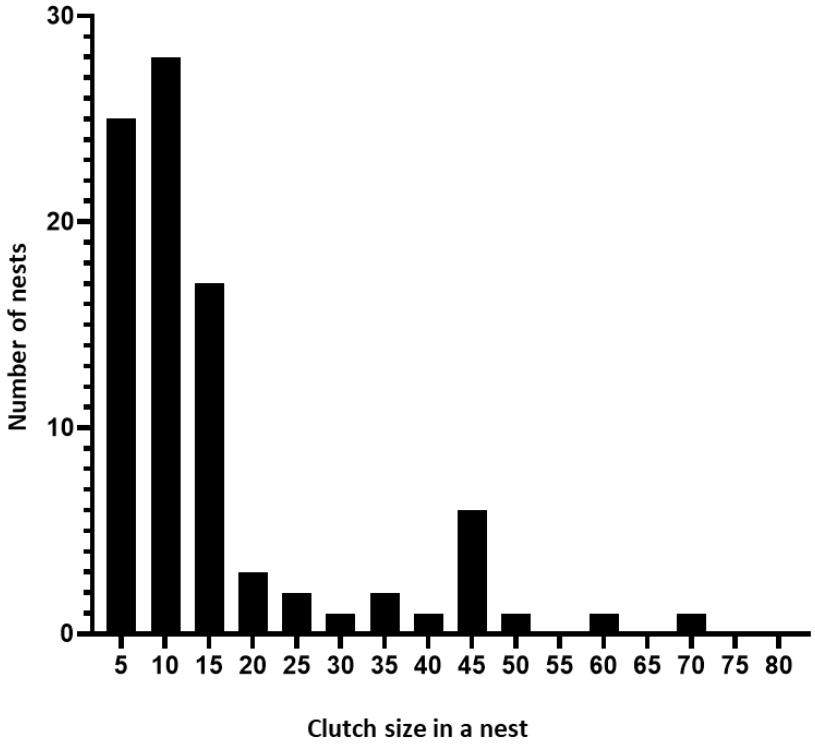

Figure S3. Number of eggs recorded in a nest during the study period.

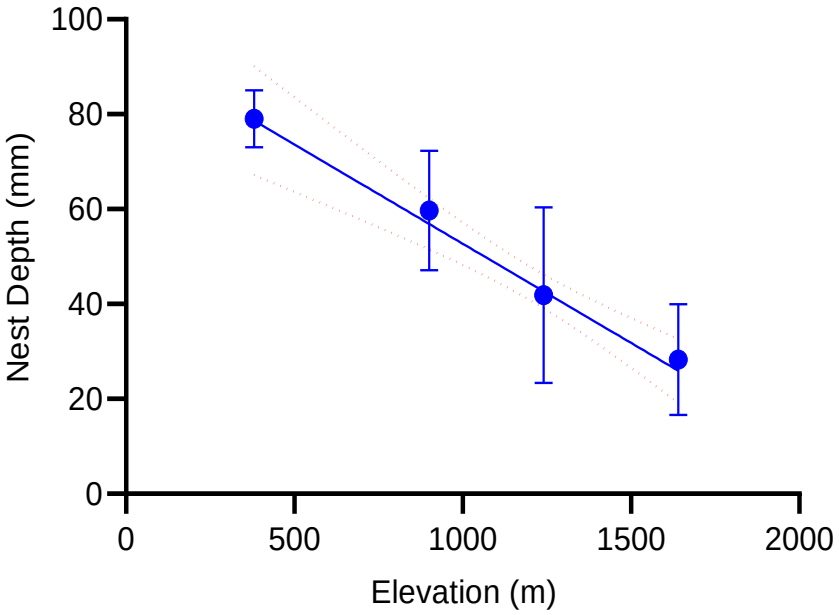

Figure S4: The pattern of increasing the mean nest depth with decreasing elevation.

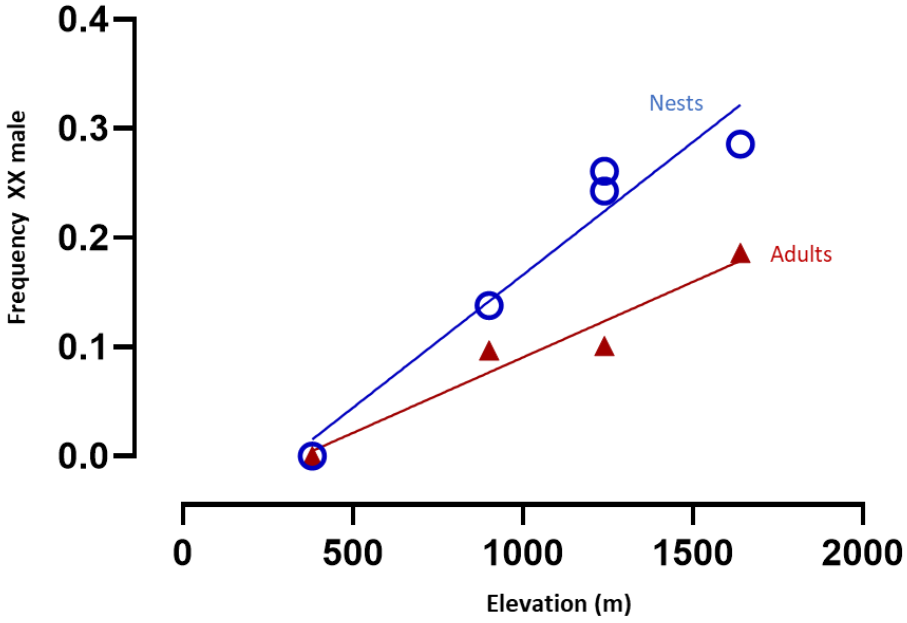

Figure S5: Frequency of XX males in two life stages in *B. duperreyi*. Adult frequency of sex reversal data from Dissanayake et al., (2021).

Table S1. The mean weekly temperature regimes in two types of nets (sex reversed found nests and normal nests).

	Levene's Test for Equality of Variances		t-test for Equality of Means					
			t	df	Sig. (2-tailed)	Mean Difference ± SEM	95% Confidence Interval of the Differences	
	F	Sig.					Lower	Upper
Mt Ginini XXmale found nest vs XXmale not found nest	1.3	0.719	0.907	16	0.377	-0.9630 ± 1.061	-3.213	1.287
PCSI XXmale found nest vs XXmale not found nest	1.264	0.75	2.057	16	0.056	1.174 ± 0.5707	-0.03564	2.384
PCSII XXmale found nest vs XXmale not found nest	1.113	0.883	1.198	16	0.248	1.136 ± 0.9490	-0.8753	3.148
Cooma XXmale found nest vs XXmale not found nest	1.8	0.42	4.089	16	0.0009	1.922 ± 0.4701	0.9256	2.919

Table S2. Pearson r and P value for Pearson correlation

Correlation	Sex reversal frequency in the nests vs.							
	Elevation	Mean nest temperature	Tmax (°C)	Tmin (°C)	Total Rain (mm)	Evaporation (mm)	Radiation (MJ/m^2)	Vapor pressure (hPa)
Pearson r	0.9797	-0.9574	-0.9952	-0.8223	0.3418	-0.9124	0.8474	-0.9874
95% confidence interval	0.3176 to 0.9996	-0.9991 to 0.04618	-0.9999 to -0.7848	-0.9961 to 0.6618	-0.9222 to 0.9807	-0.9982 to 0.3956	-0.6127 to 0.9967	-0.9997 to -0.5147
R squared	0.9597	0.9166	0.9905	0.6762	0.1168	0.8324	0.718	0.9749
P value								
P (two-tailed)	0.0203	0.0426	0.0048	0.1777	0.6582	0.0876	0.1526	0.0126
P value summary	*	*	**	ns	ns	ns	ns	*
Significant? (alpha = 0.05)	Yes	Yes	Yes	No	No	No	No	Yes

“Thus, from the war of nature, from famine and death, the most exalted object which we are capable of conceiving, namely, the production of the higher animals, directly follows. There is grandeur in this view of life, with its several powers, having been originally breathed into a few forms or into one; and that, whilst this planet has gone cycling on according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved.”

— Charles Darwin, The Origin of Species